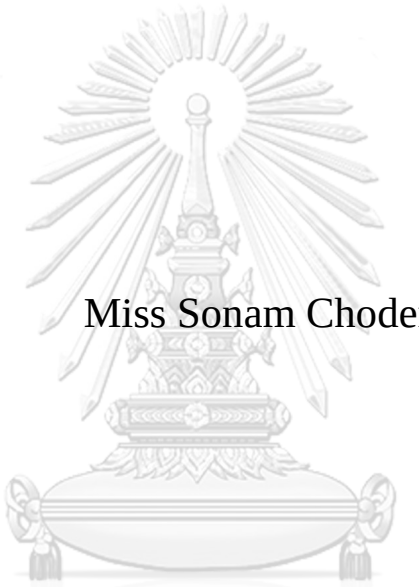
An affordable immunohistochemical approach to estimate the prevalence of BRAF V600E mutation in papillary thyroid cancer patients of King Chulalongkorn Memorial Hospital, Thailand.

Miss Sonam Choden



A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Clinical Sciences
Common Course
FACULTY OF MEDICINE
Chulalongkorn University
Academic Year 2019

การศึกษาเพื่อประเมินความชุกของการกลายพันธุ์ของยีน BRAF V600E ในมะเร็งต่อมไทรอยด์ชนิด papillary thyroid carcinoma โดยวิธีการย้อมทางอิมมูโนฮีสโตเคมีที่มีราคาไม่แพงมากเกินไปในผู้ป่วยที่เข้ารับการรักษาที่โรงพยาบาลจุฬาลงกรณ์สภากาชาดไทย

น.ส.โซนัม โชเดน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเวชศาสตร์คลินิก ไม่สังกัดภาควิชา/เทียบเท่า
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2562

Thesis Title	An affordable immunohistochemical approach to estimate the prevalence of BRAF V600E mutation in papillary thyroid cancer patients of King Chulalongkorn Memorial Hospital, Thailand.
By	Miss Sonam Choden
Field of Study	Clinical Sciences
Thesis Advisor	Associate Professor SOMBOON KEELAWAT, MD

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โซนัม โชเดน : การศึกษาเพื่อประเมินความชุกของการกลายพันธุ์ของยีน BRAF V600E **ในมะเร็งต่อมไทรอยด์ชนิด** papillary thyroid carcinoma **โดยวิธีการย้อมทางอิมมูโนฮีสโตเคมีที่มีราคาไม่แพงมากเกินไปในผู้ป่วยที่เข้ารับการรักษาที่โรงพยาบาลจุฬาลงกรณ์สภากาชาดไทย.** (An affordable immunohistochemical approach to estimate the prevalence of BRAF V600E mutation in papillary thyroid cancer patients of King Chulalongkorn Memorial Hospital, Thailand.) **อ. ที่ปรึกษาหลัก : รศ.สมบูรณ์ คีลาวัฒน์**

บทนำ: มะเร็งของต่อมไทรอยด์ชนิด Papillary thyroid carcinoma (PTC) เป็นมะเร็งชนิดที่พบได้บ่อยที่สุดของต่อมไทรอยด์ การเปลี่ยนแปลงทางพันธุกรรมที่พบได้มากที่สุดในมะเร็งชนิดนี้คือการกลายพันธุ์ของยีน BRAF ที่ตำแหน่ง V600E (*BRAF*^{V600E}) โดยการกลายพันธุ์นี้มีผลต่อการวินิจฉัยโรคและการพยากรณ์โรค อัตราการกลายพันธ์ของยีน *BRAF* ^{V600E} ยังไม่เคยมีการรายงานในประเทศไทย การวิจัยนี้มีวัตถุประสงค์เพื่อประเมินความชุกของการกลายพันธ์ของยีน *BRAF* ^{V600E} ในมะเร็งของต่อมไทรอยด์ชนิด PTC โดยทำการศึกษาในกลุ่มตัวอย่างขนาดใหญ่และใช้วิธีการประเมิน ได้แก่การย้อม Immunohistochemistry (IHC) ด้วย VE1 antibody ซึ่งมีความจำเพาะกับการกลายพันธุ์ดังกล่าว ร่วมกับการใช้เทคนิค Tissue microarray (TMA)

ระเบียบวิธีการวิจัย: ในการวิจัยนี้ TMA ประกอบด้วยชิ้นเนื้อมะเร็งของต่อมไทรอยด์ชนิด PTC จำนวนทั้งหมด 476 ราย ในจำนวนทั้งหมดนี้มีชิ้นเนื้อจำนวน 100 รายที่ได้รับการวิเคราะห์หาการกลายพันธุ์ของยีน *BRAF* ^{V600E} โดยวิธี direct sequencing และการย้อม IHC ด้วย VE1 antibody ในการศึกษานำร่องที่ผ่านมาของผู้วิจัย สำหรับชิ้นเนื้อนอกจาก 100 รายนี้จะได้รับการประเมินการกลายพันธุ์ของยีน *BRAF* ^{V600E} โดยการย้อม IHC ด้วย VE1 antibody เพียงวิธีเดียว ผู้วิจัยได้ใช้การวิเคราะห์ตัวแปรเดียว (univariate analysis) และการวิเคราะห์หลายตัวแปร (multivariate analysis) เพื่อหาความสัมพันธ์ระหว่างการกลายพันธุ์ของยีนดังกล่าวกับลักษณะทางคลินิกและพยาธิวิทยา (clinicopathological variables)

ผลการวิจัย: ในการศึกษานำร่องที่ผ่านมา ผู้วิจัยพบว่า การย้อม IHC ด้วย VE1 antibody ให้ผลเป็นที่น่าพอใจ ($\kappa = 0.884$) เมื่อเปรียบเทียบกับวิธี direct sequencing ในการตรวจหาการกลายพันธุ์ของยีน *BRAF* ^{V600E} ร่วมกับการใช้เทคนิค TMA สำหรับการวิจัยนี้พบว่าความชุกของการกลายพันธุ์ของยีน *BRAF* ^{V600E} เท่ากับ 60.9% เมื่อใช้การย้อม IHC ด้วย VE1 antibody การกลายพันธุ์ของยีนนี้พบได้บ่อยในมะเร็งของต่อมไทรอยด์ชนิด PTC ที่มีลักษณะ tall cell (92.9%) และลักษณะ classic (70.2%) การวิเคราะห์หลายตัวแปร (Multivariate analysis) พบว่ามีความสัมพันธ์ระหว่างการกลายพันธุ์ของ *BRAF* ^{V600E} กับลักษณะทางพยาธิวิทยาของมะเร็ง การแทรกซึมของเซลล์มะเร็งออกไปนอกต่อมไทรอยด์ (extrathyroidal extension) และการไม่มีการอักเสบของต่อมไทรอยด์ชนิด Hashimoto's thyroiditis ($P<0.05$)

สาขาวิชา	**เวชศาสตร์คลินิก**	**ลายมือชื่อนิสิต** .....
ปีการศึกษา	2562	**ลายมือชื่อ อ.ที่ปรึกษาหลัก** .....

6174357030 : MAJOR CLINICAL SCIENCES
KEYWORD: BRAFV600E, immunohistochemistry, papillary thyroid carcinoma, VE1

Sonam Choden : An affordable immunohistochemical approach to estimate the prevalence of BRAF V600E mutation in papillary thyroid cancer patients of King Chulalongkorn Memorial Hospital, Thailand. . Advisor: Assoc. Prof. SOMBOON KEELAWAT, MD

Background: Papillary thyroid carcinoma (PTC) accounts for the majority of diagnoses of thyroid carcinoma. *BRAF*^{V600E} mutation is the most common genetic alteration in PTC, which has diagnostic and prognostic significance. The rate of *BRAF*^{V600E} mutation in PTC from Thailand has not been reported. Our purpose was to estimate the prevalence of *BRAF* mutation in a large institutional series using an affordable approach, which combined mutation-specific immunohistochemistry (IHC) with VE1 antibody and tissue microarray (TMA).

Methods: A total of 476 PTC cases plotted on TMA were employed for determining the mutation status in this study. The cancer tissue of initial 100 cases (pilot study) were analyzed for *BRAF*^{V600E} mutation by using both direct sequencing and VE1 immunostaining. For the subsequent PTC cases, VE1 IHC was used as an alternative to direct sequencing for the detection of mutation. Univariate and multivariate analyses were done to determine the association of clinicopathological variables with *BRAF*^{V600E} mutation.

Results: In the pilot study, VE1 IHC showed excellent analytical performance ($\kappa = 0.884$) for detecting *BRAF*^{V600E} mutation in PTC TMA as compared to direct sequencing. The prevalence of *BRAF*^{V600E} in the whole cohort was 60.9% by using VE1 IHC. The mutation was commonly seen in tall cell (92.9%) and classic (70.2%) variants of PTC. Multivariate analysis ($P<0.05$) showed association of *BRAF*^{V600E} with histological type of tumor, extrathyroidal extension, and absence of Hashimoto's thyroiditis.

Conclusions: In conclusion, *BRAF*^{V600E} mutation was detected in 60.9% of Thai PTC and it was associated with several aggressive clinicopathological variables of thyroid cancer. VE1 IHC proved as a reliable method able to replace direct sequencing for the detection of the mutation. A combination of mutation-specific IHC and TMA allows conducting large cohort studies more labor-saving and cost-efficiently.

Field of Study:	Clinical Sciences	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Associate professor Somboon Keelawat, Head of Department of Pathology, King Chulalongkorn Memorial Hospital (KCMH). Without his guidance, encouragement and constructive criticism, this project would not have been possible. Andrey Bychkov, MD, Ph.D., Director of Digital pathology at Department of Pathology, Kameda Medical Center (Ex-Postdoctoral Research Fellow, Department of pathology, KCMH), has been my mentor since the commencement of this project and provided me with necessary advice, knowledge and valuable feedback. I am short of words to thank him for everything. I am thankful to Professor Chan Kwon Jung from the Department of Pathology,St. Mary's Hospital, Catholic University of Korea, Seoul, for helping me to perform direct sequencing in his hospital and also for providing me with knowledge and necessary feedback in this study. This work would have lost its charm without his tremendous contribution. I sincerely thank him for his kindness.

This work would have been impossible without the help of Ractchadapiseksompotch Fund, Faculty of Medicine, Chulalongkorn University. I remain thankful to the university for providing me with the necessary fund and allowing me to conduct this project. I am grateful to the panel of reviewers appointed by the Ethics and Research committee. They reviewed my project proposal extensively and made the necessary amendments.

I am grateful to Ms. Atthanee Chiyaphan for her patience and kindness in my repeated visits for statistical advice.

Lastly, I would like to thank all the laboratory staff, especially Mrs. Kanista Keetacheeva and Ms. Jutamas Wongphoom in the pathology department, KCMH for helping me with the laboratory work. Without their assistance, my work would not have been complete.

Sonam Choden

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List of abbreviations

ATC	Anaplastic thyroid carcinoma
BRAF	B-type RAf Kinase
C-PCR	Competitive polymerase chain reaction
CRC	Colorectal carcinoma
DS	Direct sequencing
ENE	Extranodal extension
ETE	Extrathyroidal extension
FFPE	Formalin-fixed paraffin-embedded
FC/FTC	Follicular thyroid carcinoma
FN	False negative
FP	False positive
H&E	Haemotoxylin and Eosin
IHC	Immunohistochemistry
KCMH	King Chulalongkorn Memorial Hospital
LN	Lymph node
LNM	Lymph node metastasis
LVSI	Lymphovascular invasion
MAPK	Mitogen-activated protein kinase pathway
NPP	Negative predictive valve
PDTC	Poorly differentiated thyroid carcinoma
PPV	Positive predictive value
pT	Primary Tumor staging)according to AJCC 8th edition(
PTC	Papillary thyroid carcinoma
qPCR	Quantitative polymerase chain reaction
SS	Sanger sequencing
TMA	Tissue microarray
TN	True Negative
TP	True positive
VE1	Anti-BRAF V600E monoclonal primary antibody
WS	Whole slide

Chapter 1
Introduction

1.1Background and rationale

Thyroid cancer is the most common endocrine malignancy whose incidence has been increasing dramatically over the past three decades due to increased recognition of thyroid nodules harboring carcinoma [1,2]. It is known to be the fastest-growing cancer in women worldwide, particularly in economically developed countries where high resolution imaging is widely available [1]. The rate of thyroid cancer is in Thailand is similar to average Asian and worldwide rates – according to recent estimates, thyroid carcinoma is one of the leading malignancies in Thai women, occupying seventh rank by incidence and fourth rank by prevalence [3].

Papillary thyroid cancer (PTC) is the major histological subtypes, accounting for approximately 85% of all thyroid cancers [4], which also holds true for Thailand [5]. Compared to other human malignancies, PTC carries an excellent overall prognosis with 10-year survival rate approaching 95% [4,6]. There are several risk factors associated with development of PTC, of which exposure to ionizing radiation has been well-documented environmental cause of PTC [2]. Other factors include genetic predisposition, hormonal influences, dietary components, such as iodine, nitrates, and alcohol, and more modifiers [7-9].

BRAF^{V600E} mutation is the most common somatic driver event in PTC [4,10]. *BRAF*^{V600E} is detected in almost half of PTC cases in Western cohorts (45-50%) [11]. In contrast, Asian series of PTC have much higher variation of *BRAF* incidence, which was reported ranging 31%–87% [12]. The presence of *BRAF*^{V600E} is known to be clinically relevant in terms of diagnosis, adverse prognosis, and treatment strategy [10,11,13], therefore establishing a rate of *BRAF* on the national and even institutional level is of practical significance. For example, preoperative *BRAF* testing in cytological specimen is much effective in area with high prevalence of *BRAF* mutation [14,15]. To our knowledge, there are no well-established datasets or

publications in international peer-reviewed journals available on the prevalence of BRAF mutation in Thai PTC till date.

DNA-based molecular testing methods, especially Sanger/Direct sequencing has been widely acknowledged as the gold standard for detection of oncogenes in solid tumors. However, molecular techniques are relatively expensive, time-consuming, and have certain limitations regarding specimen quality, sample adequacy, tumor heterogeneity, and more [16,17].

Recently, a new approach of detection of *BRAF* mutation by means of VE1 immunohistochemistry (IHC) has been established. VE1 is a mutation-specific monoclonal mouse antibody which enables detection of $BRAF^{V600E}$ mutated protein and its application in detection of $BRAF^{V600E}$ mutation in PTC (as well as in melanoma) was first described in 2011 by Capper et al [18].

IHC is a rapid, simple and cost-effective method that doesn't require the establishment of the molecular laboratory. Numerous studies performed on PTC, colorectal carcinoma, melanoma, and other *BRAF*-mutant tumors reported excellent concordance between VE1 IHC and molecular genotyping and suggested that VE1 IHC is a reliable method that can be used as an alternative to *BRAF* sequencing [18-24].

The main aim of this study is to estimate the prevalence of $BRAF^{V600E}$ mutation in PTC patients of King Chulalongkorn Memorial Hospital, Thailand.

Considering a need to establish a baseline rate of *BRAF* mutation in PTC from Thailand, we designed an affordable approach combining advantages of VE1 IHC and tissue microarray (TMA). The latter technology allows multiple tissue samples to be arrayed into a single paraffin block, thus significantly reducing the costs of reagents [25]. In addition to the main purpose above, we also aimed to validate the performance of VE1 immunostaining against molecular genotyping and to evaluate clinical relevance of *BRAF* mutation in the Thai series of PTC.

1.2 Research Questions

a. Is the use of IHC as a test for *BRAF* mutation correlated well with molecular studies in detection of $BRAF^{V600E}$ mutation?
b. What is the prevalence rate of $BRAF^{V600E}$ mutation in PTC patients of KCMH, Thailand?
c. Is the presence of $BRAF^{V600E}$ mutation significantly associated with adverse prognostic characteristic of PTC?

1.3 Hypothesis

a. VE1 IHC can serve as alternative to *BRAF* molecular testing in Thai PTC
b. *BRAF* mutation is seen in about half of PTC cases, similar to the findings from ASEAN countries.
c. $BRAF^{V600E}$ mutation may be associated with adverse clinicopathological characteristic of PTC.

1.4 Objectives

a. Primary objective
    - To estimate the prevalence of $BRAF^{V600E}$ mutation in papillary thyroid cancer of King Chulalongkorn Memorial Hospital, by using VE1 IHC on TMA blocks.

b. Secondary objective
    - To determine the clinical significance of $BRAF^{V600E}$ by correlating with clinic-pathological parameters
    - To compare the performance of VE1 IHC with direct sequencing (Pilot study)

1.5 Literature review

1.5.1 Papillary thyroid cancer

Papillary thyroid cancer is the most common malignant epithelial tumor of thyroid gland in both adults and children showing evidence of follicular cell differentiation [2]. PTC is characterized by a set of nuclear features, which includes nuclear enlargement and irregularity, overlapping, clearing (ground glass or Orphan Annie appearance), nuclear grooves and pseudo-inclusions [2]. In adults, PTC occur in patients between 20-50 years of age with male to female ratio of 1:4-5 [26]. There has been a dramatic increase in reported incidence of PTC worldwide since the introduction of high-resolution imaging techniques (thyroid ultrasonography) into clinical practice [2]. Several histological variants of PTC are recognized (Table 1).

*Table 1. **Papillary thyroid cancer histological variants (in alphabetical order)***

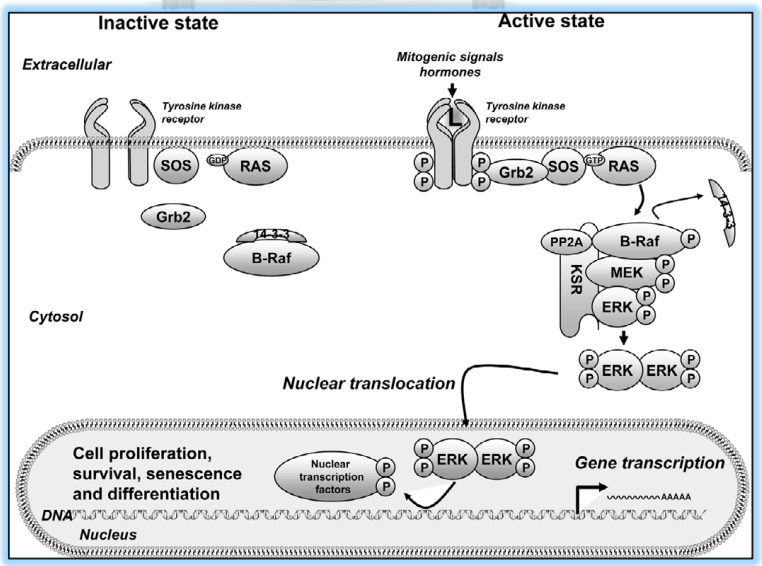
1.Classical (usual)
2.Clear cell variant
3.Columnar cell variant
4. Cribriform-morular variant
5.Diffuse sclerosing variant
6.Follicular variant
7.Hobnail variant
8.Macrofollicular variant
9.Microcarcinoma (<1 cm)
10. Oncocytic or oxyphilic variant
11. Solid variant
12.Tall cell variant
13. Warthin-like variant

(Seethala RR, Asa SL, Bullock MJ, et al; CAP: Protocol for the examination of specimens from patients with carcinomas od thyroid gland; Version 4.0.0.0; 2017)

Microfollicular, clear cell, oncocytic and warthin-like variants have a prognosis that is similar to conventional PTC but tall cell and columnar cell variants are known to have worse prognosis than conventional variant [2,26]. Solid and diffuse sclerosing variants

are mostly seen in children [26]. PTC is associated with various genetic alterations, including point mutation and rearrangements. The targets of these genetic alterations include *BRAF* and RAS (point mutation) and RET and TRK (rearrangements). All of the above mentioned genetic alteration involve signaling along mitogen-activated protein kinase (MAPK) pathway, which in turn is involved in signaling of a variety of growth factors and cell surface receptors [26,27]. The MAPK signal transduction pathway is illustrated in figure 1. The binding of ligands (L) to their membrane tyrosine kinase receptors (RTKs) leads to dimerization of the receptors and tyrosine residue autophosphorylation. The activated receptors activate RAS kinase through adaptor proteins, which then activates the phosphorylation of Raf kinases, which in turn activate the dual- specificity protein kinase: MAP kinase kinases, MEK 1 and 2. MEK 1/2 phosphorylate and activate extracellular signal-regulated kinases ERK 1 and 2. ERK1/2 regulate various transcription factors leading to gene expression [28].

Figure 1. MAPK signal transduction pathway



(Image from: Tang K T; Lee C H. BRAF mutation in papillary thyroid carcinoma: pathogenic role and clinical implications. J Chin MedAssoc. 2010; 73:113-28)

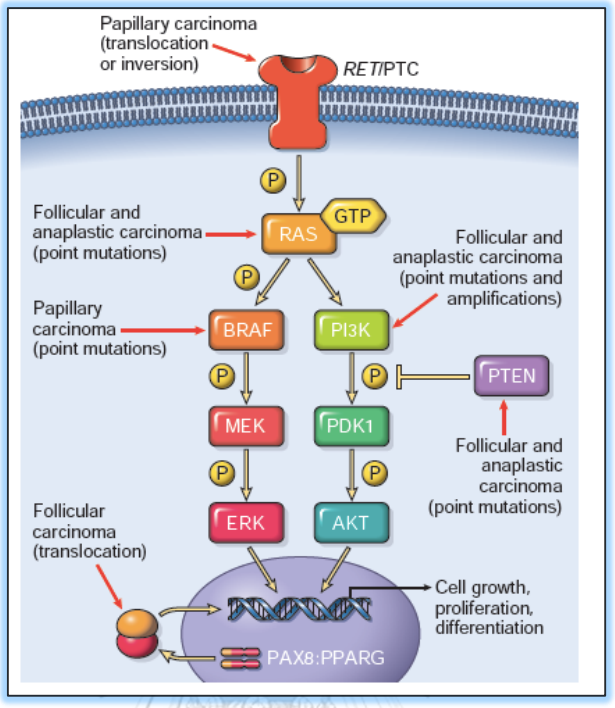
1.5.2 *BRAF*^{V600E} mutation

B-type Raf Kinase (BRAF) gene is located on chromosome arm 7q34 and it encodes B-raf, which is a part of mitogen-activated protein kinase (MAPK) signaling cascade [29]. As shown in figure. 2, MAPK pathway is involved in cell regulation and hence, activation of this pathway induce cell growth, differentiation and survival, which leads to tumor initiation, growth and progression. Although more than forty *BRAF* mutations have been identified, V600E mutation accounts for more than 90% of those mutations [10].

BRAF gene is composed of 18 exons and the most common activating mutation is seen in exon 15 at nucleotide 1799 [29]. *BRAF*^{V600E} mutation results from a transversion of thymine-to-adenine at exon 15 nucleotide (T1799A) of the *BRAF* gene, which leads to substitution of valine (V) by glutamic acid (E) at amino acid 600. Thus the abbreviation used to designate this mutation is BRAFT1799A or BRAF V600E [10,20].

BRAF mutation is associated with various solid tumors including those of thyroid carcinoma, colorectal carcinoma, brain tumors, melanoma, ovarian epithelial tumors, lung adenocarcinoma, hairy cell leukemia, renal metanephric adenoma, langerhan cell histiocytosis, and Erdheim- Chester disease [30]. *BRAF* mutation is the most frequent genetic alteration in PTC and develop exclusively in PTC and PTC-derived anaplastic thyroid cancers [28,31-33]. Other relevant genetic alterations in PTC associated with prevalence estimates from literature is illustrated in table 2.

*Figure **2**. Genetic alterations in follicular cell–derived malignancies of the thyroid gland*



(Image from: Kumar, V., Abbas, A. K., and Aster, J. C. Robbins and cotran pathologic basis of disease (9th edition.). Philadelphia: Elsevier/Saunders.2015: p1094-1096)

Table 2. Summary of the most relevant genetic alterations in papillary thyroid carcinoma with prevalence estimates from literature

PTC histotype	**BRAF**	**RET**	**RAS**	**Promoter**	**ALK**
All histotypes	30-90%	5-35%	0-35%	5-25%	0-5%
Conventional PTC	45-80% [13,34]	5-35% [35,36]	0-15% [37]	1-15% [38,39]	Not determined
Follicular variant	5-25%[13,34]	5-25% [35,36]	0-15% [37]	5-15% [38,39]	Not determined
Tall cell variant	60-95% [13,40]	35% [35,36]	15-35% [41]	5-30% [40,42]	Not determined

(Lloyd, R.V.; Osamura, R.Y.; Kloppel, G.; Rosai, J. WHO Classification of Tumors of Endocrine Organs 4th ed; International Agency for Research on Cancer (IARC): Lyon, France: 2017; Vol. 10, pp. 1-357)

1.5.3 Prevalence of *BRAF*^{V600E} in Southeast Asian PTC

According to the literature, rate of *BRAF*^{V600E} in PTC was extensively reported from USA, Europe, Australia and Middle East, with the consistent rate of 45-50% across different regions [11,12]. However, there is limited data available from South and Southeast Asian countries.

The rates from Indonesia, Hong Kong, Taiwan and India, appear to be approximately similar (40-50%) but several Chinese studies showed wide variation, ranging from 30-80% [12]. Japan and South Korea have a prevalence rate ranging from 60-90%, which stands out from the above countries [12]. The overall *BRAF* rates in Asian PTC is significantly higher than those from Western countries [36].

1.5.4 Detection of *BRAF*^{V600E} mutation

BRAF mutation test is a useful tool to determine the necessity of surgical removal of the thyroid nodule at the preoperative step [33]. *BRAF*^{V600E} mutation can be detected by molecular and immunohistochemical methods. DNA-based assays are the standard methods for the detection of *BRAF*^{V600E} mutations in papillary thyroid carcinoma [43]. Sanger sequencing is widely considered as the gold standard method for detection of point mutation.

VE1 is an anti-BRAF mouse monoclonal primary antibody, exhibiting cytoplasmic staining. It is a new method to detect *BRAF*^{V600E} mutation. Several studies have been done regarding the performance of VE1 IHC on detection of BRAF mutation, not only in PTC but also in other solid tumors associated with *BRAF*^{V600E} mutation, such as melanoma, colorectal carcinoma, ovarian carcinoma, etc. and majority of the studies showed excellent concordance of VE1 IHC with that of molecular methods, with the accuracy rate of >80 % [18,20,22-24,31,44-48]. Previous studies related to performance of VE1 IHC on detection *BRAF*^{V600E} mutation are summarized in Table 3. Immunohistochemistry gives a faster result compared to other

molecular biology techniques and thus reduces the turnaround time between the physician's request and the result. This would be of particular interest in urgent cases that require targeted therapy against an aggressive thyroid carcinoma harboring *BRAF*^{V600E} mutation [48].

An examination cost also should be taken into consideration, since an IHC method is more cost-effective than molecular biology methods. In one of the reports, the total cost ratio between IHC and a molecular biology technique was estimated to be 1:3.4 [20].

Taken together, these benefits highlight that the immunohistochemical approach can be a good alternative to the molecular biology approach for *BRAF*^{V600E} detection in papillary thyroid carcinoma patients.

Table 3. Previous studies on VE1 IHC in detection of BRAF^{V600E} *mutation*

Sources	**Cases (n)**	**TMA/ WS**	**BRAF rate by VE1(%)**	**validation**	**Sn (%)**	**Sp (%)**
Capper et al; 2011; Germany [18]	68 (47MM,21PTC)	WS	50%	DS	100%	100%
Routhier BS et al; 2013; MA [22]	152(31MM, 25lungCA, 32CRC, 35glioma, 19PTC,4FTC, 6others	WS		SNaPshot genotyping	98%	97%
Zagzag et al; 2013; USA [46]	37	TMA	89%	DS	89%	100%
Dvorak et al;2014; USA [45]	352(279CRC, 73PTC)	TMA+ WS	71.20%	SS	98.60%	99.1%
Fisher et al;2014; Atlanta, GA [43]	41 (29PTC, 1FTC, 7MC,4ATC)	WS	41.40%	Pyrosequencing	100%	76.5%
Ilie et al; 2014; France [20]	198	WS	78%	DS,PS,qPCR	98.7%	100%
Jung et al, 2014; S. Korea [49]	467	TMA	86%	qPCR, RNA ISH	95%	61%

Kim et al, 2014; S. Korea [33]	91	WS	61.50%	DS, qPCR	88.4%	68.2%
Rossi et al, 2014; Italy [50]	55	Cytology, WS	67.20	PS	82%	100%
Zimmermann et al, 2014; Switzerland [48]	55	WS	81.80%	SS	93.8%	93.8%
Jong-In Na et al, 2015; S. Korea [23]	141	WS	68%	qPCR, DS	100%	88%
Pyo et al, 2015; S. Korea [24]	1141 (Meta-analysis)	WS, TMA	79.10%	DS, SS, PS, SNaPshot assay	95%	95%
Qiu et al, 2015; Taiwan [51]	799(611CRC, 127PTC, 41MM)	WS	80%	SS,qPCR	100%	99%
Martinuzzi et al, 2016; China [52]	86	WS	71%	DS,qPCR	94.2%	92%
Zhu et al, 2016; China [47]	118	TMA	68.6%	SS	100%	82.2%
Elmageed et al,2016; Italy [44]	130	WS	97%	PCR	98%	93.3%
Fano et al, 2017; Spain [53]	82	WS		RT-PCR	100%	97%
Szymonek et al, 2017; Poland [31]	140	WS	69.90%	SS, qPCR	97.6%	81.9%
Chen et al, 2018; China [54]	40	WS	95%	qPCR	100%	66.6%
Oh HS et al, 2018; S. korea [32]	71	WS	78%	SS	100%	71.4%
Kim et al, 2018; S. korea [55]	697	TMA	90%	DS	100%	60.3%
Zhang et al, 2018; China [56]	255(123CRC, 132PTC)	WS	96.20%	SS	100%	80.8%

Kombak et al, 2019; Turkey [57]	107PTC, 19adenoma, 13normal thyroid	WS	71%	RT-PCR	90.9%	88.8%

(Abbreviations: Sn sensitivity; Sp specificity; MM malignant melanoma; FTC Follicular thyroid carcinoma; PTC papillary thyroid carcinoma, CRC colorectal carcinoma, ATC anaplastic thyroid carcinoma, CNB core needle biopsy; WS whole slide; TMA tissue microarray; PS pyro sequencing; SS sanger sequencing; DS direct sequencing; qPCR quantitative polymerase chain reaction; RT-PCR reverse transcription polymerase chain reaction).

1.5.5 VE1 immunohistochemistry and evaluation

In all of the previous reports, *BRAF*^{V600E} positive tumor cells show homogenous pattern of staining. Therefore, most of the authors used Allred scoring system to assess the intensity of VE1 cytoplasmic staining (0=negative, 1+=weak, 2+=intermediate, 3+=strong).

In our study, some of the tumor showed heterogeneous staining intensity of different proportions. Hence, we employed H-score to evaluate the VE1 staining result. H-score is a semi-quantitative system which includes proportion (0-100%) and intensity of positive cells (0, absent; 1+, weak; 2+, moderate; 3+, strong). There is knowledge gap in our literature regarding the application of H-scoring system to assess VE1 staining result.

1.5.6 Clinicopathological correlation

BRAF^{V600E} mutation in PTC is found in aggressive histological variants and PTC-derived anaplastic thyroid cancer but is rare in follicular variant, and not found in follicular thyroid carcinoma [28]. BRAF mutation is known to be associated adverse clinicopathological characteristic of PTC such as presence of ETE, lymph node metastasis and advance stages [28,58-69].

Majority of the studies demonstrated an association of *BRAF*^{V600E} mutation with aggressive clinicopathological characteristics and high tumor recurrence.

However, the results are controversial since different studies found correlation with different variables as follow:

1. Koperek et al. evaluated the expression of *BRAF*^{V600E} protein in 144 cases of PTC using novel mutation-specific antibody (VE1). The study showed expression VE1 protein more commonly in tumors with tall cell or oncocytic features but less common in tumors with follicular growth pattern. No significant correlation was seen with clinicopathological parameters of aggressiveness such as LN metastasis, peritumor infiltration or peithyroidal infiltration [21].
2. Meta-analysis of Li Carol et al., which involved 32 studies including 6372 patients showed that *BRAF* mutation is associated with LNM, stage, extrathyroidal extension, tumor size, male gender, multifocality, absence of capsule, classic and tall-cell variants of PTC [13].
3. *BRAF* positive tumors showed significantly higher rate of subsequent lymph node metastases ($p=0.035$) in the study conducted by McKelvie et al. Significant association was seen with male sex ($p=0.034$), but not with age at diagnosis, size of primary tumour, extrathyroidal extension, lymph node or distant metastases or clinical stage at diagnosis [30].
4. In retrospective cohort study of Goh et al., *BRAF*-mutated tumors were associated with an advanced T-stage ($P = 0.049$) and were more likely to have a central neck dissection ($P = 0.036$). There was no significant correlation between *BRAF* mutation status and clinical outcomes [70].
5. In another study, the presence of *BRAF* mutation was significantly associated with extrathyroid extension and multifocality ($P<0.05$), but not with age, sex, lymph node metastasis, central node metastasis, lateral node metastasis, Tumor-Node-Metastasis stage or tumor size in patients with PTC [71].

1.5.7 Tissue microarray

Tissue microarray is a recent innovation in the field of pathology and is a promising technique in evaluation of immunohistochemical markers in tumors and may be used as an alternative to conventional whole slide [72,73]. A microarray contains many small representative tissue samples from hundreds of different cases assembled on a single histological slide, and therefore allows high throughput of multiple specimens at the same time [73].

The availability of TMAs containing highly characterized tissues will enable researchers to perform studies involving thousands of tumors rapidly. Hence, TMAs will lead to a significant acceleration of the transition of basic research findings into clinical applications and it is anticipated that TMAs will soon become a widely used tool for all types of tissue-based research [74].

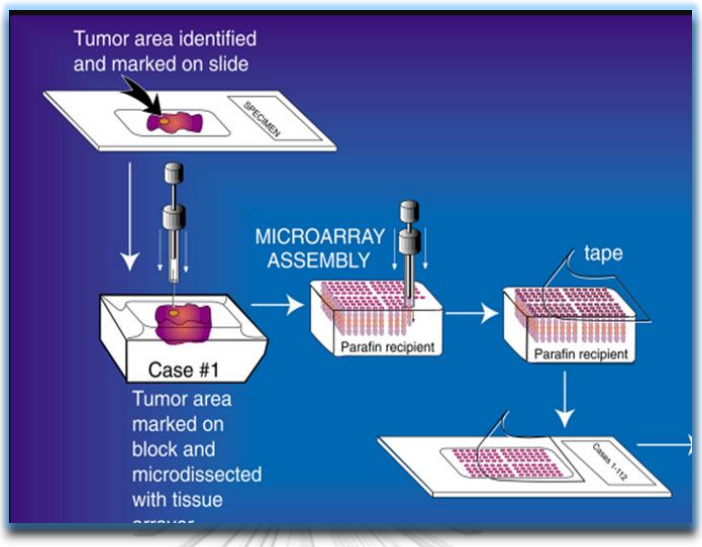
Tissue microarrays are constructed by extracting cylindrical tissue cores from different paraffin donor blocks and re-embedding these into a single recipient (microarray) block at defined array coordinates. Using this technique, up to 1000 or more of tissue samples can be arrayed into a single paraffin block.

It is an efficient method allowing simultaneous analysis of large number of specimen and also microarrays are amenable to a wide range of techniques, including immunohistochemical stains, immunological stain with either chromogenic or fluorescent visualization, in situ hybridization (mRNA ISH and FISH) [73].

Since each core in the microarray measures 2-4 mm in greatest dimension, only a small amount of reagent (a few μL) is required to analyze the entire cohort, thus reducing the assay volume, time and cost.

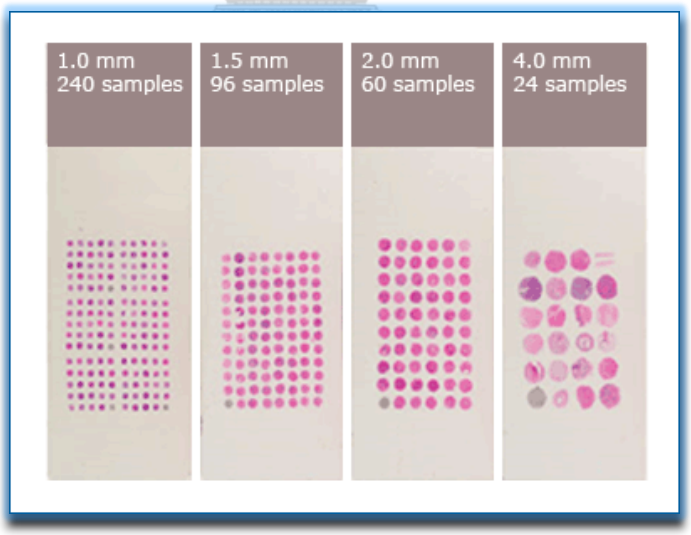
Another advantage of TMA is that, it does not destroy the original block for diagnosis and thus conserves valuable tissue [73]. Figure 3 illustrates how tissue micro array is constructed and figure 4 shows TMA composed of different core sizes.

Figure 3. Tissue microarray construction.



(Image from: http://tmalab.jhmi.edu.tma_construction.html)

Figure 4. Tissue microarray designs.



(Image from: https://www/novusbio.com/antibody-news/advantages-and-disadvantages-of-using-tissue-microarrays)

Chapter 2
Research Methodology

2.1 Study design and target populations

We conducted retrospective cross-sectional study in Department of Pathology, King Chulalongkorn Memorial Hospital (KCMH). We targeted patients with papillary thyroid cancer, surgically operated in KCMH from January 2007 to December 2017.

2.2 Inclusion criteria

Surgically resected and morphologically verified PTC of all histological variants, archived in Department of Pathology, KCMH from January 2007 to December 2017. Initial 100 PTC cases were employed for pilot study (diagnostic study, to test the quality of VE1 IHC by comparing with direct sequencing).

2.3 Exclusion criteria

We have four exclusion criteria as follows: i. Cases with missing clinical data; ii. Cases with repeat Hospital Number (HN); iii. Sample of inadequate tumor size (< 4 mm); and iv. Low quality tissue not suitable for immunohistochemical study (extensive fibrosis, calcification and hemorrhage). The exclusion criteria were same for both pilot and main study.

2.4 Sample size calculation

Pilot study:

Sample size for pilot was calculated based on "diagnostic evaluation" part (comparison of VE1with DS) focusing on specificity with the following parameters and assumptions as follows:

$$n = \frac{Z^2 P(1-P)}{d^2}$$

n =Sample size

Z = 1.96 for 95% CI

P= estimated sensitivity = 0.9 (90%, based on literature)

d= allowable error = 0.1 (10%)

So, n= 34.57 = No. of cases with positive mutation by molecular method

Since the estimated prevalence of BRAF mutation in Thai PTC = 55%

Thus, No. of patients = 34.57/0.55= 63.

We enriched the population with additional 58% of PTC cases.

Therefore, n= 1.58 x 63=99.5 (rounded to 100)

Main study:

Up to our knowledge, there was no previous study on prevalence rate of *BRAF*^{V600E} mutation in Thai PTC. Therefore, based on literature, we estimated the prevalence rate as 55% with desired precision of +/- 5%. Sample size was calculated based on "prevalence evaluation" part focusing on estimated true proportion with following parameters and assumptions as follows:

$$n = \frac{Z^2 P(1-P)}{d^2}$$

n =Sample size

Z = 1.96 for 95% CI)

P = Estimated true proportion = 0.55 (55%)

d = desired precision = 0.05 (+/- 5%)

As per the formula, the minimum number of sample required for the study is n = 381, but we enriched the population with additional 25% of PTC cases to increase the statistical power.

1.25 x 381 = 476.25 patients (rounded to 476).

2.5 Materials and methods

2.5.1 Case Selection (Patient and tissue samples):

King Chulalongkorn Memorial Hospital (KCMH), Bangkok, one of the largest tertiary referral centers for thyroid cancer in Thailand, served as a reference institution. This study was conducted with the approval of the Human Research Ethics Committee of KCMH and Chulalongkorn University Institutional Review Board (IRB No. 050/61).

Based on electronic database search, there were 1,038 patients who underwent thyroidectomy at KCMH from January 2007 to December 2017. Among them, 733 cases were diagnosed by surgical pathologists as PTC. All specimen types (total thyroidectomy, hemithyroidectomy, subtotal or near total thyroidectomy, lobectomy, and tumor excision) of primary PTC were included.

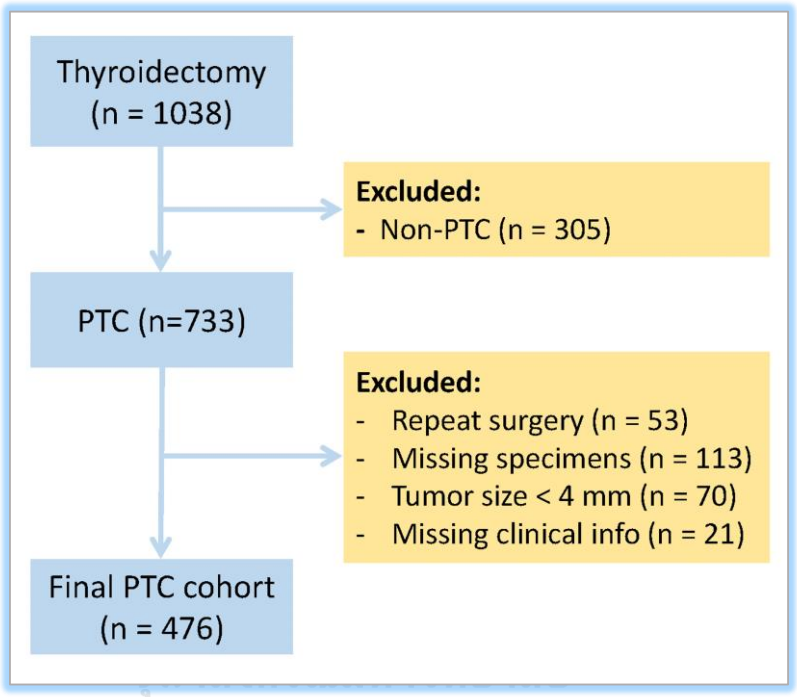
From 733 PTC cases, formalin-fixed paraffin-embedded (FFPE) blocks of 113 cases and clinical information of 21 cases were missing from our archive and database, respectively. In addition, 70 cases had tumor size insufficient for TMA preparation (< 4 mm), and 53 cases had repeated hospital number, i.e. re-operated for the same tumor. Based on our inclusion and exclusion criteria, the total number of PTC cases employed for this study was 467. The case selection process is summarized in Figure 5.

Tumor characteristics and clinical information of each patient were retrieved from the hospital pathology database, including patient's gender and age, histological variants, tumor size, laterality, multifocality, extrathyroidal extension, margins status, lymphovascular invasion, presence of Hashimoto's thyroiditis, lymph node

metastasis, distant metastasis, and pathological staging. FFPE tissue samples were retrieved from archives of the Department of Pathology, KCMH.

All pathology slides were reviewed by two pathologists (S.C. and A.B.) and classified as per the terminology and diagnostic criteria of the WHO classification of Tumors of Endocrine Organs [2]. Cancer staging was done as per the American Joint Committee on Cancer (AJCC) staging system, 8th edition [75].

Figure 5. Case selection flowchart



(Inclusion criteria were surgically resected in 2007-2017 and morphologically verified PTCs of all histological variants archieved at Department of Pathology, KCMH)

2.5.2 Histopathological Evaluation

All slides in every cases were re-evaluated (S.C and A.B). These slides were constructed from 3 mm sections of FFPE blocks and stained with hematoxylin (DAKO) and eosin (DAKO) in the automated DAKO CoverStainer as per the manufacture's recommended protocol (see appendix for full protocol).

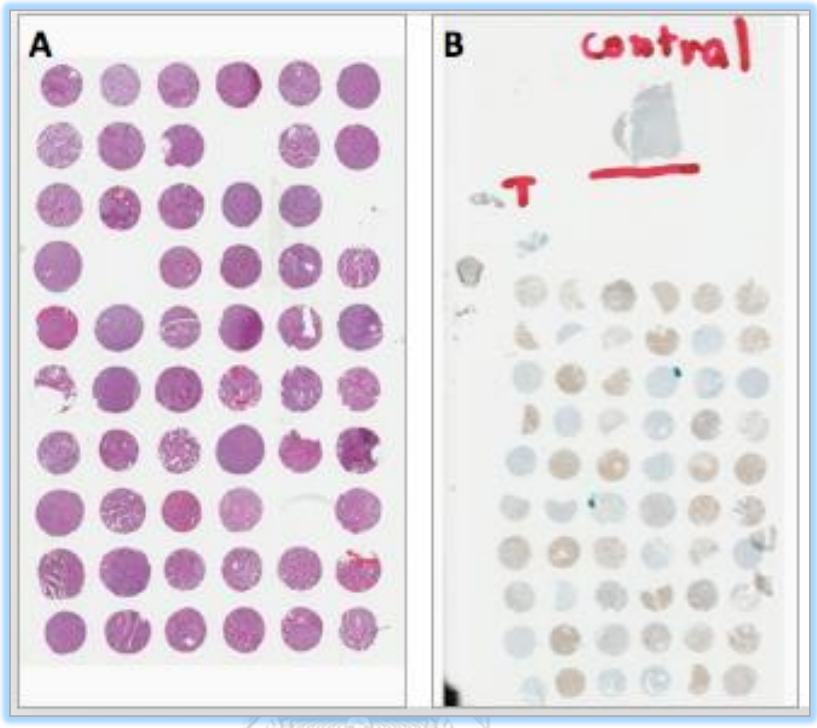
Pathological finding including histological variant, tumor size, multifocality, extrathyroidal extension, margins status, lymphovascular invasion, LN metastasis, extranodal extension, distant metastasis and staging were reevaluated to confirm the previous finding and were staged as per AJCC 2018.

2.5.3 Tissue microarray construction

TMA MASTER (3D HISTECH) tissue microarrayer was used for tissue microarray construction. All hemotoxylin and eosin (H&E) stained slides of PTC were reviewed by two pathologists (S.C and S.K) and the slide with representative tumor was selected from each case (S.C).

One area of tumor stroma interface from each cases were encircled on the conventional H&E slide and the area corresponding to the selected area on the FFPE block was marked with the felt marker. One core from each case was then cored out with 2-mm diameter needle and transferred to a recipient paraffin block for tissue microarray construction. A distance of 1-mm was kept between each cores (Figure 6).

Figure 6. TMA design of our study.



Hematoxylin & Eosin stained slide (A); VE1 IHC stained slide (B)

2.5.4 Immunohistochemistry

We performed immunohistochemistry for VE1 on 4 µm-thick tissue sections (TMA) using an automated Ventana BenchMark Ultra autostainer (Ventana Medical Systems, Tucson, AZ). Tissues sections was incubated with the anti-BRAF V600E (VE1) mouse monoclonal antibody (Ventana Medical Systems, catalog number 790-4855) for 32 min at 37°C.

Immunoreactivity of VE1 was visualized using an OptiView DAB IHC detection kit (Ventana Medical Systems) and then counterstained with Hematoxylin II

and Bluing Reagent for 8 min and 4 min respectively (see appendix for full protocol). We used human tonsil tissue as a negative control tissue for each staining run.

2.5.5 Evaluation of Immunoreactivity

All sections were examined by two pathologists (S.C and S.K). The immunoreactivity was assessed using H-scoring system and discrepancies were discussed till the consensus was reached. Slides were viewed under binocular microscope and evaluated intensity and proportions of positive cells in each case. H-score is a semi-quantitative system which includes both the proportion (0–100%) and intensity of positive cells (0, absent; 1+, weak; 2+, moderate; 3+, strong staining). The range of H-score was obtained by combining intensity and proportion scores and hence, final scores obtained were ranging from 0 to 300, as described previously [16,76].

Based on our previous study using similar method [16], where H-Score of ≥ 10 was considered positive for mutation, we found out that there was no significant association between cut off point of H-score against the *BRAF* mutation. Therefore, in the current study, positive H-score (any cytoplasmic positivity) was considered as indicative of *BRAF* on immunostaining.

2.5.6 Sanger sequencing on *BRAF* exon 15

Bidirectional Sanger sequencing of *BRAF* mutations was done for all cases from the pilot cohort at the outside facility (Department of Hospital Pathology, College of Medicine, The Catholic University, Seoul, Korea).

Total DNA was extracted from 10 μm thick paraffin-embedded whole tissue sections using RecoverAll™ Total Nucleic Acid Isolation Kit (Life Technologies, Carlsbad, CA, USA) as per the manufacturer's instruction. PCR reaction was performed using a primer pair (forward, 5′-TCATAATGCTTGCTCTGATAGGA-3′ and reverse, 5′-GGCCAAAAATTTAATCAGTGGA-3′).

Sanger sequencing was performed using the same primers and BigDye Terminator sequencing kit (Applied Biosystems, Carlsbad, CA, USA) on a 3730xl DNA analyzer (Applied Biosystems) as previously described [16].

2.5.7 Statistical analysis

For Pilot study, we assessed the *validity* **(**Sensitivity, Specificity, and Likelihood Ratio**)**, *efficacy* **(**Positive and Negative Predictive Values**)** and *reliability* **(**Percent agreement and Kappa Statistic**)** of VE1 IHC in detecting *BRAF*^{V600E} mutation by comparing with direct sequencing results **(**gold standard**).** The agreement between IHC and direct sequencing results were evaluated using Kappa coefficient (***K***).

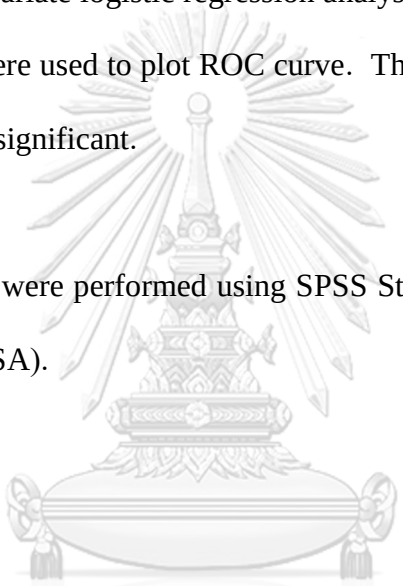
For Main cohort, Kolmogorov-Smirnov normality test was used to check if variables are normally distributed. The clinicopathological variables of the patients are presented as mean ± SD or number (%).

Categorical variables (gender, histological variants, multifocality, ETE, margin status, LVI, PNI, LN mets, distant mets, Hashimoto's thyroiditis, and tumor staging) are presented in percentage and continuous variables (age and tumor sizes) are presented in mean and median. Student's t-test (parametric test) was used to compare the mean and Mann-Whitney U-test (parametric test) was used to compare

the median of continuous variables). Pearson's chi-square-test and Fisher exact test were used to assess the differences between categorical variables as appropriate. Student *t*-test was used to compare the means of continuous variables.

Univariate and multivariate analysis were done by using logistic regression model. Variables that showed significant correlation on univariate analysis ($p < 0.25$) were included in multivariate logistic regression analysis. The variables significant on multivariate analysis were used to plot ROC curve. The *p* value of less than 0.05 was considered statistically significant.

All statistical analyses were performed using SPSS Statistical software, version 22.0 (IBM, Armonk, NY, USA).

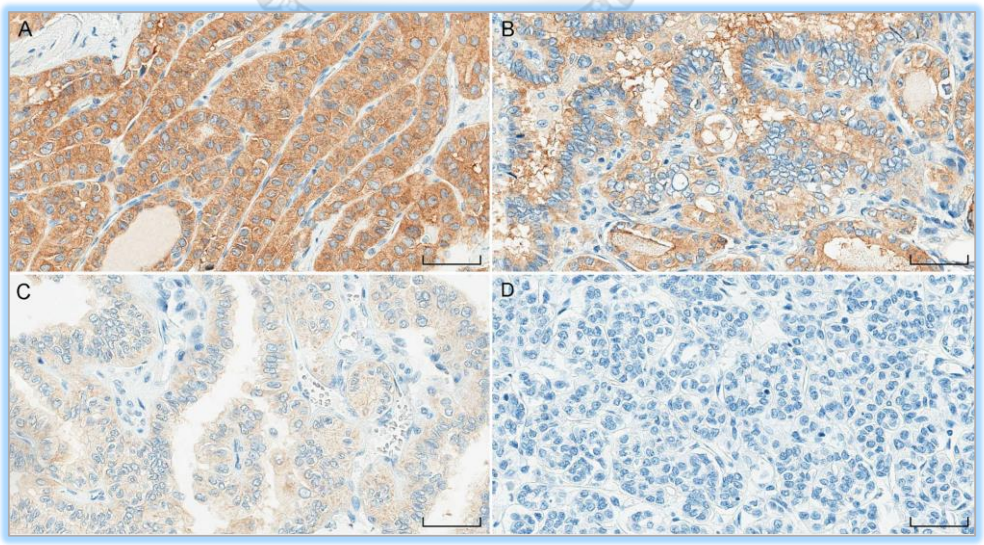


Chapter 3
Results

3.1 Analytical performance of VE1 in the pilot cohort

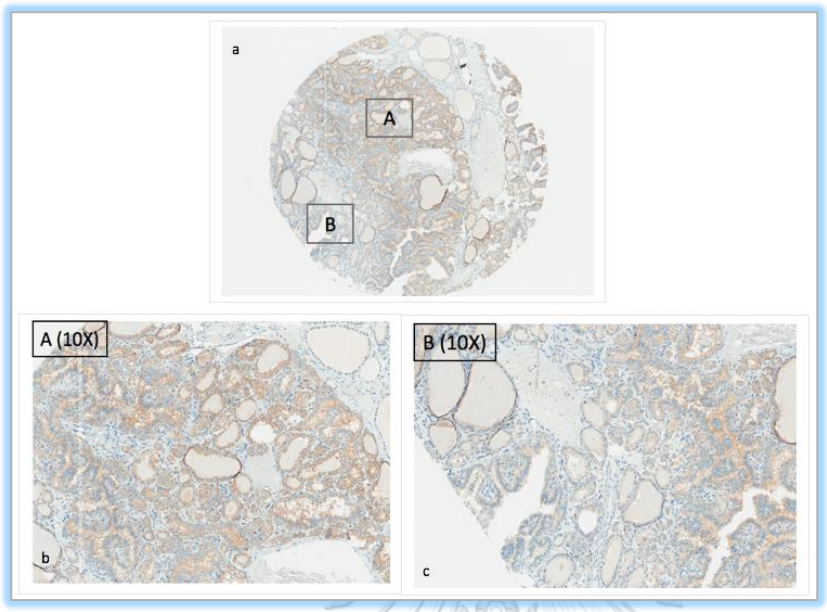
Based on sample size calculation, the minimum required sample size of our pilot cohort was 63 cases, which was further expanded to 100 PTCs (unselected continuous cohort 2009–2012) with an intention to increase the reliability of the pilot study. All PTC cases were initially assessed on hematoxylin & eosin staining to assure the presence of tumor. VE1 immunostaining of different intensities, composed of weak (1+), moderate (2+) and strong (3+) intensities were observed in the cytoplasm of the tumor cells (figure 7). Majority of the cases showed homogenous cytoplasmic staining. However, few cases showed heterogeneous distribution of stain with variable intensities and proportions (figure 8, 9). Hence, application of the H-scoring system was considered the optimal choice for evaluating staining in the pilot cohort.

Figure 7. Evaluation of VE1 immunostaining.



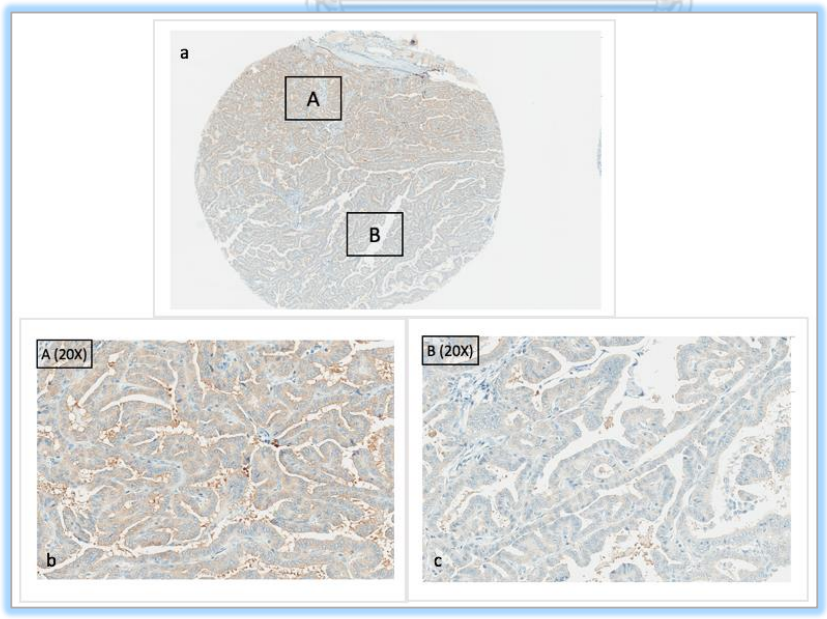
Positive IHC included strong (A), moderate (B) and weak (C) intensity in cytoplasm of tumor cells. Negative IHC had no cytoplasmic staining (D); X200

Figure 8. Case 1. Heterogeneous VE1 immunostaining on TMA.



In this core (a, x40), about 60% of tumor cells have moderate cytoplasmic staining (boxed area A) and remaining 40% have mild staining (boxed area B) intensity. Therefore, the H-score of this case is 2x60+1x40=160. High power view of boxed area (b, c), x100.

Figure 9. Case 2. Heterogeneous VE1 immunostaining on TMA.



In this core (a, x40), about 40% of tumor cells have moderate cytoplasmic staining (boxed area A) and remaining 60% have mild staining (boxed area B) intensity. Therefore, the H-score of this case is 2x40+1x60=140. High power view of boxed area (b, c), x200.

Of 100 PTC cases employed for pilot study, 69/100 (69%) were positive for VE1 expression. Direct sequencing of *BRAF* exon 15 detected $BRAF^{V600E}$ mutation in 68/100 (68%) cases and 32/100 (32%) cases were of wild type. There were 5 discordant cases. 2 cases were positive for mutation by direct sequencing but negative by VE1 (false negative), and 3 cases were negative for mutation by direct sequencing but VE1 positive on immunostaining (false positive). All of the discordant cases were of classic variant.

By considering the direct sequencing as the gold standard method, VE1 IHC showed sensitivity and specificity of 97.1% and 90.1% respectively. The receiver operating characteristic (ROC) curve demonstrated high validity of VE1 IHC in detecting $BRAF^{V600E}$ mutation in PTC specimens and found to be a comparable method with direct sequencing. This was corroborated with 93.1% area under the curve (Figure 10). The positive (9.80) and negative (0.03) likelihood ratios corresponded to the interpretation of VE1 IHC as “often useful” and “very useful”, respectively, test for detecting the mutation.

The positive and negative predictive values were 95.7 % and 93.5 % respectively. The VE1 IHC and direct sequencing results for detecting $BRAF^{V600E}$ mutation in PTC tissue showed almost perfect agreement (κ=0.884) with an overall percentage agreement of 95.0 %.

Table 4. Comparison between VE1 and direct sequencing

	DS -	DS +	
VE1 -	29	2	31
VE1+	3	66	69
Total	32	68	100

Table 5. A general interpretation guide for likelihood ratios.

+LR	**-LR**	**Shift in probability or usefulness**
> 10	<0.1	Large/very useful test
5-10	0.1-0.2	Moderate/often useful test
2-4.9	0.21-0.5	Small/sometimes useful test
1-1.9	0.51-1.0	Very small/rarely useful

(Source: http://members.nata,org/quizcenter/courses/ebb-level2/basic.cfm/)

Table 6. Kappa statistic.

	Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement Kappa	.871	.072	6.167	.0001
N of Valid Cases	81			

a. Not assuming the null hypothesis.

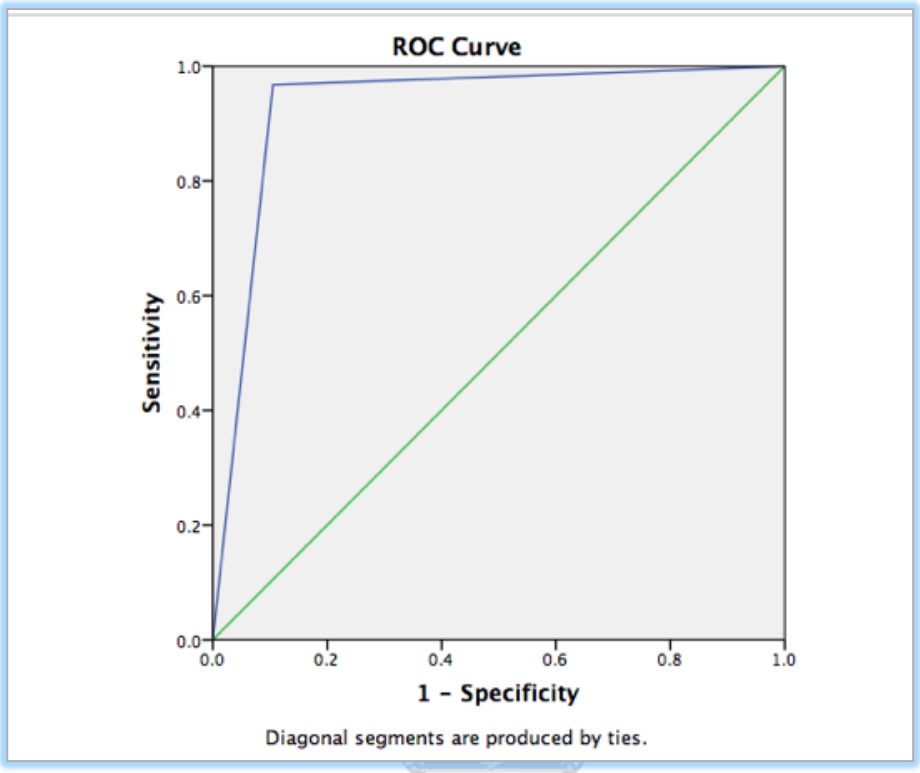
b. Using the asymptotic standard error assuming the null hypothesis.

Table 7. Kappa agreement interpretation.

<0	Less than chance agreement /no agreement
0.01-0.20	Slight agreement
0.21-0.40	Fair agreement
0.41-0.60	Moderate agreement
0.61-0.80	Substantial agreement
0.81-0.99	Almost perfect agreement

(Source: 1. Altman, Douglas G. 1999. Practical Statistics for Medical Research. Chapman; Hall/CRC Press; 2. McHugh, Mary. 2012. "Interrater Reliability: The Kappa Statistic." Biochemia Medica: Časopis Hrvatskoga Društva Medicinskih Biokemičara / HDMB 22 (October): 276-82.)

Figure 10. ROC curve for model (VE1 vs DS).



Area under curve = 0.931.

ROC curve of sensitivity and specificity of VE1 IHC in detecting BRAFV600E mutation with respect to gold standard direct sequencing method in PTC tissue specimen.

3.2 Prevalence of BRAF^{V600E} mutation in Thai PTC

We performed VE1 IHC to all of the subsequent PTC cases involved in our study as an alternative to direct sequencing. Since VE1 IHC showed excellent analytical performance in detecting *BRAF*^{V600E} mutation, any cytoplasmic immunoexpression (positive H-score) was further considered as equivalent to *BRAF*^{V600E} mutation. *BRAF*^{V600E} mutation was detected in 286/476 (60.9%), by VE1 IHC.

3.3 Clinical and pathological characteristics

The clinical and pathological characteristics of the PTC patients included in this study are summarized in table 8. Of the 476 included cases, 378/476 (79.4%)

cases were females and only 98/476 (20.6%) cases were males. The age ranged from 8 to 87 years with a mean age of 44.9 years. Surgical interventions included lobectomy, total thyroidectomy, completion thyroidectomy, subtotal thyroidectomy, and excision, which were performed in 132/476 (27.7%), 230/476 (48.3%), 7/476 (1.5%), 87/476 (18.3%) and 5/476 (1.1%) cases respectively.

Right lobe of the thyroid gland was the most common location of the tumor comprising 184/476 (38.7%) of the total cases, followed by left lobe comprising 134/476 (28.2%) of total cases. The tumor size ranged from 0.4 cm to 11 cm in the greatest dimension, with a mean size of 2.7 cm. Of the total cases involved, 25/476 (5.3%) had Hashimoto's thyroiditis as the background of thyroid cancer. Most of PTCs were of classic variant 369/476 (77.5%), followed by follicular variant 59/476 (12.4%), and tall cell variant 14 /476 (2.9%). Among the follicular variant of PTCs, 47(9.8%) and 12 (2.5%) cases were infiltrative follicular and invasive encapsulated follicular subtypes respectively.

The vast majority of cases (82.8%) belonged to the clinical stage I (AJCC 8th edition). Interestingly, this was largely contributed not by the tumor size (10.7% of microcarcinomas in the whole cohort) but rather by the age < 55 years (67% patients)

3.4 Correlation of *BRAF* mutation with clinicopathological variables

The correlation between clinicopathological variables and *BRAF*^{V600E} mutation in PTC are presented in table 8. *BRAF*^{V600E} mutation was mostly seen in older patients, tumors of larger size, and multifocality.

However, these variables showed no significant association with *BRAF* mutation. The study showed no significant difference between the *BRAF*^{V600E} rates in male and female patients. Microcarcinomas showed the rate of *BRAF*^{V600E} (57%) comparable to that in the whole PTC cohort.

Although the majority of the histological types involved in this study was of conventional variant, *BRAF*^{V600E} was frequently seen in tall cell variant (13/14, 92.9%), followed by classic variant (259/368, 70.2%). The influence of histological variants is well demonstrated by the higher rate of *BRAF*^{V600E} in the pilot series

compared to the whole cohort (69% vs. 60.9%), which was influenced by the higher prevalence of classic PTC in the former set.

Eight of 9 cases with perineural invasion were positive for *BRAF*^{V600E} mutation (88.9%). Only one case had distant metastasis (bone metastasis) at the time of diagnosis, which turned out to be positive for *BRAF*^{V600E} mutation in the primary tumor.

Table 8. Correlation of VE1 with clinicopathological variables.

Clinicopathological variables	PTC total	VE1+	VE1–	*P*
	467 (100%)	290 (60.9%)	186 (39.1%)	
Age at diagnosis (yr) mean ± SD	44.95 ± 16.06	47.22±15.83	47.22±15.84	0.314
< 55	319 (67%)	186 (58.3%)	133 (41.7%)	
≥ 55	157 (33%)	104 (66.2%)	53 (33.8%)	0.058
Sex				0.340
Male	98 (20.6%)	62 (63.3%)	36 (36.7%)	
Female	378 (79.4%)	228 (60.3%)	150 (39.7%)	
Tumor size (cm), mean ± SD	2.7±1.86	2.6±1.78	2.87±1.98	0.467
≤ 1 cm	51 (10.7%)	28 (54.9%)	23 (45.1%)	
> 1 cm	403 (84.7%)	250 (62%)	153 (38%)	0.379
cannot be determined	22(4.6%)	12 (54.5%)	10 (45.5%)	
Histological variants				**<0.0001**
1.Classic	369 (77.3%)	259 (70.2%)	110 (29.8%)	
2. Encapsulated	14 (2.9%)	4 (28.6%)	10 (71.4%)	
3. Follicular	59 (12.4%)	10 (16.9%)	49 (83.1%)	
- Infiltrative	47 (9.8%)	9 (19.1%)	38 (80.9%)	
- Invasive encapsulated	12 (2.5%)	1 (8.3%)	11 (91.7%)	
4. Diffuse sclerosing	5 (1.1%)	2 (40%)	3 (60%)	
5. Tall cell	14 (2.9%)	13 (92.9%)	1 (7.1%)	
6. Others †	15 (3.1%)	2 (13.3%)	13 (86.7%)	
Multifocality				0.184
Absent	346 (72.7%)	204 (59%)	142 (41%)	
Present	99 (20.8%)	64 (64.6%)	35 (35.4%)	
Cannot be determined	31 (6.5%)	22 (71%)	9 (29%)	
Margin				**0.022**
Negative	358 (75.2%)	209 (58.4%)	149 (41.6%)	
Positive	106 (22.3%)	74 (69.8%)	32 (30.2%)	
Cannot be determined	12 (2.5%)	7 (58.3%)	5 (41.7%)	
Lymphovascular invasion				0.198
Absent	411 (86.3%)	254 (61.8%)	157 (38.2%)	
Present	65 (13.7%)	36 (55.4%)	29 (44.6%)	
Extrathyroidal extension				**<0.0001**
Absent	292 (61.3%)	152 (52.1%)	140 (47.9%)	
Present	184 (38.7%)	138 (75%)	46 (25%)	
Hashimoto's thyroiditis				**0.009**
Absent	451 (94.7%)	281 (62.3%)	170 (37.7%)	
Present	25 (5.3%)	9 (36%)	16 (64%)	
Lymph node metastasis				0.455

Absent	37 (7.8%)	24 (64.9%)	13 (35.1%)	
Present	137 (28.8%)	85 (62%)	52 (38%)	
Nx	302 (63.4%)	181 (59.9%)	121 (40.1%)	
Distant metastasis				n/a
Absent	475 (99.8%)	289 (60.8%)	186 (39.2%)	
Present	1 (0.2%)	1 (100%)	0 (0%)	
Staging (AJCC 8th edition)				n/a
Stage I+II	461 (96.8%)	279 (60.5%)	182 (39.5%)	
Stage III+IV	3 (0.6%)	3 (100%)	0 (0%)	
Cannot be determined	12(2.5%)	8 (66.7%)	4 (33.3%)	

† includes columnar cell variant, cribriform-morular variant, solid variant, oncocytic variant, and Warthin-likr variant; IFV, infiltrative follicular variant; EFV, encapsulated follicular variant; AJCC, American Joint Committee on Cancer; SD, standard deviation; n/a, not applicable

Univariate and multivariate analysis are summarized in Table 9. On univariate analysis, *BRAF*^{V600E} was significantly associated with margin positivity (P = 0.022), extrathyroidal extension (P <0.0001), classic variant (P < 0.001), and absence of Hashimoto's thyroiditis (P = 0.009).

Variables showing a tendency of association with VE1 (P < 0.25) in the univariate analysis were included in the multivariate logistic regression model. Bivariate analysis showed association of *BRAF*^{V600E} mutation with older age of patients, absence of Hashimoto's thyroiditis, perineural invasion, extrathyroidal extension, margin positivity, classic variant and tall cell variants of PTC. Multivariate analysis showed association of *BRAF*^{V600E} mutation with classic and tall cell variants of PTC, extrathyroidal extension, and absence of Hashimoto's thyroiditis (Table 2).

The ROC curve analysis further demonstrated that the combined pathological variables listed above have a fair chance of predicting the presence of *BRAF*^{V600E} mutation in our series, which was corroborated by 75.03% area under the curve (Figure 11).

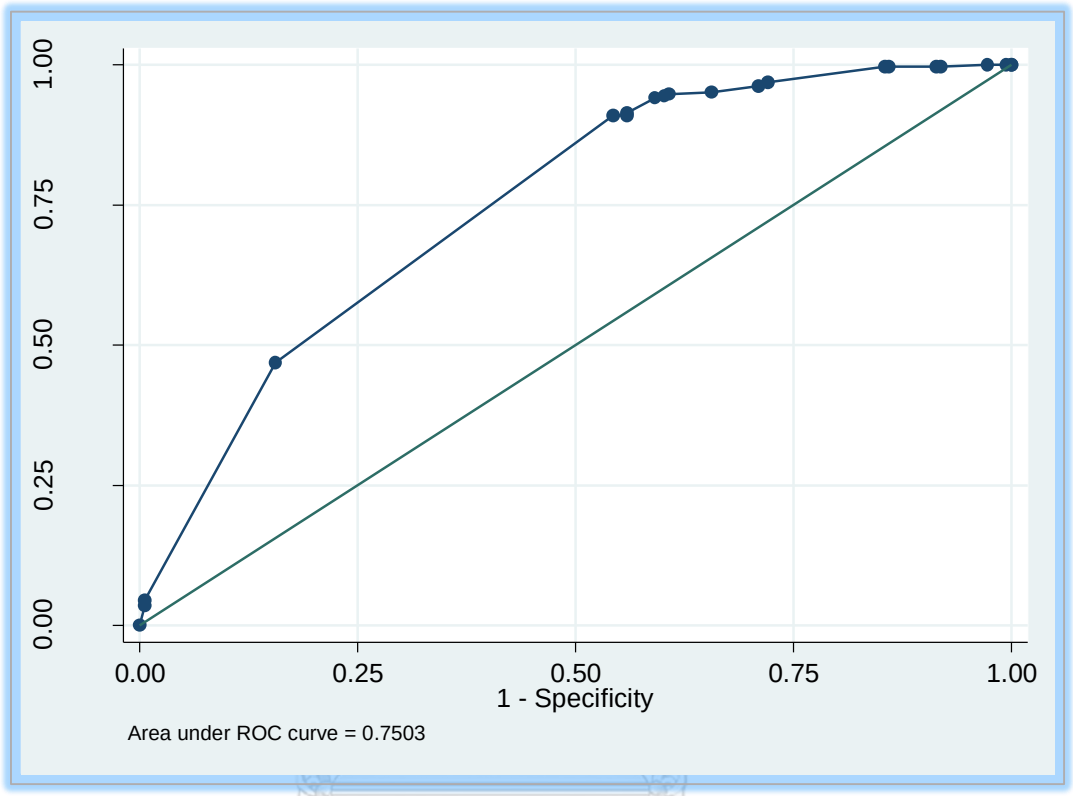
Individual variable of significance in multivariate analysis, however was not useful in predicting the mutation status on PTC, as corroborated by 10.6%, 52.4%, 43.6%, 41.0%, and 40.8% area under curve for older age (>=55), Hashimoto's thyroiditis, Histological variants, , margin status and extrathyroidal extension respectively (Figure 12).

Table 9. *Bivariate and multivariate logistic regression analysis for clinical significance of VE1 expression*

Variable	Bivariate analysis Crude OR (*P* value)	Multivariate analysis Adjusted OR (*P* value)
Age		
< 55	Reference	
≥ 55	1.403 (**0.096**)	1.147 (0.564)
Sex		
Female	Reference	
Male	1.133 (0.594)	
Histological variant		
1. Classic	15.305 (**< 0.001**)	27.631 (**0.002**)
2. Encapsulated	2.600 (0.321)	6.103 (0.134)
3. Follicular	1.327 (0.735)	2.672 (0.372)
4. Diffuse sclerosing	4.333 (**0.217**)	9.964 (0.100)
5. Tall cell	84.50 (**0.001**)	107.675 (**0.002**)
6. Other†	Reference	
Margin		
Negative	Reference	
Positive	1.649 (**0.035**)	0.970 (0.915)
Extrathyroidal extension		
Absent	Reference	
Present	2.763 (**< 0.001**)	2.026 (**0.004**)
Hashimoto's thyroiditis		
Absent	Reference	
Present	0.340 (**0.012**)	0.390 (**0.045**)

† includes columnar cell variant, cribriform-morular variant, solid variant, oncocytic variant, and Warthin-like variant

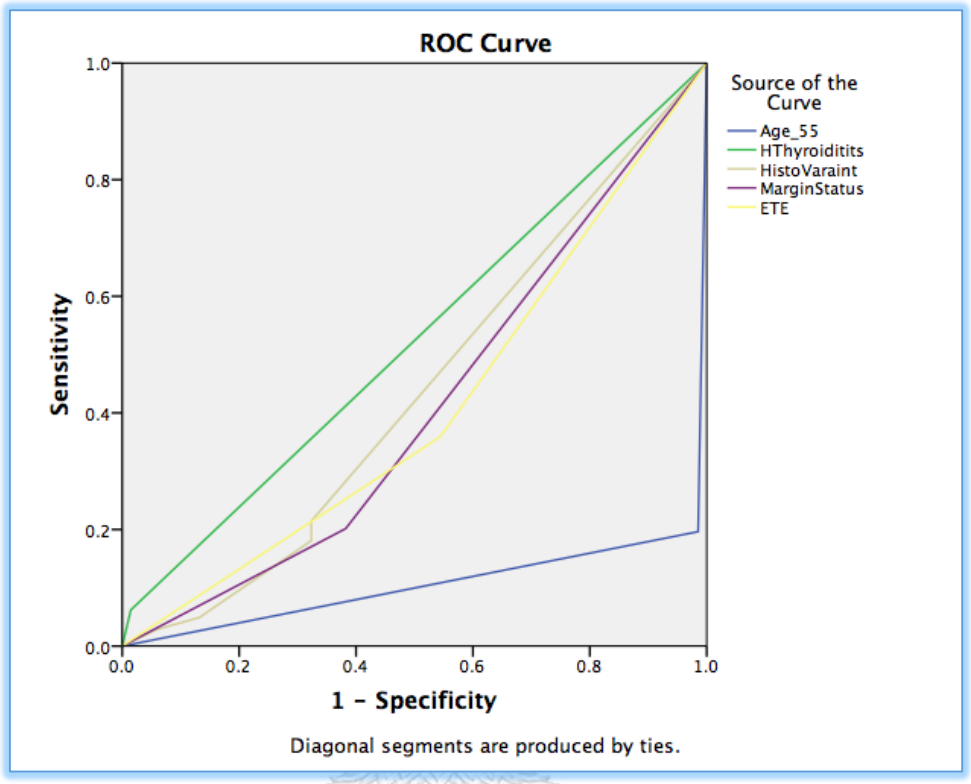
Figure 11. ROC curve of significant clinicopathological variables in multivariate analysis



Area under the curve=0.7503

ROC curve of sensitivity and specificity of clinicopathological variables in predicting *BRAF*^{V600E} mutation. ROC was calculated using clinicopathological variables which were statistically significant in multivariate analysis i.e. age>=55, histological variants, extrathyroidal extension, margin positivity and Hashimotos thyroiditis; ***P*** *value* < 0.25; Image was obtained from software STATA version 16).

Figure 12 ROC curve of significant individual variables (in multivariate analysis) in predicting the presence of BRAF^{V600E} mutation in PTC



Area under curve: Older age (≥55) =10.6%, Hashimoto's thyroiditis =52.4%, Histological varaints 43.6%, margin status = 41.0%, and extrathyroidal extension.

Chapter 4
Discussion

The *BRAF*^{V600E} mutation has been reported to be prevalent in PTC and is associated with adverse prognostic factors in this tumor [4,6,10]. It can also act as a diagnostic marker for PTC among various types of thyroid cancer [14]. In this series, we report our institutional experience on the detection of *BRAF*^{V600E} mutation on TMA of PTC specimens by using VE1 IHC and also the association of *BRAF*^{V600E} mutation with clinicopathological variables.

To validate the performance of VE1 IHC before using it for detection of *BRAF*^{V600E} mutation in this study, we initially selected 100 PTC cases to compare IHC results with the direct sequencing study. As compared to direct sequencing, VE1 IHC showed a sensitivity of 97.1% and the specificity of 90.1%, which is concordant with previous studies [18-24]. There were 5 discordant cases in our pilot cohort, which included 2 cases negative for *BRAF*^{V600E} by VE1 IHC but positive by direct sequencing (false negative result) and 3 cases positive for *BRAF*^{V600E} by VE1 IHC but was negative by direct sequencing (false positive result). We repeated IHC on the whole tissue section of all these discordant cases but the results were persistently the same as the initial result.

Although direct sequencing has been widely regarded as the gold standard method for detection of point mutation [16,55], it has been reported to have relatively lower sensitivity, requiring higher percentage of tumor cells within the samples [55] and produced more false negative results when it is used as a solo validation method [16]. In our case, the tumor size of one of the initial false positive cases was 0.4 cm

and moreover, we couldn't adopt additional molecular workup for our discordant cases since resolving discordant cases was not our objective.

We are of the opinion that our initial VE1 false positive result could be a rather false negative result of direct sequencing due to its lower sensitivity. However, we believe that employing cases with adequate tumor size and using a combination of molecular methods for validation might produce lower discordant results.

The possible explanation for VE1 IHC false negative in our study could be due to the loss of mutation antigen. It has been reported that the long-term storage of tissue sections suffers from loss of antigenicity [77,78]. The FFPE PTC tissues used for our pilot cohort were stored for more than 5 years in our archive. We believe that usage of newer sample for the study would produce less false negative rate. Another cause of false negative is heterogeneity of tumor lesion [79,80]. This may explain why VE1 immunoreactivity is not detected in some TMA tissue samples [79,81].

Nevertheless, VE1 IHC in our study produced excellent analytical performance with overall percentage agreement of 95.0% in detecting *BRAF*^{V600E} mutation in PTC as compared to direct sequencing. Knowing the status of *BRAF* mutation in PTC patients would be beneficial in order to develop therapeutic target and to predict the outcome in response to targeted therapy. The Vemurafenib is a highly selective and potent inhibitor of *BRAF*^{V600E}, and it has been approved by the U.S Food and Drug Administration for treatment of *BRAF*-mutated melanoma [82]. Vemurafenib was also found to be potentially effective and well-tolerated treatment strategy in patients with advanced PTC harboring *BRAF*^{V600E} mutation [83]. However, clinical trials are still going on regarding the usage of target therapy on PTC harboring *BRAF*^{V600E} mutation. In this study, VE1 IHC showed excellent analytic performance.

Therefore, we propose that IHC may be used as a screening strategy for *BRAF*^{V600E} mutation in patients with PTC. Similar to the study done by Farzin et al, on usage of IHC as a first screening strategy for targeted therapies of lung cancer [84], VE1 IHC may be used to triage patients with PTC for further molecular studies before offering targeted therapies in the near future.

We performed clinicopathological correlations with *BRAF*^{V600E} mutation (VE1+) and found that *BRAF*^{V600E} was associated with classic and tall cell variants of PTC, margin positivity (on univariate and bivariate analysis), presence of extrathyroidal extension, and absence of Hashimoto's thyroiditis. Several studies have shown the role of *BRAF*^{V600E} in tumor aggressiveness and inferior clinical outcome in PTC patients [10,55]. Our findings were in accordance with previous publications, which defined extrathyroidal extension, tall cell morphology, and positive surgical margin as predictors of aggressive behavior of PTC, while the association of Hashimoto's thyroiditis with outcome of differentiated thyroid cancer in currently debated [85].

BRAF^{V600E} mutation is frequently seen in tall cell (60-95%) [13,40] and conventional (45-80%) variants of PTC [13,34]. In our study, 369/476 (77.3%) PTC cases were of conventional variant, of which 259/476 (70.2%) were *BRAF*^{V600E} mutant. We believe that the association of *BRAF* mutation with classic variant of PTC seen in our study might be due to a rather high prevalence of this variant involved in the study. *BRAF* mutation has been recently introduced to the risk stratification chart of patients with differentiated thyroid cancer as a factor conveying a higher chance of tumor recurrence [85,86]. Since our access to clinical data was limited only to the

records provided in the laboratory information system, we could not evaluate further correlation with such important endpoints of PTC outcome as recurrence and mortality in this series. Additional studies are warranted to prove association of *BRAF*^{V600E} mutation with outcome of thyroid cancer in Thai patients.

This is the first large-scale study on *BRAF* rate in PTC from Thailand, and we believe that our series collected at the major tertiary referral cancer center could be representative of the whole country. A similar approach has been successfully employed recently to establish a baseline rate of human papillomavirus in head-neck cancer [87].

Our study reports the rate of *BRAF*^{V600E} mutation in Thai PTC as 60.9%. This is relatively lower than *BRAF*^{V600E} prevalence in PTC reported by the close neighbors like Vietnam (83%) and the Philippines (70.6%); however, number of the cases enrolled in these studies was as low as 53 and 17, respectively – this is definitely not sufficient to draw meaningful conclusions about the nationwide rate [88,89]. Reports from other Asian countries, such as Japan, and South Korea are much more abundant and consistently showed the high prevalence of *BRAF*^{V600E} mutation (> 70%) in PTC [12,36]. The striking differences in the prevalence rates may be attributed by geography (pollutants, iodine intake), ethnicity (genetics and habits) and other factors (histological variants involved in the study, Hashimoto's thyroiditis) [12].

It is not surprising to see such a scarce amount of data from the Southeast Asian countries, because most of them cope with limited resources. One of the major purposes of our project was to develop a low-cost testing alternative to estimate the prevalence of *BRAF*^{V600E} in large cohort studies. IHC to substitute genotyping was one way to reduce costs. Importantly, this step required initial validation with a

reference molecular test in a well-powered pilot series.

Another approach to significantly minimize expenses was a using of small-sized specimens instead of whole-tissue sections [25,90]. Finally, by combining VE1 IHC and TMA, we could afford performing a large cohort study in limited resource settings. We believe that our approach our approach can serve as a model for other institutions.

Chapter 5
Conclusion

BRAF^{V00E} mutation was detected in 60.9% of Thai PTC and it was associated with several aggressive clinicopathological variables of thyroid cancer.

VE1 IHC is a reliable method and may serve as alternative to direct sequencing for the detection of mutation within resource-limited and healthcare-cost-containment environments.

A combination of mutation-specific IHC and TMA allows conducting large cohort studies more labor-saving and cost-efficiently.

Appendix

1. H&E staining protocol

- Xylene 1 3.30 min
- Xylene 2 3.30 min
- Absolute alcohol 2 min
- 95% alcohol 2 min
- 95% alcohol 2 min
- tap water 1 min
- Hematoxylin 5 min
- Deionized water 1 min
- Bluing buffer 1 min
- Tap water 3 min
- 95% alcohol 1 min
- Eosin 5 min
- 95% alcohol 1 min
- Absolute 1 min
- Absolute 1 min
- Xylene 1 min

Reagents:

Dako Hematoxylin (ready-to-use)

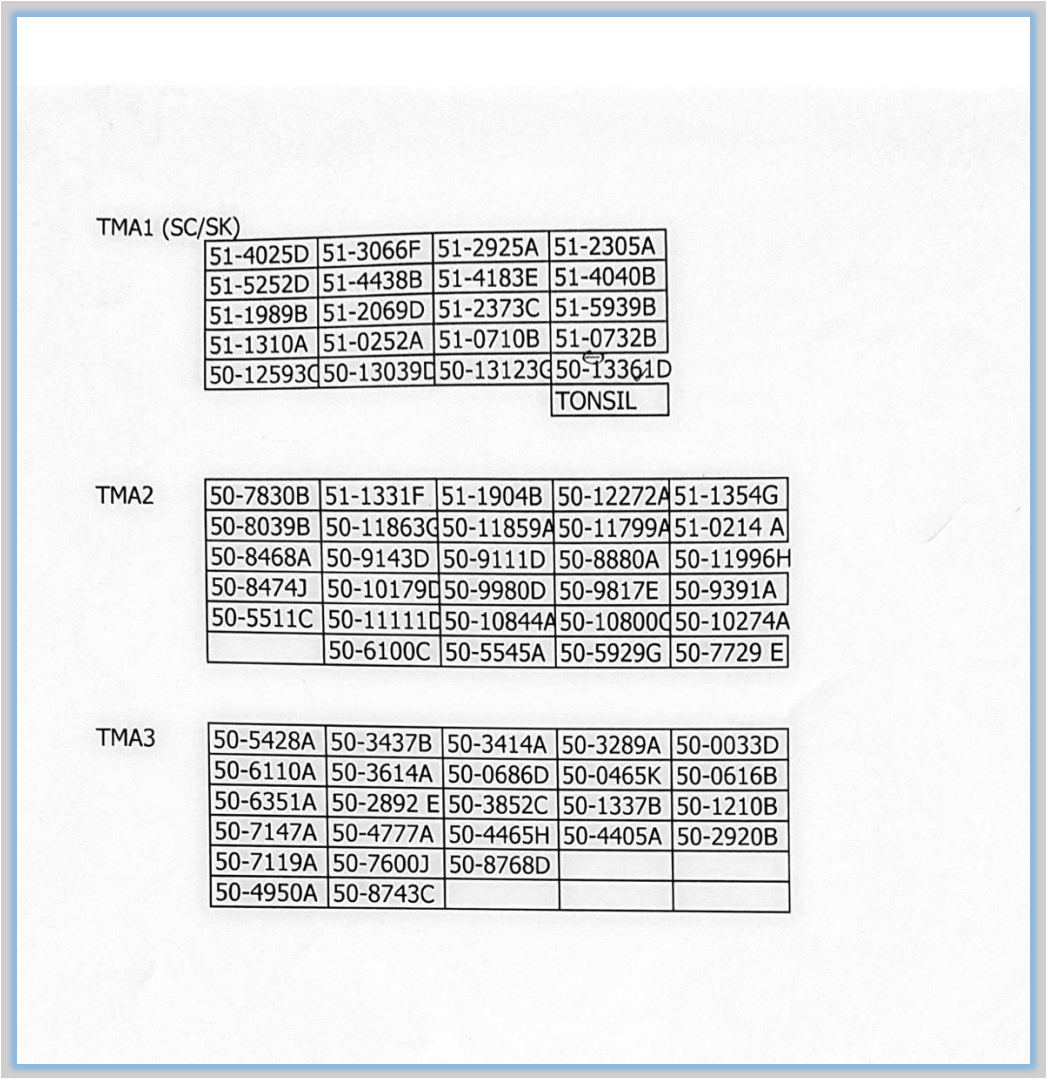
Dako Eosin (ready-to-use)

Dako Bluing Buffer (ready-to-use)

2. Protocol steps for BRAFV600E (VE1) staining

- Paraffin
- Baking (at 75° C) : 4 minutes
- Deparaffinization
- Cell conditioning
    - CC1 8 min
    - CC1 16 min
    - CC1 24 min
    - CC1 32 min
    - CC1 40 min
    - CC1 48 min
    - CC1 56 min
    - CC1 64 min
- Pre Primary Peroxidase inhibit
- Primary antibody
    - Incubation temperature: 37° C
- Antibody titration
    - Medium incubation time: 32 min
- OptiView HQ Linker
- OptiView HQ Universal Linker
    - Incubation time: 8 min
- OptiView HRP multimer
    - Incubation time: 12 min
- Counterstain
    - Hematoxylin II (2208)
- Post counterstain
    - Bluing reagent (2037)

3. TMA mapping sheet



TMA1 (SC/SK)

51-4025D	51-3066F	51-2925A	51-2305A
51-5252D	51-4438B	51-4183E	51-4040B
51-1989B	51-2069D	51-2373C	51-5939B
51-1310A	51-0252A	51-0710B	51-0732B
50-12593C	50-13039D	50-13123C	50-13361D
			TONSIL

TMA2

50-7830B	51-1331F	51-1904B	50-12272A	51-1354G
50-8039B	50-11863C	50-11859A	50-11799A	51-0214 A
50-8468A	50-9143D	50-9111D	50-8880A	50-11996H
50-8474J	50-10179D	50-9980D	50-9817E	50-9391A
50-5511C	50-11111D	50-10844A	50-10800C	50-10274A
	50-6100C	50-5545A	50-5929G	50-7729 E

TMA3

50-5428A	50-3437B	50-3414A	50-3289A	50-0033D
50-6110A	50-3614A	50-0686D	50-0465K	50-0616B
50-6351A	50-2892 E	50-3852C	50-1337B	50-1210B
50-7147A	50-4777A	50-4465H	50-4405A	50-2920B
50-7119A	50-7600J	50-8768D		
50-4950A	50-8743C			

TMA4

51-7389 A	51-7931 F	51-8122 A	51-8127 C	51-8439 B	51-8659 C
52-9235 A	51-9258 D	51-9401 B	51-9745 A	51-9951 B	51-10094 E
51-10250A	51-10425 A	51-10927A	51-11025 K	51-11351 A	51-11574 A
51-11839B	51-12058 C	51-12151A	51-12256 A	51-12436 F	51-12823 D
51-13542B	51-14056 C	52-68 A	52-147 A	52-691 A	52-852 B
52-975 C	52-1211 G	52-1569J	52-1727 K	52-1849 B	52-5055
52-3873 B	52-4238 B	52-4165H	52-4165 H	52-4705 A	52-4992 C
52-6557C	52-524 C	52-2196D	52-6566 F	52-6157 D	52-6809 G
52-6834 B	52-7608 C	52-7610B	52-5329 C	51-10276 G	

TMA5

52-8540 B	52-14290 X	52-11325 A	52-12074 C	52-12469 A	52-8119 A3
52-12788 C	52-13058 C	52-13064 J	52-13233 C	52-13400 A	52-13556 A
52-13494 B	52-13624 A	52-13642 I	52-13772 B	52-10070 K	52-14542 C
52-14733 C	52-15036 C	52-15538 A	53-336 A	53-430 E	53-507 A
53-1261 E	53-1825 A	53-1941 B	53-1372 A	53-3251 C	53-3612 C
53-3649 B	53-3650 B	53-4445 E	53-4508 C	53-4874 B	53-4875 D
53-4891 M	53-6817 E	53-5401 B	53-6783 C	53-7189 A	53-7977 G
53-7988 C	53-9287 F	53-8273 A	53-9422 I	53-9580 H	53-10732 A
53-10733 E	53-10788 G	53-10916 A	53-11720 A	53-12447 B	53-13027 B
53-13088 B	53-13270 F	53-14517 A	53-12717B	53-15668 I	53-16630 A

TMA6

50-464A	50-2892A	50-8039E	51-214B
51-6101B	51-6474B	51-6747A	51-6892B
51-6649A	51-7931B	51-8659A	51-9235E
51-9745B	51-10094C	51-10250C	51-14056D
52-1849G	52-5329A	52-8119 A1	52-11325D
52-13058A	52-13494A	52-13642A	52-13772A
52-14290W	52-15036D	53-3172C	53-3251D
53-4508D	53-10916B	53-14517B	51-7389G
TONSIL			

TMA7

54-87C	54-312G	54-1218G	54-1223J	54-1266A	54-1416N
54-1551E	54-1602C	54-2519A	54-2520A	54-3129B	54-3289C
54-3380C	54-3587A	54-3628B	54-3778A	54-4085C	54-4248 I
54-4622A	54-5062K	54-5201B	54-5727B	54-5831D	54-6193M
54-6288A	54-8650F	54-7244B	54-9109A	54-9311B	54-9903A
54-10089G	54-10197F	54-10515A	54-11048L	54-11454A	54-11949C
54-11950C	54-12073M	54-12221G	54-12340B	54-12683E	54-13222D
54-13953A	54-14029A	54-14963A	54-16252A	54-16576B	54-16612 I
54-17675B	54-17677 I	54-18561D	54-18829B	54-19271C	55-805E
55-1665D	55-2014E	55-2120F	55-2527C	55-2528B	54-5038 I

TMA8

55-4109A	55-4178C	55-4207A	55-4540A3	55-4940C
55-5031A	55-5082F	55-5509B	55-5524C	55-5626A
55-6008A	55-6734A	55-6799A	55-7005C	55-7959A
55-8315E	55-8609A	55-9301C	55-9322B	55-9736B
55-9870B	55-10081B	55-10112A	55-10251C	55-1109E
55-11382A	55-12124A	55-12503C	55-12903L	55-14109F
55-14113A	55-14249K	55-15838A	55-16221E	55-16370A
55-17821B	55-17846A	55-19044F	51-07931G	51-14709B
52-1727B	52-5248B	52-13642J	53-3172D	53-3251E
53-18035H	54-312B	54-1223F	54-4085D	54-12340A
54-14963B				TONSIL

TMA9

54-4085D	54-12340A	55-16370A	55-12903L	55-5524C	55-4540A3
56-00223C	56-00282D	56-01149A	56-01222B	56-02417B	56-02625D
56-02845G	56-04555C	56-05379B	56-05499A	56-05517C	56-06256A
56-06432F	56-08244A	56-08929A	56-09104A	56-10264D	56-10367C
56-10843A	56-10872A	56-12296C	56-12532A	56-12769I	56-13256F
56-13261D	56-14334A	56-14489A	56-14898K	56-15603G	56-15638H
56-15764E	56-16492D	56-16909B	56-17234A	56-17513C	56-17708C
56-17935A	56-18235E	56-18497A	56-19176B	56-19602D	56-21349A
56-21401C	56-21906A	57-00093B	57-00184A1	57-00590C	57-00605B
57-01333E	57-01733C	57-01941J	57-02343C	57-2452A	57-3043A
					TONSIL

TMA10

55-4540A3	57-1333E	57-1733C	57-1941J	57-2343C	57-2452A
57-3043A	57-3285C	57-3377A	57-3954G	57-5204B	57-5269A
57-5677D	57-6389A	57-6546A	57-7341B2	57-9286C	57-9563N
57-9759C	57-12417AI	57-12878A	57-13418J	57-13683C	57-14416C
57-15035G	57-15065B	57-15167E	57-15633F	57-16565G	57-17337B
57-17477B	57-18050I	57-18308D	5718330D	57-18802C	57-19019A
57-21430A	57-21489A	57-21716B	57-21928C	57-21929C	57-22012
57-22365A	59-579A	59-2888C	59-300023H	59-300152P	59-300179B
59-300781B	59-300814B	59-301340A	59-301398E	59-301966A	59-302793B
59-303948H	59-304507C	59-305352F	59-306742C	59-307256C	59-307470C
					TONSIL

TA11

59-308461B	59-308596B	59-308951D	59-310168C	59-310262F	59-310766 A1
59-311072B	59-311073C	59-311459D	59-311668D	59-311753A	59-312324H
59-312906K	60-0075 A1	60-00234A	60-0727A	60-1164B	60-1169G
60-1935B	60-2139D	60-2289F	60-2852A	60-2958C	60-3492A
60-3510A	60-3874C	60-3993A	60-4023A	60-4053K	60-4296E
60-4514A	60-4779A	60-5745A	60-5810L	60-6208A	60-6325F
60-6376C	60-6411C	60-6515B	60-6650E	60-6835AA	60-7195D
60-7306A	60-7585D	60-8371D	60-8377G	60-8503A	60-8655B
60-9020B	60-9111E	60-9261B	60-9294A	60-9758F	60-9801C
60-10093F	60-10283U	60-10337B	60-10572B	60-10761G	60-11939B
					TONSIL

TMA12

58-00162F	58-00163H	58-01001C	58-01468F	58-01641A	58-03335L
58-03468H	58-03772B	58-03962Q	58-04336K	58-04633B	58-04947B
58-05004B	58-05409C	58-06790F	58-08697C	58-09295A	58-09444D
58-10076B	58-11439F	58-11986B	58-12467F	58-12969E	58-13276C
58-18374A	58-18683A	58-20315L	58-20544A	58-21099H	58-21218A
58-21464D	58-21809E	58-22716B	60-2139B	60-12381A	60-12642A
60-13090A	60-13872D	60-14464B	60-14534T	60-14593A	60-14720G
60-14764A	60-15344A	60-15657C	60-15742D	60-15805B	60-15656E
60-16188H	60-16257I	60-16512A	60-16605A	60-16918E	60-17364A
60-18004A	60-18115A	60-18350L	60-18530E	60-18765A	60-19134B
					TONSIL

TMA13

60-19166G	60-19272B	60-19372F
60-19453A	60-19458B	60-19876G
60-20219A	60-20865G	60-21000A
60-21334A	60-21355I	TONSIL

TMA14

58-3772B	58-11986B
58-12467F	58-20315L
58-21218A	58-22716B
	tonsil

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