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BRAF V600E Immunohistochemistry Predicts Prognosis of Patients with Cutaneous Melanoma in Thai population

Jiraroch Meevassana, MD*†
Kullawit Anothaisatapon, MD‡
Surawish Subbalekha, MD*
Supitcha Kamolratanakul, MD§
Tippawan Siritientong, RPh, PhD*¶
Komkrit Ruangritchankul, MD‖
Pornthep Pungrasami, MD*‡
Kevin J Hamill, PhD**
Apichai Angsapatt, MD*‡
Nakarin Kitkumthorn, DDS, PhD††

Background: The BRAF V600E mutation in the Thai population has been identified in a considerable percentage of people with cutaneous melanoma. The objectives of this study were to determine the prevalence of this mutation in cutaneous melanomas, conduct a clinicopathological association analysis with the BRAF V600E mutation, and develop a treatment strategy for patients with this mutation that would take advantage of the medications currently available to treat them.

Methods: Anti-BRAF V600E (clone VE1) immunohistochemistry was performed on 50 pathological samples of cutaneous melanoma after excluding the samples with a low amount of pathologic tissue, a lack of clinical data, and poor follow-up. BRAF V600E expression DNA sequencing was performed to confirm the results of several cases.

Results: Anti-BRAF V600E antibody positivity was noted in 56% (28/50) of cutaneous melanoma cases. DNA sequencing results were consistent with immunohistochemistry results. In cutaneous melanoma, the BRAF V600E mutation was significantly associated with adverse prognosis of patients, including reduced overall survival and disease-free survival.

Conclusions: An increased prevalence of the BRAF V600E mutation was determined in a collection of cutaneous melanomas in the Thai population, implying that BRAF-targeted therapy may be a promising strategy for patients with BRAF-mutated cutaneous melanoma. This study revealed an association between the clinicopathological aspects of cutaneous melanoma and overall survival, disease-free survival, and overall mortality. A treatment with anti-BRAF-targeted therapy, which incorporates the already available medications, is being researched and developed.
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*From the *Center of Excellence in Burn and Wound care, Faculty of Medicine, Chulalongkorn, Bangkok, Thailand; †Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; ‡Division of Plastic and Reconstructive Surgery, Department of Surgery, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; §Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; ¶Department of Food and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; ‖Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; **Institute of Life Course and Medical Sciences, Faculty of Health and Life Sciences, University of Liverpool, Liverpool, UK; and ††Department of Oral Biology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand.*

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INTRODUCTION

Cutaneous melanoma is one of the most invasive skin cancers and has a crucial impact on the mortality of patients. It accounts for 0.6% of deaths owing to cancers of all sites and 65% of deaths owing to all skin cancers, and its prevalence is expected to increase steadily.[1,2] Furthermore, according to the 2020 statistics, the number of new cutaneous melanoma cases in the United States was 100,350 with 6,850 deaths, and the proportion is expected to further increase.[2–4]

Cutaneous melanoma is caused by abnormal melanocyte division in the epidermal basal layer.[5,6] Melanocytes produce melanin, a substance that absorbs UV radiation

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that is harmful to skin cells.[7,8] UV radiation damages DNA strands in skin cells.[9,10] The high rate of genetic mutations in cutaneous melanoma has been studied.[11,12]

BRAF is classified as a cancer-causing gene or proto-oncogene.[13,14] It has been discovered that several cancers frequently harbor the BRAF mutation, which results from glutamic acid replacement in valine at codon 600 (V600E mutation is found in up to 90% of all BRAF mutations).[13–16] BRAF mutation triggers the activity of mitogen-activated protein kinase signaling (MAPK), resulting in uncontrollable cell growth and proliferation.[17,18] Mutations in the BRAF gene are reported to be a relevant cause of cutaneous melanoma. Overall, the prevalence rates vary in different countries and ethnicities. BRAF mutation is noted in up to 40% of patients with cutaneous melanoma (54.8% of Caucasian patients and 38.2% of Asian patients).[19] The nonchronic sun damage group has the highest percentage (56%) of this mutation, whereas the chronic sun damage group has 6%.[13] The discovery of specific oncogenic aberrations guided the development of molecular targeted treatment for melanoma, which included two types of drugs: those specific for the BRAF mutation (vemurafenib and dabrafenib) and small-molecule MAPK 1 and 2 (MEK1 and MEK2) inhibitors (trametinib).[20–22]

Cancer treatment with targeted therapy drugs is becoming more common, as are studies of mutations in cutaneous melanoma. The use of BRAF V600E mutation as a predictive and prognostic biomarker for cutaneous melanoma has become widespread. However, there is limited data on the prevalence of BRAF V600E mutation in the Thai population. This sparked an exploration of the prevalence of BRAF V600E in cutaneous melanoma and its prognosis. Thus, this study aimed to assess the rate of BRAF V600E mutation in cutaneous melanomas and perform a clinicopathological correlation analysis.

Takeaways

Question: How much is the prevalence of BRAF V600E in cutaneous melanoma and its prognosis?

Findings: Anti-BRAF V600E antibody positivity was noted in 56% (28/50) of cutaneous melanoma cases. The BRAF V600E mutation significantly reduced overall survival and disease-free survival.

Meaning: Anti-BRAF-targeted therapy is a treatment strategy for people with BRAF mutations.

MATERIALS AND METHODS

Sample Recruitment

The institutional review board of Chulalongkorn University's Faculty of Medicine in Bangkok, Thailand (Med Chula IRB no. 727/64) approved this hospital-based study protocol. Patients in all age ranges undergoing preoperative treatment were recruited at the Division of Plastic and Reconstructive Surgery, Department of Surgery, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand between 2012 and 2018. The included patients provided written informed consent. None of the patients received any form of radiation therapy or chemotherapy before surgery. KR and NK confirmed the diagnosis of cutaneous melanoma and the subtype classification in all patients using hematoxylin and eosin staining. Formalin-fixed paraffin-embedded tissue blocks were selected based on tumor tissue abundance and complete clinical data. The exclusion criteria are low amounts of pathologic tissue, a lack of clinical data, and poor follow-up. We maintained a time-point follow-up record from the beginning of surgery until recurrence, final follow-up, or death. Fifty subjects were recruited for the experiments. Demographic information such as age, sex, tumor stage, recurrence time, and death time was carefully extracted from the clinical chart records. The American Joint Committee on Cancer eighth edition cancer staging manual was used for tumor and lymph node metastasis staging.[23,24]

Immunohistochemical Staining and Analysis of BRAF V600E

For immunohistochemistry (IHC), primary antibodies against BRAF V600E were used. Three μm thick sections were taken from formalin-fixed paraffin-embedded blocks. On the Ventana Benchmark XT (Ventana-Roche Diagnostics, Meylan, France) automated slide strainer, the slides were stained with BRAF V600E (VE1) primary monoclonal antibody from mouse (1 μg/100 μl, Ventana Medical Systems, catalog number 760-5095) at 37°C for 32 minutes. The BRAF V600E protein was detected using the OptiView DAB IHC Detection Kit (Ventana-Roche Diagnostics, Meylan, France). The slides were then counterstained for 8 minutes with hematoxylin I (ab245880, Abcam, UK) and 4 minutes with bluing reagent (BR-OT, Biogenost, Croatia, EU).

To ensure the reliability and validity of the staining, each examination included cutaneous melanoma samples with a known wild-type BRAF profile and BRAF V600E mutations as negative and positive controls, respectively. Staining scores were considered positive when cytoplasmic staining was evident in more than 80% of tumor cells in the sections. A negative score was assigned to the sample if nuclear staining was observed with faint staining of isolated interspersed cells or fading diffuse staining.

DNA Sequencing Analysis of BRAF V600E

DNA sequencing was used to validate five cases of positive and negative immunostaining for BRAF V600E. The QIAamp DNA formalin-fixed paraffin-embedded tissue kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions to extract genomic DNA from micro-dissected tumor tissues. BRAF exon 15 was amplified and sequenced using the following primers: forward 5'-AAATTAGATCTCTTACCTAAACTCTTCATA-3' and reverse 5'-GACCCCATCGAGATTT-3'.

Statistical Analyses

All data were analyzed using SPSS version 22 for Windows (Chicago, Ill.). Fisher exact test and Pearson chi-square test were used to determine the association between clinicopathological variables and BRAF V600E

status. Analysis of variance was used to compare more than two groups. The Kaplan–Meier method was used to determine disease-free survival (DFS) and overall survival (OS). The Cox regression model was used as the foundation for both univariate and multivariate survival analyses. A two-sided *P* value less than 0.05 was considered statistically significant.

RESULTS

Clinicopathological Characteristics

Table 1 summarizes the clinical and pathological findings of the patients. Fifty Thai patients with cutaneous melanoma were included in the study, with men (48%) and women (52%). The average age of the patients was 65 years (range, 28–94 years). Regarding the histologic subtypes of cutaneous melanoma, half of the patients (50%) had nodular subtypes, followed by acral lentiginous (40%) and superficial spreading (10%). Most patients had high-depth lesions classified as Breslow level 4 (56%) or level 3 (20%), with a minority classified as level 2 (16%) or level 1 (8%). Furthermore, patients were diagnosed as having advanced-stage melanoma (NM III–IV) (56%) and early-stage melanoma (NM I–II) (44%). The presence of ulcerated lesions in cutaneous melanoma was significantly more common (62%) than nonulcerated lesions (38%). The most common node staging for cutaneous melanoma was stage 0 (48%), followed by stages 1 (26%), 2 (12%), and 3 (14%). The average duration of follow-up was 32.25 months (range 5–96 months); two patients had recurrence and one died. No significant differences in recurrence or death were observed between the groups.

BRAF V600E and Clinicopathological Correlation

BRAF V600E staining was positive in 28 (56%) patients and was detected in the cytoplasm of tumor cells (SDC 1a). In contrast, BRAF V600E staining was negative in all tumor cells in the known wild-type BRAF samples. Melanin pigments were observed in some tumor cells (SDC 1b). Five cases with positive and five cases with negative immunostaining were chosen at random for sequencing, and all positive immunostaining cases had a BRAF point mutation (GTG > GAG, SDC 1c). All negative immunostaining cases, on the other hand, lacked this mutation (SDC 1d). There was no evidence of an association between the BRAF V600E mutation and sex or age. BRAF V600E was detected more frequently in advanced-stage melanomas (56%) than in early-stage melanomas (44%); however, the difference was not statistically significant. Patients with ulcerated lesions had higher BRAF V600E levels than those without ulcerated lesions ($P = 0.033$). In terms of histology, patients with nodular subtypes had a higher prevalence of BRAF V600E than those with acral lentiginous and superficial spreading melanoma (72% versus 35% versus 60%, $P = 0.045$). BRAF V600E mutation was noticeably more frequent in patients with high Breslow level lesions ($P = 0.029$), indicating that the BRAF V600E phenotype is invasive. (**See figure, Supplemental Digital Content 1,** which shows BRAF V600E representative figures. **http://links.lww.com/PRSGO/C202**.)

BRAF V600E and Outcome Analysis

Overall, patients in this study had a 25-month median OS and a 30-month median DFS. Patients with the BRAF V600E mutation had a shorter DFS (median, 9 versus 19 months, $P = 0.034$, Fig. 1A) and OS (median, 16 versus 41 months, $P = 0.025$, Fig. 1B) than those with the wild-type BRAF. Univariate analysis revealed that BRAF V600E was associated with poor recurrent outcomes (Table 2), whereas BRAF V600E and Breslow levels predicted death outcomes (Table 3). Furthermore, multivariate analysis (Table 3) revealed that BRAF V600E mutation was an

Table 1. Clinicopathological Characteristics of Cutaneous Melanoma Patients with BRAF V600E

Clinicopathological Characteristics		n	BRAF wt	BRAF V600E	*P*
Gender	Men	24	10	14	0.749
	Women	26	12	14	
Age	≤65	26	11	15	0.802
	>65	24	11	13	
Histological subtype	Superficial spreading	5	2	3	0.045*
	Nodular	25	7	18	
	Acral lentiginous	20	13	7	
Breslow level	1	4	2	2	0.029*
	2	8	7	1	
	3	10	5	5	
	4	28	8	20	
Tumor stage	Early stage (I–II)	22	13	9	0.057
	Advanced stage (III–IV)	28	9	19	
Ulcer	Presence	31	10	21	0.033*
	Absence	19	12	7	
Staging node	0	24	14	10	0.166
	1	13	3	10	
	2	6	3	3	
	3	7	2	5	
Recurrence*	Recurrent	29	10	19	0.110
	Not Recurrent	19	11	8	
Death*	Dead	28	10	18	0.243
	Alive	21	11	10	
Total		50	22	28	

*Remarks for missing data.

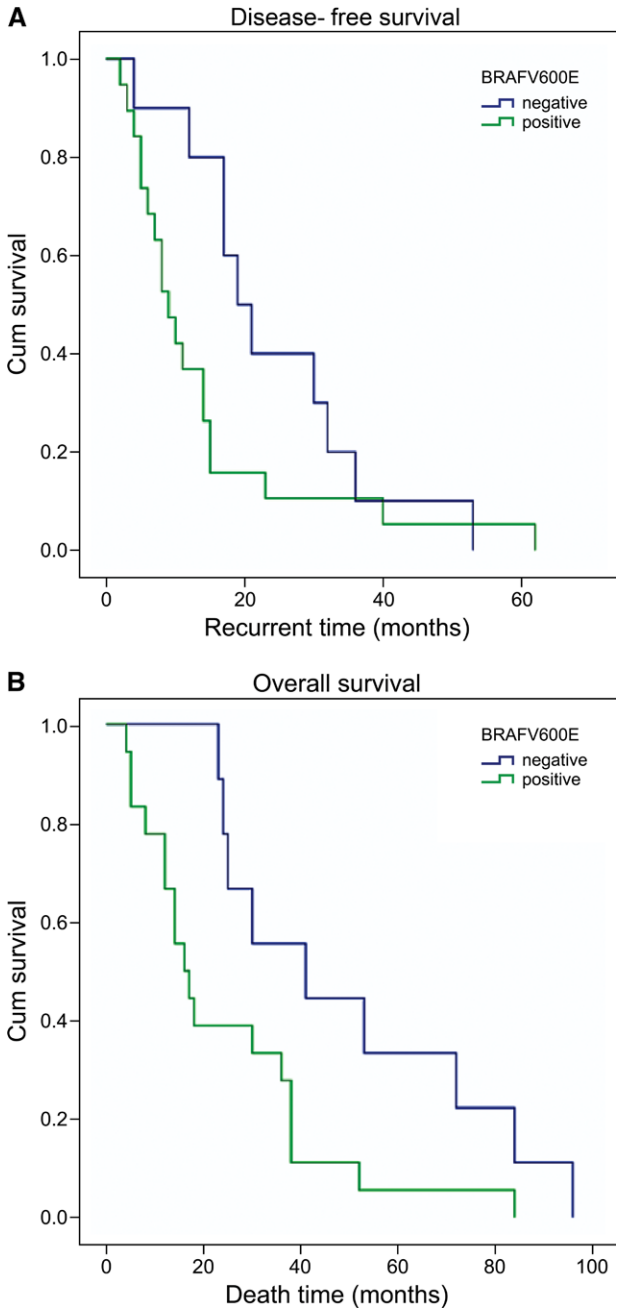

Fig. 1. A: Disease-free survival of cutaneous melanoma patients with BRAF V600E mutation and BRAF wild-type ($P = 0.106$). B: Overall survival of cutaneous melanoma patients with BRAF V600E mutation and wild-type BRAF ($P = 0.025$).

independent risk factor for OS (hazard ratio = 3.714, $P = 0.018$) in patients with cutaneous melanoma.

DISCUSSION

The cause of cutaneous melanoma is unclear despite the efforts of researchers to reveal the pathological pattern at the molecular level underlying its tumorigenesis.[25] Nowadays, targeted therapies, including BRAF or combined BRAF/MEK inhibitors, play a major role in the systemic treatment of cutaneous melanoma.[21] Both targeted therapies have been reported to improve progression-free survival and OS.[26] In contrast, in practice, few patients can undergo targeted therapy owing to high expenses.

The potential of utilizing BRAF-targeted therapy for the treatment of cutaneous melanoma is increased since the rate of BRAF mutations in this study is 56% higher than the previous report in the average Asian population of 19.5%[7] and Japan at 41.8 %.[16,19]

Several studies have reported poor melanoma-specific survival (MSS) in patients with early-stage disease.[27,28] In this study, we found a reduction in OS and DFS in patients with cutaneous melanoma with BRAF mutations. Moreover, the significance of BRAF V600E in both univariate and multivariate analyses showed that BRAF V600E could be used as a marker to predict OS in patients with melanoma (Tables 2 and 3). Multivariate analysis showed that BRAF V600E was an independent risk factor for OS (HR = 3.714, $P = 0.018$) in patients with cutaneous melanoma (Table 3). Our results correspond to those of a previous study showing that patients with heterogeneous BRAF V600E and BRAF V600E-positive melanoma had considerably lower MSS than those with BRAF V600E-negative melanoma.[27]

Regarding tumor or Breslow thickness, several studies have reported that Breslow thickness is the most significant factor that can predict MSS. The AJCC eighth edition cancer staging manual reported the 10-year MSS to be 98% for T1aN0 cases and 75% for cases with tumor thickness greater than 4.0 mm or T4bN0 cases.[29] Correlation according to the univariate analysis in this study showed that Breslow level can predict prognosis and death outcomes of patients with melanoma (Table 3).

The high rate of genetic mutations and chemotherapy resistance in cutaneous melanoma results in low efficacy of treatment.[30] A new class of drugs called "molecular targeted therapy" has been recently discovered, which focuses primarily on the biomolecular mechanism of cancer incidence and survival.[31,32] Thus far, the treatment has yielded positive results. A study is being conducted on patients with unresected stage IIIB/C or stage IV melanoma with BRAF V600E or V600K mutations. Compared with the use of a BRAF inhibitor alone, combination therapy of BRAF inhibitor with a MEK inhibitor was found to significantly improve DFS and OS.[26] Additionally, there is a study on the COMBI-AD trial involving patients with stage III (IIIA >1 mm, IIIB/C) melanoma with the BRAF V600 mutation. The effectiveness of the combination therapy was compared with that of a placebo. The combination therapy was found to significantly improve both relapse-free survival and OS.[14] The NCCN guideline version 2.2021[17] currently recommends that patients with stage IIIA or higher disease receive systemic adjuvant therapy in conjunction with surgery. Adjuvant therapy as the first-line treatment consists of anti-PD1 agents (nivolumab and pembrolizumab). Drugs for molecular targeted therapy (dabrafenib/trametinib) are recommended for patients with BRAF V600 mutation.

Table 2. Univariate Analysis Results with Cox Regression for Recurrent Patients with BRAF V600E Cutaneous Melanoma

Characteristics		Univariate Analysis		
		HR	95% CI	*P*
Gender	Men	1.0		
	Women	1.4	0.6–3.0	0.423
Age	≤65	1.0		
	>65	1.0	0.5–2.2	0.967
Histological subtype	Superficial spreading	1.0		
	Nodular	0.7	0.2–2.2	0.567
	Acral lentiginous	0.5	0.2–1.9	0.317
BRAF	BRAF wild type	1.0		
	BRAF V600E	1.9	0.9–4.2	0.116
Breslow level	1	1.0		
	2	1.0	0–49485	1.000
	3	1.0	0–47283	1.000
	4	1.0	0–46352	1.000
Tumor stage	Early stage (I-II)	1.0		
	Advanced stage (III-IV)	1.0	0.5–2.3	0.964
Ulcer	Absence	1.0		
	Presence	1.1	0.5–2.4	0.879
Staging node	0	1.0		
	1	0.9	0.4–2.4	0.884
	2	0.8	0.3–2.4	0.737
	3	2.3	0.8–6.9	0.135

Table 3. Univariate and Multivariate Analysis Results with Cox Regression for Death in Patients with BRAF V600E Cutaneous Melanoma Patients

Characteristics		Univariate Analysis			Multivariate Analysis		
		HR	95% CI	*P*	HR	95% CI	*P*
Gender	Men	1.0					
	Women	1.2	0.5–2.7	0.643			
Age	≤65	1.0					
	>65	0.5	0.2–1.2	0.120			
Histological subtype	Superficial spreading	1.0					
	Nodular	0.9	0.3–3.0	0.944			
	Acral lentiginous	0.6	0.2–2.3	0.489			
BRAF	BRAF wild type	1.0			1.0		
	BRAF V600E	2.5	1.1–5.9	0.037*	3.7	1.2 – 11.0	0.018*
Breslow level	1	1.0			1.0		
	2	0.1	0.0–1.314	0.080	0.285	0.0 – 4.1	0.354
	3	0.1	0.0–0.9	0.040*	0.075	0.0 – 0.9	0.050
	4	0.1	0.0–0.8	0.037*	0.090	0.0 – 1.0	0.052
Tumor stage	Early stage (I-II)	1.0					
	Advanced stage (III-IV)	1.6	0.7–3.8	0.273			
Ulcer	Absence	1.0					
	Presence	1.4	0.6–3.2	0.471			
Staging node	0	1.0					
	1	1.2	0.4– 3.4	0.793			
	2	1.6	0.6–4.6	0.369			
	3	1.8	0.6–5.5	0.302			

CONCLUSIONS

Our findings revealed a high prevalence of BRAF V600E mutation in Thai patients with cutaneous melanoma, increasing the likelihood of using BRAF-targeted therapy for cutaneous melanoma treatment, particularly in cases that are surgically unresectable or in cases of advanced disease stages. Among patients with cutaneous melanoma, those harboring BRAF V600E mutation had poor recurrence results, and the mutation was an independent risk factor for OS. We believe that this study's findings will be useful for understanding the disease prevalence, prognostic conditions, and future treatments.

Nakarin Kitkumthorn, DDS, PhD
Department of Oral Biology, Faculty of Dentistry
Mahidol University
Phayathai Road, Ratchathewi
Bangkok 10400, Thailand
E-mail: nakarinkit@gmail.com

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