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ORIGINAL ARTICLE

Mutation Profile of B-Raf Gene Analyzed by fully Automated System and Clinical Features in Japanese Melanoma Patients

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Abstract BRAF gene mutations have been observed in 30–50 % of malignant melanoma patients. Recent development of therapeutic intervention using BRAF inhibitors requires an accurate and rapid detection system for BRAF mutations. In addition, the clinical characteristics of the melanoma associated with BRAF mutations in Japanese patients have not been investigated on a large scale evaluation. We recently established quenching probe system (QP) for detection of an activating BRAF mutation, V600E and evaluated 113 melanoma samples diagnosed in Saga University Hospital from 1982 to 2011. The QP system includes fully automated genotyping, based on analysis of the probe DNA melting curve, which binds the target mutated site using a fluorescent guanine quenched probe. BRAF mutations were detected in 54 of 115 (47 %) including 51 of V600E and 3 of V600 K in Japanese melanoma cases. Among clinical subtypes of melanoma, nodular melanoma showed high frequency (12 of 15; 80 %) of mutation followed by superficial spreading melanoma (13 of 26; 50 %). The QP system is a simple and sensitive method to determine BRAF V600E mutation, and will be useful tool for patient-oriented therapy with BRAF inhibitors. Introduction

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Introduction

Melanoma is the leading cause of skin cancer, and incidence of the disease as well as mortality due to advanced metastatic melanoma have been increasing. Until recently, therapeutic options for treatment of metastatic melanoma were limited, with median survival for patients with stage IV melanoma ranging from 8 to 18 months after diagnosis [1]. In Japan, 1400 deaths from skin cancer including melanoma were documented in 2010, with an estimated rate of death of 1.0 in 100,000 [2]. Although various chemotherapeutic regimens have been introduced for the treatment of metastatic melanoma, no chemotherapeutic agent has surpassed dacarbazine, with a median overall survival of 5.6 to 7.8 months after the introduction of treatment [3–6]. Recently, two novel drugs were approved by the Food and Drug Administration of the United States. Ipilimumab, a monoclonal antibody that blocks cytotoxic T-lymphocyte associated antigen 4 (CTLA4), has been reported to provide improved overall survival in a phase 3 study [7, 8]. In addition, vemurafenib, a potent inhibitor of mutated BRAF, showed remarkable antitumor activity against melanoma cell lines with the BRAF V600E mutation, and improved survival of advanced melanoma patients with BRAF V600E mutation compared with dacarbazine in a phase 3 clinical trial [9]. Because approximately 40 to 60 % of melanomas carry mutations in *BRAF* that lead to constitutive activation of downstream signaling through the MAPK pathway, inhibition of the Raf-Ras-Map kinase cascade is a

promising molecular target for therapy with melanoma [10, 11]. Among them, about 80–90 % of these mutations were a substitution of glutamic acid for valine at codon 600 (BRAF V600E), although other activating mutations, such as BRAF V600 K and BRAF V600R, have also been reported [11–13]. Molecular targeted therapy using BRAF inhibitors requires an accurate and rapid detection system for BRAF mutations. In addition, the clinical characteristics of melanoma associated with BRAF mutations in Japanese patients have not been well investigated in a large-scale evaluation. Ashida et al. recently reported that BRAF mutations were found in 26.7 % of Japanese melanoma patients with a high frequency of mutation rate observed in chronic sun-induced-damage (CSD) type melanoma [14]. To solve such issues, the quenching probe (QP) system for detection of an activating BRAF mutation was established in collaboration with ARKRAY Inc. The QP system includes fully automated genotyping, based on analysis of the probe DNA melting curve, which binds the target mutated site using a fluorescent guanine quenched probe. Using this technique, we evaluated 113 melanoma samples from patients diagnosed in Saga University Hospital from 1982 to 2011, and clinical characteristics of melanoma patients carrying activating BRAF mutation were analyzed.

Materials and Methods

Human Melanoma Cell Lines

Human melanoma cell lines SK-MEL-28 and SK-MEL-2 were purchased from the American Type Culture Collection (Manassas, VA). These cells were cultured in minimum essential medium (MEM) supplemented with 10 % fetal bovine serum at 37 °C under 5 % CO2. BRAF mutational status of these cells was previously reported: SK-MEL-28 harbors the BRAF V600E mutation, whereas SK-MEL-2 carries wild-type BRAF [15].

Patients and Tumor Tissue Samples

The samples from primary and/or metastatic lesions of 113 melanoma patients diagnosed at Saga University Hospital from 1982 to 2011 were included, but patients with melanoma in situ (Clark Level I) were excluded from this study. Clinical data, such as age, sex, primary tumor site, TNM staging (American Joint Committee on Cancer: AJCC, 2009), tumor thickness, presence of ulceration, histological subtypes, and Clark level, are summarized in Table 1. The study protocol was approved by the Clinical Research Ethics Committee of Saga University (approval number: 23–48). All patients gave informed consent for obtaining tissue specimens according to the Declaration of Helsinki.

Table 1 Sensitivity for detection of *BRAF* V600E mutation using the QP method

	Threshold	
	Quantity	Ratio (%)
Plasmid DNA	2.4 copies	3.2 %*
Genomic DNA	8 pg	2.6 %†

*Percentage of control plasmid carrying the *BRAF* V600E mutation relative to the amount of wild-type plasmid in the mixture

† Percentage of genomic DNA isolated from SK-MEL-28 (V600E) in the mixture with that from SK-MEL-2 (wild-type)

DNA Isolation and Mutation Analysis

Genomic DNA was isolated from cell lines and formalin-fixed paraffin-embedded (FFPE) melanoma specimens using QIAamp® DNA mini kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was determined based on absorbance at 260 nm using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Japan). BRAF exon 15 mutations were determined by direct sequencing. Polymerase chain reaction (PCR) for exon 15 of the *BRAF* gene was performed using the following primers: BRAF exon 15 forward, 5′-TCATAATGCTTGCT CTGATAGGA-3′ and *BRAF* exon15 reverse, 5′-GGCC AAAAATTTAATCAGTGGA-3′, with PCR product lengths of 224-bp. PCR amplifications were performed in 20 μl reaction mixture containing 0.1 μM of each primer, 2.5 mM MgCl2, 0.25 mM dNTPs, and 2.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems) in 1× PCR buffer. In total, 40 amplification cycles (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s) were performed with an initial denaturation at 94 °C for 10 min and a final extension at 72 °C for 10 min. PCR products were sequenced directly using an Applied Biosystems 3130 Genetic Analyzer.

Detection of BRAF V600E Mutation Using QP Method

To detect the BRAF V600E mutation, we performed the quenching probe (QP) method using i-densy (ARKRAY Inc., Kyoto, Japan), which can detect the potential gene polymorphism with full automation [16]. Forward and reverse PCR primers used in the QP method were 5′-TGCT TGCTCTGATAGGAAAATGAGATCTAC-3′ and 5′-AAAC TGATGGGACCCACTCCAT-3′, respectively. The presence of BRAF V600E mutation in the amplified sequences was determined by monitoring the fluorescence intensity of a TAMRA-conjugated specific guanine quench fluorophore probe (QProbe, J-Bio21, Tokyo, Japan), which is complementary to the V600E mutation: 5′-GCTACAGAGAAATC TC-(TAMRA)-3′. When the QProbe is hybridized with the target DNA, its fluorescence is quenched by the guanine in

the target sequence, which is complementary to the modified cytosine at the 3′ end. In theory, the QProbe can bind to both the BRAF V600E mutant and wild type amplicons during PCR, but will bind to the mutant sequence with higher affinity. After PCR was completed, the temperature was reduced to 40 °C and then gradually increased, during which fluorescence intensity at different temperatures was measured to identify wild-type and mutant amplicons (fluorescence intensity depends on QProbe dissociation from the amplicon: wild-type dissociates at 46 °C and mutant at 53 °C (Fig. 1a).

Statistical Analysis

Fisher's exact test for 2 × 2 tables and Pearson's chi-square test for contingency tables of higher dimension were used to examine associations between BRAF mutational status and the following categorical variables: sex, primary tumor site, TNM staging, ulceration, histological subtypes, and Clark level. A t-test or Mann-Whitney U test was used to examine associations between mutational status and age or tumor thickness. All *P* values are 2-sided and P values less than 0.05 were

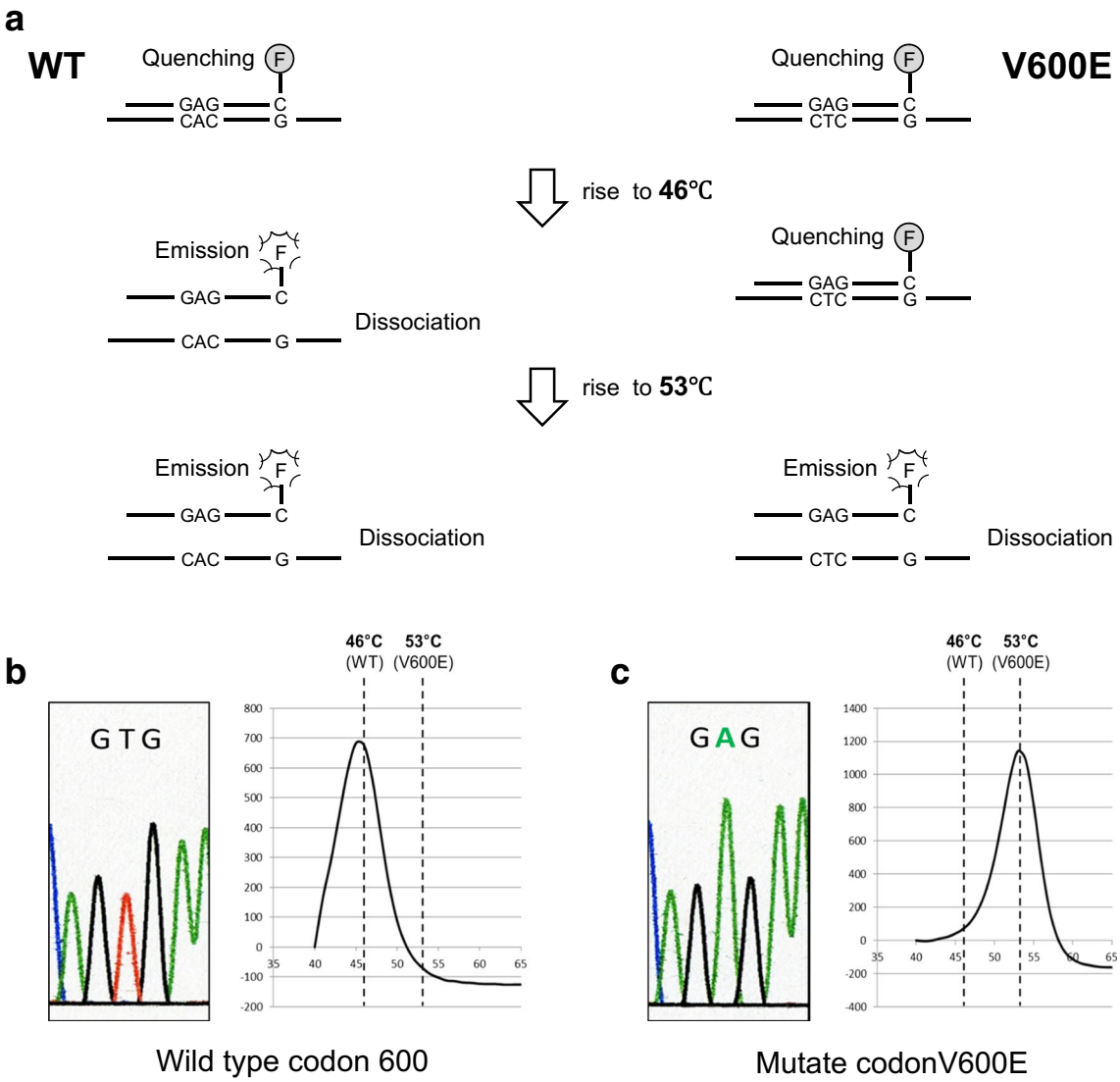

Fig. 1 Detection of BRAF V600E mutation using the QP method. (**a**) Principles of the QP method. Presence of the V600E sequence is determined by the QP-method, in which the fluorescence intensity of a TAMRA-conjugated specific guanine quench fluorophore probe complementary to V600E is monitored. When a QProbe is hybridized to target DNA, its fluorescence is quenched by the guanine in the target, which is complementary to the modified cytosine. Following PCR, the temperature is reduced to 40 °C and then gradually increased. To identify the wild-type and mutant amplicons, the fluorescence intensity at the different temperatures is measured. When the QProbe dissociates from the amplicon—wild type at 46 °C and mutant at 53 °C—the fluorescence intensity increases. (**b**) BRAF mutation analysis of human melanoma cell lines. Results with direct sequencing (left) and the QP method (right) of genomic DNA isolated from SK-MEL-2 cells. Wild-type sequence in codon 600 (GTG) is shown in direct sequencing and the peak of fluorescence intensity at 46 °C with the QP method. (**c**) Results of direct sequencing (left) and QP method (right) of genomic DNA isolated from SK-MEL-28 cells. The GAG mutation (V600E) (arrow) is shown in direct sequencing and the peak of fluorescence intensity at 53 °C with the QP method

considered statistically significant. All tests were performed using SPSS for Windows, version 17.0.0. Survival analyses were performed using Kaplan-Meier methods, and log-rank tests and Cox regression methods were used to compare the survival of different patient groups.

Results

Evaluation of QP Method for Detection of BRAF V600E Mutation

To analyze the mutation of BRAF exon15 in genomic DNA isolated from SK-MEL-28, a melanoma cell line carrying BRAF V600E mutation was used [15]. Direct sequencing confirmed that SK-MEL-28 cells harbor the V600E mutation. The QP method can detect V600E mutation by the difference of the peak of melting curve at 53 °C for mutant allele and at 46 °C for wild allele as described in Materials and Methods (Fig. 1). We determined the detection limit of V600E mutation using the QP method. Sequential dilution was conducted using plasmids containing BRAF V600E mutation or genomic DNAs isolated from SK-MEL-28. Detection limits were determined to be 2.4 copies assessed by plasmid DNAs and 8 pg assessed by genomic DNA content, respectively (Table 1). We also determined the sensitivity of the QP method by mixing two plasmids carrying BRAF exon15 with or without the V600E mutation in different ratios, and found that the detection limit for BRAF V600E was 3.2 % for mutant plasmid and 2.6 % for genomic DNAs.

BRAF Mutation Analysis in Human Melanoma Samples Using Direct Sequencing and QP Method

Using the QP method, we performed mutation analyses of BRAF exon 15 in the samples obtained from FFPE sections of primary or metastatic lesions of 113 melanoma patients, and compared the results with direct sequencing (Table 2). In direct sequencing, 26 samples (23.0 %) were not evaluable because of invalid PCR, whereas all samples could be evaluated using the QP method. Using direct sequencing we detected BRAF exon 15 mutations in 41 among 87 evaluable samples; all 41 mutations occurred at codon V600 in the *BRAF* gene. Thirty-eight of these mutations were a single base-pair substitution located at T1799 A—V600E—and three were tandem mutations located at GT1798–99AA—V600 K. Although the QP method can detect V600E mutation alone in the present system, 43 samples were positive for V600E mutation. Three samples showing V600 K mutation by direct sequencing were identified as wild-type by the QP method, and four samples called wild-type by direct sequencing were identified as V600E by the QP method. Conversely, eight samples were identified as wild-type by the QP method but were positive for V600E by direct sequencing. By combining the results for BRAF exon 15 mutation with direct sequencing and the QP method, the mutation rates of BRAF exon15 in melanoma patients in our hospital was determined to be 47.8 % (54/ 113) (Table 2).

Table 2 Mutation profiles and summary of the results of BRAF mutational status

Mutation profiles		Direct sequence			
		V600E	V600K	WT	NE
QP method	V600E	30	0	4	9
	WT	8	3	42	17
Summary of the mutation rates					
BRAF mutation	54 (47.8 %)	V600E		51 (45.1 %)	
		V600 K		3 (2.7 %)	
BRAF wild-type	59 (52.2 %)				

NE Not Evaluable

Correlation of BRAF Mutation with Clinicopathological Characteristics

The clinical and pathological characteristics of patients according to BRAF mutation status are shown in Table 3. A significant difference in the frequency of BRAF mutations between histological subtypes were observed, with a high frequency of BRAF mutations in nodular melanoma (NM) (80.0 %; 12/15) and superficial spreading melanoma (SSM) (50.0 %; 13/26). However, all other clinical parameters, which included age, sex, primary tumor site, TNM staging, tumor thickness, presence of ulceration, and Clark level, were not significantly different between patients with mutant versus wild-type *BRAF* gene.

Mutation Status and Patient Survival

Among 113 patients in our cohort, 91 (80.5 %) had complete information on clinical condition. Kaplan-Meier analyses of overall survival from the date of initial diagnosis, stratified by BRAF mutation status, are illustrated in Fig. 2. Hazard ratios are presented in Table 4. The 5-year survival was 55.4 % (95%CI, 40.3–70.5 %) for the BRAF mutation group compared with 78.8 % (95%CI, 66.4–91.3 %) for the wild-type group. Patients with BRAF mutation had significantly poorer prognosis compared to patients with wild-type BRAF (hazard ratio 2.42, 95%CI, 1.10–5.34, log-rank $P = 0.024$).

Table 3 Correlation of BRAF mutation with clinicopathological characteristics

	All patients (n = 113)	BRAF mutation (n = 54)	BRAF wild type (n = 59)	P value
Age: years				
Mean (±SD)	64.8 (±17.6)	62.2 (±17.3)	67.2 (±17.7)	0.140
Sex				
Male	56	28 (50.0 %)	28 (50.0 %)	0.708
Female	57	26 (45.6 %)	31 (54.4 %)	
Primary tumor site				
Head and Neck	16	8 (50.0 %)	8 (50.0 %)	1.000
Trunk / Extremity / Other	97	46 (47.4 %)	51 (52.6 %)	
Clinical stage (AJCC)				
I	23	12 (52.2 %)	11 (47.8 %)	0.916
II	43	20 (46.5 %)	23 (53.5 %)	
III	35	15 (42.9 %)	20 (57.1 %)	
IV	6	3 (50.0 %)	3 (50.0 %)	
Unknown	6	4 (66.7 %)	2 (33.3 %)	
Tumor thickness: mm				
Median (range)	3.15 (0.2–22.0)	2.80 (0.2–22.0)	3.40 (0.3–22.0)	0.324
Ulceration				
Absent	58	29 (50.0 %)	29 (50.0 %)	0.705
Present	53	24 (45.3 %)	29 (54.7 %)	
Unknown	2	1 (50.0 %)	1 (50.0 %)	
Histological subtypes				
NM	15	12 (80.0 %)	3 (20.0 %)	0.038
SSM	26	13 (50.0 %)	13 (50.0 %)	
ALM	57	20 (35.1 %)	37 (64.9 %)	
LMM	8	3 (37.5 %)	5 (62.5 %)	
Mucosal	2	1 (50.0 %)	1 (50.0 %)	
Unknown	5	5 (100 %)	0 (0 %)	
Clark level†				
I	20	10 (50.0 %)	10 (50.0 %)	0.743
II	17	10 (58.8 %)	7 (41.2 %)	
III	47	22 (46.8 %)	25 (53.2 %)	
IV	24	10 (41.7 %)	14 (58.3 %)	
Unknown	5	2 (40.0 %)	3 (60.0 %)	

AJCC American Joint Committee on Cancer, *NM* odular melanoma, *SSM* uperficial spreading melanoma, *ALM* acral lentiginous melanoma, *LMM* lentigo maligna melanoma

†Patients with melanoma in situ (Clark Level I) were excluded in this study

Discussion

We developed a novel, fully automated QP system for detecting the BRAF V600E mutation. The method is sensitive and can be adapted for detecting BRAF V600E mutation in DNA obtained from old FFPE samples. Among various cancer types that have been reported to carry the BRAF V600 mutation, we chose to apply the QP system in melanoma patients because a high incidence of BRAF V600 mutation has been reported in melanoma, and very few analyses of BRAF mutation have been conducted in Japanese melanoma patients. Our results suggest that the mutation rate of BRAF V600 in melanoma patients in Japan is comparable to that previously reported for western countries [1]. Therefore, molecular targeting therapy against the Ras-Raf-MAPK cascade is applicable to Japanese melanoma patients. In addition, recent investigation revealed that BRAF mutation occurs in various other cancer types, such as hairy cell leukemia, colon, lung, and head and neck cancers [10, 11, 17–21]. The BRAF mutation frequency in our cohort (47.8 %) was higher than that (26.7 %) reported by Ashida et al. Although more large scaled evaluation required, one of the reason may associated with high sensitivity of QP method. Because the QP system is simple and quick with fully automated manipulation for

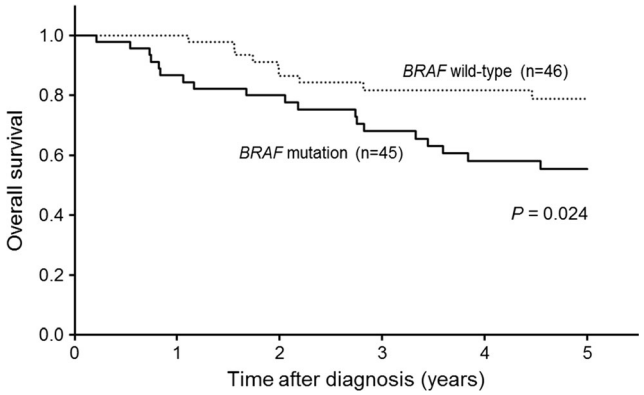

Fig. 2 Kaplan-Meier survival curves after diagnosis of malignant melanoma, with respect to presence or absence of BRAF gene mutations. Patients with BRAF mutation had a significantly poor prognosis compared with patients having wild-type BRAF (hazard ratio 2.42, 95%CI, 1.10–5.34, log-rank $P = 0.024$)

detecting the BRAF V600E mutation, it is a useful tool for evaluation of the BRAF gene in cancer cells. In addition, the sensitivity for detection of V600E mutation is acceptable for evaluating clinical samples including non-cancerous lesions. Furthermore, all DNA samples obtained from FFPE specimens including very old samples were evaluable by the QP method, which is important for mass screening including old clinical samples. However, the present BRAF detection method using the QP system can detect only V600E, not other BRAF mutations. In addition, eight samples with V600E mutant alleles detected by direct sequencing were not correctly identified as V600E mutations by the QP method. Although the assay system for detecting BRAF V600E mutation based on the QP method is useful in its present form, a more advanced system covering other BRAF mutations with enhanced sensitivity and specificity is under development.

As for clinical significance of BRAF mutations in Japanese melanoma patients, our cohort revealed that BRAF mutation is observed preferentially in NM and SSM histological types compared with other types among Japanese melanoma patients. Previous study suggested that BRAF mutation may be frequently observed in melanoma cells originating on non-chronically sun-damaged skin [22]. Although our histological data are not based on the same evaluation criteria as with previous reports, the distribution of BRAF mutations may differ between Japanese and western patients, because the frequencies of BRAF mutations did not differ between the primary sites located in head and neck, which have more chance to be exposed to sun resulting in non-chronically sun-damaged skin, and those located in other sites. In contrast to location of primary site, histological subtype showed the unique feature that BRAF mutations were common in NM and SSM histological types [22]. Although the reason why the BRAF mutation is associated with such histological types is not clear, the phenomenon may provide clues for analyzing the molecular mechanisms of melanoma in Japanese patients.

We also noted a unique observation in terms of prognosis of Japanese melanoma patients carrying BRAF mutation. In our study, melanoma patients with BRAF mutation showed apparently poor prognosis compared with patients having the wild-type BRAF gene. The prognostic value of BRAF mutation has been analyzed by a systemic review and meta-analysis [22–24]. According to the results, the hazard ratio for BRAF mutation was 1.7 in melanoma patients. Our results were comparable with those reports and indicate the importance of evaluation of BRAF mutation in Japanese melanoma patients. Because molecular targeted therapy against BRAF mutation for melanoma patients using Ipilimumab or vemurafenib are showing promising clinical power, clarifying target patients for such treatment is thought to be an important issue. We believe that the QP method is a useful detection tool for evaluation of suitable patients by detecting BRAF mutations.

Table 4 Survival outcome by multivariate Cox proportional hazards analysis for melanoma patients

Factors	Hazard Ratio (95 % CI)	*P* value
BRAF status (Mutation / Wild-type)	4.999 (1.732–14.429)	0.003
Age	1.022 (0.985–1.060)	0.256
Sex (Male / Female)	1.153 (0.389–3.417)	0.797
AJCC clinical stage (Stage III-IV / I-II)	4.812 (1.619–14.304)	0.005
Primary tumor site (Head and Neck / Trunk-Extremity-Other)	3.149 (0.429–23.132)	0.259
Tumor thickness	1.111 (0.995–1.242)	0.062
Ulceration (Present / Absent)	2.008 (0.712–5.661)	0.187
Histological subtypes		
NM	Reference	
SSM	1.296 (0.241–6.981)	0.763
ALM	1.692 (0.230–12.440)	0.606
LMM	0.359 (0.021–6.041)	0.477
Clark level (Level IV-V / II-III)	0.684 (0.222–2.107)	0.509

CI confidence interval

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Compliance with Ethical Standards

Contributions I. M, S. K., N. S., A. S., Y. N. ,T. I., N. S., A. S., Y. N. performed the analyses and obtained results. Pathological diagnosis was conducted by N.M. Y. N., S. K., and E. S. edited the paper. E.S. designed the research and wrote the paper.

Conflict of Interest All authors declare that we have no conflicts of interest about any financial and personal relationships with other people or organizations.

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