BRAF V600 mutations and pathological features in Japanese melanoma patients

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Ultraviolet radiation is a risk factor for *BRAF* V600 mutations frequently found in melanomas that cause constitutive BRAF activation. Primary sites of melanoma and the frequency of *BRAF* mutations might differ between races. Melanoma is rare in Japan (1500–2000 cases/year compared with 132 000/year worldwide) and the frequency and distribution of *BRAF* V600 mutations are unknown. We aimed to investigate the frequency of *BRAF* V600 mutations in a cohort of Japanese patients with melanoma and determine the relationship between mutations and clinical/pathologic features. DNA was extracted from 80 formalin-fixed, paraffin-embedded tumours from individuals diagnosed with melanoma. *BRAF* V600 mutations were detected using the Cobas 4800 System with z480 Analyzer and Cobas 4800 BRAF V600 Mutation Test reagents. *BRAF* V600 mutations were detected in 41.8% of tested tumours, with an invalid rate of 1.3%. The mutation rate was more than 60% in patients aged less than 60 years and more than 36% in patients with stage III/IV disease. No sex difference in the mutation rate was observed. *BRAF* V600 mutations were detected in 18.8% of acral lentiginous melanomas (ALMs), 64.7% of superficial spreading melanomas, 50.0% of lentigo maligna melanomas and 20.0% of nodular melanomas. Although the mutation rate was low in ALMs, 36.4% were mutation positive at stage III/IV compared with 9.5% at stage I/II. This study confirmed associations among *BRAF* V600 mutations, pathological features and subtypes of melanoma. *BRAF* V600 mutations were more frequent in late-stage ALMs than in early-stage ALMs. Superficial spreading melanomas had similar mutation rates at all stages. These insights suggest improved treatment predictions for stage III/IV melanoma patients. *Melanoma Res* 25:9–14 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Melanoma is the most deadly form of skin cancer, and is a major health problem worldwide. Over the last decade, the incidence and mortality rates of melanoma have increased in individuals of European origin [1]. Approximately 40–70% of melanomas contain activating mutations of the *BRAF* gene [2–5], which might be caused by prolonged periods of exposure to solar ultraviolet radiation [6–8]. However, *BRAF* mutations were detected in melanomas in nonchronic sun-damaged skin, whereas *NRAS* mutations were present in melanomas in chronic sun-damaged skin [9]. *BRAF* encodes a growth factor (B-Raf protein) that regulates the MAP kinase/ERK-signalling pathway, which is involved in cell division, differentiation and secretion. *BRAF* mutations encode mutated B-Raf proteins that have elevated kinase activity and can transform cell lines; because of this, *BRAF* was identified as a common oncogene in human melanoma and other human cancers [2]. The most common mutation in *BRAF* causes the substitution of glutamic acid for valine at amino acid 600 (*BRAF* V600), resulting in the constitutive activation of *BRAF* and uncontrollable melanocyte cell growth, eventually leading to tumour formation [10].

A number of clinical trials of treatments for melanoma have been reported, including dabrafenib, a *BRAF* inhibitor, which improved progression-free survival in metastatic melanoma patients with the *BRAF* V600 mutation [11]; ipilimumab, an anti-CTLA-4 antibody, which improved overall survival, but had a high incidence of adverse events [12]; or a MAPK kinase inhibitor, trametinib, for patients with metastatic melanoma with *BRAF* V600E/V600K mutations, which improved progression-free and overall survival [13]. Clinical trials investigating combination therapy showed that dabrafenib plus trametinib promoted longer progression-free survival with

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reduced incidence and severity of toxicity when compared with respective monotherapies [14], whereas ipilimumab plus dacarbazine, an alkylating agent, improved overall survival but showed similar adverse events to ipilimumab alone with elevated liver enzymes [15].

Three multicentre clinical trials (phase 1, 2 and 3) of a BRAF V600 kinase inhibitor, vemurafenib, in melanoma patients showed that treatment resulted in complete or partial tumour regression and improvements in both overall survival and progression-free survival in the majority of patients carrying the *BRAF* V600 mutation, thus indicating its potent antitumour activity [16–18]. Vemurafenib was approved by the Food and Drug Administration in 2011 and was codeveloped with the Cobas 4800 BRAF V600 Mutation Test (an in-vitro assay based on TaqMan real-time PCR technology) to measure *BRAF* V600 mutations with a high sensitivity for selecting patients for treatment [19]. Melanomas are categorized by the World Health organization into four subtypes as follows: acral lentiginous melanomas (ALMs), superficial spreading melanomas (SSMs), lentigo maligna melanomas (LMMs) and nodular melanomas (NMs) [20]. A meta-analysis of 19 studies of different melanoma subtypes and the incidence of *BRAF* mutations concluded that *BRAF* mutations were associated with SSM [odds ratio (OR) 2.021, 95% confidence interval (CI) 1.440–2.835, $P < 0.001$]. The ORs for BRAF mutation in LMM, ALM and NM were 0.422 (95% CI 0.291–0.614, $P < 0.001$), 0.385 (95% CI 0.237–0.626, $P < 0.001$) and 0.980 (95% CI 0.730–1.316, $P = 0.893$), respectively [9]. *BRAF* mutations were present in 49% of SSM, 41% of NM, 22% of LMM and 20% of ALM patients [9]. By contrast, a study not included in the meta-analysis indicated that both SSM and NM were significantly associated with mutant *BRAF* (OR 9.8, 95% CI 2.0–47.2, $P < 0.005$) by multivariate analysis of 137 Australian patients with metastatic melanoma [21].

Of note, in a previous study [22], *BRAF* V599E mutations in primary melanomas surgically removed from 35 Japanese patients were observed at lower frequencies than in a western study [2], and different *BRAF* V599E mutation frequencies were observed in different melanoma subtypes. *BRAF* V599E mutations were most common in SSM, but less frequent in LMM, NM and ALM [22]. One explanation for this might be a difference in the histological subtypes of melanoma between Japanese and other races.

In Japan, the occurrence of melanoma is rare; the estimated annual incidence is ~1500–2000 cases compared with the worldwide annual incidence of 132 000. One potential reason for the lower incidence might be skin colour as pigmented skin has increased protection from ultraviolet light. Currently, the frequency and distribution of *BRAF* V600 mutations among Japanese melanoma patients are unknown. However, a recent study indicated that malignant skin tumours are increasing in frequency in the Japanese population aged 30–39 years and particularly in those between 60 an 69 years of age, although the reason for this increase is unclear [23].

The present study investigated the frequency of *BRAF* V600 mutations using the Cobas 4800 BRAF V600 Mutation Test in Japanese patients with the four subtypes of melanoma (ALM, SSM, LMM and NM) as the primary endpoint. The associations between the *BRAF* V600 mutation and clinical or pathologic features were determined as the secondary endpoint.

Methods

Study design

This observational study used formalin-fixed, paraffin-embedded (FFPE) specimens from 80 Japanese melanoma patients who underwent resection or biopsy between May 2005 and December 2012. The *BRAF* mutation rate in the USA and European Union is ~40–70% [2,3,5]. Therefore, we assumed that 80 specimens would be required to provide at least one positive *BRAF* mutation in each subtype. A sample list containing patient demographic data and the incidence of melanoma subtypes in patient specimens (Table 1) from the Department of Dermatological Oncology, National Cancer Center Hospital, Tokyo, Japan, was presented to Roche Diagnostics (Tokyo, Japan) and each sample was assigned an enrolment number.

Six slides per patient containing 5-μm FFPE sections were prepared (one for haematoxylin and eosin staining and five for *BRAF* testing) at the Department of Pathology and Clinical Laboratories, National Cancer Center Hospital. Sections for *BRAF* testing were subjected to DNA extraction and mutation detection using the Cobas 4800 BRAF V600 Mutation Test (Roche Molecular Systems, Pleasanton, California, USA), which was performed at Roche Diagnostics.

Table 1 Patient demographic data

	Number of patients (total $n = 79$) (% of all patients)
Sex	
Male	42 (53.2)
Female	37 (46.8)
Age (years)	
<50	15 (19.0)
50–59	11 (13.9)
60–69	24 (30.4)
>69	28 (35.4)
Unknown	1 (1.3)
Melanoma subtype	
ALM	32 (40.5)
SSM	34 (43.0)
NM	8 (10.1)
LMM	5 (6.3)
Tumour type	
Primary	79 (100.0)
Metastatic	0 (0.0)

ALM, acral lentiginous melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma.

Patients and tissue samples

Of all Japanese patients newly diagnosed with melanoma between 2005 and 2012 at the National Cancer Center Hospital, 134 patients had samples available in the hospital archives. Therefore, 134 samples from 134 Japanese patients diagnosed with melanoma were screened.

Inclusion criteria included FFPE tissue resected in operation or biopsies and pathologically diagnosed as melanoma, and agreement for use after written informed consent from patients. Of the 134 patients, four patients refused consent, six were unable to comply with the protocol, seven were excluded because the samples from these patients were on loan to other hospitals during the study period and 37 were excluded as their specimens were deemed inappropriate by an investigator because of decalcification of the fingers ($n=35$) or because of spontaneous regression of the primary lesion ($n=2$). Thus, 80 FFPE archived samples from 80 Japanese patients were eligible. One sample was considered invalid. Thus, 79 samples were used for the analyses in this study. The study was approved by the ethics committee of each participating institute and was carried out according to the institutional review board guidelines. The diagnosis of melanoma stage III/IV disease was made according to the American Joint Cancer Committee/Union Internationale Contre le Cancer [24, 25] and melanoma subtypes were determined according to the definitions proposed by Clark *et al.* [20].

Cobas 4800 BRAF V600 Mutation Test

The Cobas 4800 BRAF V600 Mutation Test was performed as described previously [19] (Fig. 1). Briefly, DNA was extracted using the Cobas DNA Sample Preparation Kit (Roche Molecular Systems, Branchburg, New Jersey, USA). Specimens containing less than 50% tumour area were macrodissected before DNA extraction. *BRAF* mutations were detected with the Cobas 4800 (z480) System (Roche Diagnostic Systems, Branchburg, New Jersey, USA) using Cobas 4800 BRAF V600 Mutation Test reagents (Roche Diagnostic Systems), and the results were analysed using Cobas 4800 BRAF Analysis Package Software (version 2.0, Roche Diagnostic Systems). In cases of invalid results, specimen testing was repeated. An invalid result from retesting was excluded from the analysis as an invalid sample.

Study endpoints

The primary endpoint was the frequency of *BRAF* V600 mutations in Japanese patients with melanoma. The secondary endpoint was the relationships between *BRAF* V600 mutations and clinical or pathological features of melanoma.

Results

Demographic data

Patient demographic data are shown in Table 1. Of 79 melanoma patients (53.2% men) grouped by age, 35.4% (28/79) were older than 69 years. The most common melanoma subtypes observed were ALM (40.5%, 32/79) and SSM (43.0%, 34/79), whereas NM (10.1%, 8/79) and LMM (6.3%, 5/79) were less common. All tumours were primary tumours and not metastatic. The Cobas 4800 BRAF V600 Mutation Test required at least 50% tumour content in the FFPE sections used; otherwise, macrodissection was necessary before DNA extraction. The tumour content of FFPE sections was mostly less than 10% (60.8%, 48/79), with 31.6% containing 10–50% tumour content and only 7.6% with greater than 50% tumour content. Eighty FFPE specimens were collected for analysis by the Cobas 4800 BRAF V600 Mutation Test. Seventy-nine specimens were positively evaluated and one invalid specimen was excluded (1.3%, 1/80) because neither wild-type nor mutated DNA was amplified, indicating that the sample was damaged before analysis.

Overall *BRAF* V600 mutation status

Overall, *BRAF* V600 mutations were detected in 41.8% (33/79) of the tested FFPE sections. *BRAF* V600 mutations were not detected in the remaining 58.2% (46/79) of samples (data not shown). The mutation rate in men was 40.5% and that in women was 43.2%, suggesting no sex difference in the mutation rates. The mutation rate was more than 60% in patients aged less than 60 years (Table 2).

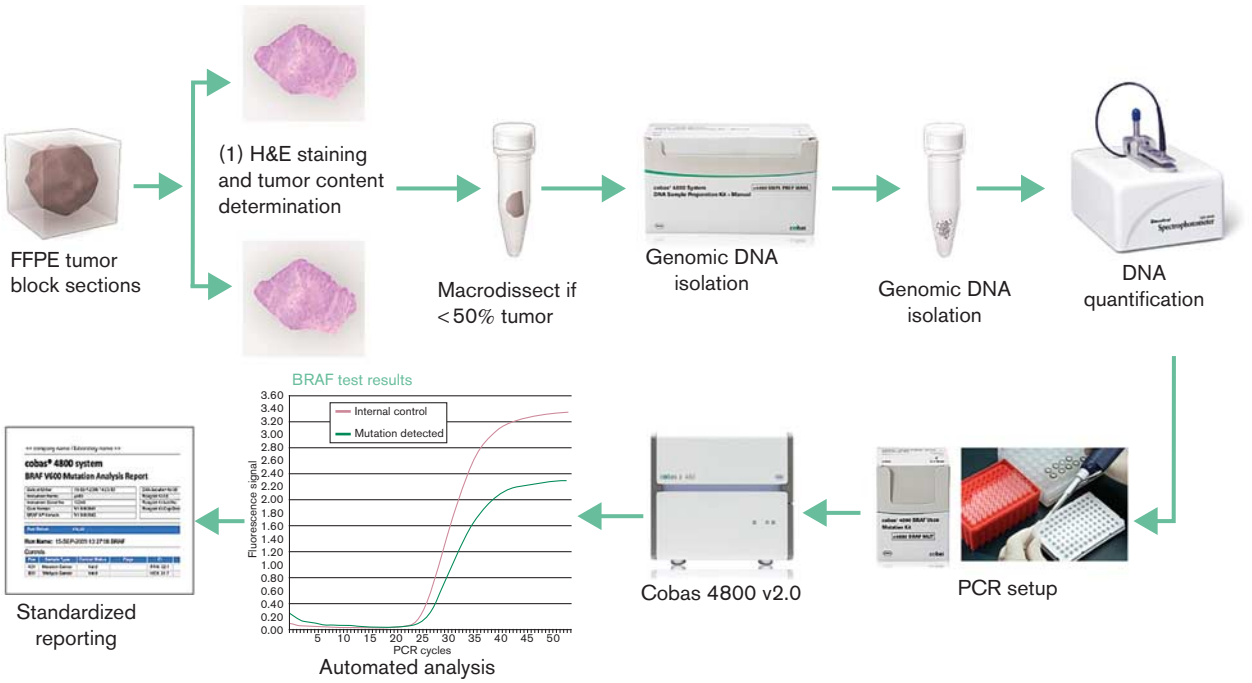
BRAF V600 mutation status stratified by melanoma subtype

When mutations were stratified by melanoma subtype, *BRAF* V600 mutations were detected in 18.8% (6/32) of the ALM samples, 64.7% (22/34) of the SSM samples, 50.0% (4/8) of the NM samples and 20.0% (1/5) of the LMM samples (Table 3).

BRAF V600 mutation status stratified by melanoma stage

Of the 79 patients, 41 were classified as having melanoma stage III/IV disease (Table 4). Although the overall mutation rate was relatively low (18.8%) among ALM patients, when the mutation rate was stratified by subtype for those with stage III/IV disease, 36.4% (4/11) of ALM samples were *BRAF* V600 mutation positive compared with 65.2% (15/23) of SSM samples, 66.7% (4/6) of NM samples and all (1/1) LMM samples (Table 4). Thus, although ALM was still the melanoma type with the lowest frequency of *BRAF* V600 mutations, the relative frequency was twice as high and much closer to those in other melanoma types with stage III/IV disease. When stratified by early melanoma stage (I/II), the *BRAF* mutation frequency was only 9.5% in patients with early-stage ALM (Table 4). By contrast, there was little difference in the *BRAF* mutation frequency between late-stage (65.2%) and early-stage SSM (63.6%).

Fig. 1



Cobas 4800 BRAF V600 Mutation Test workflow. FFPE, formalin-fixed, paraffin-embedded; H&E, haematoxylin and eosin. Reproduced with permission from [19]. Copyright Lippincott Williams & Wilkins Philadelphia, Pennsylvania, USA.

Table 2 Overall *BRAF* mutation status of sample cohort by sex and age

	Number of patients ($n=79$)	MD (n)	MND (n)	MD (% sex)
Male	42	17	25	40.5
Female	37	16	21	43.2
Age (years)				
<50	15	10	5	66.7
50–60	11	7	4	63.6
60–69	24	10	14	41.7
>69	28	6	22	21.4
Unknown	1	0	1	0.0

MD, mutation detected; MND, mutation not detected.

Table 3 *BRAF* mutation rate by melanoma subtype

	Number of patients ($n=79$)	MD (n)	MND (n)	MD (% of subtype)
ALM	32	6	26	18.8
SSM	34	22	12	64.7
NM	8	4	4	50.0
LMM	5	1	4	20.0

ALM, acral lentiginous melanoma; LMM, lentigo maligna melanoma; MD, mutation detected; MND, mutation not detected; NM, nodular melanoma; SSM, superficial spreading melanoma.

Table 4 *BRAF* mutation status at disease stage I/II and III/IV

	Mutation status at disease stage I/II			Mutation status at disease stage III/IV		
Subtypes	Number of patients ($n=38$)	MD (n)	MD (% of subtype)	Number of patients ($n=41$)	MD (n)	MD (% of subtype)
ALM	21	2	9.5	11	4	36.4
SSM	11	7	63.6	23	15	65.2
NM	2	0	0.0	6	4	66.7
LMM	4	0	0.0	1	1	100.0

ALM, acral lentiginous melanoma; LMM, lentigo maligna melanoma; MD, mutation detected; MND, mutation not detected; NM, nodular melanoma; SSM, superficial spreading melanoma.

Table 5 Positive *BRAF* mutation observed by site

	% MD in site
Head and neck	61.9
Upper limb	40.0
Trunk	86.7
Lower limb and hip	64.3
Sole, heel and foot	18.8

MD, mutation detected.

Association between the *BRAF* V600 mutation and melanoma location

Finally, the relationship between *BRAF* V600 mutations and the location of melanomas in Japanese patients was established. *BRAF* V600 mutations were commonly observed in the trunk (86.7%), lower limb and hip (64.3%), and the head and neck (61.9%), but were less frequent in the upper limbs (40%) and sole, heel and feet (18.8%) (Table 5).

Discussion

In the present study, we investigated the frequency of *BRAF* V600 mutations in Japanese patients with melanoma. We found that *BRAF* V600 mutations occurred in only 41.8% of melanomas, which is slightly lower than the percentage (49.7%) of melanomas bearing *BRAF* V600 mutations observed in a study of European and US melanoma patients [26] and higher than in a previous study of Japanese melanoma cases [22]. Interestingly, although the frequency of *BRAF* mutations in the current study was lower (26%, 9/35) than that in the USA or Europe, it was similar to the rate in Chinese melanoma patients (25.5%, 110/432) [27]. Other studies of Asian melanoma patients have reported lower *BRAF* mutation rates in melanomas of Korean patients (11.9%, 24/202) [28] and Chinese Han (14.7%, 16/109) [29], indicating a lower incidence of *BRAF* mutations in melanomas of Asian individuals.

A number of studies have shown that ALMs have a lower *BRAF* mutation frequency than do SSMs (reviewed by Platz *et al.* [10]). For example, a study in European and US melanoma patients showed that 58.1% of SSM samples had *BRAF* V600 mutations, whereas only 16.7% of ALM samples did [26]. In this study, we found a similar *BRAF* V600 mutation rate in each subtype (ALM 18.8%, SSM 64.7%). This study investigated the *BRAF* V600 mutation rate of primary melanomas. A recent study investigating differences in the incidence of the *BRAF* V600 mutation in primary and metastatic melanoma showed that *BRAF* status was related to metastatic burden and poor prognosis, and therefore, could be used to identify stage III melanoma patients [30]. This indicated that *BRAF* mutations might be involved in the spread of the primary melanoma to distant sites. However, because small sample numbers were used, these results ($n = 72$) and ours ($n = 79$) should be confirmed by future studies using larger numbers of patients.

A previous study showed that ALM was more prevalent than SSM in Japan (~45 vs. ~25%, respectively) and that the ratio of Japanese men to women with melanoma ranged from 1 : 0.97 to 1 : 1.1 [23], similar to that observed in the current study (1 : 0.88). In this study, 35 finger specimens with ALM were ineligible, requiring additional acid treatment by the investigator to soften the bones. A diagnosis of melanoma is made on the basis of pathological features; thus, acid treatment was performed on the FFPE samples, especially ALM. Unfortunately, the acid treatment caused DNA fragmentation and the samples were unsuitable for DNA testing. Therefore, the ratio of ALM decreased, and so we observed that the prevalence of ALM was similar to that of SSM (40.5 and 43.0%, respectively). We considered this to be one reason why we observed a similar *BRAF* V600 mutation rate to that in Europe and the USA. These 35 finger samples were decalcified and therefore were not appropriate for the detection of oncogenes. Thus, the samples for oncogene detection need to be managed carefully.

In the current study, when the mutation rate was stratified by melanoma subtype for those with late-stage melanoma (stage III/IV), 36.4% (4/11) of ALM tumours were found to be *BRAF* V600 mutation positive. Interestingly, the *BRAF* mutation frequency was only 9.5% in patients with early-stage ALM (Table 4). By contrast, there was little difference in the *BRAF* mutation frequency between late-stage (65.2%) and early-stage SSM (63.6%) (Table 4). To avoid missing *BRAF* V600 mutations in late-stage melanoma, we recommend performing a second biopsy.

This study had some limitations. The study had a relatively small sample size ($n = 80$) and therefore might not be reflective of the Japanese population as a whole, possibly explaining why the frequencies of SSM and ALM were similar, in contrast to previous studies.

Future studies should be carried out to determine whether the majority of V600 mutations are V600E or are other types of V600 mutations such as V600K or V600D, which might distinguish these samples from other ethnic populations. Furthermore, the status of mitogen-activated protein kinase kinase kinase 8 (MAP3K8, COT) expression in patients with *BRAF* V600 mutations should be assessed to determine whether vemurafenib would be effective in the Japanese population or if resistance would occur rapidly [26]. Finally, a comparison of the Cobas 4800 BRAF V600 Mutation Test with other techniques to identify *BRAF* V600 mutations such as circulating free DNA by amplification refractory mutation testing system [31] or immunohistochemistry [32] should be performed.

In summary, with the emergence of molecular-targeted therapeutics, genetic insights such as those provided by the present study are expected to provide more effective treatment options for the melanoma patients harbouring these mutations. Specifically, analysis using the Cobas 4800 BRAF V600 Mutation Test showed an increased frequency of *BRAF* V600 mutations in late-stage melanoma patients, especially stage III/IV ALM, who might benefit from vemurafenib treatment.

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Conflicts of interest

N.Y. has received speaking fees from Chugai Pharmaceutical Co. Ltd, Bristol-Myers K.K.,

GlaxoSmithKline K.K. and Takeda Pharmaceutical Company Limited. T.O. and A.H. are salaried employees of Roche Diagnostics K.K. N.N. is a salaried employee of Chugai Pharmaceutical Co. Ltd. For the remaining authors there are no conflicts of interest.

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