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Comparative study on driver mutations in primary and metastatic melanomas at a single Japanese institute: A clue for intra- and inter-tumor heterogeneity

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ABSTRACT

Background: Searching for driver mutations in melanoma is critical to understanding melanoma genesis, progression and response to therapy.

Objectives: We aimed to investigate the frequency and pattern of driver mutations in Japanese primary and metastatic melanomas including cases of unknown primary origin, in relation to their clinicopathologic manifestations.

Methods: Seventy-seven samples from 60 patients with melanoma were screened for 70 driver mutations of 20 oncogenes by Sequenom MelaCarta MassARRAY, and the results for primary and metastatic melanomas were compared.

Results: Of 77 tissue samples, *BRAF* V600E was detected in 21 samples (27%), *CDK4* R24C in 7, *EPHB6* G404S in 6, *BRAF* V600K in 2, *NEK10* E379K in 2, and *CDK4* R24H, *NRAS* Q61K, *NRAS* Q61R, *KRAS* G12A, *KIT* L576P, *KIT* V559A, *ERBB4* E452K, and *PDGFRA* E996K in one sample each. No driver mutations related to the MAPK cascade including *RAS* and *BRAF* were detected in the chronically sun-damaged (CSD) group of melanoma. Dual or triple driver mutations were found in four of 40 (10%) samples from the primary melanomas, and three of 37 (8%) of the metastatic melanomas. Fourteen of 26 (54%) samples of non-CSD melanoma, and 3 of 6 (50%) melanomas of unknown primary origin had the *BRAF* V600E mutation. Mutations in membrane-bound receptors including *KIT*, *ERBB4* and *EPHB6* were detected in 8 of 77 (10%) samples. Of 17 pairs of primary and metastatic melanomas from the same patient, the primary mutation pattern was changed to a novel one in three cases, and only one of the plural mutations in the primary melanoma was found in the metastatic lesions in two cases.

Conclusions: BRAF V600E is a predominant mutation in non-CSD melanoma and melanomas of unknown primary origin. Mutational heterogeneity may exist in the primary melanoma (intra-tumor heterogeneity), and between the primary and metastatic lesions (inter-tumor heterogeneity).

Keywords: melanoma, driver mutation, primary, metastasis, mutational heterogeneity, *BRAF*

1. Introduction

Recent genome sequencing has revealed that cutaneous melanomas possess a greater mutation load than other solid tumors [1-3]. The mutations in cutaneous melanomas are characterized by a distinct pattern reflecting the frequent C > T mutations caused by misrepair of ultraviolet (UV)-induced covalent bonds between adjacent pyrimidines [1]. Among the mutations, it is important to distinguish the driver mutations that are directly related to the oncogenesis of melanoma from the passenger mutations without oncogenic significance. For instance, a driver mutation frequently observed in melanoma, *BRAF* V600E, does not appear attributable to direct UV-induced damage [2]. Actually, *BRAF* V600E mutation is frequently detected in non-chronically sun damaged (non-CSD) melanoma, and often found among younger patients [3]. From the clinical view point, the search for driver mutations offers therapeutic insights for patients with melanoma. The presence of the *BRAF* V600E mutation predict the efficacy of *BRAF* inhibitors such as vemurafenib and dabrafenib [4, 5]. While, both driver and passenger mutations of melanomas may provide neoantigens targeted by cytotoxic T lymphocytes (CTLs). In addition to CD8+ lymphocyte infiltration and the presence of mismatch repair deficiency, the mutation load correlates with the response of melanomas to immunotherapy[6-8].

Mutation patterns of melanomas are known to be related to the clinical phenotypes of chronically sun-damaged (CSD), non-CSD, acral, and mucosal melanomas in Caucasians [9,10], and the frequencies of melanoma phenotypes differ according to race [11]. The first aim of the present study was to investigate the frequency and pattern of driver mutations in Japanese patients with melanoma, and to address their relation to these various phenotypes. Although *BRAF*, *NRAS* and *KIT* mutations were examined in a large series of Japanese melanoma patients [12], we have employed more comprehensive method to analyze 70 point mutations in 20 putative melanoma oncogens[13].

One type of mutation known to activate the mitogen-activated protein kinase (MAPK) signaling pathway, mainly *BRAF* mutations, is frequently detected in early lesions of melanoma and even in benign melanocytic nevi [14]. Melanoma cells may gain additional driver mutations during progression; i.e., *TERT* promotor mutations in *in situ* lesions, biallelic inactivation of *CDKN2A* in

early invasive primary tumors , and *PTEN* and/or *TP53* mutations in advanced primary melanomas. Apart from this genomic evolution model, there has been a concept of mutational heterogeneity of carcinogenesis, in which the primary tumor is thought to be composed of several neoplastic subclones with different mutation patterns [15, 16]. The second aim of the present study was to prove the possibility that plural driver mutations exist in primary tumors, and that the initial mutation pattern can be altered in the metastatic lesion. Here we report that *BRAF* V600E is the predominant mutation in Japanese non-CSD melanoma patients, and that both intra- and inter-tumor mutational heterogeneities exist in primary and metastatic melanomas.

2. Material and methods

2.1 Patients and tissue samples

The present study was performed with the ethical board committee approval of Okayama University Hospital (No. 2139, 2014), and the patients' written informed consent were obtained. Sixty patients with melanoma were enrolled in the present study. According to the anatomical sites and clinical phenotypes of the primary lesions, the melanomas were classified into four groups: CSD melanoma, 5 cases; non-CSD melanoma, 26 cases; acral melanoma, 21 cases; unknown primary origin, 6 cases; and mucosal melanoma, 2 cases (Table 1). Seventy-seven samples were obtained from 60 patients with melanoma by surgical removal for therapeutic or diagnostic purposes. Of these 60 patients, primary cutaneous lesions were examined in 23 patients, the metastatic lesions in 20 patients (lymph node; 13 patients, lung; 4, and skin; 3), and both the primary and metastatic lesions in 17 patients.

2.2 Detection of driver mutations

Sections from formalin-fixed, paraffin-embedded tissue samples were assayed for driver mutations. Before extraction of DNA, we confirmed the percentages of melanoma cells included as more than 10% of the cellular components. Somatic mutations were screened using the Sequenom MassARRAY system (MelaCarta Panel v1.0 , Agena Bioscience, San Diego), which is able to

detect 70 driver mutations in 20 oncogenes including *BRAF*, *KIT*, and *NRAS* [13]. In brief, 20 ng of genomic DNA extracted from the tissue sections was used for polymerase chain reaction (PCR) amplification. A single base-pair extension reaction was performed using iPLEX Pro chemistry (Agena Bioscience, San Diego), and resin-treated samples were spotted onto SpectroCHIP II arrays (Agena Bioscience, San Diego). Mutant alleles were then distinguished via mass spectrometry (MassARRAY System, Agena Bioscience, San Diego). Mutations were detected by a minimum 5% threshold of the mutant allele peak, and allele peaks below the 5% threshold were designated as "mutation not detected" (MND). With the present array system, at least one mutation can be found in approximately 70% of all melanomas [17].

2.3 Comparison with the patients' clinicopathologic findings and mutation profiles

The profile of driver mutations was compared with the patients' clinical data and the melanoma subtypes. In order to address the genetic evolution of melanomas, the identities of somatic mutations in primary and metastatic melanomas of the same patient were compared.

2.4 Immunophenotyping of melanoma cells

Phenotypes of melanoma cells were examined by immunohistochemistry using specific antibodies to HMB45 (clone HMB45, DAKO, Glostrup), tyrosinase (clone T311, Leica Biosystems, Nussloch) and MART-1 (clone M2-7C10, COVANCE, Dedham) on an automated immunohistochemistry staining instrument, BenchMark® XT (Roche, Basel).

2.5 Statistical analysis

We analyzed the results using Fisher's exact test (JMP® 11, SAS Institute Inc, Cary, NC). Differences were considered significant when $P < 0.05$.

3. Results

3.1. The profile of driver mutations

Of 77 tissue samples from 60 patients, including both primary and metastatic melanomas, driver mutations were detected in 38 samples: *BRAF* V600E was detected in 21 samples (27%), *CDK4* R24C in 7, *EPHB6* G404S in 6, *BRAF* V600K in 2, *NEK10* E379K in 2, and *CDK4* R24H, *NRAS* Q61K, *NRAS* Q61R, *KRAS* G12A, *KIT* L576P, *KIT* V559A, *ERBB4* E452K, and *PDGFRA* E996K in one sample each (Table 1). *BRAF* mutations were observed more frequently in the younger patient groups (<49 years; 7/12, ≥50-69 years; 8/23, ≥70 years; 5/25) (Fig 1a). Among the different stages of melanomas, *BRAF* mutations were detected in 0%, 17%, 53% and 40% in the stage I, II, III and IV melanoma, respectively (Fig 1b).

Dual or triple mutations were found in four of 40 (10%) samples from the primary lesion (cases 8, 12, 35, 36 in Table 1), and three of 37 (8%) from the metastatic lesions (cases 35, 58, 59 in Table 1). No driver mutations, designated as “mutation not detected” (MND), were found in 28 of 60 (47%) patients.

3.2. Driver mutations in melanoma subtypes

Of 60 patients, 52 patients with primary cutaneous melanomas were classified into three subtypes: non-CSD; 26 patients, CSD; five patients, and acral; 21 patients. Of the remaining patients, six were classified as unknown primary origin, and two as mucosal melanoma.

Among the melanoma subtypes, *BRAF* mutations were mainly detected in patients with non-CSD melanoma and in the unknown-primary-origin group: 14 of 26 (54%), and three of six (50%), respectively (Fig 1c). The frequency of *BRAF* mutations in the non-CSD group was significantly higher than that in the CSD group (non-CSD; 14/26, CSD; 0/5, $P = 0.0482$). There was no clear difference in the frequency of *BRAF* mutations between the primary and metastatic melanomas (primary; 10/40, metastasis; 12/37, $P = 0.6144$) (Fig 1d).

Mutations in molecules related to the MAPK signaling pathway, including *NRAS*, *KRAS* and *BRAF*, were detected in 26 of 77 (34%) samples, although no such mutations were detected in the

CSD melanoma in our series. Mutations in membrane-bound receptors including *KIT, ERBB4* and *EPHB6* were detected in seven of 60 (12%) patients: two (8%) of 26 patients with non-CSD melanoma, four of 21 (19%) patients with acral melanoma, and one of six (17%) patients with unknown origin melanoma. There was no clear difference in frequency or melanoma subtype between patients with mutations in the MAPK signaling pathway and membrane receptors.

3.3. Mutational heterogeneity in the primary melanoma

Of 40 patients with primary melanoma examined, plural driver mutations were observed in the same primary melanoma from four patients (10%) (Table 1): namely, *NRAS* Q61R + *EPHB6* G404S mutations (case 8; 85 y.o., non-CSD, stage IIIC), *BRAF* V600E + *CDK4* R24C (case 35; 71 y.o., ALM, stage IIIB), *EPHB6* G404S + *KRAS* G12A + *NEK10* E379K (case 36; 66 y.o., ALM, stage IIIA), and *CDK4* R24H + *EPHB6* G404S (case 12; 55 y.o., non-CSD, stage IIA). In the metastatic lesions, three(9%) of 33 samples revealed two or three driver mutations: namely, *BRAF* V600E + *BRAF* V600K in case 35, *BRAF* V600E + *CDK4* R24C in case 58, and *EPHB6* G404S + *PDGFRA* E996K + *ERBB4* E452K in case 59. Therefore, mutational heterogeneity can exist in both primary and metastatic melanomas. There was no duplicate mutation involving both *RAS* and *RAF* genes, both of which are gene members of the MAPK signaling pathway.

3.4. Comparison of the driver mutations in the primary and metastatic lesions in the same patient.

In 17 patients (Table 2), both the primary and metastatic lesions were examined for driver mutations. Six of those (Cases 8, 15, 16, 22, 35, 36 in Table 2) revealed at least one driver mutation in both types of lesion. One patient (case 15) possessed the same *BRAF* V600E mutation in both the primary and metastatic lesions. In three cases, the driver mutation(s) in the primary lesions were changed to other mutation(s) in the metastatic lesions: *BRAF* V600E + *CDK4* R24C was changed to *BRAF* V600E + *BRAF* V600K in case 35; and *BRAF* V600E was changed to *CDK4* R24C in two patients (case 16 and 22). Furthermore, *BRAF* V600E mutation found in the primary tumor was no longer detected in the metastatic lesions in four patients (cases 5, 6, 34, and 41). In contrast, *BRAF*

V600E mutation present in the metastases was not detected in the primary tumors in two patients (cases 7 and 26). In two patients, one of the plural mutations in the primary melanoma survived in the metastatic lesions (cases 8, and 36). These results indicate inter-tumor heterogeneity in the mutations of melanomas. No certain therapeutic regimen was noted to account for the alteration of driver mutations in the group of patients with inter-tumor heterogeneity.

3.5 Immunophenotyping in the primary melanoma and the metastatic lesion with different driver mutations

No clear difference was observed in morphological findings or the expression of HMB-45, MART-1 or tyrosinase in cases with different driver mutations in the primary and metastatic lesions (cases 8, 16, 35 and 36). (Fig. S1)

4. Discussion

Consistent with the results in previous study [12], the present study revealed that *BRAF* mutations such as *BRAF* V600E and *BRAF* V600K were predominantly observed in the non-CSD melanoma (54%), especially in the younger patient group (Fig 1a). No *BRAF* or *RAS* mutation was detected in the tissue samples from the CSD melanoma. Previous studies have shown that, in general, the non-CSD melanomas occur earlier in life and have lower mutation burdens, frequently bearing the *BRAF* V600E mutation [14]. In contrast, the CSD melanomas usually occur on the head and neck areas of the elderly, and are characterized by the presence of higher mutation burdens including *NRAS*, *NF1*, *KIT* and *BRAF* non-V600E. Our study also found that the *BRAF* V600E mutation is predominant in non-CSD melanomas; mutations other than *BRAF* V600E were detected in the CSD melanomas.

Three of 6 (50%) melanoma samples from the unknown primary origin revealed the presence of the *BRAF* V600E mutation in our series. Therefore, as reported previously in Caucasian patients [18], the mutation pattern of this group is similar to that of the non-CSD melanoma group, suggesting that

a significant proportion of these cases arise from regressed or unrecognized primary cutaneous melanomas.

It is noteworthy that *EPHB6* G404S mutation, which was not highlighted in the recent report of The Cancer Genome Atlas database (TCGA) [19], is the third common mutation (five of 60 patients) detected in our series. Since three of five patients with *EPHB6* G404S mutation were acral melanomas, the discrepancy between the TCGA and the present study might be explained by the higher proportion of acral melanoma in our series (35%). Interestingly, Jones et al [20] have recently reported significant difference of *EPHB6* G404S mutation rates in the North Island versus South Island of New Zealand (7.8% vs 0%), and speculated that the difference depends primarily on environmental risk factors, namely, differences in intermittent sun exposure or type of UV radiation. Although mutations in *EPHB6* have been observed recurrently in other cancers such as non-small cell lung cancer, the functional consequence of the G404S mutation is currently unknown [21]. Etiology and significance of this mutation need to be investigated in future studies. Melanomas harboring *NF1* mutations are classified into one of the representative genomic subgroups [19]. Unfortunately, however, our array system used for the present study is not designed for detection of the *NF1* mutations.

With the present array system, no driver mutation (the MND group), designated as the wild type (WT) elsewhere, was found in 28 of 60 (47%) patients, the frequency of which was rather high as compared with the previous data using the same method [17]. In order to explain this, we should consider the lack of sensitivity of our array system or the scanty amount of tumor cell-derived DNA. As we expected, the percentages of the MND were high in the stages I and II diseases, as compared with those in the stages III and IV diseases (Fig 1b). We believe that the high frequency of the MND group in our series might be related to the small tumor burden in the tissue sections obtained from the patients with the stages I and II, and irrelevant to the mutational evolution of melanoma cells in progression of the disease.

It has been reported that *BRAF* mutations can be detectable in the early melanoma, and even in melanocytic nevi [14]. Our study, however, indicates that *BRAF* mutations were absent or detected

in a low frequency in the stages I and II melanoma (Fig 1b), while such mutations were more frequently detected in the younger group of patients (Fig 1a), and in the certain subtypes such as non-CSD melanomas and melanomas of unknown primary origin (Fig 1c). Since the average age of patients with non-CSD melanoma (58.1 years) was younger than that of other patient groups including CSD, acral and mucosal melanomas (69.4 years), the clinical subtype of non-CSD melanoma might influence the association of *BRAF* mutations more strongly than the patient's age.

It is intriguing to note that two or more driver mutations were detected in four of the 40 (10%) primary melanomas. Mutational heterogeneity in the primary tumor was previously observed in melanoma by Sensi et al [22], and Eriksson et al [23]. Furthermore, our comparative study of driver mutations between the primary and metastatic lesions provides evidence that only one of several driver mutations in the primary lesions was selected in the process of metastasis in two patients (*NRAS* Q61R in case 8 and *EPHB6* G404S in case 36). These results are consistent with the concept of intra- and inter-tumor heterogeneity observed previously [24-27], and clearly indicate that there exist distinct neoplastic subclones harboring different driver mutations within the primary melanoma tumor, some of which may give rise to metastases [15,16].

Since *BRAF* and *NRAS* mutations are found both in melanoma and melanocytic nevus, it is postulated that these mutations are a founder event in melanomagenesis [28]. However, actual carcinogenic pathways from melanocyte to melanoma are thought to be more complex [9]. We also observed in paired samples of primary tumor and metastasis from the same patient that in six out of 17 (35%) patients with *BRAF* V600E mutation found in primary tumor was either not detected (cases 5,6,34 and 41) or was changed to *CDK4* R24C mutation (cases 16 and 22). This indicates that minor subclones without having *BRAF* V600E mutation were present in the primary tumor and evolved into metastasis. On the other hand, in cases 7 and 26, *BRAF* V600E mutation was detected only in metastasis, suggesting that acquisition of this mutation occurred later after the divergence of metastatic subclone. Thus, our observations strongly suggest that in a substantial number of patients, *BRAF* V600E mutation is not a founder mutation in melanoma development, and highlight the complex intra- and inter-tumor heterogeneity of melanoma. Alternatively, we should consider the

possibility that the low amount of melanoma cell-derived DNA in the tested samples gave a negative result for *BRAF* V600E mutations.

Intra-tumoral heterogeneity is a matter of concern for the treatment of melanoma, because the remnant of subclones resistant to the initial therapy can lead to disease relapse and metastasis. Actually, *BRAF* inhibitors elicit rapid antitumor responses in the majority of melanoma patients with the *BRAF* V600E mutation, but drug resistance occurs within several months [4]. Recent observations on cell-mediated immune responses against melanomas indicate that a high clonal neoantigen burden is associated with dense infiltration of effector T cells, and longer progression-free survival [29, 30]. Although we still do not know whether the therapeutic regimens such as radiation and cytotoxic chemotherapy are a driving force to induce selection of a certain melanoma clone, we should pay attention to the selection of subclones with metastatic potential, and to the genetic evolutionary processes involved in the introduction of additional mutations. As far as we studied, it seems difficult to know the genomic heterogeneity by routine morphological findings or immunophenotyping (Fig. S1).

In conclusion, *BRAF* V600E is a predominant mutation in non-CSD melanoma. Mutational heterogeneity may be present within the primary melanomas, and also occur between the primary and metastatic melanomas.

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Table 1. C**linical backgrounds of 60 patients and the driver mutations**

Case no.	Age	Sex	Site	Type	Stage	tumor thickness(mm)	Primary tumor	Mutant allele freque	Metastasis	Mutant allele freque
1	53	M	Abdomen	non-CSD	III B	2.3	NE		*BRAF* V600E	0.15
2	68	F	Arm	non-CSD	IV	5	NE		*BRAF* V600K	0.53
3	32	M	Thigh	non-CSD	IV	2.8	MND		MND	
4	70	F	Chest	non-CSD	II A	1.8	*BRAF* V600E	0.13	NE	
5	62	F	Lower leg	non-CSD	III C	3.4	*BRAF* V600E	0.14	MND	
6	55	F	Thigh	non-CSD	III B	3.3	*BRAF* V600E	0.12	MND	
7	34	F	Thigh	non-CSD	III C	1.2	MND		*BRAF* V600E	0.46
8	85	F	Arm	non-CSD	III C	12	*NRAS* Q61R *EPHB6*	0.32 0.16	*NRAS* Q61R	0.28
9	35	F	Thigh	non-CSD	II B	4.2	*BRAF* V600E	0.14	NE	
10	91	F	Chest	non-CSD	IV	5.9	NE		MND	
11	79	F	Arm	non-CSD	III B	4.2	NE		MND	
12	55	F	Shoulder	non-CSD	II A	2.2	*CDK4* R24H *EPHB6*	0.3 0.19	NE	
13	59	M	Abdomen	non-CSD	IV	5	NE		MND	
14	61	F	Arm	non-CSD	III B	2	NE		*BRAF* V600E	0.2
15	55	F	Arm	non-CSD	IV	1.9	*BRAF* V600E	0.39	*BRAF* V600E	0.36
16	64	M	Back	non-CSD	III B	4.2	*BRAF* V600E	0.15	*CDK4* R24C	0.06
17	59	F	Back	non-CSD	II A	3.5	MND		NE	
18	45	M	Back	non-CSD	IV	4.5	NE		*BRAF* V600E	0.08
19	71	M	Abdomen	non-CSD	III A	3	NE		MND	
20	59	M	Arm	non-CSD	I B	1.5	MND		NE	
21	62	F	Arm	non-CSD	I A	in situ	*CDK4* R24C	0.13	NE	
22	32	F	Back	non-CSD	III B	7.2	*BRAF* V600E	0.17	*CDK4* R24C	0.08
23	22	F	pople	non-CSD	IV	unknown	NE		*BRAF* V600E	0.49
24	55	M	Thigh	non-CSD	I B	1.3	MND		NE	
25	71	M	Arm	non-CSD	I A	0.9	*CDK4* R24C	0.06	NE	
26	77	F	pople	non-CSD	III B	1.3	MND		*BRAF* V600E	0.38
27	53	F	Lip	CSD	I B	1.9	*NEK10* E379K	0.38	NE	
28	59	F	Neck	CSD	IV	2.7	NE		MND	
29	51	F	Ear	CSD	II B	4.3	MND		NE	
30	92	F	Cheek	CSD	II C	4.6	MND		NE	
31	43	M	Head	CSD	I B	1.6	MND		NE	
32	77	M	Foot	acral	II B	3.6	MND		NE	
33	82	M	fifth toe	acral	IV	8	MND		MND	
34	72	M	Sole	acral	IV	15	*BRAF* V600E	0.14	MND	
35	71	M	Third toe	acral	III B	3.8	*BRAF* V600E *CDK4* R24C *EPHB6*	0.11 0.13 0.25	*BRAF* V600E *BRAF* V600K	0.17 0.12
36	66	M	Sole	acral	III A	0.7	G404S *KRAS* G12A	0.14 0.14	*EPHB6* G404S	0.12
37	56	M	First toe	acral	III B	5.5	MND		MND	
38	78	F	Sole	acral	IV	5	NE		*NRAS* Q61K	0.56
39	66	M	Sole	acral	II A	2.2	*EPHB6* G404S	0.26	NE	
40	75	F	Sole	acral	II B	2.9	MND		NE	
41	85	F	Sole	acral	III C	6.1	*BRAF* V600E	0.12	MND	
42	80	F	Sole	acral	III A	0.9	MND		MND	
43	84	F	Heel	acral	II B	2.2	*KIT* L576P	0.16	NE	
44	39	F	First finger	acral	II C	5.5	MND		NE	
45	42	F	First finger	acral	I A	0.2	MND		NE	
46	74	F	Sole	acral	I A	0.5	MND		NE	
47	93	M	Sole	acral	III B	4.9	MND		MND	
48	69	M	Sole	acral	I A	0.5	MND		NE	
49	80	F	Heel	acral	III B	6	*CDK4* R24C	0.05	NE	
50	94	F	Heel	acral	II C	10.3	MND		NE	
51	59	M	Heel	acral	III C	11	*KIT* V559A	0.8	NE	
52	50	M	First finger	acral	IV	0.5	NE		MND	
53	84	F	Conjunctiva	mucosal	IV		NE		MND	
54	70	F	Vulva	mucosal	IV		NE		MND	
55	61	M	Unknown	unknown	IV		NE		*BRAF* V600E	0.24
56	48	F	Unknown	unknown	IV		NE		MND	
57	48	M	Unknown	unknown	IV		NE		*BRAF* V600E	0.1
58	45	F	Unknown	unknown	IV		NE		*BRAF* V600E *CDK4* R24C	0.12 0.16
59	76	M	Unknown	unknown	IV		NE		*EPHB6* G404S *PDGFRA* E996K	0.33 0.08 0.12
60	82	M	Unknown	unknown	IV		NE		MND	

CSD, chronically sun-damaged melanoma; MND, mutation not detected; NE, not examined.

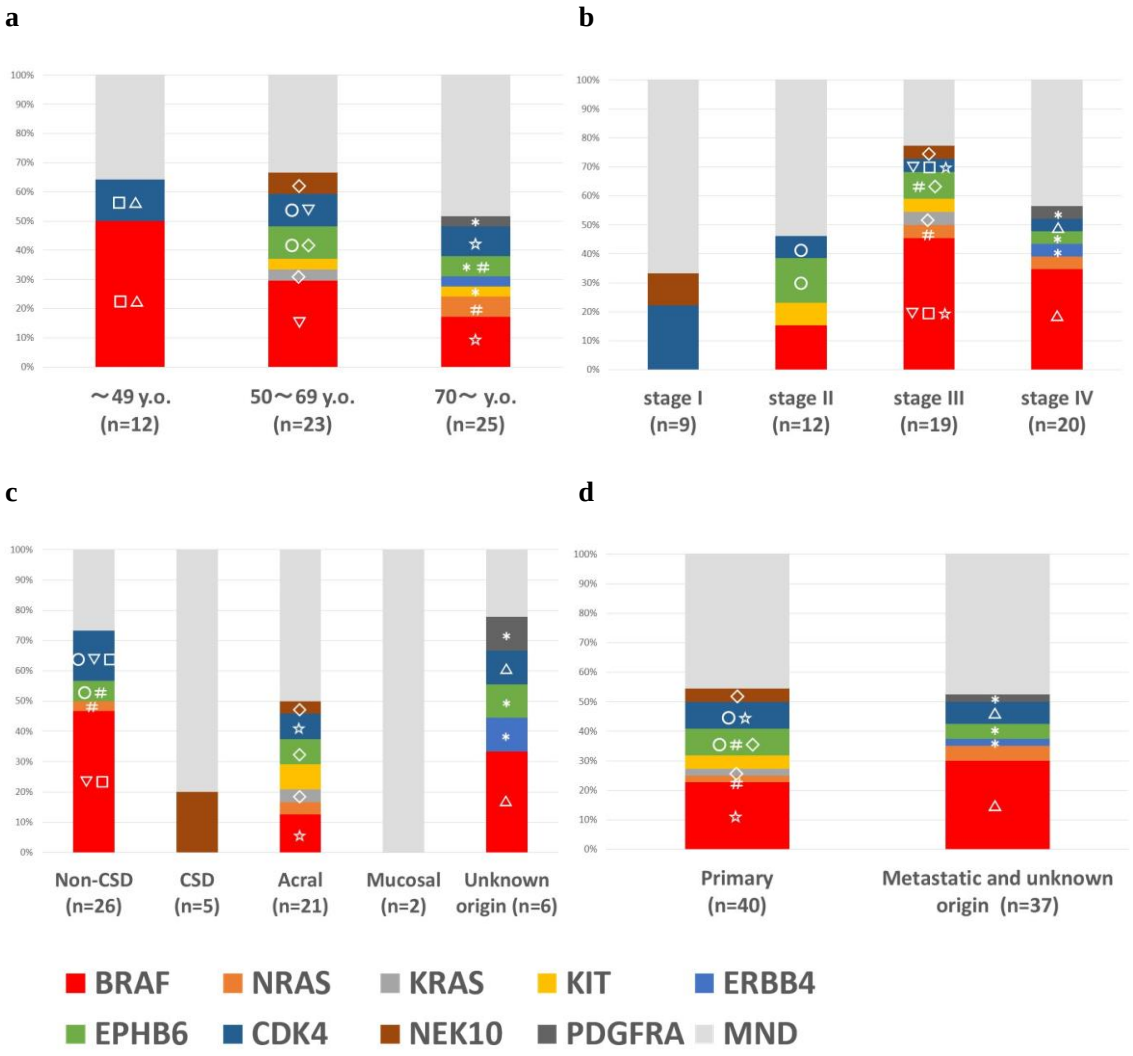
Table2. Comparison of driver mutations in the primary and metastatic lesions

Case no.	Primary tumor	Metastasis	site of metastasis	Treatment
8	*NRAS* Q61R *EPHB6* G404S	*NRAS* Q61R	lymph node	excision, LND, IFNβ
15	*BRAF* V600E	*BRAF* V600E	lymph node, lung, bone, ovary, peritoneum	excision, LND vemurafenib, nivolumab
16	*BRAF* V600E	*CDK4* R24C	lymph node	excision, LND
22	*BRAF* V600E	*CDK4* R24C	lymph node	excision, LND, IFNβ
35	*BRAF* V600E *CDK4* R24C	*BRAF* V600E *BRAF* V600K	lymph node	amputation, LND
36	*EPHB6* G404S *KRAS* G12A *NEK10* E379K	*EPHB6* G404S	lymph node	excision, LND, D-IFNβ 6 course, IFNβ
5	*BRAF* V600E	MND	lymph node	excision, LND, IFNβ
6	*BRAF* V600E	MND	lymph node	excision, LND, IFNβ
34	*BRAF* V600E	MND	lymph node, brain	excision, LND, DAC-Tam-IFN 3 course, IFNβ
41	*BRAF* V600E	MND	lymph node	excision, LN resection, vemurafenib
7	MND	*BRAF* V600E	lymph node	excision, LND, DAC-Tam-IFN 4 course, IFNβ
26	MND	*BRAF* V600E	lymph node	excision, LND, IFNβ
3	MND	MND	ymph node, lung, ski	excision, LND, D-IFNβ 6 course
33	MND	MND	lymph node, bone	amputation, LND, ipilimumab
37	MND	MND	lymph node	amputation, LND
42	MND	MND	lymph node	excision, LND, D-IFNβ 5 course, IFNβ
47	MND	MND	lymph node	excision, LN resection

MND, mutation not detected; LND, lymph node dissection; IFNβ, interferonβ local injection; D-IFNβ, DTIC intravenous injection + interferonβ local injection; DAC-Tam-IFN, DTIC,ACNU,CDDP intravenous injection + TAM oral treatment + interferonβ local injection.

Legends for figures

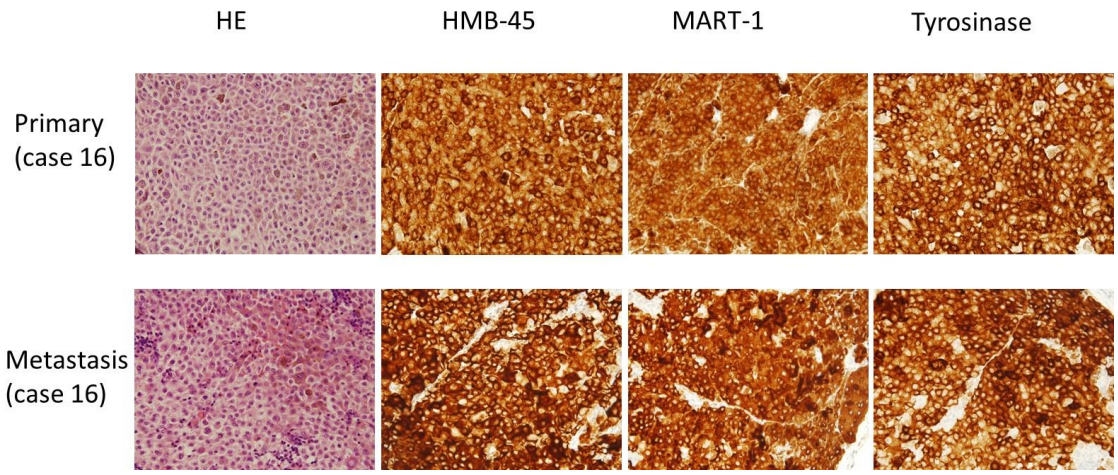
Fig 1. Percentages of driver mutations detected



BRAF mutations are detected in the younger patient group (< 49 years) more frequently than in the elderly group (a). *BRAF* mutation is absent in patients with the stage I disease, and less frequently observed in the stage II. In the stages III and IV, the relative frequencies of BRAF mutations are increased (the stages III and IV include 6 primary (Pr.) and 5 metastatic (Me.) lesions, and 2 Pr. and 7 Me. lesions, respectively) (b). *BRAF* mutations are frequently associated with non-CSD melanomas and melanomas of unknown primary origin. Note that the average age of patients with non-CSD melanoma (58.1 years) is younger than that of other patient group (69.4 years) (c). No clear difference is observed in frequency of *BRAF* mutations among the primary, metastatic and

unknown origin melanomas. (d). Eight cases harbor a combination of mutations as follows: # NRAS Q61R+EPHB6 G404S, ○CDK4 R24C+EPHB6 G404S, ▽BRAF V600E+CDK4 R24C, □BRAF V600E+CDK4 R24C, ☆BRAF V600E+BRAF V600K+CDK4 R24C, ◇ EPHB6 G404S+KRAS G12A+NEK10 E379K, △BRAF V600E+CDK4 R24C, ＊EPHB6 G404S+PDGFRA E996K+ERBB4 E452K.

Legends for supplementary data (Fig S1)



No clear difference is observed in the morphologic findings or the expression of HMB-45, MART-1 or tyrosinase in cases with different driver mutations in the primary and metastatic lesions (case 16).