Detecting copy number alterations of oncogenes in cell-free DNA to monitor treatment response in acral and mucosal melanoma

Asuka Mikoshiba, Atsuko Ashida, Kaori Sakaizawa, Yukiko Kiniwa, Ryuhei Okuyama

Department of Dermatology, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto, 390-8621 Japan

Running title: Plasma copy number ratio as a melanoma marker

Key words: acral melanoma, circulating tumor DNA, copy number alteration, droplet digital PCR, mucosal melanoma

Conflict of interest disclosure statements: None declared.

Funding sources: This study was supported by JSPS KAKENHI Grant Number 18K1605400 (K.S.).

IRB approval status: Reviewed and approved by the Institutional Review Board of the Ethics Committee of the School of Medicine, Shinshu University; approval certificate #612.

***Correspondence**: Ryuhei Okuyama, Department of Dermatology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. Tel: +81-263-37-2647. FAX: +81-263-37-2646. E-mail: rokuyama@shinshu-u.ac.jp

Word count = 3499

Number of figures = 4, Supplemental Figures = 1

Number of tables = 1, Supplemental Tables = 1

Number of references = 25

Abstract

Background: Reliable biomarkers are necessary for assessment of treatment responses. Acral and mucosal melanomas are commonly associated with copy number (CN) alterations rather than specific point mutations, with CN alterations in *KIT*, *CDK4*, and *CCND1* occurring frequently. Cell-free DNA is released to peripheral blood by both normal and tumor cells, and therefore contains the same genetic alterations present in the source tumor.

Objective: To investigate the usefulness of detecting CN alterations in oncogenes in cell-free DNA for monitoring treatment response in acral and mucosal melanomas.

Methods: We isolated cell-free DNA from peripheral blood and assessed the CN alterations in the cell-free DNA. Using droplet digital PCR, we examined CN alterations of *KIT*, *CDK4*, and *CCND1* in tumors from 37 melanoma patients (acral, n = 27; mucosal, n = 10) and peripheral blood from 24 melanoma patients (acral, n = 17; mucosal, n = 7).

Results: CN gain was detected in at least one of the genes examined in 62.9% (17/27) of acral melanomas and 70% (7/10) of mucosal melanomas. CN gains were also detected in the plasma of some patients. Furthermore, plasma CN ratio

was correlated with clinical condition. This correlation was especially clear in patients with high CN ratios in tumors and high tumor burdens.

Conclusion: Plasma CN ratios may be useful for evaluating treatment responses in patients with acral and mucosal melanoma.

Introduction

Substantial progress has been made in the treatment of advanced melanoma. In particular, immune checkpoint inhibitors and small-molecule targeted medicines, have yielded remarkable long-term benefits. However, these agents have increased the complexity of treatment strategies, and accurate and reliable biomarkers are necessary for assessing treatment responses and predicting early recurrence. Serum lactate dehydrogenase (LDH) is closely associated with tumor burden in cancer patients, including melanoma patients [1], and is widely used as a marker during anticancer treatments. However, LDH levels are occasionally elevated nonspecifically.

Cell-free DNA is released from both normal cells and tumor cells in peripheral blood via a variety of mechanisms, including apoptosis and necrosis. Circulating tumor DNA (ctDNA), the fraction of cell-free DNA derived from tumor cells, contains the same genetic alterations present in the source tumor [2]. *EGFR* and *KRAS* mutations can be detected in the peripheral blood of patients with lung cancer, and alterations in ctDNA levels may represent novel biomarkers for evaluation of tumor features [3, 4]. In melanoma, ctDNA studies have focused on detecting point mutations in *BRAF* and *NRAS* [5, 6].

The genetic alterations in melanoma differ among ethnic groups. *BRAF* (50–60%) and *NRAS (*15–25%) mutations are common in Caucasians and are mostly observed on the trunk or extremities. In Asian populations, these mutations are less common, occurring in 20–30% (*BRAF*) and 7–10% (*NRAS*) of individuals [7-10]. This difference in mutation frequencies reflects a difference in the distribution of melanoma subtypes: *BRAF* and *NRAS* mutations are infrequent in acral and mucosal melanomas, which have different genomic landscapes from other types of melanoma, with a lower burden of point mutations and a greater prevalence of large-scale structural variants, including copy number (CN) alterations [11, 12]. Acral and mucosal melanomas have high frequencies of amplifications in genes such as *KIT*, *CDK4*, *CCND1*, *TERT,* and *PDGFRA* [8, 13, 14], and *KIT*, *CDK4*, and *CCND1* amplifications have been identified as crucial for the activation of the mitogen-activated protein kinase pathway in melanoma [14-16]. In acral and mucosal melanomas with *KIT* mutations and/or amplifications, the tyrosine kinase receptor KIT promotes proliferation [15, 17]. In addition, CDK4 and its homolog CDK6 form heterodimers with CCND1 and play a role in cell-cycle regulation. Consistent with this, dysregulation of the CDK4/6–CCND1 pathway increases proliferation in many types of cancers, including melanoma [16, 18]. Thus, CN

alterations of *KIT*, *CDK4,* and *CCND1* are associated with melanoma formation. Acral and mucosal melanomas represent approximately half of cases in Japanese and other Asian patients [19], warranting studies of these CN alterations in peripheral blood for the purpose of assessing tumor burden. In this study, we monitored plasma CN alterations of *KIT*, *CDK4*, and *CCND1* during treatment of patients with acral and mucosal melanomas, and sought to determine whether the measurements could be used to evaluate treatment response.

Materials and Methods

Patients and tumor tissue samples

We examined melanomas from 37 patients (acral, n = 27; mucosal, n = 10) enrolled between September 2013 and March 2017, and treated at the Department of Dermatology of Shinshu University Hospital. Of the 37 melanomas, 34 were primary lesions and three were metastatic lesions. Objective response to therapies was assessed by computed tomography (CT) using the Response Evaluation Criteria in Solid Tumors (RECIST; version 1.1) criteria. Normal skin samples that did not include melanoma cells were collected from the periphery of

tumor lesions and used as controls. This study was approved by the Ethics Committee of the Shinshu University School of Medicine (approval number: 612) and was conducted according to Institutional Review Board guidelines. All melanoma patients in the study provided written informed consent for the use of their tumor tissues and peripheral blood.

Mutation analysis of tumor lesions

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissues, followed by PCR amplification of the mutational hotspot areas (*BRAF* exon 15, *NRAS* exons 2 and 3, and *KIT* exons 11, 13, and 17) and sequencing as described previously [7, 10].

Immunohistochemical analysis of protein expression

Immunohistochemical analysis was performed using the Dako EnVision System (Agilent, Santa Clara, CA). Paraffin sections were dewaxed and incubated in 3% H_2O_2. Antigen retrieval was performed by autoclaving, and the sections were incubated with antibodies against KIT (Agilent), CDK4 (Cell Signaling Technology, Danvers, MA), or CCND1 (Thermo Fisher Scientific, Waltham, MA).

Immunostaining was graded according to an immunoreactivity score (IRS) calculated by multiplying a score based on the percentages of positive cells (0, negative; 1, 1–20%; 2, 21–50%; and 3, 51–100%) by a score based on staining intensity (1, weak; 2, moderate; and 3, strong). The final expression was evaluated as follows: negative (-), IRS 0; low (+), IRS 1 or 2; intermediate (++), IRS 3 or 4; and high (+++), IRS 6 or 9 [17].

Cell-free DNA extraction

Cell-free DNA was extracted from 2 mL aliquots of plasma using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, German). Plasma samples were obtained from 24 patients with melanoma (acral, n = 17; mucosal, n = 7) and 20 healthy control subjects. Blood samples (7 mL) were collected in tubes containing ethylenediaminetetraacetic acid and centrifuged at 1,900 *g* for 10 min; the supernatant was centrifuged a second time for 10 min at 16,000 *g* to separate plasma. Plasma samples were stored at -80°C until analysis.

CN analysis

CN of *KIT*, *CDK4*, and *CCND1* DNA was analyzed in tissue samples and cell-free DNA derived from plasma samples, and was quantified by droplet digital PCR (ddPCR; QX200 Droplet Digital PCR System; Bio-Rad, Hercules, CA). The gene encoding apolipoprotein B (*APOB*; Riken Genesis, Yokohama, Japan) was used as a reference [20].

Reaction mixtures (20 µL) contained ddPCR Supermix (Bio-Rad), amplification probes specific for *KIT*, *CDK4*, or *CCND1* (Riken Genesis), *APOB*, restriction enzyme (*Mse*I), and template DNA. Droplets were generated from 20 µL reaction mixtures using a QX200 Droplet Generator (Bio-Rad). PCR was performed in a C1000 Touch thermal cycler (Bio-Rad) using the following cycling conditions: 95°C for 10 min; 40 cycles of 94°C for 30 s; 58°C for 1 min; 98°C for 10 min; and a final hold at 4°C. Droplets were read using a QX200 Droplet Reader (Bio-Rad) and analyzed using the QuantaSoft software (Bio-Rad). Reactions were performed in duplicate.

CN was calculated as the ratio of *KIT*, *CDK4*, or *CCND1* DNA concentration to the reference gene concentration multiplied by 2 to account for the presence of two copies of each gene in a normal diploid genome. A CN ratio greater than the cutoff value was recorded as a CN gain.

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics software 24. CN ratios were compared between paired tissue samples using the Wilcoxon signed-rank test, and between the two individual groups using the Mann–Whitney *U*-test. Differences and correlations were considered significant when $p < 0.05$.

Results

Mutation and CN analysis in tissue samples

Clinical features, genetic alterations, and histological observations are summarized in Table S1. Point mutations of *BRAF*, *NRAS*, and *KIT* were mutually exclusive in all patients. In acral melanomas, *BRAF*, *NRAS*, and *KIT* mutations were present in 7.4% (2/27), 25.9% (7/27), and 11.1% (3/27) of cases, respectively (Fig. 1A). The frequencies of *BRAF* and *KIT* mutations were similar to those reported in previous studies, whereas *NRAS* mutations were more prevalent [7, 10]. Point mutations were not detected among patients with mucosal melanomas, except for an *NRAS* mutation in one case. More than half of the

patients (55.6% for acral and 90% for mucosal melanoma) did not have any of these point mutations.

Next, we investigated CN alterations in the *KIT*, *CDK4*, and *CCND1* in 37 melanoma tissues (acral, n = 27; mucosal, n = 10) and their corresponding normal tissues (the peripheries of the tumor lesions that did not contain melanoma cells: acral, n = 22; mucosal, n = 4). In 26 pairs of samples, CN ratios were significantly higher in the melanoma tissues (*KIT*, $p = 0.021$; *CDK4*, $p = 0.003$; and *CCND1*, $p = 0.038$; Fig. 1B). Cutoff values have not been established for CN ratios in normal tissues. To minimize the false positive rate, we defined the upper limit of CN ratios of normal tissue as the cutoff value: *KIT*, 2.80; *CDK4*, 2.70; and *CCND1*, 2.40.

Using these cutoff values, acral melanoma lesions had CN gains in *KIT, CDK4*, and *CCND1* in 29.6% (8/27), 29.6% (8/27), and 29.6% (8/27) of cases, respectively. Mucosal melanoma lesions had CN gains in *KIT*, *CDK4*, and *CCND1* in 60% (6/10), 20% (2/10), and 30% (3/10) of cases, respectively. All three patients with *KIT* point mutations (Cases 3, 4, and 7) had CN gains in *KIT* (Fig. 1A, Table S1). Fifteen of 37 patients had CN gains in a single gene, whereas some patients had CN gains in multiple genes: seven patients in two genes and

two patients in three genes, consistent with a previous report [13]. The overall frequencies of CN gains in more than one gene were 62.9% (17/27) in acral melanoma and 70% (7/10) in mucosal melanoma (Fig. 1C). The result indicated that CN gains were more common than specific point mutations in acral and mucosal melanomas.

Associations between CN ratios and protein expression levels

Next, to determine whether CN gains were related to increases in protein expression, we analyzed protein expression in tumor tissues. KIT was expressed in cell membranes and cytoplasmic regions, whereas CDK4 and CCND1 were expressed in nuclear and cytoplasmic regions (Fig. 2A). According to IRS, immunostaining was graded as negative (-), low (+), intermediate (++), or high (+++). Acral melanomas expressed KIT, CDK4, and CCND1 in 51.9% (14/27), 44.4% (12/27), and 55.6% (15/27) of cases, respectively. Mucosal melanomas expressed KIT, CDK4, and CCND1 in 37.5% (3/8), 12.5% (1/8), and 62.5% (5/8) of cases, respectively (Fig. 1A, Table S1).

CN ratios of *KIT* and *CDK4* were higher in IRS-high (+++) tumors than in IRS-negative (-) tumors (*KIT*, $p = 0.001$; *CDK4*, $p < 0.001$; Fig. 2B). On the other

hand, CN ratios for *CCND1* were not correlated with CCND1 protein expression. CN gains in *KIT* and *CDK4*, but not in *CCND1*, were associated with elevated protein expression in acral and mucosal melanomas.

Analysis of CN ratios in plasma samples

CN ratios of *KIT*, *CDK4*, and *CCND1* may have been altered in peripheral blood due to DNA release from tumor lesions in association with tumor progression, as evidenced by the observation that their CN was often increased in acral and mucosal melanoma lesions. In addition, based on the relationship between CN ratio and protein expression, CN gains in *KIT* and *CDK4* seemed to be connected with tumor formation. Therefore, plasma CN ratios may be helpful for disease monitoring. However, peripheral blood usually contains only a small amount of ctDNA. We examined CN ratios in cell-free DNA from the peripheral blood of 24 melanoma patients with higher CN ratios than the cutoff values described above (acral, n = 17; mucosal, n = 7) and 20 healthy controls. We set a cutoff for plasma CN ratio of each gene based on the maximum CN ratio in the controls (*KIT*, 2.09; *CDK4*, 2.06; *CCND1*, 2.43). The plasma CN ratios of *KIT* and *CCND1* were significantly higher in melanoma patients than in healthy controls (*KIT*, $p = 0.008$;

CCND1, $p < 0.001$; Fig. 3A). The plasma CN ratio of *CDK4* was elevated in only three patients, and no significant difference was detected between patients and controls ($p = 0.074$). Thus, plasma CN of *KIT*, *CDK4*, and *CCND1* seemed to be elevated in some melanomas.

The amount of ctDNA is closely correlated with tumor burden [5, 6], and plasma CN gains may reflect tumor progression. Melanoma patients with organ metastasis (except regional LN metastasis) had higher ratios of plasma CN of at least one of three genes than patients without organ metastasis ($p = 0.008$, Fig. 3B), suggesting that the plasma CN ratio reflects tumor burden and that oncogene DNA fragments are released into the peripheral blood from tumor tissue in association with tumor progression. Furthermore, in cases with organ metastasis, patients with tumor CN ratios >5 tended to have higher ratios of plasma CN than those with tumor CN ratios ≤5 ($p = 0.042$, Fig. 3B). These results suggest that plasma CN ratios are useful for monitoring tumor progression in patients with high CN ratios in tumor tissues.

Monitoring of CN ratios in plasma samples

We examined plasma CN ratios in 24 patients and progression of plasma CN ratios during treatment in 16 of these patients to determine whether plasma CN ratios reflect tumor progression. At first, we focused on Cases 7, 11, and 14, who had organ metastasis with tumor CN ratios >5 (Table 1).

Case 11 was a 66-year-old male with a melanoma on his sole; the CN ratio of *CDK4* was 16.90 in the primary lesion (Fig. 4A). He presented with multiple metastases and was treated with a combination of ipilimumab and nivolumab (Day 0). Although treatment was canceled after the second administration due to drug-induced liver dysfunction on Day 22, tumor response was classified as stable disease (SD) on Day 42 and maintained this status on Day 91, as determined by CT imaging. After combination therapy, the plasma CN ratio of *CDK4* decreased from 4.97 (Day 1) to 1.79 (Day 44) and remained in the normal range thereafter (2.01 on Days 86 and 119). Similarly, LDH levels (normal range, <230 IU/L) were reduced after the therapy. In this case, CN ratios and LDH levels were well correlated with the treatment response.

Case 14 was a 51-year-old female with a melanoma on her sole; the CN ratio of *CCND1* was 8.83 in the primary lesion (Fig. 4B). The patient presented with multiple lung metastases and was treated with pembrolizumab (Day 0). Although

the tumor response was classified as progressive disease (PD) on Days 83 and 173 because of the new appearance of a metastasis in one lymph node, lung metastases were stable in CT imaging. Subsequently, new liver metastases developed on Day 334 and enlarged rapidly. The plasma CN ratios of *CCND1* remained around the normal upper limit until Day 281. Subsequently, the CN ratios increased from 2.93 (Day 355) to 6.41 (Day 425), along with the LDH level. Thus, plasma CN ratio seemed to reflect the clinical condition, because plasma CN ratios approximately paralleled tumor burden in the lung and liver.

Case 7 was an 87-year-old female with a melanoma on the nail apparatus; the CN ratio of *KIT* was 5.69 in the primary lesion (Fig. 4C). She presented with multiple metastases and was treated with nivolumab (Day 0). She responded well until Day 660, and her tumor status was classified as SD on Days 77~392, and partial response (PR) on Day 595. The plasma CN ratio of *KIT* increased slightly on Day 464, returned to a normal value on Day 560, and was stable until Day 651. LDH levels increased substantially on Day 464, reflecting tissue damage caused by endoscopic retrograde cholangiopancreatography rather than tumor burden, because CT imaging did not show tumor progression. Plasma CN ratio reflected tumor burden without significantly being affected by the tissue damage.

On the other hand, we did not observe plasma CN alteration in association with tumor burden in the cases with tumor CN ratios ≤5 even after tumor progression (6 cases) or in the absence of organ metastasis (7 cases) (Table 1). Case 3 was a 52-year-old female with a melanoma; *KIT* and *CDK4* CN ratios were 3.83 and 3.97, respectively (Supplementary Fig. 1A). She presented with multiple metastases and was treated with cisplatin (Day 0). However, multiple new metastases appeared, and tumor response was classified as PD on Day 75; however, plasma CN ratios were not significantly altered. In Cases 6 (tumor CN ratio: *CCND1*, 3.83) and 30 (tumor CN ratios: *CDK4*, 4.00 and *CCND1,* 2.97), the plasma CN ratios were not substantially altered, despite tumor enlargement and the appearance of new metastases (Supplementary Fig. 1B, C).

Thus, plasma CN ratios seemed to reflect tumor progression in cases with tumor CN ratios >5 but not with tumor CN ratios ≤5.

Discussion

In contrast to other subtypes of melanoma, acral and mucosal melanomas often harbor structural alterations in the genome. In this study, we focused on CN alteration of three oncogenes, *KIT*, *CDK4*, and *CCND1*. More than half of acral

and mucosal melanomas had CN gains in at least one of these genes. Furthermore, the CN gains in these genes were detectable in the peripheral blood of some melanoma patients. In particular, the plasma CN ratios of *KIT*, *CDK4*, and *CCND1* tended to be elevated in the patients who had organ metastasis with high CN ratios in tumor tissues, and seemed to reflect tumor burden to some extent. These results suggest that plasma CN ratio is helpful for evaluating tumor burden and treatment response in acral and mucosal melanomas.

Recent progress in the analysis of ctDNA and circulating tumor cells in blood samples has provided minimally invasive “liquid biopsy” surrogates that can give us important information about treatment response, recurrence, and drug resistance in cancer patients [2, 21]. In particular, tumor progression is frequently reflected by alterations in the peripheral blood levels of DNA fragments containing point mutations [3-6]. In melanomas, efforts to detect ctDNA have largely focused on point mutations in *BRAF* and *NRAS* [5, 6]. However, we cannot use point-mutated DNA as the marker in the majority of acral and mucosal melanomas. Accordingly, we focused instead on alteration of CN ratios in peripheral blood. Our results revealed that the plasma CN ratios of *KIT*, *CDK4*, and *CCND1* reflected tumor burden to some extent in the case of tumor cells with high CN

ratios of oncogenes. Similarly, plasma CN ratios of *HER2* seem to reflect tumor burden in gastric cancer [22, 23]. Although CT and other diagnostic imaging modalities are commonly used for assessment of treatment response and recurrence, they are usually performed every 2–3 months. On the other hand, plasma CN ratios can be evaluated more frequently and could therefore be useful for evaluating tumor condition in real time. The tumor CN ratio of at least one of the oncogenes (*KIT*, *CDK4*, and *CCND1*) was increased in more than half the patients with acral or mucosal melanomas, suggesting that it could have potential as a trigger for the initiation of early evaluations with imaging modalities and if required, a timely switch to more appropriate therapeutic interventions.

We found that monitoring of plasma CN ratios was useful for patients who had tumor CN ratios >5 with organ metastasis. Plasma CN ratios were altered in association with tumor burden in Cases 11 (tumor CN ratio: *CDK4*, 16.90), 14 (tumor CN ratio: *CCND1*, 8.83), and 7 (tumor CN ratio: *KIT*, 5.69). By contrast, plasma CN ratio was under the cutoff value in Case 9 (tumor CN ratio: *CDK4*, 20.10), who had lymph node metastasis but not organ metastasis (Supplementary Table 1). These results suggest that plasma CN ratio reflects tumor burden in cases with relatively large amounts of tumor but not in those with

small amounts. Because cell-free DNA in the peripheral blood includes DNA from tumor and normal cells, alteration of CN ratio is difficult to detect in the presence of large amounts of DNA from normal cells. Thus, the CN ratio is not highly sensitive at this moment. However, it will be helpful as a marker for evaluating organ metastasis in patients who have high CN ratios in tumor tissues. In addition, the high CN ratios of *KIT* and *CDK4* tended to be related to high levels of protein expression, suggesting that the CN ratio is helpful in acral and mucosal melanomas with high expression of KIT or CDK4.

LDH is widely used as a surrogate marker of tumor progression in melanoma [24]; elevated LDH level is associated with high disease burden and poor survival. However, its value as a biomarker is limited, as LDH occasionally remains within the normal range despite tumor progression. Furthermore, LDH increases in response to many conditions, such as infections and liver dysfunction. In Case 7, LDH was increased with endoscopic retrograde cholangiopancreatography whereas CT imaging did not detect tumor progression. The plasma CN ratio was more consistent with tumor progression on CT imaging than LDH. In addition, LDH levels are frequently increased in cases with adverse events caused by therapeutic medicines. By contrast, plasma CN ratio reflects tumor burden

without being significantly affected by other conditions [25]. For evaluation of the progression of melanoma, LDH measurements could potentially be made more clinically relevant if they were combined with assays for additional biomarkers, such as plasma CN ratios.

Our results suggest that the plasma CN ratios of *KIT*, *CDK4*, and *CCND1* provide information that could help monitor acral and mucosal melanomas in patients who have high CN ratios in tumor tissues, especially if the CN ratios are combined with LDH measurements. However, one limitation of the present study was the small number of patients. Further prospective analysis is required to assess the value of plasma CN ratio as a biomarker of clinical condition.

Acknowledgments

We thank Ms. A. Uchiyama and Ms. M. Ozawa for technical assistance and Professor M. Koinuma (Teikyo Heisei University) for support with statistical analysis.

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Figure legends

Fig. 1. Genetic alterations and protein expression levels in tissue samples of acral and mucosal melanomas. (A) Point mutations (*BRAF*, *NRAS*, and *KIT*), copy number (CN) alterations (*KIT*, *CDK4*, and *CCND1*), and immunoreactivity score (IRS) for KIT, CDK4, and CCND1. Samples were primary lesions (acral, n = 27; mucosal, n = 10) except in Cases 3, 13, and 36. CN ratios are as follows: (++), >5; (+), ≤5 and >cutoff; and (−), ≤cutoff. IRS is depicted as follows: (+++), IRS 6 or 9; (++), IRS 3 or 4; (+), IRS 1 or 2; and (−), negative. (B) CN ratios of *KIT*, *CDK4*, and *CCND1* in tumor and normal tissue. CN ratios were quantified using droplet digital PCR in tumor (acral, n = 27; mucosal, n = 10) and normal tissue (acral, n = 22; mucosal, n = 4). Horizontal dashed bars indicate cutoff values of tumor CN ratios (*KIT*, 2.80; *CDK4*, 2.70; and *CCND1*, 2.40). Significant differences were identified using paired Wilcoxon signed-rank test: $*p < 0.05$ and $***p < 0.005$. (C) Proportion of genetic alterations, including somatic mutations (*BRAF*, *NRAS*, and *KIT*) and CN gains (*KIT*, *CDK4*, and *CCND1*) in patients with acral and mucosal melanomas. IHC, immunohistochemistry.

Fig. 2. Immunohistochemical analysis of KIT, CDK4, and CCND1 in tissue samples. (A) Representative KIT, CDK4, and CCDN1 expression in Cases 31, 10, and 1, respectively. Scale bars, 100 μm. (B) Associations between copy number ratios and immunohistochemical results. Boxes indicate first and third quartiles, and horizontal lines show the median. Whiskers represent 1.5 times the distance between the first and third quartiles. Points outside the whiskers are considered outliers (◦) or extreme values (•). Horizontal dashed bars indicate cutoff values (*KIT*, 2.80; *CDK4*, 2.70; *CCND1*, 2.40). Significant differences were identified using Mann–Whitney *U*-test: $^{**}p < 0.01$; $^{***}p < 0.005$.

Fig. 3. Quantification of plasma copy number (CN) ratios in 24 melanoma patients (acral, n = 17; mucosal, n = 7) with tissue CN ratios that exceeded cutoff values. (A) Plasma CN ratios of *KIT*, *CDK4*, and *CCND1* were quantified using droplet digital PCR in melanoma patients (*KIT*, n = 12; *CDK4*, n = 10; and *CCND1*, n = 11) and healthy controls (n = 20). Horizontal dashed bars indicate cutoff values of plasma CN ratios (*KIT*, 2.09; *CDK4*, 2.06; and *CCND1*, 2.43). Significant differences were identified using Mann–Whitney *U*-tests: $^{**}p < 0.01$, $^{***}p < 0.005$, and ns, not significant. (B) Difference in plasma CN ratio between presence and

absence of organ metastasis. Significant differences were identified using Mann–Whitney *U*-test: *$p < 0.05$, **$p < 0.01$, and ns, not significant.

Fig. 4. Plasma copy number (CN) ratios in melanoma patients during the treatment. (A) Monitoring of plasma CN ratios of *CDK4* with clinical follow-up in Case 11. The graph shows the alteration of *CDK4* CN ratios and LDH levels. The green and black horizontal dashed bars indicate the upper limit of the CN ratio (2.06) and the normal range of LDH (230 IU/L), respectively. The arrowheads in CT imaging indicate intramuscular metastasis on Days -35 and 91 (upper panel). (B) Monitoring of plasma CN ratios of *CCND1* with clinical follow-up in Case 14. The graph shows the alteration of *CCND1* CN ratios and LDH levels. The red and black horizontal dashed bars indicate the upper limit of the CN ratio (2.43) and the normal range of LDH, respectively. The arrowheads in CT imaging indicate liver metastasis on Days 334 and 441 (upper panel). (C) Monitoring of plasma CN ratios of *KIT* with clinical follow-up in Case 7. The graph shows the alteration of *KIT* CN ratios and LDH levels. The blue and black horizontal dashed bars indicate the upper limit of the CN ratio (2.09) and normal LDH, respectively. The arrowheads in CT imaging indicate skin metastasis on Days 660 and 722 (upper

panel). PR, partial response; SD, stable disease; PD, progressive disease; and ERCP, endoscopic retrograde cholangiopancreatography.

Table 1. Monitoring of plasma CN ratios during treatment

Case	Sample	Time point (Day)†	Copy number ratio			RECIST	Treatment	Metastatic organs
			KIT	*CDK4*	*CCND1*			
3	Tissue		3.83	3.97	2.08			
	Plasma	75				PD	(-)	Lung, bone, brain, skin
		77	2.28	2.71			(-)	
		117				PD	(-)	Lung, bone, brain‡, skin, liver‡
		130	3.17	2.86			(-)	
		155	3.14	3.24			(-)	
		216	3.21	3.59		PD	(-)	Lung, bone, brain, skin‡, liver
6	Tissue		2.96	1.97	3.83			
	Plasma	-11			2.46		Niv	LN
		133			2.49		Niv	
		142				SD	Niv	LN
		339				SD	Niv	LN
		385			2.82		Niv	
		463				PD	Niv	LN‡
		570			2.49		Niv	
		591	2.14	1.83	3.03		Niv	
7	Tissue		5.69	2.07	1.68			
	Plasma	0	1.88				Niv	Lung, brain, liver, LN
		77				SD	Niv	Lung, brain, liver, LN
		119	2.07				Niv	
		175				SD	Niv	Lung, brain, liver, LN
		238	1.95				Niv	
		259				SD	Niv	Lung, brain, liver, LN
		392				SD	Niv	Lung, brain, liver, LN
		464	2.66				Niv	
		560	2.08				Niv	
		595				PR	Niv	Lung, brain, liver, LN
		651	2.09				Niv	
		660				PD	Niv	Lung, brain, liver, skin‡, adrenal gland‡, LN
		679	2.79				Niv	
		714	3.66	1.70			Niv	
		722				PD	(-)	Lung, brain, liver, skin, adrenal gland, mediastinum‡
11	Tissue		2.15	16.90	1.89			
	Plasma	-158		1.76			(-)	Lung, liver, intramuscular, LN
		1		4.97			Ipi +Nivo	
		42				SD	(-)	Lung, liver, intramuscular, LN
		44		1.79			(-)	
		86		2.01			(-)	
		91				SD	(-)	Lung, liver, intramuscular, LN
		119		2.01			(-)	
14	Tissue		2.49	2.60	8.83			
	Plasma	-15			2.44		(-)	Lung
		21			2.49		Pem	
		83				PD	Pem	Lung, LN‡
		112			2.42		Pem	
		173				PD	Pem	Lung, LN
		281			2.51		Pem	
		334				PD	Pem	Lung, liver‡, LN
		355			2.93		Pem	
		425			6.41		(-)	
		441				PD	(-)	Lung, liver, peritoneal cavity‡, LN
30	Tissue		2.64	4.00	2.97			
	Plasma	27		1.88	2.48		Niv	Lung, liver, LN
		52				PD	Niv	Lung, liver‡, LN
		143				PD	Niv	Lung, liver‡, LN
		231		1.54	2.58		Niv	
		234				PD	Niv	Lung, liver‡, LN
		267		1.34	3.18		Niv	

† Day 0, Starting day of the treatments. ‡ New metastasis.

Abbreviations: (-), no treatment; Ipi, ipilimumab; LN, lymph node; Niv, nivolumab; PD, progressive disease; Pem, pembrolizumab; PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumors; and SD, stable disease.

Fig. 1.

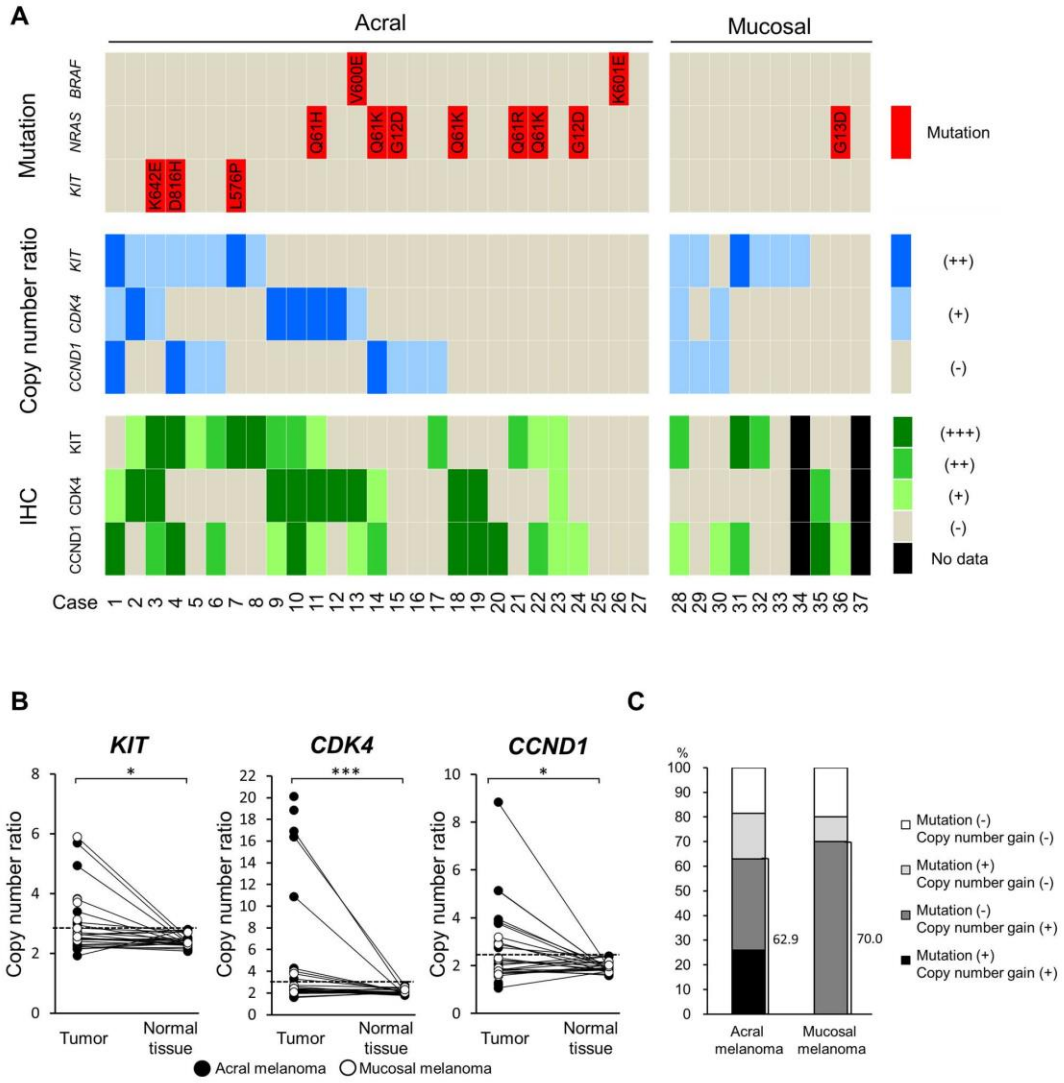

A
Acral
Mucosal
Mutation
BRAF
NRAS
KIT
V600E
K601E
Q61H
Q61K
G12D
Q61K
Q61R
Q61K
G12D
G13D
K642E
D816H
L576P
Mutation
Copy number ratio
KIT
CDK4
CCND1
(++)
(+)
(-)
IHC
KIT
CDK4
CCND1
(+++)
(++)
(+)
(-)
No data
Case 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37
B
KIT
CDK4
CCND1
*
***
*
Copy number ratio
Tumor
Normal tissue
Acral melanoma
Mucosal melanoma
C
%
62.9
70.0
Acral melanoma
Mucosal melanoma
Mutation (-) Copy number gain (-)
Mutation (+) Copy number gain (-)
Mutation (-) Copy number gain (+)
Mutation (+) Copy number gain (+)

Fig. 2.

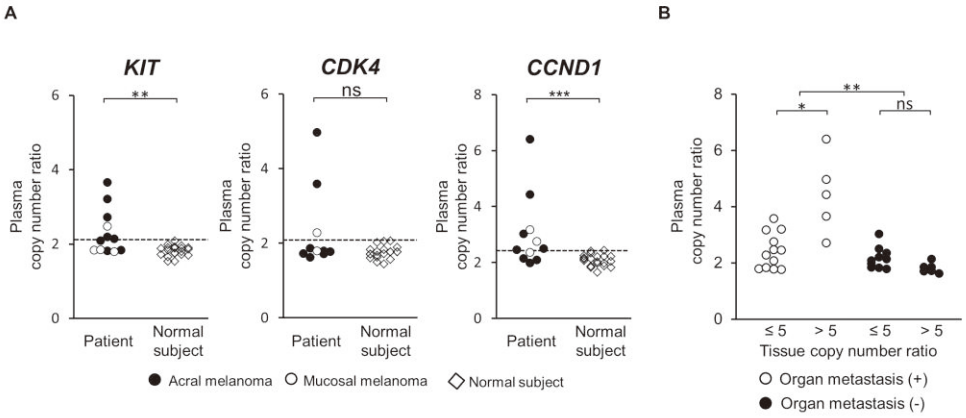

A
KIT
CDK4
CCND1
Plasma copy number ratio
Patient
Normal subject
Acral melanoma
Mucosal melanoma
Normal subject
B
Tissue copy number ratio
Organ metastasis (+)
Organ metastasis (-)

Fig. 3.

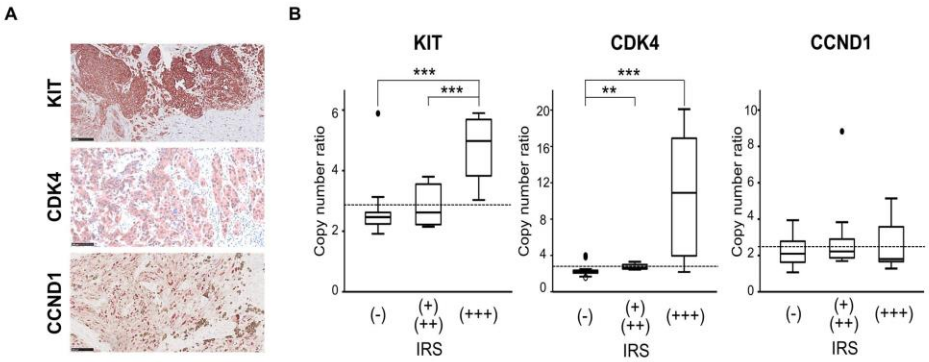
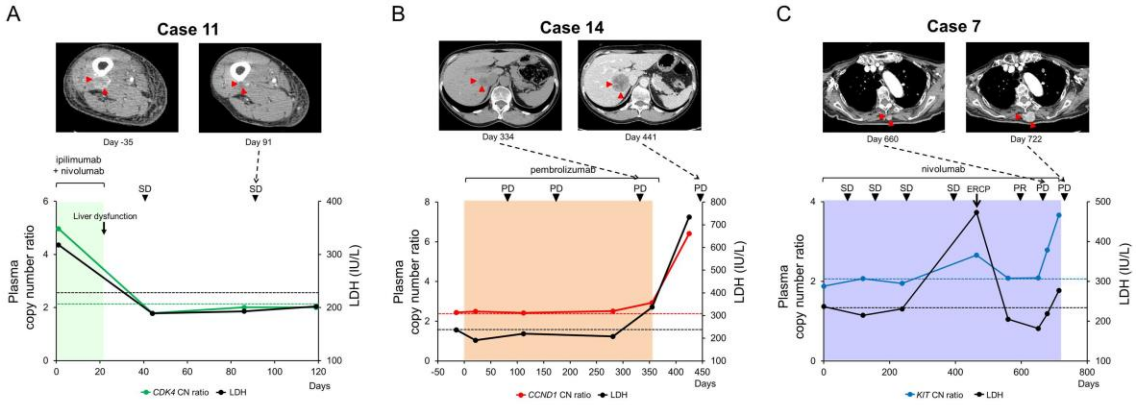

A
KIT
CDK4
CCND1
B
KIT
CDK4
CCND1
Copy number ratio
IRS

Fig.4.


A
Case 11
Day -35
Day 91
ipilimumab
+ nivolumab
SD
SD
Liver dysfunction
Plasma copy number ratio
LDH (IU/L)
Days
CDK4 CN ratio
LDH
B
Case 14
Day 334
Day 441
pembrolizumab
PD
PD
PD
PD
Plasma copy number ratio
LDH (IU/L)
Days
CCND1 CN ratio
LDH
C
Case 7
Day 660
Day 722
nivolumab
SD
SD
SD
SD
ERCP
PR
PD
PD
Plasma copy number ratio
LDH (IU/L)
Days
KIT CN ratio
LDH