CLINICAL REPORT

Circulating Tumour DNA for Monitoring Treatment Response to Anti-PD-1 Immunotherapy in Melanoma Patients

Atsuko ASHIDA, Kaori SAKAIZAWA, Hisashi UHARA and Ryuhei OKUYAMA
Department of Dermatology, Shinshu University School of Medicine, Matsumoto, Japan

Anti-programmed cell death-1 (anti-PD-1) antibody shows high therapeutic efficacy in patients with advanced melanoma. However, assessment of its therapeutic activity can be challenging because of tumour enlargement associated with intratumoural inflammation. Because circulating tumour DNA (ctDNA) correlates with tumour burden, we assessed the value of ctDNA levels as an indicator of tumour changes. Quantification of ctDNA (*BRAF*^{mutant} or *NRAS*^{mutant}) levels by droplet digital PCR in 5 patients with *BRAF* or *NRAS* mutant melanoma during the treatment course showed dynamic changes corresponding to radiological and clinical alterations. In 3 cases in which the anti-PD-1 antibody was effective, ctDNA levels decreased within 2–4 weeks after treatment initiation. In 2 cases in which the anti-PD-1 antibody was ineffective, ctDNA levels did not decrease after treatment initiation. ctDNA could be a useful biomarker to predict early response to treatment in patients with advanced melanoma treated with anti-PD-1 immunotherapy.

Key words: melanoma; circulating tumour DNA; anti-programmed cell death-1 antibody; BRAF; NRAS; droplet digital PCR.

Accepted Jul 5, 2017; Epub ahead of print Jul 6, 2017

Acta Derm Venereol 2017; 97: 1212–1218.

Corr: Ryuhei Okuyama, Department of Dermatology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. E-mail: rokuyama@shinshu-u.ac.jp

In the advanced stages, melanoma is among the most aggressive and treatment-resistant cancers and its incidence is increasing worldwide. Substantial progress has been made recently in the treatment of advanced melanoma. Immune checkpoint blockade is recommended as the first-line therapy for patients with metastatic or unresectable melanoma. Monoclonal antibodies against programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) showed remarkable long-term benefits in 40% and 10% of patients, respectively (1, 2). These antibodies act directly on the immune system, rather than on the tumour, and the kinetics of tumour regression may be delayed. In some cases, tumours assessed using computed tomography (CT) imaging appear to enlarge during therapy before regressing. In other patients, new tumours appear during therapy and later regress. The apparent tumour progression may, in some cases, reflect intratumoural inflammation rather than actual tumour growth. Despite considerable progress in the treatment of melanoma, reliable markers to predict treatment response or evaluate early recurrence are lacking. Non-invasive indicators of changes in tumour burden could provide early information about potential therapeutic outcomes, thereby preventing potentially serious immune-related adverse events in patients with true disease progression.

Serum lactate dehydrogenase (LDH) is widely used as a surrogate marker of tumour progression in melanoma (3). Elevated LDH level is associated with a high disease burden and poor survival. However, because LDH occasionally remains within the normal range despite tumour progression, its value as a biomarker is limited. In addition, LDH increases in response to many conditions, such as infections, liver dysfunction, and myocardial disorders among others, and in malignancies other than melanoma.

Circulating tumour DNA (ctDNA) in the peripheral blood is a novel biomarker for evaluating tumour features in patients with advanced cancer. Cell-free DNA is released from both normal and tumour cells via a variety of mechanisms, including apoptosis and necrosis. ctDNA contains the same genetic alterations present in the source tumour (4–6). In particular, ctDNA with mutations in *EGFR*, *KRAS*, or *BRAF* is detected in cancer patients, and the detection of mutations in ctDNA is under investigation as a specific biomarker for the diagnosis and monitoring of patients with different cancer types (7–11). Mutations of *BRAF* and *NRAS* are common in melanoma: the frequency of *BRAF* mutations, chiefly *BRAF*^{V600E}, is 50–60% in Caucasians and 20–30% in Asian populations (12–14); and that of NRAS mutations is 15–25% in Caucasians and 7–10% in Asian populations (14–16). Therefore, *BRAF*-mutated and *NRAS*-mutated ctDNAs are potentially useful biomarkers for melanoma.

In the present study, dynamic changes in ctDNA were analysed in parallel with measurement of LDH levels in melanoma patients during treatment with anti-PD-1 antibody. Droplet digital PCR (ddPCR) technology, which can be readily used to quantify mutant DNA copies, was used to examine changes in mutant ctDNA levels across different time-points. The results indicated that *BRAF*-mutated and *NRAS*-mutated ctDNAs are potentially useful biomarkers of treatment response to anti-PD-1 immunotherapy in patients with melanoma.

doi: 10.2340/00015555-2748
Acta Derm Venereol 2017; 97: 1212–1218

Table I. Characteristics of patients

Case	Age, years/ Sex	Primary site	Mutation	Metastatic organs at baseline	Pre-treatment of nivolumab	Post-treatment of nivolumab
1	63/F	Upper eyelid	*BRAF*^{V600E}	Brain, liver, skin, soft tissue, peritoneal cavity, and LN	Dacarbazine	Vemurafenib
2	47/F	Head	*BRAF*^{V600E}	Bone, skin, soft tissue, and LN	BRAF inhibitor (clinical trial)	Ipilimumab
3	74/F	Head	*BRAF*^{V600K}	Lung	(–)	Ongoing
4	51/M	Neck	BRAF^{V600E}	Pleural cavity, peritoneal cavity, and LN	DAC	(–)
5	83/M	Foot	*NRAS*^{Q61K}	Lung, liver, skin, and LN	(–)	(–)

LN: lymph node; DAC: dacarbazine, nimustine, and cisplatin.

PATIENTS AND METHODS

Cell culture

Four melanoma cell lines were used in this study: Mel-2 (*BRAF*$^{wild\text{-}type}$, *NRAS*$^{wild\text{-}type}$), A375 (*BRAF*^{V600E}, *NRAS*$^{wild\text{-}type}$), WM3282 (*BRAF*^{V600K}, *NRAS*$^{wild\text{-}type}$) and WM3406 (*BRAF*$^{wild\text{-}type}$, *NRAS*^{Q61K}). Mel-2 was established from Japanese patients, and the other lines were established from Caucasian patients. Mel-2 was obtained from Professor Y. Kawakami (Keio University School of Medicine, Tokyo). A375 was obtained from Professor T. Nagatani (Tokyo Medical University School of Medicine, Tokyo). Mel-2 and A375 were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2. WM3282 and WM3406 cells were purchased from Rockland (Limerick, PA, USA) and cultured in 80% MCDB153 and 20% Leibovitz's L-15 supplemented with 2% heat-inactivated foetal bovine serum and 1.68 mM $CaCl_2$ at 36°C in a humidified atmosphere of 5% CO_2. Cells were fed twice per week and passaged by exposure to 0.25% trypsin prepared in ethylenediaminetetraacetic acid (EDTA).

Patients and mutations in tumour specimens

Five patients with *BRAF*-mutated and *NRAS*-mutated melanoma who had metastatic disease were evaluated. Patients were treated with the anti-PD-1 antibody nivolumab at 2 mg/kg every 3 weeks. Nivolumab was followed by vemurafenib (BRAF inhibitor) 1,920 mg/day and ipilimumab (anti-CTLA-4 antibody) at 3 mg/kg every 3 weeks in Patients 1 and 2, respectively. CT imaging was performed at 2- or 3-month intervals, and tumour responses to treatment were evaluated using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (CR, complete response; PR, partial response; SD, stable disease; and PD, progressive disease) (17). Patient characteristics are summarized in **Table I**.

The patients were treated in the Department of Dermatology of Shinshu University Hospital. Diagnosis of melanoma was pathologically confirmed by haematoxylin and eosin staining, and the presence of mutations was confirmed in tumour tissues. In brief, genomic DNA was extracted from formalin-fixed, paraffin-embedded tumour specimens. Exon 15 of *BRAF* and exons 2/3 of *NRAS*, which include mutational hot spots, were amplified by PCR and sequenced (12, 16). All melanoma patients enrolled in the study provided written informed consent for the use of their peripheral blood and resected tumour tissues. Twenty-eight healthy subjects were studied as control samples. Previous data on the *BRAF*^{V600K} signal in the peripheral blood of healthy subjects were also included in the analysis (18). The study was approved by the Ethics Committee of the Shinshu University School of Medicine and conducted according to Institutional Review Board guidelines.

Cell-free DNA extraction and mutation analysis

Blood samples (7 ml) were collected in tubes containing EDTA. Within 2 h after collection, blood samples were centrifuged at 1,900 g for 10 min, followed by a further 10 min of centrifugation at 16,000 g to separate plasma, and then stored at −80°C until analysis. Cell-free DNA was extracted from 2 ml of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Tokyo). *BRAF*-mutated and *NRAS*-mutated ctDNAs were quantified by PrimePCR™ ddPCR™ mutation assay kit (Bio-Rad, Hercules, CA, USA: For manufacturer's information, see http://www.bio-rad.com/en-jp/product/primepcr-pcr-primers-assays-arrays). The reaction mixture (20 µl) contained ddPCR Supermix (Bio-Rad), amplification primer/probe mix specific for *BRAF* or *NRAS* (Bio-Rad), template DNA, and water. Droplets were generated from 20 µl of reaction mixture using a QX200 Droplet Generator (Bio-Rad). Next, droplets were transferred to PCR plates, and the PCR reaction was performed in a C1000 Touch thermal cycler

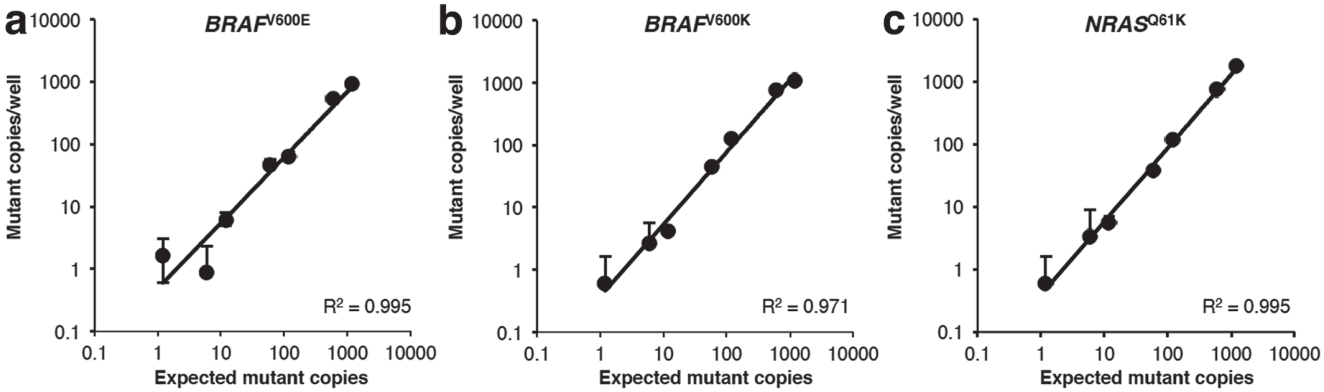

Fig. 1. Analytical sensitivity of droplet digital PCR (ddPCR). (a) Genomic DNA from the A375 cell line, which harbours the *BRAF*^{V600E} homogenous mutation, was serially diluted in a constant amount of genomic DNA from the Mel-2 cell line, which harbours wild-type *BRAF*. (b) Genomic DNA from the WM3282 cell line, which harbours the *BRAF*^{V600K} homogenous mutation, was serially diluted. (c) Genomic DNA from the WM3406 cell line, which harbours the *NRAS*^{Q61K} homogenous mutation, was serially diluted. The mutant DNA was diluted in 4 ng wild-type genomic DNA. Analysis was performed in triplicate, and experiments were repeated 3 times.

(Bio-Rad) using the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, 98°C for 10 min, and a final incubation at 4°C. Droplets were read using a QX200 Droplet Reader (Bio-Rad), and data were analysed using QuantaSoft analysis software version 1.7 (Bio-Rad). Reactions were performed at least in duplicate, and all runs included no-template controls. DNA samples from Mel-2, A375, WM3282, and WM3406 were used to represent homozygous *BRAF*$^{\text{wild-type}}$/*NRAS*$^{\text{wild-type}}$, homozygous *BRAF*$^{\text{V600E}}$, homozygous *BRAF*$^{\text{V600K}}$, and homozygous *NRAS*$^{\text{Q61K}}$, respectively.

Statistical analysis

Correlation analyses were performed with the Spearman rank correlation coefficient.

RESULTS

ctDNA quantification in an in vitro *model experiment*

The DNA samples used for assay validation were obtained from the human melanoma cell lines Mel-2, A375, WM3282, and WM3406. To validate the limits of detection, DNA from the A375 (*BRAF*$^{\text{V600E}}$: homozygous), WM3282 (*BRAF*$^{\text{V600K}}$: homozygous), and WM3406 (*NRAS*$^{\text{Q61K}}$: homozygous) lines was serially diluted into DNA from Mel-2 (*BRAF*$^{\text{wild-type}}$, *NRAS*$^{\text{wild-type}}$) cells, and the alleles were quantified using ddPCR. The mutated DNA was diluted within a total of 4 ng of DNA template because at least 4 ng of cell-free DNA (>1 ng/µl) was obtained from patients and control samples for each ddPCR plate well. *BRAF*$^{\text{V600E}}$ was detected at concentrations as low as approximately 1.6 copies/well in 4 ng of DNA template, and the assay was linear over the entire tested range ($R^2=0.995$, $p<0.01$; **Fig. 1**a). Similarly, as low as 1 copy/well of *BRAF*$^{\text{V600K}}$ ($R^2=0.971$, $p<0.01$; Fig. 1b) and *NRAS*$^{\text{Q61K}}$ ($R^2=0.995$, $p<0.01$; Fig. 1c) was detected in 4 ng of DNA template. Wild-type and mutant alleles were not detected in blank samples without DNA.

Quantification of the mutated ctDNA from the peripheral blood of melanoma patients and healthy subjects

Next, cell-free DNA was extracted from the peripheral blood of 5 patients with melanoma who were treated with nivolumab, an anti-PD-1 antibody (Table I). ddPCR was used to quantify ctDNA, which was detected in all 5 patients during their follow-up (**Table II**). The copy number of *BRAF*$^{\text{V600E}}$ ctDNA was 0–12,750 copies/ml, that of *BRAF*$^{\text{V600K}}$ ctDNA was 0–146 copies/ml, and that of *NRAS*$^{\text{Q61K}}$ ctDNA was 0–348 copies/ml. In addition, 28 healthy control subjects were examined. A *BRAF*$^{\text{V600E}}$ signal/well (9 copies/ml) was detected in 2 subjects, a *BRAF*$^{\text{V600K}}$ signal/well (15 copies/ml) in 1 subject, and a *NRAS*$^{\text{Q61K}}$ signal/well (7.5 copies/ml) in 2 subjects. However, the signals were not reproducible in healthy control subjects when the assay was repeated.

Table II. Analysis of ctDNA in plasma of 5 patients with metastatic melanoma

Case	Time-points (day)	ctDNA[a] (copies/ml of plasma)	LDH (IU/l)	RECIST
1	−22	180[c]	290	
	0[b]	NA	321	
	21	0	95	
	58	NA	NA	PR
	65	0	210	
	105	NA	NA	PR
	156	12	144	
	185	NA	NA	PR
	213	12	157	
	240[d]	NA	NA	PD
	268	105	164	
	286	288	168	
	310	105	229	
	331	507	157	PD
	351	444	313	
	359	84	265	
	365	15	243	
	384	12	157	
2	−7	480[c]	166	
	0[b]	NA	281	
	7	500	232	
	19	39	230	
	26	50	226	
	56	5	197	
	82	0	221	
	96[d]	NA	222	PD
	103	28	237	
	124	210	256	
	147	95	299	
	166	150	294	
	187	300	427	PD
3	0[b]	146[c]	262	
	12	110	240	
	54	NA	259	SD
	82	17	245	
	117	0	229	SD
	159	0	245	
	194	6	236	
	222[d]	0	250	PD
	229	0	245	
	250	15	239	
	281	NA	255	
	327	0	245	
	362	15	239	
4	−12	1,170	181	
	0[b]	1,050[c]	180	
	9	1,500	196	
	23	5,640	200	
	30	10,140	214	
	35	NA	263	PD
	48	12,750	295	PD
5	−42	0	278	
	0[b]	0[c]	210	
	27	48	371	
	70	348	567	PD

[a]The ctDNA levels are mean. [b]Starting nivolumab. [c]Baseline ctDNA level. [d]New metastasis.
NA: not available; RECIST: Response Evaluation Criteria in Solid Tumor; PR: partial response; PD: progressive disease; SD: stable disease.

Alteration in ctDNA levels after treatment with anti-PD-1 antibody

The levels of ctDNA and another marker, LDH (normal range, <230 IU/l), were assessed in patients during the treatment course.

Patient 1 (*BRAF*$^{\text{V600E}}$ mutation in the primary lesion) presented with multiple metastases, and *BRAF*$^{\text{V600E}}$ ctDNA was detected in plasma DNA on day −22 (180

copies/ml, **Fig. 2**a). Nivolumab administration (day 0) resulted in a decrease in the level of *BRAF*^{V600E} ctDNA to undetectable levels on day 21 (Fig. 2a and b). Similarly, LDH levels decreased to the normal range on day 21. A CT scan revealed a significant reduction in tumour size, and tumour response was classified as PR in the RECIST assessment on days 58, 105, and 185 (Fig. 2c). Although the level of *BRAF*^{V600E} ctDNA was low until day 213, new lesions were detected in the bladder on day 240, and tumour response was categorized as PD. Furthermore, new lesions appeared in the brain, and the *BRAF*^{V600E} ctDNA level was increased on day 268 (105 copies/ml), although the LDH level remained within the normal range. The LDH level exceeded the upper limit on day 351. Changes in ctDNA levels preceded those in LDH. Treatment was changed to vemurafenib on day 352. ctDNA and LDH levels decreased significantly at 1 week after commencing vemurafenib treatment. The patient died on day 427.

Patient 2 (*BRAF*^{V600E} mutation in the primary lesion) developed PD with multiple metastases, and *BRAF*^{V600E} ctDNA was detected in plasma DNA on day −7 (480 copies/ml, **Fig. 3**a). The level of *BRAF*^{V600E} ctDNA was significantly decreased on day 19 because of nivolumab administration (39 copies/ml). Although the ctDNA level

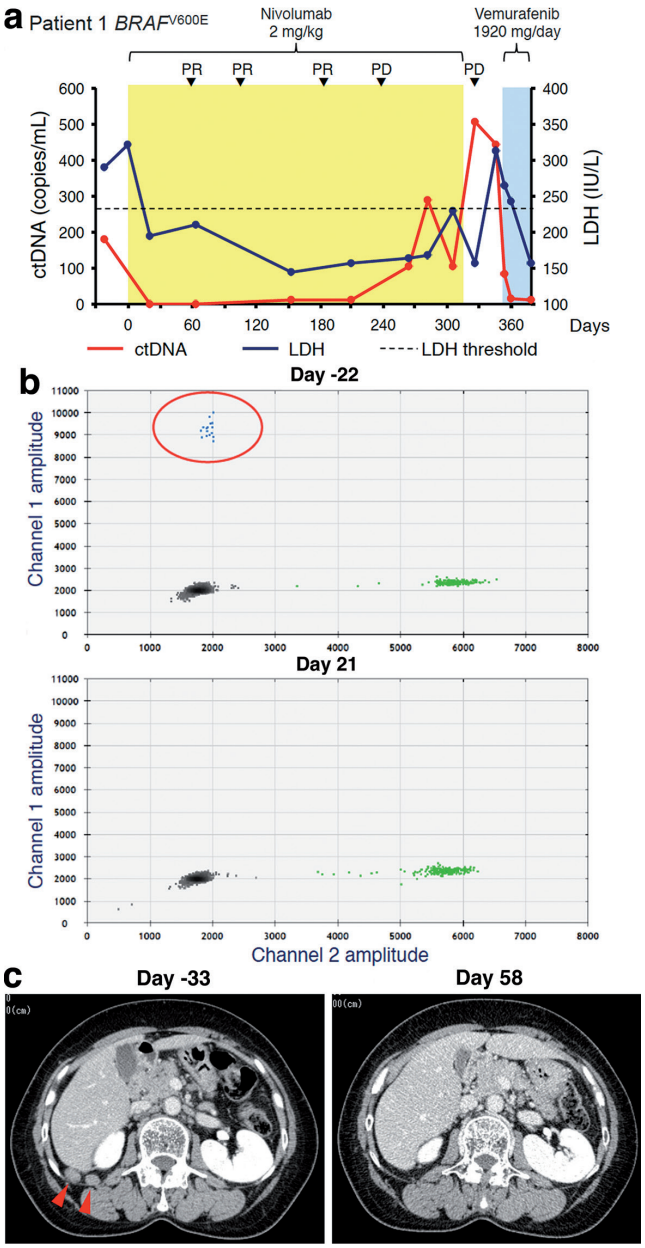

Fig. 2. Monitoring of ctDNA and lactate dehydrogenase (LDH) levels in Patient 1 with droplet digital PCR (ddPCR) results and radiological findings. (a) The graph shows the alteration of the *BRAF*^{V600E} mutant copy number as well as that of LDH in Patient 1. The upper limit of the normal LDH level is 230 IU/l. PR: partial response; PD: progressive disease. (b) ddPCR plots at 2 clinical time-points. Following initiation of nivolumab, *BRAF*^{V600E} ctDNA was significantly decreased (*red ellipse*). (*Blue dots* are *BRAF*^{V600E}, *green dots* are *BRAF*$^{wild\text{-}type}$, and *grey dots* are droplets without DNA interest.) (c) Computed tomography (CT) images at 2 clinical time-points. *Red arrows* indicate peritoneal dissemination on day −33.

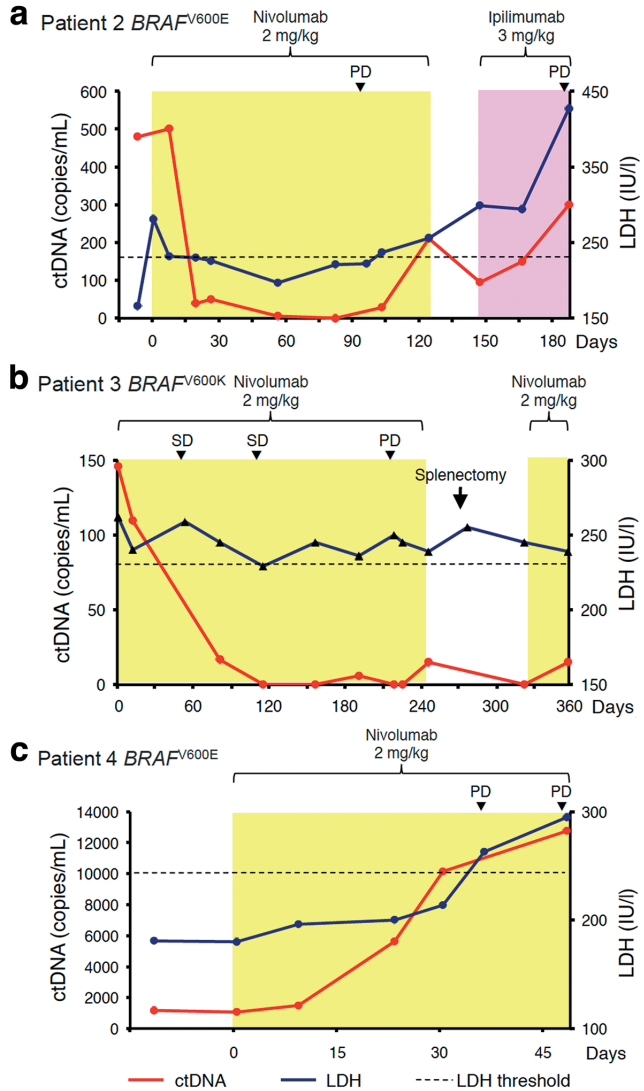

Fig. 3. Monitoring of ctDNA and lactate dehydrogenase (LDH) levels with clinical follow-up in Patients 2 (a), 3 (b), and 4 (c). Graphs show the alteration of the *BRAF*^{V600E} mutant copy number in Patients 2 (a) and 4 (c) as well as that of the *BRAF*^{V600K} mutant copy number in Patient 3 (b). SD: stable disease.

remained low until day 82, a CT scan revealed multiple new lesions in the brain on day 96, which represented PD in the RECIST assessment. The ctDNA level was substantially increased after day 103. Treatment was changed to ipilimumab on day 147.

Patient 3 (*BRAF*^{V600K} mutation in the primary lesion) presented with multiple lung metastases. *BRAF*^{V600K} ctDNA was detected in plasma DNA at the time of nivolumab initiation (day 0; 146 copies/ml, Fig. 3b). The level of *BRAF*^{V600K} ctDNA started to decrease on day 12, decreasing further thereafter and remaining low, with undetectable levels between days 82 and 229. A CT scan showed SD in the RECIST assessment on days 54 and 117. Although the lung metastases were stable, a new lesion appeared in the spleen on day 222, and the clinical evaluation was PD. The patient underwent resection of the spleen metastasis on day 281. Her condition was clinically stable, and the *BRAF*^{V600K} ctDNA remained undetectable or low before and after the resection. The LDH level remained above the upper limits of the normal range during monitoring. The ctDNA levels seemed to be consistent with the patient's clinical condition.

Patient 4 (*BRAF*^{V600E} mutation in the primary lesion) developed PD with multiple metastases, and *BRAF*^{V600E} ctDNA was detected in plasma DNA on day −12 (1,170 copies/ml, Fig. 3c). Despite nivolumab administration, pleural effusion and ascites progressed and the patient deteriorated rapidly. The *BRAF*^{V600E} ctDNA level did not decrease in response to nivolumab. *BRAF*^{V600E} ctDNA increased to 10,140 copies/ml on day 30. Although the LDH level increased, it remained within the normal range until day 30. A CT scan showed PD on day 48. The patient died on day 84.

Patient 5 (*NRAS*^{Q61K} mutation in the primary lesion) presented with multiple metastases and *NRAS*^{Q61K} ctDNA was not detected in plasma DNA at the time of nivolumab initiation (day 0, **Fig. 4**a and b). The patient's clinical condition deteriorated despite nivolumab administration. *NRAS*^{Q61K} ctDNA and LDH levels increased to 48 copies/ml and 371 IU/l on day 27, and increased further according to the patient's condition. A CT scan showed PD on day 70 (Fig. 4c). The patient died on day 122.

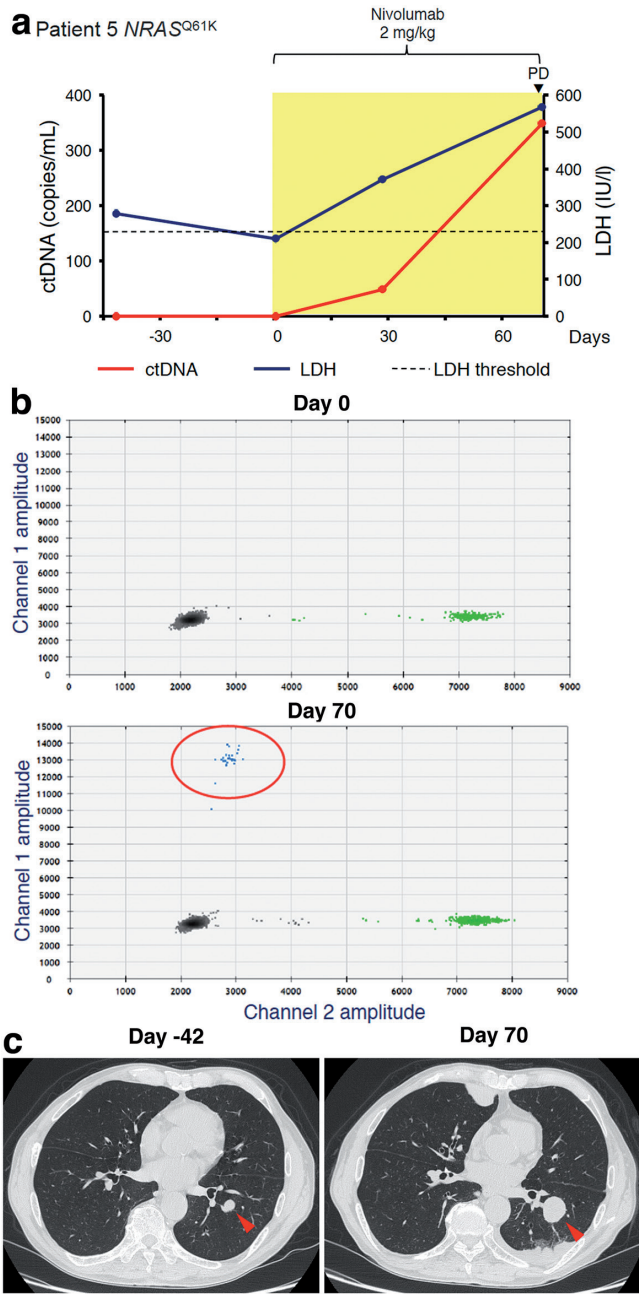

Fig. 4. Monitoring of ctDNA and lactate dehydrogenase (LDH) levels in Patient 5 with droplet digital PCR (ddPCR) results and radiological findings. (a) The graph shows the alteration of the *NRAS*^{Q61K} mutation copy number as well as that of lactate dehydrogenase (LDH) in Patient 5. (b) ddPCR plots at 2 clinical time-points. *NRAS*^{Q61K} ctDNA appeared in the peripheral blood despite the initiation of treatment with nivolumab (*red ellipse*). (*Blue dots* are *NRAS*^{Q61K}, *green dots* are *NRAS*$^{wild\text{-}type}$, and *grey dots* are droplets without DNA interest.) (c) Computed tomography (CT) images at 2 clinical time-points. *Red arrows* point to lung metastasis on day −42 and day 70.

DISCUSSION

Monitoring the response to treatment is essential to determine the benefit of therapies and avoid the prolonged use of ineffective and potentially toxic treatments. There is an unmet need to identify biomarkers for measuring treatment response in melanoma. Here, we used ctDNA with *BRAF* and *NRAS* mutations in the peripheral blood to monitor the response to anti-PD-1 immunotherapy in 5 patients with advanced melanoma. ctDNA levels were approximately correlated with the response to treatment with anti-PD-1 antibody, suggesting the value of ctDNA as a blood-based biomarker.

The clinical management of patients undergoing immune checkpoint blockade therapy can be challenging. Radiographic changes on conventional CT scans can be misleading because the tumour may show apparent enlargement or new lesions may appear before later regressing. Furthermore, prolonged disease stabilization

may contribute to overall survival benefits. However, many cancers such as melanoma have no validated tumour marker. Genetic alterations in human cancers, such as point mutations, can be useful as personalized biomarkers because they distinguish ctDNA from normal DNA. ddPCR has high sensitivity and specificity for the detection of a small amount of DNA. In the present study, ddPCR detected 1–2 copies of mutant *BRAF* and *NRAS* DNAs in 4 ng DNA. However, ddPCR also detected mutated DNAs in a few healthy subjects, and the occurrence of false-positive reactions associated with polymerase-induced errors needs to be considered (19). However, the detection of ctDNA in healthy subjects is not usually reproducible because the false-amplification rate is low. In patients treated with BRAF inhibitors, low levels of BRAF ctDNA are associated with a high overall response rate and long progression-free survival (20, 21). As ddPCR capable machines are increasingly available, this approach can be used to monitor tumour burden in melanoma patients in a minimally-invasive manner.

Although molecular-targeted agents, such as BRAF and MEK inhibitors, exert a rapid effect on melanoma, immunotherapy is occasionally associated with a latency of its anti-tumour effect. The molecular-targeted agents attack melanoma cells directly, and ctDNA levels decrease within several days after the administration in cases of favourable response (4, 20). By contrast, in the present study, the decline of ctDNA required 2–4 weeks after the first administration of nivolumab in patients with controlled disease. Cellular tumour activity is inhibited by the immune-mediated effects of the immune checkpoint blockade, and tumour destruction may take a longer time. In addition, the ctDNA levels increased within 2–4 weeks in patients in which the disease was not controlled. An increasing ctDNA level indicates persistent tumour activity and hence worse prognosis compared with patients who have undetectable or falling ctDNA levels. Changes in ctDNA levels in melanoma patients treated with immune checkpoint blockade were recently reported by other groups (22, 23). Consistent with the present results, the therapeutic effect of the immune checkpoint blockade was reflected in the ctDNA levels several weeks after the initiation of treatment.

Secondary resistance occasionally develops during immunotherapy, and it is difficult to determine whether immune checkpoint blockade should be continued. Increased ctDNA may reflect a state of secondary resistance to the treatment in which tumour cells have evaded the immune checkpoint blockade. The levels of ctDNA increased in Patient 1 and Patient 2 despite low and undetectable levels immediately after nivolumab initiation. The rebound in ctDNA levels correlated with treatment failure. On the other hand, ctDNA levels remained low or undetectable in Patient 3 despite the definition of PD on day 222 because of spleen metastasis, and her clinical condition remained stable for more than 6 months after splenectomy, suggesting that nivolumab had an effect on tumour activity. With frequent monitoring, ctDNA may be detected simultaneously with or before radiological detection of progression. Although CT scans are usually performed every 2–3 months, an increase in ctDNA levels could potentially serve as a trigger for early evaluation with imaging modalities, leading to a timely switch to more appropriate therapeutic interventions.

Measurement of LDH levels has been incorporated into the management of melanoma patients, and an elevated level is associated with high disease burden and decreased survival. Recently, LDH was reported as a useful marker to predict early response to nivolumab in patients with melanoma (24). However, LDH is neither sensitive nor specific, and it can be slow in reflecting changes in disease status (23). The ctDNA level, as measured by ddPCR, was a better indicator of treatment response and the emergence of treatment resistance than LDH levels in the present study.

Longitudinal analysis of ctDNA during treatment demonstrated that ctDNA levels detect treatment response and the emergence of resistance and may allow for an alternative treatment to be introduced before a major decline in health parameters. Taking a "wait-and-see" approach may cost a patient valuable time during which suitable alternative interventions could be tried. A limitation of the present study was the small number of patients. Further prospective analysis is required to assess the value of ctDNA as a biomarker of clinical outcome in patients receiving immune checkpoint blockade therapy.

ACKNOWLEDGEMENTS

The authors would like to thank Ms. Aya Uchiyama for technical assistance.

The authors declare no conflict of interest.

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