Usefulness of Monitoring Circulating Tumor Cells as a Therapeutic Biomarker in Melanoma with BRAF Mutation

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Abstract

**Background:** Circulating tumor cells (CTCs) are non-invasive biomarkers that could be used to characterize changes in tumors. In this study, we monitored the number of CTCs, as well as the *BRAF* genotype of individual CTCs.

**Methods:** CTCs were isolated from peripheral blood using a high-density dielectrophoretic microwell array, followed by labeling with melanoma-specific markers (MART-1 and/or gp100) and a leukocyte marker (CD45).

**Results:** Examination of patients with stage 0–III melanoma detected CTCs even in patients with early disease (stage 0 or I). Next, we analyzed CTCs in five patients with stage IV melanoma during treatment with BRAF/MEK inhibitors. The number of CTCs fluctuated in association with the drug response in four of the five patients, suggesting that the total number of CTCs usually reflected the drug response. Interestingly, one of those patients had CTCs with seven different *BRAF* genotypes, and the mutated CTCs disappeared upon treatment with BRAF/MEK inhibitors, except for those harboring *BRAF*<sub>A598V</sub>.

**Conclusions:** CTCs are present even in the early stage of melanoma, and the number of CTCs seems to reflect drug response during treatment with BRAF/MEK inhibitors. Furthermore, genetic heterogeneity of *BRAF* may contribute to drug resistance of BRAF/MEK inhibitor. Our findings demonstrate the usefulness of CTC analysis in melanoma for monitoring targeted therapies and understanding mechanism of drug resistance.

Background

Molecularly targeted therapies and immune checkpoint inhibitors have improved the prognosis of advanced melanoma. Although the objective response rates to those treatments range from 40–70% in clinical trials [1], real-world outcomes are inferior [2]. Therefore, prediction of drug response and optimization of treatment order are required. Pretreatment tumor biopsies provide useful information, including driver mutations, expression levels of programmed death-ligand 1, infiltration of CD8-positive T-cells within tumors, and microsatellite instability. However, those markers are insufficient for predicting response to treatment because they reflect tumor status at a single time point. Moreover, although additional biopsies may be desired during treatment, tissue biopsies may not accurately reflect systemic tumor status due to intertumoral and intratumoral heterogeneity [3].

Baseline factors, including lactate dehydrogenase (LDH) levels, the number of metastatic organs, and Eastern Cooperative Oncology Group performance status, have been reported to be well-correlated with survival in clinical trials for advanced melanoma [4]. However, LDH also reflects side effect of drugs and infection, thus it may not become an accurate biomarker that correlates with disease status. Liquid biopsy, including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and circulating microRNA, have attracted attention as potential biomarkers [5–7]. ctDNA, which is released from dead tumor cells, is present in peripheral blood as cell-free DNA and is a useful tool for monitoring real-time
disease status. Although ctDNA is quite specific for tumors, it is rapidly degraded, decreasing the sensitivity of assays that are based on it. In addition, some mutated cell-free DNA is produced in clonal hematopoiesis [7].

CTCs are released from primary and/or metastatic tumors into the peripheral blood. Several strategies have been used to detect CTC, including a microbead-sorting method, flow cytometry, microfluidics, and filtration-based devices [8–11]. In combination with negative selection for leukocyte specific markers, various markers are used to detect melanoma cells in peripheral blood, including CD146, melanoma-associated chondroitin sulfate proteoglycan, ATP-binding cassette subfamily B member 5, CD271, and receptor activator of NF-κB. Assuming that the CTC population represents the distributed tumor burden and biological features, characterization of these cells could provide a complementary sample for the monitoring of tumor characteristics [11–13]. CTCs are a promising source of material because they can be obtained via routine blood sampling and can provide real-time information about the characteristics of tumors over time. CTC characterization can reveal the early response to immune checkpoint inhibitors in melanoma [14] and identify genetic heterogeneity in BRAF V600 status [15].

According to recent long-term observations, patients treated with combinations of BRAF/MEK inhibitors exhibit favorable outcomes. In particular, patients with complete remission achieve longer progression-free survival and overall survival [4]. However, the majority of patients with partial response or stable disease exhibit a short-duration response and experience recurrence within several months after initiation of therapy. Therefore, it is necessary to establish biomarkers that enable early detection of resistance and evaluation of treatment response. To this end, as well as to elucidate the mechanisms of drug resistance, analysis of CTCs may be useful. Hence, in this study, we monitored the number of CTCs along with the BRAF genotype during treatment with BRAF/MEK inhibitors.

**Methods**

**Blood and tissue samples**

Peripheral blood was obtained from patients with melanoma and from healthy individuals. For metastatic setting, blood was collected at several time points before and during the BRAF-targeted therapy. Formalin-fixed paraffin-embedded tissues were used for pathological diagnosis and genotyping in BRAF V600. The Cobas 4800 BRAF Mutation Test (Roche Molecular Diagnosis, Basel, Switzerland) or Oncomine Dx Target Test (Thermo Scientific, Waltham, MA, USA) using primary tumor was positive in all metastatic patients treated with BRAF/MEK inhibitors (Table S1).

**Identification of CTC**

To analyze tumor features, we monitored CTCs using a high-density dielectrophoretic microwell array. The principles underlying identification and capture of CTCs were described previously [16]. In brief, peripheral blood mononuclear cells were resuspended in 300 mM mannitol solution, a solution with
suitable conductivity for dielectrophoresis. The suspension was loaded into the cell entrapment chamber, and the cells were entrapped in the microwells by dielectrophoretic force. The trapped cells were labeled with antibodies against the melanoma-specific markers MART-1 (BioLegend, San Diego, CA, USA) and gp100 (DAKO, Santa Clara, CA, USA), followed by anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Life Technologies, Eugene, OR, USA). To exclude leukocytes, we used an anti-CD45 antibody conjugated to phycoerythrin (Beckman Coulter, Marseille, France). Subsequently, fluorescence microscopic images were captured from the chambers for analyzing cells entrapped into each well. MART-1/gp100-positive and CD45-negative cells were counted as CTCs. In addition, spike-in experiment was performed and the detail information were described in Supporting information. Finally, in BRAF<sup>V600E/K</sup> patients, CTCs were captured by micromanipulation and subjected to DNA sequencing.

**Isolation and mutation analysis of CTC**

For single-cell sequencing of CTCs, captured cells from the cell entrapment chambers were singly collected in tubes containing 20 µL nuclease-free water. For DNA sequencing, genomic DNA was extracted from each cell, followed by PCR amplification of BRAF exon 15 and Sanger sequencing as described previously [17]. For detail information of DNA sequencing was described in Supporting information.

**Statistical analysis**

For statistical analysis, the Student’s t-test was used to compare the number of CTCs between patients with different stages. Differences and correlations were considered significant when $p < 0.05$.

**Results**

**CTC detection in patients with stage 0–III melanoma**

Peripheral blood was collected from melanoma patients with stage 0–III disease before surgical resection of the primary tumor and sentinel node biopsy (Table S2). Melanoma stage was determined based on the American Joint Committee on Cancer Staging Manual (8th edition). To distinguish tumor cells from white blood cells, CTCs were defined as positive for melanoma-specific markers (MART-1 and/or gp100) and negative for CD45. We defined the intensity threshold of each parameter to minimize false positivity, using a mixture of melanoma cell lines with normal blood cells. Sensitivity and specificity were 12.6–60.6% (Fig. S1) and 99.9% (data not shown), respectively; sensitivity differed among cell lines. The number of CTCs per 4 mL blood in stage 0–I, stage II, and stage III disease was 3–16 (median, 8; interquartile range, 6), 3–10 (median, 7; interquartile range, 5), and 6–18 (median, 11; interquartile range, 5), respectively (Fig. 1a; Table S2). The number of CTCs was not well correlated with tumor thickness. Notably, we detected five cells per 4 mL blood in a patient with melanoma in situ. By contrast, zero or one
cell meeting the criteria for CTCs was present per 4 mL blood in healthy individuals. In primary tumors (stage I), bulky nests of melanoma cells in the dermis may have been the source of CTCs (Fig. 1b, c).

**Monitoring CTC in *BRAF*-mutated advanced melanoma**

To evaluate the usefulness of CTCs as biomarkers correlated with responsiveness to treatment, we next analyzed blood from five patients with *BRAF*-mutated melanoma (MMbraf1–5), who were treated with BRAF/MEK inhibitors (Table S1). Objective response to therapies was assessed by computed tomography (CT) scan using the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (CR, complete response; PR, partial response; SD, stable disease; and PD, progressive disease).

MMbraf1–4 were treated with dabrafenib and trametinib for unresectable metastases. We monitored the number of CTCs at four time points. In MMbraf1, administration of dabrafenib and trametinib (Day 0) resulted in a decrease in the number of CTCs on Day 82, and lactate dehydrogenase (LDH; upper limit of normal, 230 U/L) levels increased moderately at the same timepoint (Fig. 2a). A CT scan revealed a significant reduction in tumor size, and tumor response was classified as PR. Subsequently, the number of CTCs increased on Day 126, and on Day 183 the tumor response was categorized as PD based on the appearance of a novel metastatic lesion in the left lung. In MMbraf2, the number of CTCs began to decrease on Day 36, and the metastatic lesion had partially regressed on Day 49, corresponding to SD (Fig. 2b). LDH levels increased slightly at the same timepoint. In MMbraf3, the number of CTCs increased on Days 14 and 42, but suddenly decreased on Day 49 (Fig. 2c). LDH levels increased at these timepoints, and CT scan revealed enlargement of a metastatic lesion in the liver on Day 56, corresponding to PD. In MMbraf4, CTCs were less abundant on Days 20, 56, and 70 than at the beginning (Fig. 2d). The LDH level increased slightly on Day 20. Thereafter, although LDH level was stable, it remained above the upper limit of the normal range. A CT scan revealed PR on Day 70.

**Monitoring *BRAF*-mutated CTC during *BRAF* targeted therapy**

In MMbraf5, *BRAFV600E* mutation was identified in a primary tumor but not in a lymph node metastasis (Fig. 3a), suggesting heterogeneity of the *BRAF* genotype. Therefore, we decided to investigate the *BRAF* genotype of CTCs at the single-cell level. When a lung metastasis was detected by CT scan, the patient was initially treated with nivolumab. Because the lung metastasis was enlarged 9 months later, nivolumab was switched to dabrafenib and trametinib. Before switching the therapy, the total number of CTCs was 310 /mL with 58.1 /mL *BRAFV600E*-mutated CTCs. On Day 8, the numbers of both total and *BRAFV600E*-mutated CTCs decreased to 62 /mL and 5 /mL, respectively (Fig. 3b, c, d; Table 1). In addition, *BRAFV600R*, *BRAFV600M*, *BRAFV600A*, *BRAFK601E*, *BRAFK601R*, and *BRAFV598V* CTCs were found in the blood (Fig. 3b, c). After initiation of dabrafenib and trametinib, *BRAFV600E* CTCs gradually decreased and finally disappeared on Day 92 (Fig. 3d). Similarly, *BRAFV600R*, *BRAFV600M*, *BRAFV600A*, *BRAFK601E*, and *BRAFK601R*
CTCs disappeared until Day 120. On the other hand, the number of total CTCs decreased but never disappeared. Interestingly, \(BRAF^{A598V}\) and \(BRAF\) wild-type CTCs were still detected even after other \(BRAF\)-mutated CTCs disappeared (Table 1). A CT scan on Day 70 revealed that tumor response was classified as PR due to a reduction of lung metastasis. By contrast, LDH levels did not decrease during treatment, probably due to an adverse event. Because the patient was diagnosed with drug-induced interstitial pneumonia on Day 148, dabrafenib and trametinib were suspended. Thereafter, \(BRAF^{V600E}\)-mutated CTCs reappeared and the number of total CTCs increased (Fig. 3d; Table 1).

**Discussion**

CTCs have been actively studied in the context of solid tumors. Here, we analyzed CTCs in melanoma using a high-density dielectrophoretic microwell array, followed by labeling of CTC with markers specific for melanoma and leukocytes [15, 16]. Because the relatively straightforward assay, from labeling to detection, can be performed on the same plate, this method is useful for isolation and characterization of small numbers of cells at the single-cell level.

Our results demonstrated that CTCs were present even in stage 0 or I melanoma. Although CTCs are present in limited numbers [9], they exist even in the early stages of melanoma, as well as in other diseases such as breast and lung cancer [18–20] Primary tumors with minimal invasion may exert their metastatic potential via releasing CTCs.

In addition, we found that the number of CTCs was not well correlated with tumor thickness. Tumor cells usually transform to an invasive and metastatic phenotype in response to hypoxia, genetic instability, and activation of oncogenes [21]. Because hypoxia in tumor tissues elicits angiogenesis, the formation of a bulky mass can cause local hypoxia and activate the potential to migrate to and access blood vessels even in early-stage disease. Notably in this regard, we detected CTCs even in a case with melanoma \(in\ \text{situ}\). Theoretically, melanoma \(in\ \text{situ}\) should not develop metastasis. Such cases may have occult invasive lesions within tumor [22, 23], and serial sectioning deeper into the tissue block may show the invasion.

Although we analyzed a small number of cases, we found that the number of CTCs was not associated with clinical stage, consistent with previous studies of melanoma and lung cancer [11, 18]. Interestingly, detection of CTCs is associated with overall survival in stage II–III patients with melanoma [24]. Thus, the number of CTCs may be a prognostic biomarker among patients with the same staging.

The results of this study demonstrated that alteration of CTC number is associated with a clinical response to BRAF/MEK inhibitors. LDH levels were correlated with clinical response in two out of five patients, and the number of CTCs seemed to reflect the response in four out of five patients, suggesting that CTC count could be a useful biomarker for advanced melanoma treated with BRAF/MEK inhibitors. In a recent study that combined analysis of CTCs and ctDNA, CTC number was strongly associated with the level of ctDNA; moreover, the number of CTCs prior to systemic therapies was negatively correlated
with overall and progression-free survival [12]. These observations, along with our results, indicate that
detection of CTCs during treatment provides useful information that supports imaging studies, such as
CT scans, in the prediction of drug response and prognosis.

Heterogeneity of protein expression and genetic alteration in CTCs has been reported in melanoma [10, 
11, 15, 25]. In some patients, CTCs are heterogenous with respect to *BRAF* genotype [11, 15]. In this study,
BRAF/MEK inhibitors seemed to be effective against melanoma cells with various mutations in V600 and
K601 residues of BRAF. However, CTCs with *BRAF*<sup>A59V</sup> persisted throughout treatment, implying a
potential mechanism of drug resistance. Future studies should test this possibility.

**Conclusions**

CTC analysis is useful for evaluating disease status during molecularly targeted therapies, and analysis
at the single-cell level may provide information for overcoming drug resistance. In addition, CTCs with
certain properties may develop into metastases, suggesting that analysis of CTCs could shed light on the
metastatic signature.

**Abbreviations**

LDH, lactate dehydrogenase; CTC, circulating tumor cells; ctDNA, circulating tumor DNA; RECIST,
Response Evaluation Criteria in Solid Tumors; CR, complete response; PR, partial response; SD, stable
disease; PD, progressive disease.

**Declarations**

**Acknowledgments**

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**Authors’ contributions**

YK performed identification and isolation of CTC, and genetic analysis. KN and AM prepared peripheral
blood mononuclear cells. YA and AM conducted preliminary experiments to set the labeling conditions for
CTC. YK and RO wrote manuscript with support from AA. All authors read and approved the final
manuscript.

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**Availability of data and materials**
The datasets used and analyzed during the current study are available from the first author Dr. Yukiko Kiniwa on reasonable request.

**Ethics approval and consent to participate**

The study was approved by the Ethics Committee of the Shinshu University School of Medicine (approval numbers 358, 430, and 634). All patients approved to participate this research and provided written consent forms.

**Consent for publication**

*Not applicable*

**Competing interests**

Y.A. and A.M. are employees of Tosoh. Tosoh have applied for patents related to the dielectrophoretic microwell array system. This does not alter the authors’ adherence to all policies of *BMC Cancer* on sharing data and materials.

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**Table**

**Table 1.** BRAF status of CTCs detected in MMbraf5 during treatment with dabrafenib and trametinib

| BRAF mutation status of CTC | Day 60 | Day 8 | Day 15 | Day 36 | Day 57 | Day 92 | Day 120 | Day 148 | Treatment suspended |
|-----------------------------|--------|-------|--------|--------|--------|--------|---------|---------|---------------------|
| Wild type                   | 34     | 9     | 14     | 15     | 14     | 6      | 3       | 8       | 17                  |
| V600E                       | 9      | 2     | 2      | 1      | 1      | 0      | 0       | 0       | 0                   |
| V600R                       | 0      | 1     | 5      | 2      | 1      | 0      | 0       | 0       | 0                   |
| V600M                       | 0      | 0     | 0      | 1      | 0      | 0      | 0       | 0       | 0                   |
| V600A                       | 0      | 1     | 0      | 0      | 0      | 0      | 0       | 0       | 0                   |
| K601E                       | 4      | 2     | 0      | 1      | 1      | 1      | 0       | 0       | 0                   |
| K601R                       | 1      | 0     | 0      | 0      | 0      | 0      | 0       | 0       | 0                   |
| A599V                       | 0      | 2     | 0      | 0      | 1      | 0      | 1       | 3       | 0                   |

The number of isolated CTC

| Day 60 | Day 8 | Day 15 | Day 36 | Day 57 | Day 92 | Day 120 | Day 148 |
|--------|-------|--------|--------|--------|--------|---------|---------|
| 48     | 17    | 21     | 20     | 18     | 7      | 4       | 11      | 23     |

*Day, day before or after treatment started.*

*We did not analyze BRAF genotype of all isolated CTC at each time point.*

**Figures**
Figure 1

Number of circulating tumor cells (CTCs) at stages 0–I, II, and III. (a) Number of CTCs in healthy individuals and melanoma patients at stages 0–I, II, and III. CTCs were counted using blood samples collected before surgical resection of the primary tumor and sentinel node biopsy. (b, c) Pathologic features of stage I primary melanoma. Atypical melanocytes invaded the dermis in a nodular and diffuse manner. Tumor thicknesses were 0.6 (b) and 0.8 mm (c). Clark levels were III (b) and IV (c) (×100, hematoxylin/eosin staining).
Figure 2

Number of CTCs during treatment with BRAF/MEK inhibitors. MMbraf1, MMbraf2, MMbraf3, and MMbraf4 were diagnosed with metastatic BRAFv600E/K melanoma. (a) Monitoring the number of CTCs during the clinical course in MMbraf1. The graph shows the number of CTCs and the LDH level (upper limit of normal, 230 IU/L). Arrows indicate lung metastases in computed tomography (CT) imaging. (b) Monitoring the number of CTCs during the clinical course in MMbraf2. Circles indicate a right axillary lymph node (LN) metastasis in CT imaging. (c) Monitoring the number of CTCs during the clinical course in MMbraf3. Arrowheads indicate a liver metastasis in CT imaging. (d) Monitoring the number of CTCs during the clinical course in MMbraf4. Arrows indicate a right inguinal LN metastasis in CT imaging.
Figure 3

BRAF genotype and the number of CTCs in MMbraf5. (a) BRAF sequence chromatograms of the primary tumor and a metastatic lymph node. (b) BRAF sequence chromatograms of CTCs during treatment with BRAF/MEK inhibitors. (c) Diversity of BRAF mutations surrounding codon 600 in MMbraf5. (d) Number of CTCs during treatment and clinical outcome response in patient MMbraf5. Arrowheads indicate lung metastasis in CT imaging on Days -40, 70, and 148. Far-right CT imaging shows the appearance of...
interstitial pneumonia on Day 148. In the upper graph, solid and dotted lines indicate the number of total CTCs and the LDH level, respectively, during the clinical course in MMbraf5. In the lower graph, pink and purple lines indicate the numbers of BRAFV600E CTCs and total CTCs with BRAF mutations, respectively.

**Supplementary Files**

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