

Serial Monitoring of Circulating Tumor Cells Predicts Outcome of Induction Biochemotherapy plus Maintenance Biotherapy for Metastatic Melanoma

Kazuo Koyanagi¹, Steven J. O'Day², Peter Boasberg², Michael B. Atkins⁵, He-Jing Wang³, Rene Gonzalez⁶, Karl Lewis⁶, John A. Thompson⁷, Clay M. Anderson⁸, Jose Lutzky⁹, Thomas T. Amatruda¹⁰, Evan Hersh¹¹, Jon Richards¹², Jeffrey S. Weber⁴, and Dave S.B. Hoon¹

Abstract

Purpose: Molecular biomarkers in blood are promising for assessment of tumor progression and treatment response. We hypothesized that serial monitoring of circulating tumor cells (CTC) with the use of multimarker quantitative real-time reverse transcriptase-PCR assays could be a surrogate predictor of outcome for melanoma patients enrolled in a multicenter phase II clinical trial of biochemotherapy (BCT) combined with maintenance biotherapy (mBT).

Experimental Design: Blood specimens were collected from 87 patients before and during induction BCT and mBT for stage IV melanoma. Expression of five melanoma-associated CTC biomarkers (MART-1, GalNAc-T, PAX-3, MAGE-A3, and Mitf) was assessed by quantitative real-time reverse transcriptase-PCR, and correlated with treatment response and disease outcome.

Results: The number of positive CTC biomarkers decreased overall during induction BCT ($P < 0.0001$). CTC biomarker detection after two cycles of BCT was correlated with treatment response ($P = 0.005$) and overall survival ($P = 0.001$): an increase in the number of CTC biomarkers was associated with poor response ($P = 0.006$) and overall survival ($P < 0.0001$). Multivariate analyses with the use of a Cox proportional hazards model identified the change in CTC biomarkers after two cycles of BCT as an independent prognostic factor for disease progression (risk ratio, 12.6; 95% confidence interval, 4.78-33.4; $P < 0.0001$) and overall survival (risk ratio, 6.11; 95% confidence interval, 2.37-15.7; $P = 0.0005$).

Conclusion: Serial monitoring of CTC during induction BCT may be useful for predicting therapeutic efficacy and disease outcome in patients receiving BCT and mBT for stage IV melanoma. *Clin Cancer Res*; 16(8); 2402-8. ©2010 AACR.

Circulating tumor cells (CTC) in blood are a promising surrogate biomarker of treatment response and outcome in metastatic melanoma (1-6). We successfully used a multimarker quantitative real-time reverse transcriptase-PCR (qRT-PCR) assay to correlate CTC detection with treatment outcome in patients receiving neoadjuvant biochemotherapy (BCT) before complete surgical resection of

American Joint Committee on Cancer stage III melanomas (7, 8). This assay relied on serial blood sampling to quantify biomarker expression levels at specific time points and to identify biomarker changes during treatment. The same approach might be useful to monitor response to treatment for American Joint Committee on Cancer stage IV melanoma because surgical resection of distant metastases is often limited, and systemic regimens are prolonged, potentially toxic, and increasingly complex. Moreover, the median survival of patients with stage IV melanoma is only 6 to 9 months (9, 10), so early determination of treatment efficacy might enable timely treatment modification.

The heterogeneous expression of melanoma-related genes in blood favors a multimarker qRT-PCR assay that uses melanoma-associated biomarkers or therapeutic agents that are functionally distinct and therefore nonoverlapping (1, 11, 12). Our sensitive and specific qRT-PCR assay for detection of CTC in blood uses five such biomarkers: melanoma antigen recognized by T cells-1 (MART-1), melanoma antigen gene-A3 family (MAGE-A3), $\beta 1 \rightarrow 4$ -N-acetylgalactosaminyltransferase (GalNAc-T), paired box

Authors' Affiliations: ¹Department of Molecular Oncology, John Wayne Cancer Institute; ²The Angeles Clinic and Research Institute, Santa Monica, California; ³Department of Biostatistics, University of California; ⁴University of Southern California, Los Angeles, California ⁵Harvard/Beth-Israel Medical Center, Boston, Massachusetts; ⁶University of Colorado Cancer Center, Aurora, Colorado; ⁷University of Washington, Seattle, Washington; ⁸University of Missouri, Columbia, Missouri; ⁹Mount Sinai Comprehensive Cancer Center, Miami Beach, Florida; ¹⁰The Humphrey Cancer Center, Robbinsdale, Minnesota; ¹¹University of Arizona, Tucson, Arizona; and ¹²Oncology Specialist, S.C., Park Ridge, Illinois

Corresponding Author: Dave S.B. Hoon, Department of Molecular Oncology, John Wayne Cancer Institute, 2200 Santa Monica Boulevard, Santa Monica, CA 90404. Phone: 310-449-5267; Fax: 310-449-5282; E-mail: hoon@jwci.org.

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Translational Relevance

There is a lack of validated blood tests for assessment of malignant melanoma patients during multimodal therapy. In this study, circulating tumor cells (CTC) were assessed to monitor serial blood from a phase II multicenter clinical trial of biochemotherapy followed by maintenance biotherapy. The established multimarker melanoma biomarkers assessed by quantitative real-time PCR were sensitive to detect CTCs in blood directly. The assay robustness allows monitoring of patients in a multicenter setting. The monitoring of CTC during early stage therapy was predictive of disease progression. These studies show that CTCs have clinical utility in monitoring melanoma patients in multimodal therapy.

homeotic gene transcription factor 3 (PAX-3), and microphthalmia transcription factor (Mitf; refs. 7, 13, 14). In this study, we used this assay to detect CTC biomarkers in blood specimens from a recently reported cohort of patients undergoing a novel regimen of BCT and maintenance biotherapy (mBT) for stage IV melanoma (15). We hypothesized that the response to treatment would be inversely correlated with the CTC biomarkers detected at various time points during treatment and that this correlation would predict overall survival.

Patients and Methods

Patients and treatment. Subjects for this qRT-PCR study were enrolled in a recently reported prospective multicenter phase II trial of concurrent decrescendo BCT (4-6 cycles) followed by mBT (up to 12 cycles; ref. 15). The BCT regimen was repeated every 21 d and comprised cisplatin (20 mg/m² i.v. on days 1-4), dacarbazine (800 mg/m² i.v. on day 1), vinblastine (1.5 mg/m² i.v. on days 1-4), interleukin 2 (Chiron Corporation, Emeryville, CA; continuous infusion of 18 MU/m² over 24 h on day 1, 9 MU/m² over 24 h on day 2, and 4.5 MU/m² over 24 h on days 3 and 4), IFN- α 2b (Schering-Plough, Kenilworth, NJ; 5 MU/m² s.c. on days 1-5), and granulocyte macrophage colony-stimulating factor (Amgen Inc., Thousand Oaks, CA; 500 μ g s.c. on days 6-15). The mBT regimen was a 28-d cycle of low-dose interleukin 2 (2 MU/m² s.c. daily) and granulocyte macrophage colony-stimulating factor (250 μ g s.c. on days 1-14), which included intermittent pulses of high-dose decrescendo interleukin 2 (18 MU/m², continuous infusion over the first 6 h, the next 12 h, and the final 24 h) on days 1 to 2 of mBT cycles 1 to 6, 8, 10, and 12 (15).

Response was assessed by Response Evaluation Criteria in Solid Tumors every 6 wk (two cycles) during BCT and every 3 mo during mBT. Patients with stable disease, partial response, or complete response continued with the study treatment; those with progressive disease during

BCT or mBT did not receive further study treatment but were followed for survival. Response Evaluation Criteria in Solid Tumors for progression on mBT was modified from a 20% to a 30% increase to allow nonclinically significant progression without deterioration of performance status (<5% of the study population). Patients who developed new central nervous system (CNS) lesions were allowed to remain in the study if they had stable or responding systemic disease (non-CNS).

All blood specimens were coded for double-blind study and processed within 24 h. Peripheral blood was drawn regularly during treatment, processed, and cryostored until used as previously described (8). Patients gave written informed consent for the use of their blood specimens before treatment, and the qRT-PCR study was approved and carried out in accordance with guidelines set forth by the individual Institutional Review Board committees and reporting recommendations for tumor marker prognostic studies [Reporting recommendations for tumor marker prognostic studies (REMARK) criteria; ref. 16].

Blood processing for qRT-PCR assay. We selected four sampling times for the multimarker qRT-PCR study: immediately before the first cycle of BCT (BCT1; $n = 87$), before the third cycle of BCT (BCT3; $n = 87$), before the first cycle of mBT (mBT1, $n = 64$), and before the third cycle of mBT (mBT3; $n = 54$). The interval between each of the four sampling times was ~6-to-8 wk. The interval between BCT3 and mBT1 was ~9-to-12 wk in patients who received five or six cycles of BCT.

Peripheral blood specimens (10 mL) were collected in sodium citrate-containing tubes, and the first several milliliters were discarded to eliminate skin-plug contamination, as previously described (11, 17). Nucleated cell fractions were isolated from blood specimens with the use of the Purescript RBC Lysis Solution (Gentra, Minneapolis, MN) and cryopreserved in liquid nitrogen until thawed for the study, as previously described (18).

TRI Reagent (Molecular Research Center, Cincinnati, OH) was used to isolate total cellular RNA from blood specimens, as previously described (11). RNA was quantified and assessed for purity by UV spectrophotometry. Reverse transcriptase reactions were done with the use of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) with oligo(dT) primer (19).

Multimarker qRT-PCR assay measured mRNA levels of MART-1, MAGE-A3, GalNAc-T, PAX-3, and Mitf. In previous studies, we validated the sensitivity and specificity of this qRT-PCR assay for detection of CTC in blood specimens (7, 8, 13, 14). In those studies, all five CTC biomarkers were frequently detected in melanoma cell lines but were not detected in blood specimens from healthy donors. In the present study, the qRT-PCR assay was done with the use of an ABI Prism 7900HT thermocycler (Applied Biosystems, Foster City, CA; ref. 18). We transferred cDNA from 200 ng total RNA to individual wells of a 384-well PCR plate; 0.5 μ mol/L of each primer, 0.3 μ mol/L probe, and 5 μ L *iTaq* custom supermix with ROX (Bio-Rad Laboratories, Hercules, CA) were added to a final volume of

Table 1. Detection of biomarkers in blood sampled at specific intervals during treatment

Biomarkers	BCT1	BCT3
	N = 87 (%)	N = 87 (%)
MART-1	35 (40)	18 (21)
MAGE-A3	24 (28)	13 (15)
GalNAc-T	38 (44)	30 (34)
PAX-3	20 (23)	20 (23)
Mitf	22 (25)	25 (29)
No. of biomarkers tested		
0	21 (24)	30 (35)
1	21 (24)	24 (28)
2	25 (29)	20 (23)
3	13 (15)	10 (11)
≥4	7 (8)	3 (3)

Abbreviation: BCT, biochemotherapy.

10 μ L. Samples were amplified with a pre-cycling hold at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and then 1 min of annealing/extension at 55°C for glyceraldehyde-3-phosphate dehydrogenase, at 59°C for MART-1, at 58°C for MAGE-A3 and Mitf, and at 62°C for GalNAc-T and PAX-3. The standard curve was generated by using a threshold cycle of seven serial dilutions of plasmid templates (10^0 - 10^6 copies). The threshold cycle of each sample was interpolated from the standard curve, and the number of mRNA copies was calculated. PCR efficiency, assessed from the slopes of the curves, was 90% to 100%. The correlation coefficient for all standard curves (threshold cycle versus log copy number) was ≥ 0.99 .

Each qRT-PCR assay was done at least twice, and included marker-positive and marker-negative controls and reagent controls (reagent without RNA or cDNA). If only one of the duplicates was positive, the qRT-PCR assay was repeated. Any specimen with inadequate mRNA copies ($< 1 \times 10^4$) of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene was excluded. The mean mRNA copy number was used for analysis. Blood processing, RNA extraction, qRT-PCR assay setup, and post-qRT-PCR product analysis were carried out in separate designated rooms to prevent cross contamination.

Biostatistical analysis. Biomarker change and overall survival were primary end points; marker expression, treatment response, and progression-free survival were secondary end points.

Wilcoxon signed rank test was used to compare the number of biomarkers detected before and during treatment. χ^2 test and exact t test were used to examine the association between biomarker detection and treatment response. Survival was measured from the start of BCT (BCT1). The correlation between biomarker detection, biomarker change,

and survival was examined by log rank test. Survival curves were generated with the use of the Kaplan-Meier method. A Cox proportional hazards model was developed to examine the association of biomarker detection with progression-free and overall survival, and used for multivariate analysis.

McNemar's test compared the detection of individual CTC biomarkers between any two time points. Mann-Whitney *U* test assessed CTC biomarker differences according to site of metastasis. The analysis was done with the use of the SAS statistical software, and all tests were two-sided with a significance level ≤ 0.05 .

Results

Patients assessed for qRT-PCR. Our qRT-PCR study included 87 of 133 patients participating in the clinical trial biomarker assay. The 133 patients comprised 94 males and 39 females, with a median age of 50 years (range, 18-76 years); our cohort comprised 60 males and 27 females, with a median age of 48 years (range, 18-73 years). The patient cohort had histopathologically confirmed M_{1a} ($n = 8$), M_{1b} ($n = 19$), or M_{1c} ($n = 60$) melanoma. The cycles of BCT received were 2, 3, 4, and 6 in 10, 2, 65, and 10 patients, respectively. The cycles of mBT were 1 to 3, 4 to 6, and 7 to 12 in 18, 23, and 23 patients, respectively. Twenty-three patients did not receive mBT. These distributions paralleled those in the parent population. All 87 patients had BCT1 and BCT3 blood specimens; the remaining 46 patients were excluded because of lack of blood procurement and inadequate samples to process at BCT1 or BCT3. Of the 87 patients, 64 had mBT1 blood specimens, and 23 patients had no blood specimens at mBT1 (11 patients developed progressive disease after BCT, 6 patients did not receive mBT for other reasons, 3 had inadequate specimens for qRT-PCR analysis, and 3 had no blood sampling at mBT1). Of 64 patients with blood samples at mBT1, 54 patients had blood samples at mBT3, 5 discontinued mBT because of progressive disease before mBT3, and 5 had no sampling at mBT3. Overall, 292 blood samples were studied from 87 patients that participated or qualified for entry into the companion biomarker assay.

Detection of CTC biomarkers in blood. Before treatment, blood specimens from 66 (76%) of 87 patients expressed at least one CTC biomarker, and specimens from 45 (52%) patients expressed > 1 CTC biomarker. Overall, the number of CTC biomarkers gradually decreased during treatment: at mBT3, specimens from 24 (44%) patients had no CTC biomarkers, and only 11 (21%) specimens expressed > 1 CTC biomarker ($P < 0.0001$). However, this decrease was significant only during the first two cycles of BCT among the specified sampling intervals (Table 1).

MART-1 and Mitf mRNA detection rates dropped significantly during overall treatment (BCT1 versus mBT3; MART-1: $P = 0.03$; Mitf: $P = 0.005$), reflecting a gradual and significant decrease during each sampling interval except for a nonsignificant decrease in Mitf detection during the first two cycles of BCT. However, there were no significant changes in CTC biomarker detection except for a

Table 2. Correlation of CTC biomarkers with BCT response

BCT response	No. of CTC biomarkers (%)							
	BCT1				BCT3			
	0	1	≥2	P*	0	1	≥2	P*
CR/PR (n = 44)	10 (23)	12 (27)	22 (50)	0.24	18 (41)	16 (36)	10 (23)	0.005
SD (n = 32)	6 (19)	9 (28)	17 (53)		10 (31)	8 (25)	14 (44)	
PD (n = 11)	5 (45)	0 (0)	6 (55)		2 (18)	0 (0)	9 (82)	

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

* χ^2 test, exact *P*-value.

significant decrease in MAGE-A3 detection between BCT1 and BCT3.

Association between CTC biomarkers and treatment response. The number of patients who achieved complete response, partial response, stable disease, and progressive disease as a best response to BCT was 8, 36, 32, and 11, respectively. Also, these distributions almost paralleled those in the parent study population cohort (data not shown). The number of CTC biomarkers before treatment (BCT1) did not predict treatment response (Table 2). However, the number of CTC biomarkers during BCT was significantly lower in the complete/partial response group ($P = 0.005$). Twenty-two patients had an increase in the number of CTC biomarkers; seven had an increase of at least two biomarkers (Table 3). The number of biomarkers increased in 10 (31%) and 4 (36%) patients with stable and progressive disease, respectively, compared with only 8 (18%) patients with complete/partial response ($P = 0.006$).

At a median follow-up of 15.4 months (range, 2.1-43.1 months), the best response to BCT and mBT was complete response, partial response, stable disease, and progressive disease in 9, 25, 18, and 30 patients, respectively. Five patients received elective surgery after induction BCT and were excluded from the analysis. As with the best response to BCT, the best response to BCT + mBT was significantly correlated with the number of CTC biomarkers detected at BCT3 ($P = 0.01$; Table 4) but not with the number of biomarkers detected at BCT1 (data not shown). The number of CTC biomarkers detected in the last available blood specimen significantly correlated with best response to BCT + mBT ($P = 0.006$; Table 4).

CTC biomarkers as a predictor of disease progression. Before treatment, there was no correlation between CTC biomarker detection and patient sex, age, Eastern Cooperative Oncology Group performance status, or previous treatment status. Of the 87 patients, 8 had M_{1a} disease, 19 had M_{1b} disease, and 60 had M_{1c} disease before treatment; and 41 patients developed CNS metastasis during treatment. There was no correlation between CTC biomarker detection/increase, and distant metastases or CNS metastasis.

During follow-up, 78 patients showed disease progression, and the other 9 patients were free from disease progression. At BCT1, patients with >1 CTC biomarker tended to have shorter survival than patients with no or one CTC bio-

marker (data not shown). At BCT3, progression-free survival significantly decreased when a CTC biomarker was positive (Fig. 1A). The size of the decrease was directly correlated with the number of positive CTC biomarkers. Median progression-free survival was 10.02 months [95% confidence interval (95% CI), 7.10-15.90], 8.10 months (95% CI, 6.67-11.10), and 5.68 months (95% CI, 4.50-7.20) for patients with no positive CTC biomarkers ($n = 30$), one positive CTC biomarker ($n = 24$), and ≥2 positive CTC biomarkers ($n = 33$), respectively ($P = 0.002$).

Between BCT1 and BCT3, 65 patients showed no increase or decrease in number of CTC biomarkers, 15 had an increase of one CTC biomarker, and 7 had an increase of >1 CTC biomarker. Median progression-free survival was 8.18 months (95% CI, 6.93-9.46), 7.82 months (95% CI, 5.88-9.82), and 3.68 months (95% CI, 2.17-4.50) for patients with an increase of 0, 1, and ≥2, respectively ($P < 0.0001$; Fig. 1B). Clinical factors did not correlate with disease progression in univariate analyses. A Cox proportional hazards regression model that used a stepwise procedure to assess BCT3 versus standard clinical variables showed that only the number of CTC biomarkers at BCT3 was an independent prognostic factor for progression-free survival (risk ratio, 2.58; 95% CI, 1.50-4.43; $P = 0.0006$). A similar analysis showed that only the change in the number of positive CTC biomarkers was an independent prognostic factor for progression-free survival (risk ratio, 12.6; 95% CI, 4.78-33.4; $P < 0.0001$).

CTC biomarkers as a predictor of overall survival. During follow-up, 72 patients died of disease progression. Overall

Table 3. Correlation of changes in CTC biomarkers with BCT response

BCT response	Increase in CTC biomarkers between BCT1 and BCT3 (%)			
	≤0	1	≥2	P*
CR/PR (n = 44)	36 (82)	6 (14)	2 (4)	0.006
SD (n = 32)	22 (69)	9 (28)	1 (3)	
PD (n = 11)	7 (64)	0 (0)	4 (36)	

* χ^2 test.

Table 4. Correlation of CTC biomarkers with overall response

Overall response	No. of CTC biomarkers (%)							
	BCT3				Last blood sampling			
	0	1	≥2	<i>P</i> *	0	1	≥2	<i>P</i> *
CR/PR (<i>n</i> = 34)	16 (47)	13 (38)	5 (15)	0.01	20 (59)	9 (26)	5 (15)	0.006
SD (<i>n</i> = 18)	6 (33)	5 (28)	7 (39)		6 (33)	4 (22)	8 (45)	
PD (<i>n</i> = 30)	8 (27)	5 (17)	17 (56)		6 (20)	7 (23)	17 (57)	

* χ^2 test.

survival showed a significant inverse correlation with the number of positive CTC biomarkers at BCT3 (Fig. 1C). Median overall survival was 22.3 months (95% CI, 19.5-28.6), 15.4 months (95% CI, 13.5-19.1), and 9.8 months (95% CI, 7.7-13.5) for patients with 0, 1, and ≥2 positive CTC biomarkers, respectively (*P* = 0.001). Median overall survival was 15.4 months (95% CI, 14.2-20.2), 19.1 months (95% CI, 11.1 to not available), and 8.0 months (95% CI, 4.5-9.8) for patients with an increase of 0, 1, or ≥2 CTC biomarkers, respectively (*P* < 0.0001; Fig. 1D). Clinical factors did not correlate with survival in univariate analyses. A Cox proportional hazards regression model that used a stepwise procedure to assess BCT3 and clinical factors found that only the number of CTC biomarkers at BCT3 was an independent prognostic factor for overall survival (risk ratio, 2.71; 95% CI, 1.56-4.73; *P* = 0.0004).

A similar analysis revealed an independent prognostic significance of a change in the number of CTC biomarkers from BCT1 to BCT3 for overall survival (risk ratio, 4.33; 95% CI, 1.91-9.84; *P* = 0.0005).

Discussion

Systemic BCT for stage IV melanoma has promise, but results from phase III trials have been mixed and inconsistent, (20-24), in part because responses to BCT are not durable and do not prevent CNS progression. The novel regimen of mBT and induction BCT/mBT received by patients in our correlative study was developed to amplify and prolong the systemic antitumor immune response elicited by BCT (25). As recently reported, this regimen seems to extend progression-free and overall survival

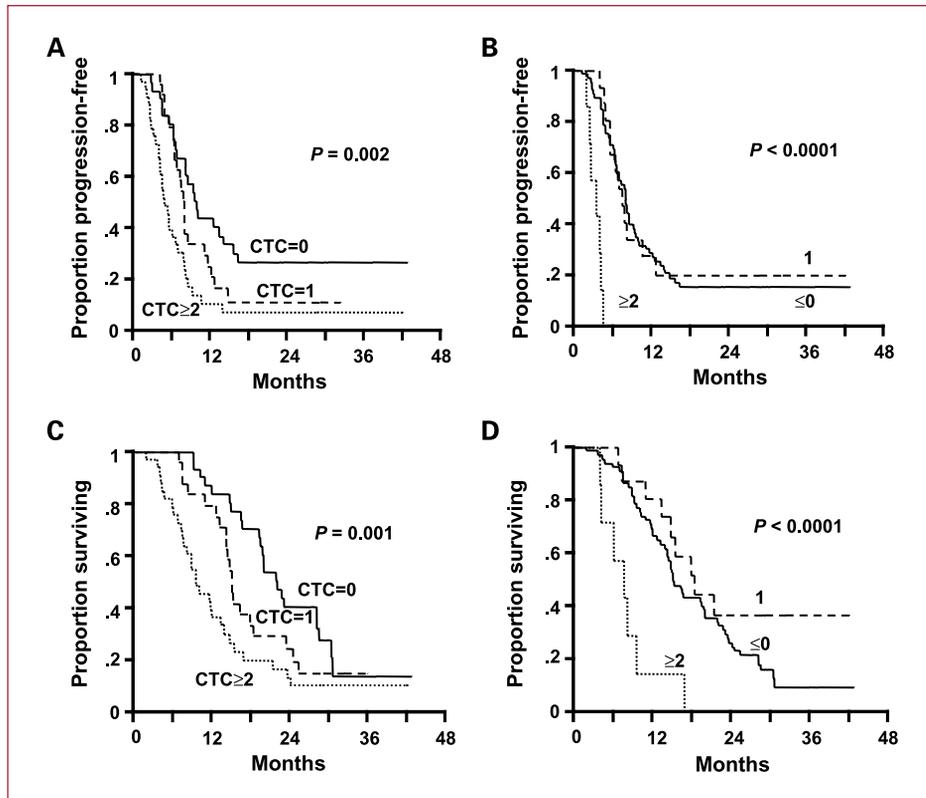


Fig. 1. A, Kaplan-Meier curves of progression-free survival based on CTC biomarker detection after two cycles of induction BCT. B, Kaplan-Meier curves of progression-free survival based on changes in CTC biomarker detection between BCT1 and BCT3. C, Kaplan-Meier curves of overall survival based on CTC biomarker detection after two cycles of induction BCT. D, Kaplan-Meier curves of overall survival based on changes in CTC biomarker detection between BCT1 and BCT3. Solid line, 0 CTC biomarkers; broken line, 1 CTC biomarker; dotted line, ≥2 CTC biomarkers.

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compared with regimens in recent multicenter trials of BCT or chemotherapy (15).

The present study showed a significant relation between prolonged survival and decreased number of CTC biomarkers during therapy. Although most investigations of CTC to predict disease progression report a single biomarker measured at a single point, a single-biomarker assay is limited by the heterogeneous expression of melanoma-related genes (11, 19, 26), particularly in advanced disease (27). However, the efficacy of a multiple-biomarker assay depends on the careful selection of CTC biomarkers (28), serial rather than single-point assessment (7, 8, 14), and quantification of CTC to compensate for ectopic and background mRNA (29). The assay system used in the study was the same as that used to assess stage III melanoma patients receiving neoadjuvant BCT (8). Our assay system showed the clinical utility and robustness of CTC biomarkers in patients with stage IV as well as stage III melanoma who received systemic therapies in phase II multicenter trials.

Our results confirm recent reports that changes in CTC during treatment may indicate therapeutic efficacy for metastatic cancer (30–32). The detection of CTC biomarkers before treatment (BCT1) was not an indicator of treatment response. Subclinical tumor metastasis, particularly to the CNS system, and tumor heterogeneity are likely related to the findings. The metastasis to CNS system is critical and occurred in almost half of our patients. Treatment effects to chemotherapeutic or immunotherapeutic agents are often different among the metastatic tumors within an individual patient. Thus, the prediction of tumor response and survival before treatment may be difficult in further advanced metastatic melanoma patients with multiple lesions and metastasis at specific organ sites. If the number of CTC biomarkers increased during treatment, we observed a poor prognosis. Of the five patients who underwent elective surgery after BCT, all had no increase in biomarker detection after surgery, and three survived for 42, 30, and 29 months, respectively. Although the number of positive biomarkers decreased across the sampling intervals, however, this decrease was significant only during the first two cycles of BCT. These significant findings may reflect reduced drug efficacy with increasing duration of treatment. These findings are important, as metastatic melanoma patients with CTC at the start of treatment may have a poor outcome.

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As treatment regimens become multimodal and multiphasic, CTC detection in serial blood specimens might be used to determine which component of treatment is most effective and which needs to be improved.

Current prognostic systems, such as the tumor-node-metastasis staging criteria and molecular features of primary tumors, are probably inadequate for managing metastatic melanoma patients receiving systemic chemotherapy and/or immunotherapy. CTC detection may be a better tool to monitor the treatment efficacy because blood assessment can be serially done, and changes in CTC may be observed immediately after administration of systemic therapy (31, 32). Serial qRT-PCR assay can assess CTC changes during different phases of treatment, and this makes CTC assessment a promising method to evaluate treatment efficacy in controlling systemic disease. In patients being treated in these clinical trials, the benefit of changing therapy in the early course of the treatment with the use of CTC detection as a surrogate of responsiveness needs to be further examined in clinical trials.

This study shows CTC change as an independent surrogate for survival and treatment efficacy in metastatic melanoma patients who received mBT after BCT. CTC blood biomarkers could be used as surrogates in developing multimodal therapeutic trials. The strategy including CTC change for early identification of treatment-resistant patients may be important not only to discontinue non-effective systemic therapy but also to develop individualized treatment regimens in metastatic melanoma patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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