Prospective Molecular Profiling of Circulating Tumor Cells from Patients with Melanoma Receiving Combinatorial Immunotherapy.

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Prospective Molecular Profiling of Circulating Tumor Cells from Patients with Melanoma Receiving Combinatorial Immunotherapy

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Abstract

BACKGROUND—Blood molecular profiling of circulating tumor cells (CTCs) can enable monitoring of patients with metastatic melanoma during checkpoint inhibitor immunotherapy (CII) and in combination with targeted therapies. We developed a microfluidics-based CTC platform to explore CTC profiling utility in CII-treated patients with melanoma using a melanoma messenger RNA (mRNA)/DNA biomarker panel.

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METHODS—Blood samples (n = 213) were collected prospectively from 75 American Joint Committee on Cancer-staged III/IV melanoma patients during CII treatment and those enriched for CTCs. CTC profiling was performed using 5 known melanoma mRNA biomarkers and BRAF V600E DNA mutation. CTC biomarker status associations with clinical outcomes were assessed.

RESULTS—CTCs were detected in 88% of blood samples from patients with melanoma. CTC-derived biomarkers and clinical variables analyzed using classification and regression tree analysis revealed that a combination of lactate dehydrogenase, CTC-mRNA biomarkers, and tumor BRAF–mutation status was indicative of clinical outcomes for patients with stage IV melanoma (n = 52). The panel stratified low-risk and high-risk patients, whereby the latter had poor disease-free (P = 0.03) and overall survival (P = 0.02). Incorporation of a DNA biomarker with mRNA profiling increased overall CTC-detection capability by 57% compared to mRNA profiling only. RNA sequencing of isolated CTCs identified significant catenin beta 1 (CTNNB1) overexpression (P <0.01) compared to nondisease donor blood. CTC-CTNNB1 was associated with progressive disease/stable disease compared to complete-responder patient status (P = 0.02). Serial CTC profiling identified subclinical disease in patients who developed progressive disease during treatment/follow-up.

CONCLUSIONS—CTC-derived mRNA/DNA biomarkers have utility for monitoring CII, targeted, and combinatorial therapies in metastatic melanoma patients.

Patients with metastatic melanoma have access to targeted therapies and checkpoint inhibitor immunotherapy (CII) for disease management. However, robust biomarkers are lacking for patient stratification and assessment of CII efficacy alone or in combination with targeted therapies (1). Serial tumor biopsies to monitor evolving tumor profiles and treatment efficacy are not feasible. Circulating tumor cells (CTCs), indicative of subclinical disease, may enable minimally invasive monitoring of patients with metastatic melanoma (2, 3). Previous multicenter phase II/III studies (4–8) demonstrated the prognostic utility of a defined CTC-derived messenger RNA (mRNA) biomarker panel (9) during follow-up and disease outcome. However, the utility of CTCs for CII and combinatorial therapies is not fully established as emerging CTC-derived, CII-related melanoma biomarkers have yet to be validated in clinical trials.

Difficulties in identifying robust CII-related CTC biomarkers in blood are attributed to low CTC abundance and the heterogeneous metastatic melanoma landscape. To address melanoma heterogeneity (10, 11) and to improve monitoring of disease status during therapy, we developed a CTC molecular profiling assay following nonepithelial cellular adhesion molecule-based CTC enrichment (12) utilizing a panel of known mRNA and DNA melanoma blood biomarkers. This CTC-derived biomarker panel was applied to prospectively collected blood samples from metastatic melanoma patients actively receiving CII, combinatorial modern therapies, or during follow-up of therapy to explore the potential

6Nonstandard abbreviations: CII, checkpoint inhibitor immunotherapy; CTC, circulating tumor cell; mRNA, messenger RNA; SJHC, Providence Saint John’s Health Center; AJCC, American Joint Committee on Cancer; PD, progressive disease; SD, stable disease; qRT-PCR, quantitative reverse transcriptase-PCR; WGA, whole-genome amplification; CTC-DNA, CTC-derived DNA; WT, BRAF wild-type; RNA-seq, RNA sequencing; OS, overall survival; DFS, disease-free survival; LDH, lactate dehydrogenase; URL, upper reference limit; CART, classification and regression tree; CTC-BRAF, CTC-derived BRAF V600E mutation; BRAF-V600E mutation, BRAF mutation; HR, hazard ratio.
of CTC profiling for assessing CII therapeutic efficacy. Here we describe the potential utility for CTC-mRNA/ DNA multimarker monitoring in patients with melanoma receiving CII.

Materials and Methods

PATIENT SAMPLES AND CELL LINES

All tissue and blood samples were obtained from consented patients with melanoma treated at Providence Saint John’s Health Center (SJHC) between 2015 and 2017 in accordance with the Western Institutional Review Board (MORD-RTPCR-0995). Current American Joint Committee on Cancer (AJCC; 8th ed) staging guidelines were applied. Prospective blood samples (n = 213) from 75 patients with melanoma were included (Table 1). Fifty patients provided 2 to 9 serial samples per patient. Patients and respective therapies are shown in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol66/issue1. Treatment response was assessed by computerized tomography/magnetic resonance imaging every 3 months, determined by a single physician (S.J. O’Day) according to Response Evaluation Criteria In Solid Tumors 1.1 criteria denoting progressive disease (PD), stable disease (SD), partial response, complete response, or no evidence of disease.

Established metastatic melanoma cell lines (EB, M14, and HM-0525) with known mRNA/DNA biomarker status (13) from melanoma patients who received surgery at John Wayne Cancer Institute were used as controls in quantitative reverse transcriptase-PCR (qRT-PCR) assays and CTC spike-in experiments.

CTC ENRICHMENT

Blood was collected in Cell-Free DNA BCT® tubes (Streck) and processed within 48 h (6). Ten milliliters of blood was prepared for the FX1 system (14) for automated label-free CTC enrichment as previously described (15). EpiCentre MasterPure™ Complete DNA and RNA purification kit was used to extract nucleic acids from enriched CTCs according to the manufacturers’ protocols with 2 modifications comprising an overnight incubation at −80°C after addition of lysis buffer and addition of 1 μL Pellet Paint® NF Co-Precipitant (Millipore Sigma) during nucleic acid precipitation.

qRT-PCR ASSAYS

Nucleic acids extracted from enriched-CTC samples were evaluated in a qRT-PCR assay containing 5 biomarkers (CTC-mRNA panel), MAGE family member A3 (MAGEA3)7, melan-A (MLANA), beta-1,4-N-acetylgalactosaminyltransferase 1 (B4GALNT1), PAX3 paired box 3 (PAX3), and tRNA-Pro (anticodon AGG) 2–6 (DCT), previously established in multiple phase II/III melanoma clinical trials (4–8). Assays were performed (6, 7, 9, 16) in accordance with Reporting Recommendations for Tumor Marker Prognostic Studies criteria.

7Human genes: MAGEA3, MAGE family member A3; MLANA, melan-A; B4GALNT1, beta 1,4-N-acetylgalactosaminyltransferase 1; PAX3, paired box 3; TRP-AGG2-6, RNA-Pro (anticodon AGG) 2-6; CTNNB1, catenin beta 1; B2M, beta-2-microglobulin; SDHA, succinate dehydrogenase complex flavoprotein subunit A.
The novel CTC-mRNA catenin beta 1 (CTNNB1) biomarker was evaluated with the Integrated DNA Technologies’ predesigned CTNNB1 qRT-PCR assay and was performed according to the manufacturer’s specifications. The qRT-PCR assay with a positive 32–cycle cutoff was established based on assessment of 10 healthy donor leukocytes post-FX1 enrichment in triplicate. The assay was performed with positive (LF0023, M14, and EB cell lines), negative (mouse line NIH3T3), and no template controls and beta-2-microglobulin (B2M) (17) and succinate dehydrogenase complex flavoprotein subunit A (SDHA) (18) housekeeping genes for internal control and RNA integrity verification. Samples that amplified before the 32-cycle cutoff were considered positive for CTNNB1.

**BRAF V600E MUTATION DIGITAL DROPLET PCR ASSAY**

Nucleic acids extracted from enriched-CTCs underwent whole-genome amplification (WGA) using the True-Prime™ WGA kit (Expedeon) followed by purification using Genomic DNA Clean & Concentrator™ (ZYMO Research Corp) according to the manufacturers’ specifications. WGA of melanoma CTC-derived DNA (CTC-DNA) isolated in vitro and from patients with melanoma were subjected to the RainDrop Digital Droplet PCR BRAF V600E Assay (RainDance™ Technologies) and performed according to the manufacturer’s protocol. The assay contained at least a 0.01% mutant sensitivity when a reconstituted BRAF V600E DNA mutant standard of 0.01% was assessed (see Fig. 1 in the online Data Supplement) and a limit of blank cutoff of ≤2 mutant-positive droplets was applied. The assay’s limit of blank was established from healthy donor leukocytes (n = 10) and CTCs (n = 25) from tumor BRAF wild-type (WT) patients in accordance with the manufacturer’s protocol. All CTC WT control samples were negative for BRAF V600E mutation detection postenrichment. Samples with insufficient CTC-DNA or unknown tumor BRAF V600E mutation status at the time of blood collection were excluded from digital droplet PCR analysis.

**IMMUNOFLUORESCENCE STAINING**

CTCs isolated from melanoma patients were identified by immunofluorescence staining for MLANA, a known melanoma-associated antigen (7), using an Alexa Fluor 488® conjugated (1:50; Santa Cruz Biotechnology) antibody. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific). Images were obtained using a Nikon Eclipse Ti microscope as previously described (18).

**RNA IN SITU HYBRIDIZATION**

CTC-matched formalin-fixed, paraffin-embedded tumor tissues were evaluated by RNA in situ hybridization for CTNNB1 expression (Hs-CNNTB1 probe #311731) using the RNAscope Multiplex Fluorescent Kit V2 (Advanced Cell Diagnostics) according to the manufacturer’s instructions. Immunofluorescence staining was observed under the fluorescence microscope (Nikon) and representative images were taken for each condition. Assays for formalin-fixed, paraffin-embedded tissues were developed and optimized as previously described (19). Images were analyzed based on ACD guidelines for in situ hybridization scoring. 3-plex negative (#320871) and positive (#320861) probes served as controls and target bacterial dihydrodipicolinate reductase gene and housekeeping genes,
respectively. In situ hybridization staining images for specific genes were assessed only when proper positive and negative controls were identified.

**CTC RNA SEQUENCING**

Post-CTC enrichment, RNA extracted from 3 patients’ CTCs underwent RNA sequencing (RNA-seq) at the John Wayne Cancer Institute Sequencing Center (20). CTC RNA-seq next-generation sequencing reads were mapped to the GENCODE v19 human genome reference using STAR v2.4.2a with default parameters and read counts generated using the “--quantMode GeneCounts” function. CTC results were normalized and compared to 6 healthy donors blood leukocytes RNA-seq data (Gene Expression Omnibus project numbers GSE53655; SRR1060788, SRR1060789, SRR1060790, SRR1060791, SRR1060792, SRR1060793) (21). Expression of CTNNB1 in nondiseased tissues was explored using the Genotype-Tissue Expression Portal (22). Significantly differentially expressed genes were identified using the DESeq2 bioconductor package with a model controlling for sample sex. RNA-seq data and read counts from single sequencing runs were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (GSE100565).

**BIOSTATISTICAL ANALYSIS**

Fisher exact test was performed for categorical variables. Overall survival (OS) was calculated from time of first sample analysis until death or last contact. Disease-free survival (DFS) was the time to melanoma disease recurrence or death. Lactate dehydrogenase (LDH; U/L) using a cutoff of ≤1.5 x (low LDH) or >1.5 x (high LDH), the upper reference limit (URL) (23, 24), were compared for clinical outcomes. CTC-derived molecular biomarkers and clinical variables were evaluated at the collection time in all 75 patients. CTC-mRNA was defined as low CTC-mRNA (n <2) or high CTC-mRNA (n [mtqu]2) as previously reported (7); CTC-CTNNB1 mRNA and CTC BRAF V600E mutation were defined as negative or positive as described above.

To evaluate the association of CTC-derived molecular biomarkers and clinical variables with OS and DFS, we performed classification and regression tree (CART) analysis (25) using statistical R package “rpart” (26). CART builds a decision tree that hierarchically determines which CTC-derived molecular biomarkers and clinical variables fall into homogeneous subgroups based on a binary recursive partitioning method, classifying patients into high- and low-risk subgroups with respect to each outcome. The high- vs low-risk subgroups of CII-treated patients identified by CART analysis were further evaluated and compared for DFS and OS using Kaplan–Meier and Cox proportional hazard-regression models, adjusted for age, sex, and M category (27). All statistical analyses were performed using R version 3.5.0 (28).

**Results**

**DUAL mRNA/DNA MELANOMA BIOMARKER DETECTION IN ISOLATED CTCS**

We evaluated for the presence of melanoma CTCs post FX1 enrichment from whole patient blood (see Fig. 2A in the online Data Supplement). We performed immunofluorescence staining on cells obtained post-FX1 isolation using MLANA antibody. MLANA expression...
is a reported diagnostic immunohistochemistry marker for lymph node melanoma micrometastasis and primary melanoma (29, 30). We successfully detected MLANA-positive cells postenrichment in 100% of patients with stage IV melanoma evaluated (n = 6; see Fig. 2B in the online Data Supplement). The detection capability of our dual mRNA/DNA CTC assay was evaluated using cells from 3 different melanoma cell lines that were spiked in at 100, 50, and 25 cells in 10 mL of healthy donor leukocytes across 3 replicate experiments. Representative results (see Table 2) demonstrated a detection capability of mRNA/DNA biomarkers from as little as 25 melanoma cells in 10 mL of blood.

DETECTION OF CTC-DERIVED mRNA/DNA BIOMARKERS IN PATIENTS WITH MELANOMA

CTC mRNA/DNA biomarker status was evaluated in prospectively collected blood samples (n = 213) from 75 patients with known clinical status. BRAF V600E DNA mutation was assessed in CTC-derived DNA because it is the most frequent DNA mutation reported in cutaneous melanoma (31, 32). Also, BRAF V600E mutation was selected because it is a well-established hotspot mutation that is druggable (i.e., dabrafenib/vemurafenib/encorafenib) compared to other frequent gene mutations found in melanoma. Detection of CTC-mRNA biomarkers and/or CTC-derived BRAF V600E mutation (herein referred to as CTC-BRAF) in respective paired BRAF-mutated (positive) metastatic tumors from these patients is summarized in Table 3 in the online Data Supplement; 88% of the 213 blood samples contained CTCs. 66 blood samples collected from 24 of 26 patients with BRAF-V600E mutation (herein referred to as BRAF mutation) tumors were analyzed for the mutation hotspot. CTCs collected from 2 patients were omitted in the analysis owing to insufficient DNA quantities. 38% of CTC samples (n = 25 of 66) contained detectable BRAF mutation (mean CTC-BRAF mutation fraction, 0.05%) and were detected in 13 of 24 patients with tumor BRAF-mutated melanoma.

Of the 63 CTCs isolated from blood samples with available LDH values, 50 CTCs contained corresponding low LDH (≤1.5 × URL) and 13 CTCs contained corresponding high LDH (>1.5 × URL) in the blood. 36% of CTCs with low LDH (n = 18 of 50) and 46% of CTCs with high LDH (n = 6 of 13) contained detectable CTC-BRAF (see Table 4 in the online Data Supplement). CTC-BRAF was detected in 57% of CTCs (n = 4 of 7) negative for CTC-mRNA biomarkers, indicating increased sensitivity of CTC biomarker detection when performing dual mRNA/DNA biomarker profiling. There was no significantly different association of CTC-BRAF detection in patients receiving BRAF/MEK inhibitor therapy compared to those who were not (P = 0.55, Fisher exact test). Overall, CTC-derived biomarkers were detected in 88% of samples evaluated. Dual mRNA/DNA biomarker profiling increased the detection of CTC biomarkers in CTC-mRNA negative samples by 57%, which increased the detection of CTCs during therapy.

CTNNB1 OVEREXPRESS IN CTCs AS A POTENTIAL IMMUNE EVASION CII BIOMARKER

The CTNNB1 pathway reportedly plays a critical role in therapeutic immune evasion (33, 34). To assess CTNNB1 expression in isolated CTCs as a potential tumor biomarker, we performed RNA-seq analysis on isolated CTCs from 3 patients with stage IV melanoma.
receiving CII. RNA-seq revealed a highly overexpressed gene, CTNNB1, in melanoma CTCs not present in healthy blood referenced in Genotype-Tissue Expression (see Fig. 3 in the online Data Supplement). We verified the CTNNB1 expression in the isolated CTC-mRNA (n = 3) through CTNNB1 qRT-PCR assay. We assessed the detection frequency of CTNNB1 expression in CTC-mRNA by an established qRT-PCR assay utilizing samples with available CTC-mRNA (n = 122). We detected CTNNB1 overexpression in 48% of CTC-mRNA (n = 58 of 122) and none in 10 healthy donor leukocytes post-FX1 enrichment. This is the first detection of CTNNB1 overexpression in CTCs isolated from patients receiving CII. To verify if CTC-derived CTNNB1 can serve as a surrogate tumor marker, we evaluated the CTNNB1 expression in CTC-matched formalin-fixed, paraffin-embedded tumors through RNA in situ hybridization (see Fig. 4 in the online Data Supplement) and identified an 87% concordance rate (n = 15; see Table 5 in the online Data Supplement) with discordance possibly due to low overall CTC enrichment from blood. Lastly, we identified that CTNNB1 expression was significantly lacking in CTCs from patients with complete response (n = 18) compared to PD/SD (n = 74) while on CII (P = 0.02).

**PREDICTORS OF CLINICAL OUTCOMES IN CII-TREATED PATIENTS**

CTC-derived molecular biomarkers may be predictive of clinical outcomes during treatment. We focused on patients with stage IV melanoma (n = 52) in our CART analysis, which included CTC biomarkers and clinical variables with respect to DFS and OS. Patient treatment regimens are summarized in Table 1 in the online Data Supplement. LDH, tumor BRAF mutation, and CTC-mRNA were identified as potentially predictive of DFS (Fig. 1A) and OS (Fig. 1B) based on the optimal split by CART. Specifically, LDH partitioned patients into highvs low-risk subgroups. CTC-mRNA biomarkers further partitioned subgroups followed by tumor BRAF mutation status. Interestingly, we found that 76% of patients with low LDH (n = 34) contained detectable CTC-mRNA (see Fig. 5A in the online Data Supplement), indicating presence of melanoma that may otherwise be missed by using LDH only. Not surprisingly, 82% of patients with high LDH (n = 11) had detectable CTC-mRNA (see Fig. 5B in the online Data Supplement). Subsequently, these variables (LDH, tumor BRAF mutation, and CTC-mRNA) were designated as a “disease outcome panel,” in which “high-risk” and “low-risk” melanoma patient subgroups were defined (Fig. 1). The high-risk subgroup contained patients with “high LDH” or “low LDH with CTC-mRNA (≥2 mRNA biomarker positive) and tumor BRAF WT.” The low-risk subgroup contained patients with “low LDH and low CTC-mRNA (<2 mRNA biomarker positive)” or “low LDH, CTC-mRNA (≥2 mRNA biomarker positive), and tumor BRAF mutation positive.” The high-risk subgroup had significantly worse DFS (P = 0.03; Fig. 2A) and OS (P = 0.02; Fig. 2B). This significant association of the panel for DFS [hazard ratio (HR) = 2.65; 95% CI, 1.10–6.39; P = 0.03] and OS (HR = 4.31; 95% CI, 1.20–15.41; P = 0.02) was also identified in a multivariable analysis controlled for age, sex, and M category (Table 2).

**CTC PROFILING IN CII-TREATED MELANOMA PATIENTS**

To determine if serial CTC biomarker status is indicative of subclinical disease in blood, we highlight 6 out of 50 patients who experienced PD during CII (Fig. 3). Patients #1 and #2 experienced PD while receiving treatment (Fig. 3A and B). Patient #3 with a BRAF-mutated tumor had a detectable CTC-BRAF mutation load reflecting PD while receiving nivolumab/
dabrafenib/trametinib (Fig. 3C). Interestingly, the dynamic levels of CTC-BRAF were captured post-dabrafenib/trametinib treatment. Patient #4 experienced PD (intracranial progression) during the start of serial bleed monitoring (Fig. 3D) and again at week 122 of treatment. Patient #5 with multiple metastases (lung, liver, and spleen) experienced disease progression to their brain after 8 weeks (Fig. 3E). Patient #6, who was disease-free following complete right groin lymph node dissection, had recurrence. Overall, CTCs were consistently detected during monitoring of all 6 patients despite fluctuating Response Evaluation Criteria In Solid Tumors 1.1–defined disease burden. Therefore, serial CTC biomarker status can indicate presence of subclinical disease during combinatorial therapies in addition to computerized tomography/magnetic resonance imaging, potentially providing biomarker-associated real-time data to guide CII treatment follow-up.

Discussion

Management of melanoma patients undergoing CII or combinatorial therapies can be challenging without real-time biomarker(s) predictive of treatment response. Treatment efficacy on the basis of clinical symptoms or computerized tomography/magnetic resonance imaging can be ambiguous or delayed because tumors can enlarge (pseudo-progression) before responding/regressing. Minimally invasive monitoring can enable real-time assessment of response to treatment. Utilizing a next-generation microfluidics–based CTC enrichment approach, we developed a CTC microfluidic molecular workflow for assessing CTC biomarker status in melanoma patients during treatment. We showed robust CTC profiling and demonstrated concurrent mRNA/ DNA biomarker detection in patients with melanoma with high detection capability for CTCs during immunotherapy treatment. CTC profiling of dual mRNA/ DNA biomarkers detected subclinical disease in a real-time manner despite lack of disease-related symptoms. This study verified our melanoma biomarkers previously used in a direct-blood CTC assay (6, 7) and enabled molecular CTC profiling during CII treatment that identified new biomarkers and clinical applications for active monitoring of disease status/prognosis.

We focused on monitoring during CII to assess patient clinical outcomes during treatment. CART analysis identified a promising disease outcome blood molecular biomarker panel consisting of LDH, CTC-mRNA, and tumor BRAF for DFS/OS. LDH as a predictive variable during CII for clinical outcome is in agreement with previous reports in metastatic melanoma (23, 24). Novel CTC biomarkers enhance the application of real-time patient minimally invasive monitoring. Serial monitoring detected subclinical disease in patients who experienced PD. By extension, CTC monitoring may detect residual disease and assist in assessing tumor resistance/ progression (35).

CTNNB1 overexpression was identified as a potential CTC biomarker indicative of immune evasion, which supports studies in human melanoma immune invasiveness (33, 34). This suggests the potential of CTC escape during immune surveillance. In addition, changes in CTC-MLANA expression were associated with PD because it was frequently detected in preoperative, non-CII-treated patients with melanoma yet was only detected during follow-up in 2 patients who recurred or experienced PD during CII (data not shown). MLANA is an antigenic target for active-specific immunotherapy (36), suggesting that CTC-MLANA
positivity could be killed during effective CII activation of antitumor immunity, whereas, in patients with poor antitumor immunity CTC-MLANA, positive cells escape successfully. In summary, CTC biomarker profiling during CII allows for better understanding of tumor blood metastasis dynamics beyond CTC enumeration and single biomarker assessment and for patient treatment stratification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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30. Shidham VB, Qi DY, Acker S, Kampalath B, Chang C-C, George V, Komorowski R. Evaluation of micrometastases in sentinel lymph nodes of cutaneous melanoma: higher diagnostic accuracy with


Fig. 1. CART analysis for disease free survival and OS in patients with stage IV melanoma (n = 52).

(A), disease free survival and (B), OS analysis using 5 variables of which LDH, tumor-derived BRAF mutation, and CTC-mRNA were identified as potentially predictive of DFS and OS. A complexity parameter of 0.001 was used. The numerator indicates number of disease/death (A), or death (B), and the denominator indicates total number of patients in the corresponding subcategory. The estimated relative event rate compared to the root node is indicated.
Fig. 2. Kaplan–Meier analysis of CART-identified subgroups with DFS and OS in patients with stage IV melanoma receiving CII.
(A), DFS and (B), OS based on CART-identified subgroups in patients with stage IV melanoma (n = 52). High-risk subgroup, patients with high LDH or low LDH with CTC-mRNA (≥2 mRNA biomarkers+) and tumor WT; low-risk subgroup, patients with low LDH and low CTC-mRNA (<2 mRNA biomarkers+) or low LDH and increased CTC-mRNA and tumor BRAF mutation positive.
Fig. 3. CTC biomarker status in melanoma patients during combinatorial therapy with disease progression or recurrence.

(A), patient 1 with left calf in-transit melanoma. Patient experienced PD with metastasis to upper left groin on pembrolizumab despite partial response at left calf lesions. (B), patient 2 with multiple pelvic lymph nodes and brain metastasis. Brain lesions displayed partial response, but pelvic lymph node disease progressed on bevacizumab treatment. (C), patient 3 with metastases to right groin lymph nodes and gluteal region. Melanoma progressed to new multifocal region in left buttock area while on nivolumab and dabrafenib/trametinib. BRAF V600E mutation in CTCs were detected upon PD despite receiving dabrafenib/trametinib. (D), patient 4 with metastatic melanoma in chest/back/buttock. While on nivolumab/dabrafenib/trametinib treatment, PD occurred (i.e. intracranial progression). (E), patient 5 with multiple metastases (lung, liver, and spleen). Patient experienced progression to the brain and in the liver after 8 weeks despite partial response to nivolumab and dabrafenib/trametinib by clinical standards. (F), patient 6 following complete right groin lymph node dissection. Patient recurred with multiple dermal metastases after a 10 month
disease–free period. Pink panels denote PD according to Response Evaluation Criteria in Solid Tumors 1.1 criteria. Abbreviations: Wks, weeks; *Ipi, ipilimumab; NED, no evidence of disease; red dashed line, LDH activities; blood-CTCs, detectable mRNA biomarkers (≥2 biomarkers) in isolated CTCs; BRAF mutation, detectable BRAF mutation in isolated CTCs.
Table 1.
Characteristics of AJCC patients with stage III/IV metastatic melanoma.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number (%)</th>
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<tr>
<td><strong>Sex</strong></td>
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<tr>
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<td>54 (72)</td>
</tr>
<tr>
<td>Female</td>
<td>21 (28)</td>
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<tr>
<td><strong>Age, y</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Median (Q1, Q3)</td>
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<td>Stage IV M1A/B/C/D</td>
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<td><strong>Tumor BRAF V600E</strong></td>
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<td><strong>CTC-mRNA, n (%)</strong></td>
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</tr>
<tr>
<td>High (≥2 CTC mRNA)</td>
<td>59 (78.7)</td>
</tr>
<tr>
<td><strong>CTC-BRAF</strong></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>21 (28)</td>
</tr>
<tr>
<td>Positive</td>
<td>13 (17.3)</td>
</tr>
<tr>
<td>NA</td>
<td>41 (54.7)</td>
</tr>
<tr>
<td><strong>CTC-CTNNB1</strong></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>16 (21.3)</td>
</tr>
<tr>
<td>Positive</td>
<td>10 (13.3)</td>
</tr>
<tr>
<td>Missing</td>
<td>49 (5.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Age at the time of the 1st specimen collection.
Table 2.

Multivariable Cox proportional regression analysis of CART-identified disease outcome panel and prognostic factors on DFS and OS for patients with stage IV melanoma (n = 52).

<table>
<thead>
<tr>
<th>Prognostic Factor</th>
<th>DFS</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age, y</td>
<td>1.00</td>
<td>(0.97, 1.03)</td>
</tr>
<tr>
<td>Sex, male</td>
<td>0.55</td>
<td>(0.23, 1.32)</td>
</tr>
<tr>
<td>M category (AJCC 8)</td>
<td>0.99</td>
<td>(0.65, 1.51)</td>
</tr>
<tr>
<td>LDH/tumor BRAF/CTC-mRNA\textsuperscript{a}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-risk\textsuperscript{b}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-risk\textsuperscript{c}</td>
<td>2.65</td>
<td>(1.10, 6.39)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Predictor from CART with regard to DFS and OS.

\textsuperscript{b}Low LDH and low CTC-mRNA or low LDH and high CTC-mRNA and tumor BRAF mutation positive.

\textsuperscript{c}High LDH or low LDH and high CTC-mRNA and tumor BRAF WT.