Genome-Wide Characterization of Circulating Tumor Cells Identifies Novel Prognostic Genomic Alterations in Systemic Melanoma Metastasis

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BACKGROUND: Circulating tumor cells (CTC) have been found in patients with metastatic melanoma and are associated with advanced melanoma stage and poor patient outcome. We hypothesize that CTC harbor genomic changes critical in the development of distant systemic metastasis. Here, we present the first genome-wide copy-number aberration (CNA) and loss of heterozygosity (LOH)-based characterization of melanoma CTC.

METHODS: CTC were isolated from peripheral blood monocytes of 13 melanoma patients with regional metastasis stage IIIB/C using antibodies against melanoma-associated cell surface gangliosides.

RESULTS: We characterized 251 CNA in CTC. Comparative analysis demonstrated >90% concordance in single-nucleotide polymorphism profiles between paired CTC and tumor metastases. In particular, there were notable recurring CNA across patients. In exploratory studies, the presence of several top CTC-associated CNA was verified in distant metastasis (stage IV) from 27 patients, suggesting that certain genomic changes are propagated from regional metastasis to CTC and to distant systemic metastases. Lastly, an exploratory biomarker panel derived from 5 CTC-associated CNA (CSMD2 (CUB and Sushi multiple domains 2), 1p35.1; CNTNAP5 (contactin associated protein-like 5), 2q14.3; NRDE2 (NRDE-2, necessary for RNA interference, domain containing), 14q32.11; ADAM6 (ADAM metallopeptidase domain 6, pseudogene), 14q32.33; and TRPM2 (transient receptor potential cation channel, subfamily m, member 2), 21q22.3) conferred prognostic utility for melanoma recurrence [hazard ratio (HR), 1.14; CI, 1.00–1.44; \( P = 0.0471 \)] and death (HR, 2.86; CI, 1.23–4.42; \( P = 0.0014 \)) in 35 patients with stage IIIIB/C melanoma, with a 5-year disease-free survival of 13% vs 69% (\( P = 0.0006 \)) and overall survival of 28% vs 94% between high-risk and low-risk groups defined by the biomarker panel, respectively.

CONCLUSIONS: This study provides the first detailed CNA-based profile of melanoma CTC and illustrates how CTC may be used as a novel approach for identification of systemic metastasis.

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Successful treatment of melanoma requires understanding of the metastatic process and identification of patients with tumors at risk for developing aggressive metastatic disease. Circulating tumor cells (CTC) have been detected in the blood of patients with melanoma (1, 2) and other solid cancers (3–5). Previously, we have demonstrated that CTC biomarkers increase with metastasis and tumor burden in melanoma patients (6). We also have shown that CTC are associated with increased risk of tumor recurrence and poor survival in melanoma patients (1, 6). Measurement of CTC has also been used as an indicator of patient response to systemic therapy in melanoma (1). Thus, CTC may be an important predictor of patient outcome and may represent an approach for early detection of “subclinical systemic metastasis” (7).

A detailed genomic characterization of CTC in melanoma has not been reported to date. However, genome-
wide profiling studies have been carried out in melanoma tumor samples with identification of important genomic aberrations (8–10). Single-nucleotide polymorphism (SNP)-derived copy number aberrations (CNA) [copy number gains (CNG), copy number losses (CNL)], and loss of heterozygosity (LOH) are important genetic aberrations in cancer progression. High-density SNP microarrays have been applied to detailed genome-wide identification of CNA events in cancer and represent an informative platform for the discovery of functional tumor-related genes (11).

Melanomas are heterogeneous, with both variant and common genotypes and phenotypes (12). We postulated that genomic signatures could occur in CTC that may represent particularly aggressive stage IIIB/C metastases capable of evolving into clinical distant organ metastasis. Genomic profiling of CTC may allow improved understanding of their role in real-time cancer metastasis events and may represent a novel approach in identification of genomic aberrations associated with melanoma prognosis and treatment.

In this study, we performed an array-based genome-wide CNA characterization of melanoma CTC. We successfully identified 251 CNAs in melanoma CTC from 13 patients and showed high genomic concordance in CTC–tumor metastasis pairs. There were notable recurring CNA in paired CTC and tumor metastases across patients. In subsequent exploratory studies, we verified the significance of CTC-associated CNA by their presence in 27 distant organ melanoma metastases [American Joint Committee on Cancer (AJCC) stage IV] and as biomarkers of poor prognosis in 35 patients with regional melanoma metastasis (stage IIIB/C).

Materials and Methods

PATIENTS

Three nonoverlapping cohorts of patients with metastatic melanoma were included in this 2-phase study. In the CTC discovery phase, tumor-involved nodal tissue and peripheral blood mononuclear cell (PBMC) samples were obtained from 13 patients with stage IIIB/C regional melanoma metastasis. In the verification phase, the clinical relevance of CTC-associated CNA was verified in distant stage IV metastasis tumor tissue of 27 patients with stage IV melanoma involving systemic metastases to the brain (n = 15), lung (n = 4), and gastrointestinal tract (n = 8) and in cell lines derived from 35 melanoma patients with regional metastasis (stage IIIB/C melanoma). M1 and M14 metastatic melanoma cell lines were used to verify the sensitivity of the CTC immune-capture assay.

All operative tissue samples were collected and processed at the John Wayne Cancer Institute and cryopreserved in liquid nitrogen. All cell lines were

from human tumor tissue obtained at the time of surgery. Early passaged melanoma lines (<12 passages) were used. This study was approved by the John Wayne Cancer Institute/Saint John’s Health Center and Western Institutional Review Board. Informed consent was obtained from all patients.

MELANOMA CTC IMMUNOCAPTURE ASSAY

Human IgM monoclonal antibodies (mAb) to ganglioside GM3 (L612) (13), ganglioside GM2 (L55) (14), and ganglioside GD2 (L72) (15) were developed by R.I. from an established Epstein–Barr virus–transformed human B-cell line, cloned, extensively purified, and verified and quality controlled under good laboratory practice (GLP) conditions. These 3 mAb were coupled to Dynabeads M-450 tosylactivated (Life Technologies), according to the manufacturer’s instructions.

VERIFICATION OF MELANOMA CTC IMMUNOCAPTURE ASSAY

M1 and M14 melanoma cells were serially diluted in PBMC from healthy donors (100, 50, 10, 1, and 0 melanoma cells in 10⁶ PBMC). Previously assessed CTC mRNA melanoma reverse transcription-quantitative PCR (RT-qPCR) biomarkers by our group (6, 16–18) were used for verification of capture of melanoma cells, including tyrosine-related protein 2 (TRP-2), melanoma antigen recognized by T cells 1 (MART-1), melanoma antigen gene-A3 family (MAGE-A3), and paired box homeotic gene transcription factor 3 (PAX-3). Additionally, immunocaptured cells were stained with anti-MART-1 mAb (GeneTex, Clone # GTX75965), followed by incubation with Alexa Fluor® 555 goat antimouse IgG2b (Invitrogen) and were mounted onto glass slides and stained with ProLong® Gold antifade reagent with DAPI (Invitrogen). The cells were analyzed by Leica microscopy to verify captured melanoma CTC.

ISOLATION OF MELANOMA CTC FROM PBMC

PBMC were isolated by Ficoll-Hypaque, collected, and suspended in freezing medium and cryopreserved in liquid nitrogen. IgM Ab-coupled Dynabeads were added to each sample (10⁶ cells/mL) followed by incubation at 4 °C (1 h). For the immunocapture assay, samples were then placed in the Dynal® MPC-6 appliance (Invitrogen), allowing aspiration of unbound cells. beads with bound cells were then washed using 0.1% BSA/PBS with 2 mmol/L EDTA. Immunocaptured cells were subjected to immunofluorescent staining with MART-1 mAb or to DNA or RNA extractions with DNAzol and Tri-Reagent (Molecular Research Center), respectively. The RT-qPCR assay was performed with the iCycler iQ Real-Time Thermocycler Detection System (Bio-Rad Laboratories) with
a beta-2-microglobulin (B2M) control housekeeping gene. The sequences of primers and fluorescence resonance energy transfer probe of B2M were as follows: primers, 5’-TGTCACAGCCCAAGATAG-3’ and 5’-CAAGCAGCAATTTGGAA-3’ and probe, 5’-FAM-TCCATGATGCTGCTTACATGTCTCGA-BHQ-1-3’ (6).

DNA EXTRACTION AND WHOLE-GENOME AMPLIFICATION
DNA extraction from frozen tumor tissues and cell lines was carried out with the QIAamp DNA mini kit (QIAGEN). DNA extraction from CTC was carried out using DNAzol. DNA quantification was performed using Quant-iT™ PicoGreen® double-stranded DNA reagent and kits (Invitrogen). Extracted DNA from CTC and paired melanoma tissue in the discovery cohort was further subjected to whole-genome amplification (WGA) using a REPLI-g mini kit (QIAGEN). The reaction was performed at 30 °C for 16 h, followed by heat inactivation at 65 °C for 3 min. Amplified DNA was used for Affymetrix SNP 6.0 array analysis. DNA isolated for the CNA verification cohort (27 stage IV melanoma metastases, 35 stage IIIIB/C regional melanoma metastases) was not subjected to WGA.

GENOME-WIDE SNP ARRAY ANALYSIS
DNA obtained after WGA was assessed on the SNP array for genotyping analysis and the procedures were performed at the University of Southern California/Children’s Hospital of Los Angeles Genome Core Laboratory. Genotyping of samples was performed using Affymetrix Genotyping Console 4.0 with the Birdseed v2 algorithm under GLP conditions (19). Before genotyping, QC of arrays was conducted using the contrast QC algorithm with a minimal call rate of >95%. For copy number analysis QC, individual arrays must have a median of the absolute values of all pairwise differences below 0.35. CNA analyses were performed using Affymetrix Genotyping Console 4.0 with regional QC correction and default software settings. HapMap reference, release 30, was used as the reference model for SNP analysis, release 30, was used as the reference model for SNP analysis, eliminating most validation cases and a false-positive rate of <10%. The minimum LOH size was set to 100 kb, eliminating >70% of the copy number–neutral LOH found in HapMap samples, and a minimum of 10 SNP markers within the region that report LOH were used.

The methods we have been characterized previously to be highly robust and accurate when identifying large (≥100 kb) CNA and LOH (20–22). Data generated by Genotyping Console were used to identify frequent CNA regions among CTC–tumor metastasis pairs. CNA on the sex chromosomes were excluded from analysis, and autosomal CNA were kept in the final analysis. The CNA reported were all on the size order of a cytoband.

BIOSTATISTICAL ANALYSIS
The biostatistical analysis methods are described in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol60/issue6.

RESULTS
CTC ISOLATION
A CTC immunocapture was developed utilizing IgM human mAb against 3 melanoma-related gangliosides [GM3 (13), GM2 (14), and GD2 (15)] coupled to immunomagnetic beads. Tumor-associated gangliosides are sialyl glycosphingolipids present on the outer surface of the cell plasma membrane (23). Melanoma-associated ganglioside expression has been previously reported by our group using biochemical assays and patient Ab analysis (13, 23, 24), and gangliosides have been successfully used as targets for mAb-based therapy in melanoma (14, 25). Use of multiple cell-surface Ab for immunocapture of CTC in melanoma patients addresses CTC capture sensitivity, tumor heterogeneity, and identification of aggressive CTC phenotypes. IgM human mAbs were used because they provide stronger capture efficiency than IgG mAbs. To evaluate the detection limit of our immuno-CTC capture assay, we spiked serially diluted cultured melanoma cells (100, 50, 10, 1, and 0 cells) in 10^7 PBMCs from healthy donors and retrieved by our immunocapture technique (Fig. 1A). Total RNA was extracted from captured melanoma cells and subjected to RT-qPCR as previously described (26). Captured CTC were verified by detection of 4 known melanoma-associated RT-qPCR biomarkers (TRP-2, MAGE-A3, MART-1, and PAX-3) that we previously identified in tumors and CTC from metastatic melanoma patients. The immunocapture assay sensitivity of capturing melanoma
Fig. 1. Verification of CTC isolation utilizing antiganglioside IgM mAb capture. Capture of CTC from PBMCs of patients with melanoma. (A), Serial dilutions of M1 melanoma cells expressing cell surface GM3 and M14 melanoma cells expressing cell surface GM2 and GD2 spiked in PBMC from healthy donors were performed to verify assay sensitivity. Immunocapture of circulating melanoma cells was verified by RT-qPCR detection of mRNA for 4 melanoma-associated antigens (TRP-2, MAGE-A3, MART-1, PAX-3). The y axis shows mRNA copies of antigens relative to a control housekeeping gene, B2M. (B), Representative analyses of CTC immunocaptured by beads from PBMC from a stage IV melanoma patient. (i), CTC are surrounded by beads in the microscopy bright field. Left, 100×; right, 600×. (ii), 4′,6-Diamidino-2-phenylindole (DAPI) indicates nuclear staining of CTC. Left, 100×; right, 600×. (iii), Red indicates anti-MART-1 mAb and Alexa Fluor 555–labeled secondary Ab staining of CTC. Left, 100×; right, 600×. (iv), Merged (DAPI + anti-MART-1 mAb) staining of CTC. Left, 100×; right, 600×. 

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CTC from PBMCs was approximately 1 to 5 melanoma cells in \(10^7\) PBMCs (Fig. 1A). The specificity of the assay for isolation of melanoma cells was verified by staining captured CTC with a melanoma-related antigen, MART-1 (Fig. 1B).

Whole-genome SNP-based analysis using the Affymetrix Human Genome-Wide SNP 6.0 microarray was carried out on captured CTC and paired metastatic tissue from 13 patients with melanoma regional palpation.
ble metastases (stage IIIB/C). CTC were evaluated for CNA using Genotyping Console 4.0 against the HapMap 270 reference. There were several notable recurring CNA across patient CTC. We identified 251 CNA present in 50% of CTC (Table 1). Of these, CNG at 20 loci were present in 90% of CTC, and CNG aberrations at 2 loci (20q31.3, 14q23.3) were seen in 100% of CTC. Furthermore, 100% of CTC had CNL at 9 loci (4p16.3, 6q25.3, 9p34.3, 10p15.3, 10q26.3, 14q32.33, 16p13.3, 18q23, 19p13.3), and an additional 14 CNL were present in >90% of CTC. Additionally, >90% of CTC had LOH at 14q23.3, and an additional 23 LOHs were detected in >50% of CTC.

**PAIRED CTC AND REGIONAL METASTASES SHOWED GENOMIC CONCORDANCE**

To examine the relevance of CTC-associated genomic aberrations, we compared the genomic profiles of CTC to those for paired tumor tissue samples from 13 melanoma patients with stage III B/C regional metastases. All 13 sample pairs showed SNP status concordance of >90%, and 10 pairs had SNP concordance of ≥97%.

<table>
<thead>
<tr>
<th>Percentage of patients with genomic variation in CTC at loci*</th>
<th>CNG loci</th>
<th>CNL loci</th>
<th>LOH loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% 2q35, 16p13.3</td>
<td>4p16.3, 6q25.3, 9q34.3, 10p15.3, 10q26.3, 14q32.33, 16p13.3, 18q23, 19p13.3</td>
<td>14q23.3</td>
<td></td>
</tr>
<tr>
<td>50%–59% 1p34.2, 1p34.3, 1p35.2, 1p36.11, 1p36.32, 1q25.3, 2q21, 2q37.1, 3p14.1, 3q22.3, 4q12.2, 5q13.1, 5q23.3, 6p21.3, 6p22.1, 6q15, 7q36.1, 8p23.1, 9q33.3, 10q24.31, 10q25.2, 11p11.2, 12q12, 12q13.3, 12q24.13, 13q14.12, 13q32.11, 16p13.11, 20q12, 22q11.22</td>
<td>1p13.2, 1q31.1, 1q44, 4q25, 4q28.1, 5q15, 5q35.2, 6q16.3, 9p13.1, 10p14.21, 10p12.2, 10q25.2, 11p15.1, 14q11.2, 15q22.31, 19p12, 21q22.11</td>
<td></td>
<td></td>
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</table>

* n = 13 patients; CNA loci in >50% of CTC are shown.

Table 1. Summary of loci with CNAs in melanoma CTC.
(see online Supplemental Table 1). Furthermore, in a genome-wide comparative analysis of CNA states defined by gene segment, the 13 CTC–tumor metastasis pairs showed 71% to 96% concordant CNA changes among 17 599 gene coding regions and 80% to 98% concordant CNA changes among 30 994 coding and noncoding 100-kb gene segments (see online Supplemental Table 1). By use of the Cohen \( \kappa \) statistical analysis, all CTC and tumor pairs showed a \( \kappa \) coefficient of >0.7, showing high agreement between CTC and tumor genotype and CNA.

Melanomas are heterogeneous, and we hypothesized that CTC harbor genomic CNA representative of particularly aggressive tumor clones. To this end, a detailed CNA analysis at SNP loci was carried out on each CTC–tumor metastasis pair to determine regions of common genomic change. Common loci CNA are presented in Fig. 2. Ten loci CNG and 14 loci CNL were identified in paired CTC–tumor metastasis in the discovery cohort. Defined by CNG and CNL common in 50% of paired CTC–tumor metastasis pairs, and LOH at 12q24.13, 16q11.2, and 1p34.3 were indeed present at a significantly higher frequency in stage IV metastases (see online Supplemental Table 2). These findings verified that certain CTC-associated CNA were also present in systemic metastases, and suggest that CTC may contain specific genomic aberrations important in development of systemic metastasis.

**CTC GENOMIC ABERRATIONS IDENTIFIED IN DISTANT ORGAN METASTASES**

Because distant organ metastases are CTC derived, we postulated that CTC-associated CNA may represent genomic regions of importance in melanoma metastasis. We sought to verify our hypothesis by evaluation of CTC-associated genomic changes in a subsequent exploratory study of 27 patients who had undergone surgical resection of distant organ tumor metastasis (stage IV patients). We evaluated the top CNA \((n = 37)\) identified in paired CTC–tumor metastasis in the discovery cohort (Fig. 2), defined by CNG and CNL common in >80% of paired CTC–tumor metastasis and LOH common in >50% of paired CTC–tumor metastasis. Of these 37 loci, genomic aberrations at 15 loci were identified in >50% of distant-organ melanoma metastases in this verification study.

To further verify whether these CNA were indeed present in metastatic melanomas, we assessed the presence of CNA spanning the 37 putative CNA from melanoma lymph node metastases \((n = 169)\) and distant metastases \((n = 35)\) from TCGA. For the CNGs, 4 were present in over 24% of the TCGA cohort, and 6 were present in >40% of the cohort. All CNLs reported were present in >40% of the TCGA cohort. LOH data were not publicly available.

To further verify whether the CNA verified in the stage IV patient cohort were indeed linked to the systemic metastatic potential of CTC rather than overall melanoma metastasis, we assessed the presence of the CNA in regional metastasis \((n = 6)\), stage IIIIB/C melanoma patients with 10-year disease-free survival (DFS) after surgical resection to render the patients disease free. Using a Pearson test to compare these verified CNA/LOH to those present in 6 patients with 10-year DFS, we found that CNGs at 14q32.33 and 1q25.1 as well as LOH at 12q24.13, 16q11.2, and 1p34.3 were indeed present at a significantly higher frequency in stage IV metastases (see online Supplemental Table 2). These findings verified that certain CTC-associated CNA were also present in systemic metastases, and suggest that CTC may contain specific genomic aberrations important in development of systemic metastasis.

**CTC GENOMIC ABERRATIONS IDENTIFIED PATIENTS WITH POOR OUTCOME**

To determine the relevance of CTC-associated CNA in predicting progression of regional melanoma, CTC-associated genomic aberrations were verified in an exploratory biomarker study of cell lines derived from regional metastatic tissue of 35 patients with stage IIIIB/IIIC melanomas. The top CNA \((n = 37)\) identified in CTC–tumor metastasis pairs in the discovery cohort (Fig. 2) were evaluated. Single-genomic site analyses and genomic panel analyses were carried out. Survival analyses of individual genomic sites did not reach statistical significance after correction for multiple testing in this pilot study analysis. Using stepwise Cox regression, we developed a 5-marker CNA panel with the greatest prognostic utility, consisting of CNG at 1p35.1, CNG at 2q14.3, CNG at 14q32.33, CNL at 14q32.11, and CNL at 21q22.3. To determine whether these events were common in metastatic melanomas, the presence of CNA in these cytobands was assessed in the TCGA cohort. Of the 5 CNA reported, a CNG at 2q14.3 was present in 26% of the cohort, with the CNA at the other markers present in 40%–51% of the cohort.

A risk score was generated that classified patients into high-risk and low-risk groups for melanoma recurrence and death. The high-risk group classification conferred a significantly worse cancer outcome compared to the low-risk group, with 5-year DFS rates of 13% vs 69% \((P = 0.0006)\) and overall survival of 28% vs 94% \((P = 0.0006)\).
Fig. 2. Summary of genome-wide SNP-based analysis of paired melanoma CTC and regional stage IIIb/C metastasis. Comparative SNP analysis was carried out between CTC and their corresponding melanoma regional metastases for CNG (A), CNL (B), and LOH (C). Each chromosome is represented by a portion of the circle and is indicated by chromosomal number. Thirteen rungs are shown, each representing a single patient. For each patient, CNA analyses were carried out on patient CTC (inner half of rung) and matched regional metastatic tissue (outer half of rung). All CNA are indicated by solid-colored bars within each rung. Open rectangular bars highlight the top CNA, defined as CNG and CNL common in >80% of paired CTC–tumor metastasis and LOH common in >50% of paired CTC–tumor metastasis. The top CNA indicated in the figure are described in the accompanying Table 1.

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Fig. 2. Continued.

Human genes: (A), CXCR2, chemokine (C-X-C motif) receptor 2 (IL8RB, previous symbol); TNP1, transition protein 1 (during histone to protamine replacement); TNS1, tensin 1; TNR, tenasin R; FSHR, follicle stimulating hormone receptor; LHCGR, luteinizing hormone/choriogonadotropin receptor; KIAA0125, KIAA0125 (FAM30A, previous symbol); CSMD2, CUB and Sushi multiple domains 2; HMGB4, high mobility group box 4; ASTN1, astrotactin 1; PAPPA2, pappalysin 2; TNF, tumor necrosis factor; MSH5, mutS homolog 5; CALCOCO1, calcium binding and coiled-coil domain 1; CBX5, chromobox homolog 5; CCDC33, coiled-coil domain containing 33; ISLR, immunoglobulin superfamily containing leucine-rich repeat; STRA6, stimulated by retinoic acid 6; (B), ARID1B, AT rich interactive domain 1B (SWI1-like); NOTCH1, notch 1; TRAF2, TNF receptor-associated factor 2; AKT1, v-akt murine thymoma viral oncogene homolog 1; JAG2, jagged 2; MTA1, metastasis associated 1; ABCA7, ATP-binding cassette, sub-family A (ABC1), member 7; KISS1R, KISS1 receptor; STK11, serine/threonine kinase 11; CTBP1, C-terminal binding protein 1; FGFR3, fibroblast growth factor receptor 3; FLJ14051, hypothetical protein FLJ14051; CALM1, calmodulin 1 (phosphorylase kinase, delta); PKD1, polycystic kidney disease 1 (autosomal dominant); TSC2, tuberous sclerosis 2; ATP9B, ATPase, class II, type 9B; NFATC1, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1.

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**Fig. 2.** Continued.

*DIP2C, DIP2 disco-interacting protein 2 homolog C (Drosophila); MGMT, O-6-methylguanine-DNA methyltransferase; AIRE, autoimmune regulator; TRPM2, transient receptor potential cation channel, subfamily M, member 2; NKD2, naked cuticle homolog 2 (Drosophila); SLC12A7, solute carrier family 12 (potassium/chloride transporter), member 7; SLC6A19, solute carrier family 6 (neutral amino acid transporter), member 19; DDX4, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; SLC38A9, solute carrier family 38, member 9; CYBA, cytochrome b-245, alpha polypeptide; DPEP1, dipeptidase 1 (renal); MVD, mevalonate (diphospho) decarboxylase; (C), ATP6V1D, ATPase, H+/H11001 transporting, lysosomal 34kDa, V1 subunit D; PLEK2, pleckstrin 2; BRAP, BRCA1 associated protein; ERP29, endoplasmic reticulum protein 29; PTPN11, protein tyrosine phosphatase, non-receptor type 11; TFAP2E, transcription factor AP-2 epsilon (activating enhancer binding protein 2 epsilon); FOLH1, folate hydrolase (prostate-specific membrane antigen) 1; ABCC11, ATP-binding cassette, sub-family C (CFTR/MPR), member 11; SIAH1, siah E3 ubiquitin protein ligase 1; DOCK3, dedicator of cytokinesis 3; RASSF1, Ras association (RalGDS/AF-6) domain family member 1; PTPRJ, protein tyrosine phosphatase, receptor type, J; GPT2, glutamic pyruvate transaminase (alanine aminotransferase) 2; MYLK3, myosin light chain kinase 3; ORC6L, origin recognition complex, subunit 6; ELAVL4, ELAV like neuron-specific RNA binding protein 4; MCM6, minichromosome maintenance complex component 6; EPM2A, epilepsy, progressive myoclonus type 2A, Lafora disease (laforin); FBXO30, F-box protein 30; GRM1, glutamate receptor, metabotropic 1; SNTG1, syntrophin, gamma 1.

*Top regions shown, defined by LOH common in >50% of paired CTC tumor metastasis.*

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**Table:**

<table>
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<tr>
<th>Cytoband</th>
<th>Start</th>
<th>End</th>
<th>No. of Pairs (n = 13) (%)</th>
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<td>1q34.3</td>
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<td>7 (53.8%)</td>
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The risk score generated from the 5-marker CNA panel was then placed in a multivariable Cox model with known melanoma prognostic factors. In a multivariable analysis of the CNA risk score and lymph node positivity, the CNA risk-score was an independent prognostic factor for 5-year melanoma recurrence (hazard ratio, 1.14; CI 1.00–1.44; \( P = 0.0471 \)) and death (hazard ratio, 2.86; CI 1.23–14.42; \( P = 0.0014 \)).

Discussion

CTC are an attractive alternative to tumor tissues for genomic profiling and biomarker analysis. CTC can be readily extracted from PBMC obtained from a blood draw without the antecedent risks of and need for an invasive surgical resection or percutaneous tissue biopsy for tumor sampling. Furthermore, tumor tissue may not always be available for analysis, particularly when metastases are small or when biopsy or surgery is not technically feasible. CTC can provide a real-time evaluation of subclinical melanoma spread and a method for monitoring patient response to therapy. A comprehensive genomic profile and analysis of melanoma CTC have not been reported to date. Our genome-wide SNP-based characterization of CTC revealed 251 CNA, which may represent candidate genomic aberrations important in melanoma progression and metastasis. Herein, we independently report on several known melanoma-associated SNP loci.

SNP loci identified by CTC were used to generate an exploratory 5-marker CNA gene panel: CUB and Sushi multiple domains 2 (\( \text{CSMD2} \), 1p35.1); contactin associated protein-like 5 (\( \text{CNTNAP5} \), 2q14.3); NRDE-2, necessary for RNA interference, domain containing (\( \text{NRDE2} \), 14q32.11); ADAM metallopeptidase domain 6, pseudogene (\( \text{ADAM6} \), 14q32.33); and transient receptor potential cation channel, subfamily M, member 2 (\( \text{TRPM2} \), 21q22.3), with the ability to differentiate melanoma patients with good and poor outcome. Four of these 5 genes have been reported in cancer or have been implicated in cell adhesion and metastasis. The study of genomic aberrations in CTC may provide insight into genomic characteristics and mechanisms by which tumor cells establish distant organ metastasis and lead to the development of strategies to target CTC to prevent or control systemic melanoma metastasis.

A comprehensive genomic profile and analysis of melanoma CTC have not been reported to date. Our genome-wide SNP-based characterization of CTC revealed 251 CNA, which may represent candidate genomic aberrations important in melanoma progression and metastasis. Herein, we independently report on several known melanoma-associated SNP loci.

SNP loci identified by CTC were used to generate an exploratory 5-marker CNA gene panel: CUB and Sushi multiple domains 2 (\( \text{CSMD2} \), 1p35.1); contactin associated protein-like 5 (\( \text{CNTNAP5} \), 2q14.3); NRDE-2, necessary for RNA interference, domain containing (\( \text{NRDE2} \), 14q32.11); ADAM metallopeptidase domain 6, pseudogene (\( \text{ADAM6} \), 14q32.33); and transient receptor potential cation channel, subfamily M, member 2 (\( \text{TRPM2} \), 21q22.3), with the ability to differentiate melanoma patients with good and poor outcome. Four of these 5 genes have been reported in cancer or have been implicated in cell adhesion and metastasis. TRPM2 is a calcium channel activated by oxidative stress that regulates susceptibility to cell death and has been implicated in prostate cancer and melanoma. In a recent report by Orfanelli et al. on aberrant sense and antisense transcripts derived from global hypomethylation studies in melanoma, several transcripts within the \( \text{TRPM2} \) locus were enriched in melanoma. qPCR confirmed upregulation of \( \text{TRPM2-AS} \) and \( \text{TRPM2-TE} \) transcripts within the
TRPM2 region, with corresponding methylation status of a shared CpG island in melanoma. Overexpression of wild-type TRPM2 and functional knockout of TRPM2-TE by stable transfection increased cellular apoptosis and necrosis in melanoma cells. Further, CSMD2 has been reported to be hypermethylated in pancreatic cancer and is a putative tumor suppressor gene (34). Also, CNTNAP5 is a transmembrane protein involved in cell adhesion (35). Comparative CNA and gene-based analyses showed the association of CNTNAP1 (member of CNTNAP family) with breast cancer risk (36). Lastly, cancer metastasis requires the ability to dissociate from neighboring cells or the extracellular matrix. ADAM is a family of membrane proteins involved in cell–cell adhesion and cell–matrix adhesion (37, 38). It is characterized by a disintegrin and metalloprotease domain with an epidermal growth factor–like region and harbors both adhesion and proteolytic domains implicated in integrin function and matrix degradation (38, 39). Verification of the biomarker panel in a large independent patient cohort would be valuable and warrants further study. Additionally, there may be other genes relevant to melanoma progression spanning the regions identified in the 5-marker CNA panel. The genes mentioned are major reported genes that have potential importance in metastasis and/or melanoma.

Our study was limited in that the immunocapture approach targeted different gangliosides with several mAbs, so some melanoma CTC that expressed low amounts or none of the gangliosides on their surface may be missed. Additionally, the verification cohorts used were pathologically well-defined stage IIIB/C and IV melanomas. Given that melanoma tumors may be heterogeneous, these samples were representative of the tumor removed beyond tissue necessary for standard pathological assessment.

This study provides the first detailed genome-wide SNP- and CNA-based analysis of melanoma CTC. The results confirm the close genomic relation between CTC and tumor metastases. There were notable recurring CNA across patient groups, which were subsequently demonstrated to be highly represented in systemic metastatic melanomas and to be of prognostic utility. The genomic study of CTC, may be an important and novel approach in the identification of pro-metastatic genes in melanoma patients with regional stage IIIB/C disease whose risk of distant metastasis development is high and time frame of recurrence is unknown.

Data Access

All array data are deposited in NCBI’s Gene Expression Omnibus (GEO) data bank and are accessible through GEO Series accession file GSE43934. In particular, the samples with 10-year DFS are O871, O870, O857, O844, O848, and O866.

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References

Genomic Characterization of Circulating Melanoma Cells


