Circulating Tumor Cells and Early Relapse in Node-positive Melanoma

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ABSTRACT

Purpose: There is a need for sensitive, reproducible biomarkers for patients with stage III melanoma to guide clinical decision making. Circulating tumor cells (CTCs) can be detected in patients with melanoma; however, there are limited data regarding their significance in stage III disease. The aim of this study was to determine whether CTCs are associated with early relapse in stage III melanoma.

Experimental Design: We prospectively assessed CTCs at first presentation in clinic (baseline) for 243 patients with stage III melanoma. CTCs were measured using the CellSearch System. Relapse-free survival (RFS) was compared between patients with one or more baseline CTC versus those with no CTCs. Log-rank test and Cox regression analysis were applied to establish associations of CTCs with RFS.

Results: At least one baseline CTC was identified in 90 of 243 (37%) patients. Forty-five (19%), 67 (28%), 118 (49%), and 13 (5%) patients were stage IIIA, IIIB, IIIC, or IIID, respectively. CTC detection was not associated with substage, or primary tumor characteristics. Multivariable analysis demonstrated that the detection of ≥1 baseline CTC was significantly associated with decreased 6-month RFS [log-rank, $P < 0.0001$; HR, 3.62, 95% confidence interval (CI), 1.78–7.36; $P < 0.0001$] and 54-month RFS (log-rank, $P = 0.01$; HR, 1.69; 95% CI, 1.13–2.54; $P = 0.01$).

Conclusions: ≥1 CTC was independently associated with melanoma relapse, suggesting that CTC assessment may be useful to identify patients at risk for relapse who could derive benefit from adjuvant therapy.

Introduction

Cutaneous melanoma results in 75% of skin cancer–related deaths (1), and the incidence has been increasing worldwide over the last decade. This trend is also seen in the United States, with an estimated 96,480 new melanoma cases and more than 7,200 deaths expected in 2019 (1). Despite the development of several targeted and immune-based therapies for patients with melanoma, the prognosis is quite heterogeneous for patients with stage III melanoma overall, with 5-year survival rates ranging from 93% to 32% for patients with stage IIIA–IIID, respectively, according to the American Joint Commission on Cancer (AJCC) 8th edition (2). These survival statistics highlight the need for better prognostic markers to identify patients at increased risk for relapse. Because adjuvant therapies are approved for all patients with stage III melanoma (not necessarily just high-risk patients), there is the potential to overtreat those with low-relapse risk.

Circulating tumor cells (CTCs) have been studied for more than 30 years in patients with melanoma, although most studies primarily used PCR methodologies (3, 4). Although some of these studies have demonstrated that CTC presence has prognostic significance (5–11), the varied methodologies employed and the lack of standardization have impeded the utilization of CTCs as a melanoma biomarker in the clinic. The semiautomated CellSearch system (Menarini Silicon Biosystems) is a standardized, FDA-approved methodology for CTC detection for metastatic breast, colon, and prostate cancers. The CellSearch CTC Assay has been studied extensively for a variety of patients with solid tumors. However, to date, only three studies have reported on the prognostic significance of CTCs in patients with melanoma using the recently developed melanoma-specific CellSearch CTC Assay; these studies included only patients with stage IV melanoma (12–14).

In this study, we utilized a melanoma-specific CTC kit to evaluate the identification rate and prognostic significance of CTC detection in patients with stage III cutaneous melanoma. We present one of the first prospective studies designed to determine whether CTCs are significantly associated with relapse in patients with stage III melanoma. We hypothesized that identification of CTCs within the blood at baseline would independently predict shorter survival, irrespective of standard primary tumor (T), distant metastases (M), regional nodes (N; TNM) factors, or the extent of lymph node metastases. If this hypothesis is validated, it would warrant larger studies to facilitate the incorporation of easily obtained, blood-based CTC assessments into standard practice. This would be of great clinical interest as there are currently no melanoma-specific, blood biomarker tests approved by the National Comprehensive Cancer Network or by the American Society of Clinical Oncology (ASCO) to identify patients with stage III melanoma who are at high risk for relapse.

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Translational Relevance

In this prospective study of 243 patients with cutaneous melanoma, the detection of one or more CTCs per 7.5-mL tubes of blood at first presentation in clinic (baseline) independently predicted relapse within 6 months of baseline, as well as at 54-month follow-up. CTC assessment at first presentation in clinic may be useful to identify patients with stage III melanoma who could derive benefit from rigorous imaging surveillance, or adjuvant systemic therapy. These data support future trials to further identify the predictive value for liquid biopsy in the optimal treatment of patients with melanoma.

Materials and Methods

Patients

This study included patients diagnosed with stage III cutaneous melanoma between February 2012 and June 2017 at The University of Texas MD Anderson Cancer Center (Houston, TX). Patients with mucosal and uveal melanoma and patients with metastatic disease proven by biopsy and/or PET/CT imaging were ineligible to participate in this study. We obtained informed consent from all patients prior to blood collection and this study was conducted in accordance with ethical guidelines set forth by the Belmont Report. Individual patient results were blinded from investigators by use of a random number system as the unique patient identifier. The Institutional Review Board (IRB) at The University at Texas MD Anderson Cancer Center (Houston, TX) approved this prospective study (LAB-11-0314; principal investigator: A. Lucci). The IRB-approved protocol is ongoing to increase accrual, and to permit sequential CTC measurements during routine follow-up visits.

Staging and classification

The primary TNM staging and tumor grade was designated according to the criteria set by the 8th edition AJCC and is reported as pathologic stage after completion of surgical therapy, unless otherwise noted (3). Regional lymph node status was determined by the presence or absence of lymph node metastasis as reported at the time of operation, either by sentinel lymph node biopsy or lymphadenectomy. Seventy-six percent of patients with a positive sentinel node biopsy underwent completion lymph node dissection of the sentinel node–positive basin. However, in most patients (75%), the baseline blood draw was taken after the finding of a positive sentinel node, before any additional lymph node surgery. All patients had baseline PET/CT or CT scans of the chest, abdomen, and pelvis at diagnosis of stage III disease to rule out clinically occult metastatic disease. Relapse was defined as recurrence of melanoma at the primary site, nodal basin, or distant metastatic sites for patients on observation.

CTC analysis

No patients reported adverse events or complications from blood collection. Serial peripheral venous blood draws were collected; the first (baseline) sample was collected within 3 months of documenting a positive sentinel lymph node biopsy in 75% of patients, or a node dissection revealing positive nodal metastases in the remaining 25%. We used one 10-mL tube of blood containing CellSave preservative for the detection of CTCs using the CellSearch Circulating Melanoma Cell Assay. CTC assessments were performed within 72 hours of blood collection at the University of Texas MD Anderson Cancer Center (Houston, TX), following the manufacturer's protocol as described previously (12). Briefly, the CellSearch Circulating Melanoma Cell test uses ferrofluids coated with CD146 antibodies to immunomagnetically enrich melanoma cells, and a fluorescently labeled melanoma-specific antibody (HMW-MAA; clone 9.2.27) for CTC detection, as well as anti-CD45, and CD34 for white blood cell and endothelial cell exclusion. A semiautomated fluorescence-based microscope system is utilized to identify CTCs: $CD146^+$, $HMW\text{-}MAA^+$, $CD45^-$, $CD34^-$, and nucleated ($DAPI^+$) cells. All results were reviewed by a trained and highly experienced laboratory scientist (C. Hall) who was blinded to all patient clinical data. In a previously published study, we observed negligible tube-to-tube variability in melanoma CTC detection for each patient sample using CellSearch; the CTC detection rate kappa inter-rater agreement was 0.88 between three 7.5-mL tubes of blood (15). Therefore, CTC detection levels in this study are reported as the number of CTCs per single 7.5-mL tube of blood. To date, we have assessed CTC positivity in 91 healthy control samples and found CTCs are rare; CTCs were detected in two of 91 (2.2%) of healthy control blood samples (mean 0.04 ± 0.2 cells per 7.5-mL tubes of blood).

Statistical analyses

We used χ^2 or Fisher exact tests to evaluate associations between presence of CTCs and clinical factors. Relapse-free survival (RFS) was defined as the time elapsed between date of baseline CTC assessment and either the date of clinical disease relapse, death, or the last follow-up. A Cochran–Armitage test was used to identify relapse trends in patients with 0, ≥1, ≥2, and ≥3 CTCs. Log-rank tests were used to compare RFS between groups. Kaplan–Meier curves were derived using STATA/IC 13.1 (StataCorp) and R version 3.6.0 was used to generate box-and-whisker plots for comparison of RFS between groups of patients stratified by the presence of one or more baseline CTCs. The Cox proportional hazards regression model was used to estimate univariate and multivariable hazard ratios for RFS. *P* values were two-tailed, and values <0.05 were considered statistically significant. An ROC curve was generated using the R version 3.6.0 (survivalROC) package.

Results

Patient characteristics

A total of 243 patients were enrolled in this study, and their demographic data are reported in **Table 1**. Mean age was 57 years (range, 20–88), and median follow-up time was 17 months (range, 1–64 months). A total of 149 patients were male (61%) and 94 were female (39%). Median Breslow tumor thickness was 2.3 mm, 83 patients (34%) had ulcerated tumors, eight patients (3%) had <1 mitotic figures per mm^2, 33 patients (14%) had 1–2 mitotic figures per mm^2, 96 patients (40%) had 3–10 mitotic figures per mm^2, and 49 patients (20%) had more than 10 mitotic figures per mm^2. Seventy-three patients (30%) had tumors with a B-Raf proto-oncogene serine/threonine-protein $kinase^{V600E}$ ($BRAF^{V600E}$) mutation. Forty-five patients (19%) were classified as stage IIIA, 67 patients (28%) stage IIIB, 118 patients (49%) were stage IIIC, and 13 (5%) were stage IIID (AJCC 8th edition). Ninety patients of 243 (37%) had one or more CTCs (range, 0–34), 41 of 243 patients (17%) had two or more CTCs, and 12 of 243 patients (5%) had three or more CTCs per 7.5-mL tubes of blood. Thirty-three patients (14%) experienced disease relapse within 6 months of baseline CTC assessment.

Table 1. Patient demographics.

Variables	Overall cohort		CTCs at first blood draw	
	Numbers of patients	%	1 or more (%)	None (%)
Total patients	243			153 (63)
≥1 CTCs			90 (37)	
≥2 CTCs			41 (17)	
≥3 CTCs			12 (5%)	
Relapse				
Within 6-months of draw 1	33	14	21/90 (23)	12/153 (8)
Within full 54-month follow-up	99	41	43/90 (48)	56/153 (37)
Median Follow-up, months (range)	17 (<1–64)		17 (<1–64)	18 (1–57)
25% Follow-up (months)	8		7	9
75% Follow-up (months)	32		32	32
Age in years, mean (range)	57 (20–88)		57 (23–83)	57 (20–88)
Gender				
Male	149	61	55/90 (61)	94/151 (62)
Female	94	39	35/90 (39)	59/151 (39)
Lymph node involvement				
1 LN	91	37	35/90 (39)	56/153 (37)
2–3 LN or in transit without nodes	65	27	23/90 (26)	42/153 (27)
>3 LN, matted or in transit with nodes	83	34	30/90 (33)	53/153 (35)
Missing	4	2	2/90 (2)	2/153 (1)
Stage III substage (AJCC 8th edition)				
IIIA	45	19	12/90 (14)	33/153 (22)
IIIB	67	28	28/90 (39)	39/153 (25)
IIIC	118	49	48/90 (47)	70/153 (46)
IIID	13	5	2/90 (2)	11/153 (7)
Histologic subtype				
Superficial spreading	86	35	33/90 (37)	53/153 (35)
Nodular	47	19	13/90 (14)	34/153 (22)
Acral lentiginous	18	7	7/90 (8)	11/153 (7)
Lentigo maligna	8	3	2/90 (2)	6/153 (4)
Unclassified	55	23	23/90 (26)	32/153 (21)
Missing	29	12	12/90 (13)	17/153 (11)
Breslow thickness (mm), median	2.3		2.1	2.4
Missing	53	22	22/90 (24)	31/153 (20)
Clark level				
Level II	2	<1	0/90 (0)	2/153 (1)
Level III	14	6	4/90 (4)	10/153 (7)
Level IV	154	64	54/90 (60)	100/153 (65)
Level V	16	7	6/90 (7)	10/153 (7)
Missing	57	23	26/90 (29)	31/153 (20)
Ulceration present	83	34	30/90 (33)	54/153 (35)
Missing	50	21	22/90 (24)	28/153 (18)
Mitotic figures		74	63/90 (70)	116/153 (76)
<1/mm^2	8	3	2/90 (2)	6/153 (4)
1–2/mm^2	33	14	10/90 (11)	23/153 (15)
3–10/mm^2	96	40	34/90 (38)	62/153 (41)
>10/mm^2	49	20	19/90 (21)	30/153 920)
Missing	57	23	26/90 (29)	32/153 (21)
Lymphovascular invasion present	53	22	18/90 (20)	35/153 (23)
Missing	74	30	30/90 (33)	44/153 (29)
BRAF mutation positive[a]	73	30	28/90 (31)	45/153 (29)
Missing	80	33	25/90 (28)	55/153 (36)
LDH (mean U/L) at draw 1[b]	98	40	447 (306–633)	456 (36–908)
Initial treatment after blood draw 1	132	54		
Chemotherapy	6	5	1 (2)	5 (6)
Targeted therapy	18	14	7 (14)	10 (13)
Immunotherapy	56	42	26 (52)	29 (36)
Other	52	39	16 (32)	36 (45)

Abbreviation: LN, lymph node

[a]BRAF, B-Raf proto-oncogene serine/threonine-protein kinase^{V600E}.

[b]LDH, serum lactate dehydrogenase (U/L). LDH levels were available for 98 of 243 patients.

CTC detection and primary tumor characteristics

No primary tumor characteristic, including Breslow thickness ($P = 0.90$), Clark level ($P = 0.53$), ulceration ($P = 0.52$), mitotic range ($P = 0.67$), $BRAF^{V600E}$ mutation status ($P = 0.40$), or histologic subtype ($P = 0.68$), was associated with the presence of one or more CTCs at baseline draw (**Table 1**).

CTC detection and substage

The presence of CTCs at baseline draw was not associated with AJCC 8th edition stage III substage (IIIA, IIIB, IIIC, or IIID; $P = 1.2$; **Table 1**).

CTC detection at baseline and adjuvant therapies

A total of 132 of 243 (54%) patients received adjuvant therapy. Six patients received chemotherapy (cisplatin, vinblastine, dacarbazine, and taxanes), 18 received targeted therapy (dabrafenib, trametinib, vemurafenib, dasatinib, sorafenib, regorafenib, and sunitinib), 56 received immunotherapy (IFNα, ipilimumab, nivolumab, and pembrolizumab), and 52 patients received other therapies (IL2, talimogene laherparepvec, and GP-100). We observed no significant associations between whether adjuvant therapy was administered, or the type of adjuvant therapy administered, and CTC detection at baseline (**Table 1**).

CTC analysis and timing of blood collection

The first (baseline) peripheral venous blood draw sample was collected within 3 months of documenting a positive sentinel lymph node biopsy, or a node dissection revealing positive nodal metastases. We identified no significant associations between CTC detection and ±3 month timing of blood collection, or relapse rates within 6 months, or full 54-month follow-up (box-and-whisker plots are included in Supplementary Data).

CTC numbers and relapse proportion

Eight percent of patients with zero CTCs at baseline relapsed, 22.4% with one CTC relapsed, 24.1% with two CTCs relapsed, and 25% of patients with ≥3 CTCs relapsed. The Cochran-Armitage test $P = 0.002$, indicates there is a trend in the proportion of relapse with increasing CTC numbers. However, in this cohort of patients without metastasis, only 17% had ≥2 CTCs and 5% of patients had ≥3 CTCs.

CTCs and ROC curve

We generated a table depicting the sensitivity and specificity of CTC detection using various CTC cut-off values. We generated an ROC curve that demonstrates the sensitivity/specificity of one or more CTC detection (Supplementary Table and ROC curve in Supplementary Data).

CTCs and RFS

Univariate analysis demonstrated that patients with one or more CTCs at baseline showed significantly decreased RFS within 6 months [log-rank $P < 0.0001$; HR, 3.62; 955 confidence interval (CI), 1.78–7.36; $P < 0.0001$; **Table 2**; **Fig. 1A**). Twenty-one (23%) of the 90 patients who had at least one or more CTCs at baseline relapsed within 6 months, compared with 12 (8%) of 153 patients who had no CTCs. The RFS rate 6 months after baseline was much lower (76%) in this group than in patients who had no CTCs (92%; log-rank $P < 0.0001$). Multivariable Cox regression confirmed that one or more CTC at baseline remained an independent predictor of relapse within 6 months, after adjusting for pathologic nodal stage, sex, age, Breslow thickness, ulceration, and lymphovascular invasion (HR, 3.13; 95% CI, 1.21–8.09; $P = 0.018$; **Table 2**).

Univariate analysis also demonstrated that patients with one or more CTCs at baseline showed significantly decreased RFS

Table 2. Cox regression analyses of 6-month RFS associated with presence of CTCs at baseline.

	Univariate Cox models			Multivariable Cox model		
Variables	**HR (95% CI)**	***P***	**Number in model**	**HR (95% CI)**	***P***	**Number in model**
≥1 CTC at baseline	3.62 (1.78–7.36)	<0.0001	243	3.13 (1.21–8.09)	0.018	162
Pathologic nodal stage			232			162
N1	1 (ref)	—		1 (ref)	—	
N2	2.12 (0.60–7.52)	0.24		0.97 (0.09–10.89)	0.98	
N3	6.81 (2.36–19.69)	<0.0001		10.81 (2.41–48.60)	0.002	
Histologic subtype			211			—
Superficial spreading	1 (ref)	—		—	—	—
Nodular	1.48 (0.54–4.09)	0.45		—	—	—
Acral lentiginous	2.37 (0.71–7.89)	0.16		—	—	—
Lentigo maligna	1.14 (0.14–9.15)	0.90		—	—	—
Unclassified	1.69 (0.65–4.37)	0.28		—	—	—
Sex (female)	1.55 (0.74–3.25)	0.25	243	0.85 (0.32–2.26)	0.74	162
Age at draw 1	1.01 (0.99–1.03)	0.43	243	1.02 (0.98–1.06)	0.30	162
Breslow depth in mm	0.98 (0.87–1.12)	0.82	190	0.95 (0.77–1.17)	0.66	162
Clark level[a]			184			—
Level 2–3	1 (ref)	—		—	—	—
Level 4–5	2.27 (0.31–16.88)	0.42		—	—	—
Ulceration present	1.30 (0.57–2.95)	0.63	193	0.92 (0.34–2.48)	0.87	162
Lymphovascular invasion (LVI) present	1.79 (0.72–4.44)	0.21	169	1.82 (0.62–5.36)	0.28	162
BRAF mutation positive[b]	2.16 (0.95–4.94)	0.07	163	—	—	—

[a]Small *n* in baseline group resulted in inability to estimate HR in multivariable model.
[b]BRAF, B-Raf proto-oncogene serine/threonine-protein kinase^{V600E}.

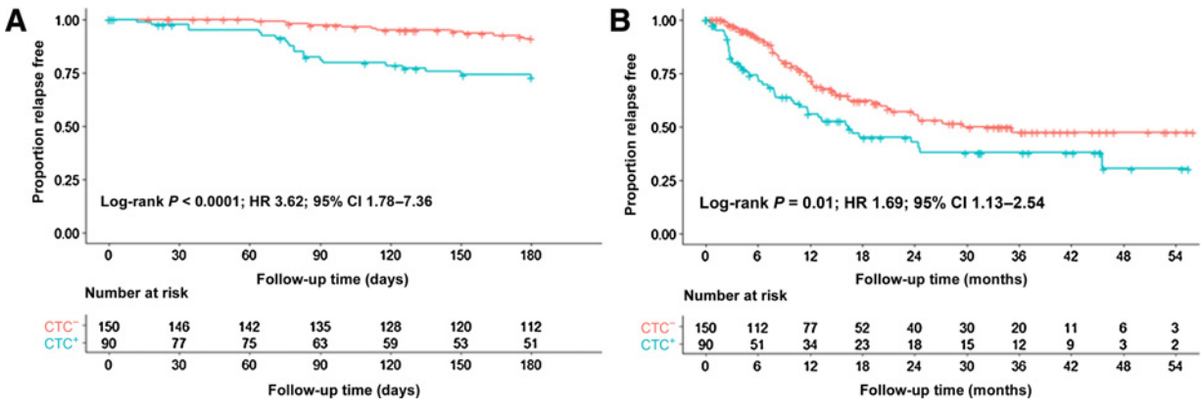

Figure 1.

Kaplan–Meier survival estimates of probabilities of RFS according to CTCs at baseline. **A,** The probability of 6-month RFS in patients with CTC count ≥1 at baseline (HR, 3.62; 95% CI, 1.78–7.36; log-rank $P < 0.0001$) versus patients with no CTCs at baseline. **B,** The probability of 54-month RFS in patients with CTC count ≥1 at baseline (HR, 1.69; 95% CI, 1.13–2.54; log-rank $P = 0.01$) versus patients with no CTCs at baseline.

within 54 months (log-rank $P = 0.01$; HR, 1.69; 95% CI, 1.13–2.54; $P = 0.01$; **Table 3**; **Fig. 1B**). Forty-three (48%) of the 90 patients who had at least one or more CTCs at baseline relapsed during the full follow-up period, compared with 56 (37%) of 153 patients who had no CTCs. Multivariable Cox regression confirmed that one or more CTC at baseline remained an independent predictor of relapse within 54 months, after adjusting for pathologic nodal stage, sex, age, Breslow thickness, ulceration, and lymphovascular invasion (HR, 2.25; 95% CI, 1.25–4.06; $P = 0.006$; **Table 3**).

CTCs, AJCC 8th edition staging, and RFS

Univariate analysis suggested that patients with one or more CTCs at baseline showed significantly poorer RFS within 6 months of baseline draw (log-rank $P < 0.0001$; HR, 3.71; 95% CI, 1.82–7.54; **Table 4**). Multivariable Cox regression confirmed that baseline CTC detection remained an independent predictor of decreased RFS within 6 months of baseline draw after adjusting for AJCC 8th edition substage (HR, 4.57; 95% CI, 2.08–10.04; $P < 0.0001$; **Table 4**). Baseline CTC detection remained significantly associated with relapse within the 54-month follow-up period. Univariate analysis observations (log-rank $P = 0.01$; HR, 1.69; 95% CI, 1.13–2.54) were confirmed with multivariable analyses (HR, 1.88; 95% CI, 1.23–2.87; $P = 0.004$). Multivariable analysis revealed a 6-month RFS HR of 3.92 for patients with stage IIIB melanoma compared with patients with stage IIIA, however, this association did not reach statistical significance ($P = 0.21$). This was likely due to the small number of relapses within the

Table 3. Cox regression analyses of 54-month RFS associated with presence of CTCs at baseline.

	Univariate Cox models			Multivariable Cox model		
Variables	**HR (95% CI)**	***P***	**Number in model**	**HR (95% CI)**	***P***	**Number in model**
≥1 CTC at baseline	1.69 (1.13–2.54)	0.01	240	2.25 (1.25–4.06)	0.006	152
Pathologic nodal stage						
N1	1 (ref)		220	1 (ref)	—	152
N2	1.43 (0.83–2.47)	0.19		1.57 (0.75–3.28)	0.23	
N3	3.57 (2.19–5.81)	<0.001		5.46 (2.77–10.76)	<0.001	
Histologic subtype						—
Superficial spreading	1 (ref)		212	—	—	—
Nodular	1.61 (0.89–2.89)	0.11		—	—	—
Acral lentiginous	2.53 (1.27–5.03)	0.008		—	—	—
Lentigo maligna	0.31 (0.04–2.25)	0.24		—	—	—
Unclassified	1.84 (1.08–3.13)	0.02		—	—	—
Sex (female)	0.59 (0.38–0.91)	0.01	240	0.60 (0.31–1.13)	0.11	152
Age at draw 1	1.02 (1.01–1.04)	<0.01	240	1.02 (1.00–1.04)	0.02	152
Breslow depth in mm	1.06 (1.01–1.11)	0.01	187	1.04 (0.97–1.11)	0.27	152
Clark level[a]						—
Level 2–3	1 (ref)			—	—	—
Level 4–5	1.71 (0.69–4.24)	0.25	183	—	—	—
Ulceration present	1.33 (0.84–2.11)	0.23	190	0.97 (0.55–1.71)	0.91	152
Lymphovascular invasion present	1.36 (0.81–2.27)	0.25	166	1.18 (0.65–2.15)	0.59	152
BRAF mutation positive[b]	1.92 (1.17–3.14)	0.01	160	—	—	—

[a]Small *n* in baseline group resulted in inability to estimate hazard ratio in multivariable model.
[b]BRAF, B-Raf proto-oncogene serine/threonine-protein kinase^{V600E}.

Table 4. Cox regression analyses of RFS associated with presence of CTCs at baseline and AJCC 8th edition pathologic stage.

	Relapse within 6 months					
	Univariate Cox model			Multivariable Cox model		
Variables	HR (95% CI)	*P*	Number in model	HR (95% CI)	*P*	Number in model
≥1 CTC at baseline	3.71 (1.82–7.54)	<0.0001	243	4.57 (2.08–10.04)	<0.0001	243
Pathologic stage			243			243
IIIA	1 (ref)	—		1 (ref)	—	
IIIB	4.51 (0.54–37.50)	0.16		3.92 (0.47–32.64)	0.21	
IIIC	9.11 (1.22–67.62)	0.03		7.82 (1.05–58.16)	0.04	
IIID	16.91 (1.89–151.37)	0.01		30.91 (3.31–288.81)	0.003	

	Relapse within 54 months					
	Univariate Cox model			Multivariable Cox model		
Variables	HR (95% CI)	*P*	Number in model	HR (95% CI)	*P*	Number in model
≥1 CTC at baseline	1.69 (1.13–2.54)	0.011	240	1.88 (1.23–2.87)	0.004	233
Pathologic stage			233			233
IIIA	1 (ref)	—		1 (ref)	—	
IIIB	2.76 (1.10–6.93)	0.03		2.63 (1.05–6.61)	0.03	
IIIC	4.99 (2.15–11.59)	<0.001		4.87 (2.10–11.32)	<0.001	
IIID	11.08 (3.80–32.33)	<0.001		13.92 (4.70–41.29)	<0.001	

IIIB substage group within 6 months. When we considered the full follow-up period, multivariable analysis demonstrated that baseline CTC detection was significantly associated with relapse among patients with stage IIIA and B melanoma (HR, 2.63; $P = 0.03$). Multivariable analyses also confirmed an increased 6-month relapse risk for patients with stage IIIC (HR, 7.82; $P = 0.04$) and IIID (HR, 30.91; $P = 0.003$), as well as a 54-month relapse (HR, 4.87; $P < 0.001$ for patients with stage IIIC and HR, 13.92; $P < 0.001$ for patients with stage IIID) when compared with those with stage IIIA (**Table 4**). Because the number of relapses were low for the stage IIIA (seven relapses), we combined the stage IIIA and III B (and IIIC and IIID) groups together for the survival analyses. Patients with stage IIIA–B disease with one or more CTCs at baseline were more likely to experience relapse compared with those who had stage IIIA–B with no CTCs at baseline (HR 5.40 for 6 months and HR 2.13 for 54 months; **Table 5**). At both 6- and 54-month follow-up, patients with stage IIIA–B as well as those with IIIC–D with one or more CTCs detected at baseline had lower relapse-free probability than patients with stage IIIA–B with no CTCs at baseline (log-rank $P < 0.0001$; **Fig 2A** and **B**).

Discussion

Over the past 20 years, several groups have utilized a variety of indirect, molecular methodologies, such as reverse transcriptase-PCR (RT-PCR) and qRT PCR, to identify CTCs in the blood of patients with melanoma. Indirect methods are based on the assumption that, as melanocytes do not circulate, the detection of melanocyte-associated transcripts should be suitable for use as a surrogate for direct CTC assessment. Published reports of CTC detection in melanoma using various markers and RT-PCR–based methodologies range from 6% to

Table 5. Cox regression analysis of RFS associated with CTCs and AJCC 8th edition substage.

	Relapse within 6 months		
	Univariate Cox model		
Interaction groups ($n = 243$)	HR (95% CI)	*P*	Number in group
CTC^- and stage IIIA–IIIB	1 (ref)	—	72
CTC^- and stage IIIC–IIID	4.56 (0.99–20.82)	0.050	81
CTC^+ and stage IIIA–IIIB	5.40 (1.05–27.82)	0.044	40
CTC^+ and stage IIIC–IIID	14.59 (3.35–63.50)	<0.001	50

	Relapse within 54 months		
	Univariate Cox model		
Interaction groups ($n = 234$)	HR (95% CI)	*P*	Number in group
CTC^- and stage IIIA–IIIB	1 (ref)	—	66
CTC^- and stage IIIC–IIID	3.17 (1.66–6.06)	<0.001	78
CTC^+ and stage IIIA–IIIB	2.13 (0.97–4.67)	0.05	40
CTC^+ and stage IIIC–IIID	5.07 (2.58–9.95)	<0.001	50

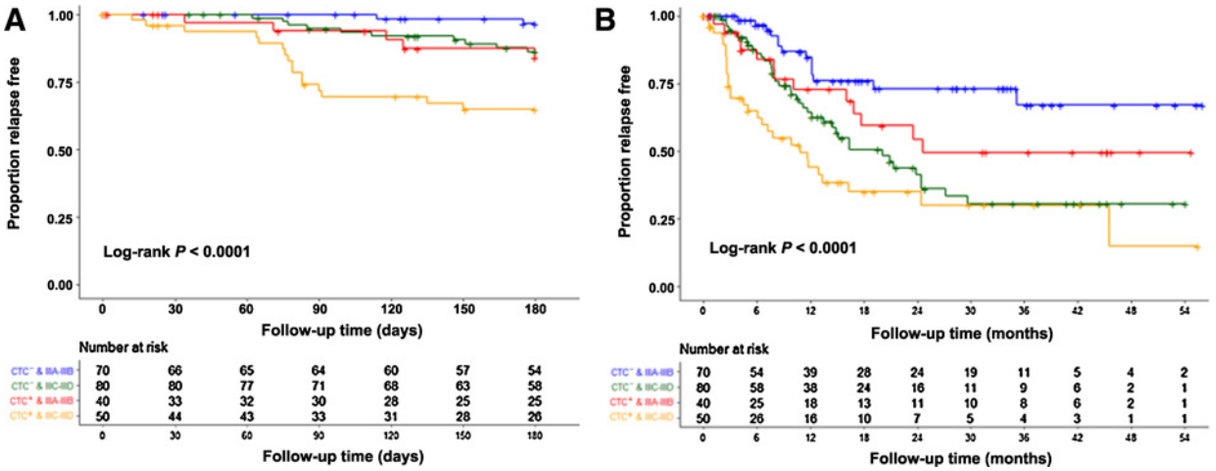

Figure 2.

Kaplan–Meier survival estimates of probabilities of RFS according to CTCs at baseline and AJCC 8th edition staging. **A,** The probability of 6-month RFS in patients with CTC count ≥1 at baseline with AJCC 8th stage IIIA–IIIB and stage IIIC–IIID disease, and patients with no baseline CTCs with AJCC 8th stage IIIA–IIIB and stage IIIC–IIID disease (log-rank $P < 0.0001$). **B,** The probability of 54-month RFS in patients with CTC count ≥1 at baseline among patients AJCC 8th stage IIIA–IIIB and stage IIIC–IIID disease, and patients with no baseline CTCs with AJCC 8th stage IIIA–IIIB and stage IIIC–IIID disease (log-rank $P < 0.0001$).

93% (16). Although many of these reports have demonstrated associations between CTC detection and outcome, none of these assays have been incorporated in clinical laboratories. This is likely due to the lack of standardization of markers and assay conditions used, which could result in reproducibility issues in a clinical laboratory setting. The CellSearch Circulating Melanoma Cell assay uses a single-enrichment marker, CD146 (MCAM and MUC-18) antibody-coated ferrofluids, to enrich CTCs from the blood. This might result in the CellSearch methodology missing some CTCs that do not express CD146. Several published reports have demonstrated a significant association between increased CD146 expression and advanced stage melanoma, as well as disease progression, however, up to 15% of tumors may lack CD146 expression altogether (17). In addition, CD146 is also expressed in various normal tissues, such as smooth muscle, subpopulations of activated T lymphocytes, vascular endothelia, Schwann cells, ganglion cells, and myofibroblasts (18), which could result in the enrichment of nonmelanoma-associated cells. The CellSearch assay utilizes human high molecular weight-melanoma associated antigen [HMW-MAA, Chondroitin Sulfate Proteoglycan 4 (CSPG4)] as the identification marker following CD146 enrichment. HMW-MAA is highly expressed on over 90% of human melanomas, but has a restricted distribution in normal tissues (19). The CellSearch Melanoma Cell assay also uses two exclusion identification markers, CD45 for white blood cell exclusion, and CD34 for endothelial cell exclusion. In addition, validated control reagents for the Circulating Melanoma Cell assay are available from the manufacturer, which provides additional assay standardization.

This study is the first report to demonstrate that a simple blood-based test based on semi-automated, melanoma-specific enrichment, independently predicted relapse for patients with node-positive melanoma. This is important because this technology is standardized, and can be easily assimilated to other centers to provide risk stratification for a group of patients where there are no biomarkers currently available to guide adjuvant treatment. Our investigation of 243 newly diagnosed patients with stage III melanoma demonstrated that the presence of one or more CTCs (per 7.5-mL tubes of blood) was significantly associated with disease relapse within 6 months of baseline CTC measurement. Such a blood test would have obvious applicability to a significant number of patients with stage III melanoma. In our sample cohort, we found that 37% of patients with node-positive melanoma had at least one CTC at baseline blood draw. We were unable to detect a significant association between CTC presence and current parameters used to predict relapse in patients with stage III melanoma, such as Breslow thickness, ulceration, or nodal status. Importantly, using multivariable analysis, CTCs independently predicted relapse within 6 months of baseline, regardless of these parameters. Thus, it appears that CTCs add important prognostic information for patients with node-positive melanoma that goes beyond that offered by standard clinicopathologic factors. As of January 2018, patients with melanoma are staged using the new 8th edition AJCC guidelines, which go beyond TNM staging and incorporate new evidence-based prognostic factors (20). However, even using the AJCC 8th edition staging system, CTCs would not be accounted for, and in this study, offered prognostic information that would be complementary to the added 8th edition staging clinicopathologic factors.

The surgical treatment of patients with stage III melanoma has recently changed based upon data obtained from the Multicenter Selective Lymphadenectomy Trials I and II (MSLT-I and MSLT-II; refs. 21, 22), and the Complete Lymph Node Dissection versus Watchful Waiting in Patients with Malignant Melanoma (DeCOG-SLT) trial (23). The MSLT-I trial showed that patients with intermediate thickness (1.2–3.5 mm) melanomas who underwent sentinel node biopsy had fewer relapses than patients who underwent wide excision and nodal observation (22). It also validated the use of sentinel node biopsy for accurately staging patients with intermediate thickness or thick (>3.50 mm) primary melanomas, and has been incorporated into ASCO and the Society for Surgical Oncology guidelines (22). Both the MSLT-II (1,939 patients) and DeCOG-SLT trials (473 patients) found no statistical differences in 3-year melanoma-specific survival between the sentinel lymph node dissection followed by observation (ultrasonography) group, versus the sentinel lymph node dissection and completion lymphadenectomy cohorts. Consequently, following the publication of the MSLT-II and DeCOG-SLT studies, most surgeons have implemented a much more selective approach to

completion lymph node dissection following a positive sentinel lymph node biopsy. This selective use of completion lymph node dissection will most certainly result in an increase in the number of patients with node-positive melanoma who do not have a complete lymph node dissection, because completion lymph node basin dissection was the standard for many years. Almost all of the melanoma adjuvant therapy trials included patients who had undergone a completion lymph node dissection. Thus, it will become more difficult to use the status of the nonsentinel lymph nodes as a factor to identify those at risk for distant relapse and worse outcome, as was demonstrated in the MSLT-II trial (22). Thus, a blood-based biomarker that identifies those at high risk for disease relapse can potentially add important prognostic information that simply cannot be accessed with the advent of the new, limited node dissection protocols.

Because of the rapid evolution of effective systemic therapies against melanoma, it is increasingly important to identify patients who are at high risk for relapse, and also to identify those who are at a lower risk, and who wish to avoid systemic adjuvant therapy and its inherent potential side effects. Reports from Madu and colleagues and other groups, have compared the 7th and 8th editions of the AJCC melanoma staging system. They reported that survival in both the 7th and 8th editions is heterogeneous for patients with stage III melanoma, and could be improved by adding other discriminators, such as sentinel node tumor burden (2, 24). Currently, there is no good blood-based biomarker to identify which patients are most likely to relapse, and thus treatment decisions are based on clinical experience and clinicopathologic factors. In our study, CTCs clearly identified a group of patients with stage III melanoma at high risk for relapse. This would be clinically very significant as an independent risk factor to help identify patients with stage III disease who would benefit most from adjuvant systemic therapy. This is critical because patients with stage III disease have a heterogeneous risk profile based on clinical staging criteria alone (2, 24). Use of CTCs to identify and subsequently treat those patients at greatest risk would obviously have significant cost/benefit advantages as well.

Finally, the utility of adjuvant systemic therapy has been demonstrated by RFS benefit in the adjuvant nivolumab versus ipilimumab (CheckMate-238), adjuvant pembrolizumab versus placebo (KEYNOTE-054), and adjuvant dabrafenib plus trametinib versus placebo (COMBI-AD) studies for resected stage III/IV melanoma (25–27). Incorporating CTC analysis at diagnosis could help refine the population at greatest risk of relapse within 6–12 months, thus identifying optimal candidates for systemic adjuvant therapy. Similarly, this stratification could spare low-risk patients from unnecessary toxicity associated with adjuvant therapy. Our study preceded the routine use of effective checkpoint blockade and targeted therapy regimens that are currently in use. Thus, it is unlikely that the adjuvant therapies administered during the period of enrollment for this study would have significantly affected the survival outcomes. In fact, in our patient cohort, more than half (54%) of the patients were observed without adjuvant treatment, 12% received single-agent ipilimumab, 9% of patients received IFN, and the remaining patients were on clinical trials using vaccines or other investigational agents. The objective of this study was not to determine systemic therapy benefit, but rather, to determine whether CTC detection at baseline provided significant risk stratification information. We performed sensitivity and specificity analyses and generated an ROC curve depicting our findings (Supplementary Data). Consistent with other published reports investigating the sensitivity and specificity of CTC detection (28, 29), we found CTCs have a high sensitivity and positive predictive value, and a lower specificity and negative predictive value (ROC AUC = 0.660).

Two recent studies have investigated the predictive value of circulating tumor DNA (ctDNA) in patients with stage II/III melanoma. A retrospective analysis of the AVAST-M adjuvant trial, which included 161 patients with stage II/III melanoma, used droplet digital PCR to identify BRAF and neuroblastoma ras viral oncogene homolog (NRAS) mutations in ctDNA at a single time point from patients with BRAF- and NRAS-mutated tumors. Although the reported ctDNA mutation sensitivity was relatively low in this study [≥1 copy of mutant BRAF was identified in 15/132 (11%) of patients with BRAF-mutated tumors and 4/29 (14%) of patients with NRAS-positive tumors], the ctDNA assay specificity was high. Detection of either BRAF- or NRAS-mutated ctDNA was independently associated with poor survival (30). A subsequent prospective study investigating serial ctDNA monitoring in 133 patients with stage III melanoma published by the same group the following year reported similar findings. ctDNA was detected in 37 of 99 (37%) of patients with known BRAF, NRAS, or telomerase reverse transcriptase mutations, but detection was independently predictive of poor outcome (31). Interestingly, in this study, a small subset of patients received adjuvant immune checkpoint inhibitors, and the authors demonstrated that ctDNA dynamics can predict checkpoint inhibitor benefit. It is becoming evident that serial liquid biopsy monitoring can provide valuable prognostic and predictive information that could improve patient management. We are currently conducting prospective trials of serial CTC and ctDNA measurements in patients with high-risk stage II and stage IIIA melanoma receiving immunotherapy, to determine whether combining CTC and ctDNA information in a longitudinal manner can provide predictive information in regards to treatment benefit. Our hope is that we will be able to more rationally recommend treatment directed at those who will benefit most, and avoid unnecessary toxicities in those who likely will not benefit.

We believe this study is important because, to our knowledge, this is the first published prospective study of patients with node-positive melanoma using a semi-automated liquid biopsy technique to show that CTC identification predicts relapse within 6 months. The data from this study provides support for the future pursuit of liquid biopsy techniques to help identify optimal candidates for adjuvant systemic therapy. In current practice, there is no clear consensus regarding adjuvant systemic therapy for patients with node-positive melanoma, thus information on CTCs and early relapse provides a basis for future clinical trials of adjuvant treatments and enhanced imaging in those patients at the highest risk for relapse. Other strengths include that this study was a prospective study with regular patient follow-up involving a relatively high number (243) of patients with node-positive melanoma.

Limitations of the study include that at the time of patient accrual, extensive tumor molecular profiling was not routinely performed for patients with stage III melanoma, so we were not able to determine whether CTC detection was associated with any specific tumor genomic mutation or molecular signature. In addition, effective checkpoint blockade and targeted therapy regimens were not widely employed during the time of this study, so we were not able to determine whether current adjuvant therapies effect CTC detection and outcome for these patients. We continue to prospectively enroll patients with stage III melanoma to address these points and to add predictive benefit of liquid biopsy to the prognostic information presented in this report.

Disclosure of Potential Conflicts of Interest

S.P. Patel reports receiving speakers bureau honoraria from Merck. J.A. Wargo is an employee for Merck and Biothera Pharma; and reports receiving speakers

bureau honoraria from Bristol-Myers Squibb, Illumina, Roche/Genentech, Novartis, AstraZeneca, Merck, Imedex, Omniprex, Gilead, Peerview, PER, Medimmune, Exelixis, and Dhome. I.C. Glitza is a paid consultant for Bristol-Myers Squibb, ARRAY, and Novartis, and reports receiving other commercial research support from Bristol-Myers Squibb and Merck. M.K.K. Wong is a paid advisory board member for Merck Pharmaceuticals, Bristol-Myers Squibb, Pfizer, and Regeneron. H.A. Tawbi is a paid consultant for Bristol-Myers Squibb, Merck, Array, Novartis, and Genentech, and reports receiving commercial research grants from Bristol-Myers Squibb, Merck, Novartis, Celgene, and GlaxoSmithKline. M.A. Davies is a paid consultant for Bristol-Myers Squibb, Novartis, Array, Roche/Genentech, and Sanofi Aventis; reports receiving commercial research grants from AstraZeneca, Roche/Genentech, Sanofi Aventis, Oncothryreon, and GlaxoSmithKline; and holds ownership interest (including patents) in NanoString. J.E. Gershenwald is a paid consultant/advisory board member for Merck, Novartis, and Bristol-Myers Squibb. P. Hwu holds ownership interest (including patents) in Dragonfly and Immatics, and is an unpaid consultant/advisory board member for Dragonfly, GlaxoSmithKline, Immatics, and Sanofi. M.I. Ross is a paid advisory board member for Merck and AMGEN. No potential conflicts of interest were disclosed by the other authors.

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