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Detection of circulating melanoma cells in the blood of melanoma patients: a preliminary study

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Abstract

Objectives—Significant prognostic heterogeneity exists within the sub-stages of melanoma; therefore, novel prognostic biomarkers are needed to provide information regarding recurrence risk. Limited available data suggests prognostic significance for circulating melanoma cells (CMCs); there is a need for a sensitive, reproducible, and standardized identification technique. Using a semi-automated technology, we sought to determine whether CMCs could reliably be identified in stage I–IV melanoma patients and if CMC presence correlated with known prognostic factors.

Methods—CMCs were detected in the peripheral blood (7.5cc) of patients with stage I–IV melanoma (n=89) using the CellSearch® system (Janssen, Raritan NJ). CD146⁺ cells were immunomagnetically enriched; nucleated HMW-MAA⁺/CD45⁺/CD34[−] cells were considered CMCs.

Results—One or more CMCs was detected in 45% of all patients, varying with stage of disease (Stages I/II, III, and IV: 35%, 44%, and 86%, respectively; p=0.03, for stage I/II vs. stage IV); 55% had one CMC, 32% had two CMCs and 13% had three or more CMCs identified. The presence of CMCs in the blood was associated with histologic subtype, particularly in patients with Stage I/II disease (superficial spreading 18% vs. acral lentiginous 75%).

Conclusions—Using a semi-automated technique, CMCs can be identified in a significant number of melanoma patients. These data support further study with longer follow-up and longitudinal/serial time points to better determine the identification rates and prognostic significance of CMCs in stage I–IV melanoma patients.

Keywords

circulating melanoma cells; melanoma; CellSearch

Introduction

The incidence of malignant cutaneous melanoma has increased significantly worldwide over the last 20 years, with an estimated 232,000 new cases in 2012 and over 55,000 deaths.[1, 2] Although the majority of patients are diagnosed with early stage disease, significant prognostic heterogeneity exists within each of the sub-stages of melanoma.[1] While the importance of prognostic variables such as ulceration and mitotic activity have become apparent, a significant number of patients diagnosed with melanoma develop recurrent disease.[3, 4] Therefore, novel prognostic markers are needed to better identify this subset of patients.

Circulating melanoma cells (CMCs) were first identified in the blood of melanoma patients 30 years ago, generating great interest in the clinical and biological significance of these cells.[5–7] Unfortunately, there is significant variability regarding the prognostic significance of CMCs in the limited number of published reports to date.[5–26] The varied, non-standardized methodologies utilized for detecting and quantitating rare CMCs likely contribute to these disparate findings. A sensitive, reproducible, standardized CMC identification technique is needed to accurately assess the prognostic significance of CMCs. [27]

To date, the semi-automated CellSearch® system is the only FDA-approved methodology for the identification and enumeration of circulating tumor cells (CTCs) in whole blood (metastatic prostate, breast, and colon cancers). Three landmark studies prompted FDA approval of the CellSearch® methodology. A study by de Bono *et al.* showed that 5 circulating tumor cells/7.5ml blood detected at baseline was an independent prognostic factor for overall survival in metastatic castration-resistant prostate cancer.[28] This study also demonstrated that circulating tumor cell enumeration before and after treatment was a better predictor of overall survival than prostate-specific antigen values. Similar findings were reported in metastatic breast cancer, whereby 5 or more CTCs/7.5 ml blood at baseline (before starting a new systemic chemotherapy), and at each follow-up time point during therapy predicted both progression-free and overall survival.[29] In patients with metastatic colorectal cancer, 3 circulating tumor cells/7.5mL blood at baseline and during follow-up predicted progression-free and overall survival.[30] Data are emerging regarding the predictive significance of CTCs in patients with non-metastatic breast cancer. Results from the French REMAGUS trial,[31] the German SUCCESS trial,[32] and our group[33] have demonstrated that the identification of one or more circulating tumor cell predicts progression-free and overall survival in patients with non-metastatic breast cancer. However, the CellSearch® system has not been widely utilized for CMC detection in patients with cutaneous melanoma. A handful of studies have been published demonstrating the prognostic significance of CMCs using the CellSearch® in the metastatic setting, whereby patients with two or more CMCs/7.5mL peripheral blood throughout treatment had shortened overall survival than those patients with less than two CMCs throughout treatment. [19, 34, 35]

In the present study, we investigated the identification of CMCs in the peripheral blood of patients with stage I–IV melanoma using the CellSearch® system. We determined if any

associations exist between CMC identification and known prognostic variables, such as tumor size, lymph node status, Clark level, AJCC stage, histologic subtype, ulceration and mitotic rate.

Methods

Patients

Patients with clinical stage IB-IV cutaneous melanoma and healthy control participants were enrolled from January 2012 to December 2012 in an institutional review board-approved protocol (University of Texas M.D. Anderson Cancer Center LAB 11-0314; Principal Investigator, A. Lucci) for the detection of minimal residual disease in peripheral venous blood. In order to increase the likelihood of CMC identification, patients with Breslow thickness $< 0.75\text{mm}$ and no unfavorable features (mitotic figures, ulceration) were excluded. We obtained informed consent from all patients and age-matched control participants prior to blood collection. Patient blood samples were drawn at the time of primary tumor diagnosis or at relapse; control participant blood samples were obtained immediately following informed consent. Individual patient and control participant results were blinded from investigators by use of a random number system as the unique patient identifier.

Staging and Classification

Clinicopathologic data was obtained from the medical record. Data analyzed included: tumor size, lymph node status, Clark level, AJCC stage[36], ulceration, mitotic activity, and histologic subtype. We designated the primary TNM staging (primary tumor [T], regional nodes [N], distant metastasis [M]) in accordance with the criteria set by the American Joint Commission on Cancer, and is reported as pathologic stage after completion of surgical therapy, unless otherwise noted.[36] Pathologic review of the primary melanoma, including histologic subtype, Clark level, the presence of ulceration, and mitotic figures was performed at UT MD Anderson for the entire patient cohort. 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 as clinically appropriate.

Circulating melanoma cell analysis

Peripheral venous blood was collected in one (10 mL glass) tube containing CellSave™ preservative for the detection of CMCs by the CellSearch® CMC assay. Following manufacturer's protocol, the CellSearch® CMC assay was performed within 96 hours of blood collection at the University of Texas MD Anderson Cancer Center. The CellSearch® test uses ferrofluids coated with CD146 antibodies to immunomagnetically enrich melanoma cells, which are further identified using fluorescently labeled antibodies to detect a combination of high molecular weight melanoma associated antigen (HMW-MAA; clone 9.2.27, Janssen, LLC), CD45, and CD34.[19] Circulating melanoma cells were defined as CD146⁺, HMW-MAA⁺, CD45⁻, CD34⁻, nucleated (DAPI⁺) cells as previously described.[19] Circulating melanoma cell results were reviewed by a qualified laboratory technician who was blinded to any patient or control participant information. In addition,

random samples were also independently reviewed by another qualified laboratory technician to validate CMC identification.

Statistical Analysis

For categorical variables, descriptive statistics were compared using χ^2 or Fisher's exact test; Wilcoxon's rank sum test (t-test) and the Kruskal-Wallis test (ANOVA) were used to compare the distributions of continuous variable among different groups. Kappa inter rater analysis was used to determine tube-to-tube variability. Statistical analyses were performed using SPSS version 19 statistical software (IBM Corp., Armonk, NY).

Results

Clinicopathologic Features

The clinicopathologic features of the patients are described in Table 1. Eighty-nine patients were included in the study with a median age of 55 years. Fifty-two of 89 (58%) patients were male and 85/89 (96%) were white. Twenty-one of 89 (26%) patients had pathologic stage I disease, 18/89 (21%) were stage II, 39/89 (46%) were stage III, and 7/89 (8%) had stage IV disease. Thirty-one of 61 (51%) patients had positive sentinel lymph nodes (SLN), 30/61 (49%) were SLN negative, and the SLN status was unknown for 28 patients. The most common histologic type was superficial spreading (28/89 patients, 32%), with 18/89 (20%) classified as nodular, 7/89 (8%) acral lentiginous, the remainder were either other (13%), unknown primary (12%), or unclassified (15%). Fifteen of 70 (21%) patients had tumors with Clark level I/II, the majority of patients (52/70, 74%) had Clark level IV tumors, 4% of patients had Clark level V, and the Clark level was unavailable for 19 patients. Sixty-one of 67 (91%) patients had one or more mitotic figure, 6/67 (9%) had less than one mitotic figure and the number of mitotic figures was unavailable for 22 patients. Ulceration was present in 35% of patients. One or more CMCs were identified in 21/89 (21%) of patients, two or more CMCs were identified in 12/89 (13%) of patients, and 5 patients (6%) had three or more CMCs.

Circulating Melanoma Cells

Figure 1 is an illustration of a representative CMC from the peripheral blood from a patient with clinical stage II disease detected by the CellSearch[®] assay. One or more circulating melanoma cells (CMCs) were detected in 38 of 89 (43%) of patients within our patient cohort, although the number of CMCs varied significantly based on stage of disease (Table 2). One or more CMCs were identified in 6 of 21 (29%) of patients with stage I disease, 7 of 18 (39%) with stage II, 17/39 (44%) with stage III and 6 of 7 patients (86%) with stage IV disease (Table 2; $p=0.019$ stage I vs. stage IV). Two or more CMCs were identified in 3 of 21 (14%) stage I patients, 3 of 18 (17%) stage II patients, 7 of 39 (18%) stage III, and 2 of 7 (29%) stage IV patients. For the entire patient cohort, CMCs were equally prevalent in patients with the presence or absence of standard prognostic and predictive markers. CMCs were detected in 14 of 39 patients (36%) with pN0 disease and 18 of 39 patients (46%) with N+ disease (Table 2; $p=0.638$). We observed no statistically significant differences between the occurrences of CMCs and macroscopic versus microscopic involvement in the lymph nodes. Primary tumor ulceration was not associated with the presence of CMCs as CMCs

were identified in 17 of 45 patients (38%) with primary tumor ulceration and 10 of 24 patients (42%) without ulceration (Table 2; $p=0.753$).

CMCs and Lymph Node Positivity

CMCs were detected in 48% of patients with clinical stage I or II disease who underwent sentinel lymph node biopsy followed by lymphadenectomy for pN+ disease compared to a 32% CMC positivity rate for clinical stage I or II patients with pN0 disease (Figure 2; $p=0.11$).

CMC Detection in Healthy Control Participants

We performed CMC assessment on 70 age-matched, healthy control participants. The overall positivity rate for healthy control participants was 2.9%; one control subject of seventy (1.4%) had one CMC and one control subject of seventy (1.4%) had two CMCs. We are continuing to enroll and monitor control participants to establish the positivity rates for healthy individuals. We will monitor healthy participants closely to determine if they develop melanoma or other types of cancers. To date, none of the control participants have developed any carcinomas.

Tube-to-Tube Variability

We performed duplicate CMC analyses on 31 samples. Because low numbers of CMCs were identified within our patient cohort, we included both patient samples as well as healthy control samples for our tube-to-tube variability analyses. The resultant kappa inter rater agreement was 0.88.

Discussion

The process of metastasis is a multi-step process that begins with the invasion of malignant cells through the basement membrane, intravasation into the blood stream, followed by extravasation, implantation and proliferation in distant sites.[37] The rate of tumor cell shedding into the blood stream is estimated to be $\sim 3 \times 10^6$ cells/gram of tissue/24 hours.[38]

The ability to detect circulating cells routinely in the blood of melanoma patients has recently become available.[5–7] A wide variety of methodologies, utilizing CMC size (isolation by size of epithelial tumor cells, ISET),[39] mRNA (rtPCR) [5–10, 12–18, 20–24, 26, 40, 41, 43–45] and protein expression, [19, 34,35] have been employed for CMC detection, making the interpretation of available studies difficult. Nevertheless, several studies have shown the detection of CMC may represent a biomarker that can identify metastatic disease spread.[8, 9, 11, 13, 16–18, 21, 24–26, 40–43] Polymerase chain reaction (PCR)-based techniques have been used to monitor CMCs for treatment response in metastatic melanoma patients receiving chemotherapy and biochemotherapy.[12, 14, 15, 20, 22, 23] However, the reproducibility of PCR-based detection methods is low,[44, 45] which hinders their reliability and implementation in routine clinical practice.

To our knowledge, this study is one of the first reports utilizing the semi-automated CellSearch® system to detect CMCs in stage I–IV melanoma patients. Since only limited

reports have been published employing CellSearch® for the detection of CMCs in melanoma (all in the metastatic setting), we performed a blinded study of CMC assessment in healthy control subjects as well as tube-to-tube variability analysis to validate the sensitivity and reproducibility of the CellSearch® methodology. The CMC 2.9% detection rate we observed for healthy control subjects is in agreement with the 6% reported by Rao, et al. (the only study to date that has report CMC values for control subjects using the CellSearch methodology).[19] We also assessed tube-to-tube variability for 31 random patients/control subjects by comparing the CMC counts in duplicate tubes; our observed kappa integer value was 0.88. Therefore, we used a single 7.5mL CellSave tube for CMC assessment.

In the present study, CMCs were identified in 38 of 89 (43%) patients with melanoma. Circulating melanoma cells were detected in 86% of patients with stage IV disease compared to 29% of patients with stage I ($P=0.019$) disease, however, CMC detection in stage II (39%, $P=0.063$) and stage III (44%, $P=0.083$) patients was not significantly different compared to stage IV patients. This is likely due to the limited number of patients included in this study. Previous studies have reported CMC identification rates of 8–70% in patients with stage I–III disease.[8, 10, 18,26] This variability is likely related to the vast array of isolation, enrichment, and detection methodologies previously used to identify CMCs.

We did not observe any significant associations between CMC identification and histologic subtype. Although the study was not powered to conduct formal statistical analyses comparing CMC identification based upon histologic subtype, we noted that 31% of patients with superficial-spreading melanoma had CMCs identified in peripheral blood, while CMCs were identified in 71% of patients with acral lentiginous and 44% of patients with nodular melanoma histologic subtypes. Although histologic subtype has not previously been associated with differences in prognosis, the observed increased incidence of CMCs in patients with non-superficial spreading subtypes warrants further investigation. We did not find any association of other well-known prognostic factors, such as tumor thickness, Clark Level, ulceration, or mitotic activity with the presence of CMCs in the blood. However, the small number of patients with 0 mitotic figures (9%) in our cohort limited the power of statistical analysis that was performed to evaluate this important prognostic marker with CMC detection.

Although AJCC stage remains the best predictor of recurrence risk in patients with melanoma, there is a large degree of heterogeneity even within stage groups with regard to outcome and/or disease progression.[4, 36] Currently available prognostic factors are inadequate at identifying the population of patients likely to recur and would thus benefit from adjuvant therapy. To determine if any associations existed between CMC presence and lymph node positivity, we evaluated the identification of CMCs in patients who were diagnosed with clinical stage I or II disease who had negative sentinel lymph nodes (SLN) at surgery compared with those who had positive SLNs at surgery (pathologic stage III). Interestingly, 32% of SLN negative patients had CMCs detected in the blood, compared to 48% of SLN-positive patients, which trended toward, but did not reach statistical significance (Figure 2; $p=0.11$). It is possible that statistical significance was not achieved due to the small numbers of patients within these subgroups. It is also plausible to hypothesize that there are two distinct routes of dissemination, hematogenous and

lymphatic, which can occur independently. This hypothesis is supported by published studies that did not identify a significant correlation between circulating tumor cell detection and lymph node positivity in non-metastatic breast cancer patients,[33, 46] and reports indicating that 10–15% of melanoma patients with lymph node negative disease develop recurrences.[47–49] These data suggest that CMC assessment might provide additional prognostic information to better identify patients at risk of regional metastasis, and support further investigation into the utility of CMCs as an independent prognostic variable.

Our study is limited by the relatively small sample size. Since the CMC detection rates have not been established for early-stage melanoma patients using the CellSearch® methodology, for this initial proof of principle study, the patient cohort was skewed toward a higher-risk population. In addition, patients in this study were enrolled at various time-points in their care; (initial diagnosis, time of recurrence, etc.) making it difficult to apply these data to a broad patient population. However, these data demonstrate that CMCs can be consistently identified in all stages of melanoma with the CellSearch® system. Furthermore, these data support prospective studies with longer follow-up and longitudinal/serial time points to establish CMC detection rates, and to better assess the prognostic significance of CMCs in melanoma patients. To achieve these objectives we are continuing to enroll patients and have modified our original protocol to include longer follow up and longitudinal CMC analyses. In conclusion, using this semi-automated technique, CMCs were identified in a significant number of melanoma patients, and their presence correlates with advancing AJCC stage. In patients with early stage melanoma, the presence of CMCs may also be related to histopathologic subtype, although the clinical significance of this finding is unknown at this time. These data highlight the need for further prospective evaluation of CMCs in patients with cutaneous melanoma.

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Figure 1.

Circulating melanoma cells are identifiable in all stages of melanoma. These pseudo-colored images show a circulating melanoma cell from a patient with clinical stage II disease detected by the CellSearch system using a CMC kit. Cells with positive staining for the melanoma marker high molecular weight–melanoma-associated antibody (HMW-MAA, PE; green) and nuclear DAPI staining (pink) with no staining for CD45/CD34 were considered CMCs. DAPI: 4',6-diamidino-2-phenylindole, PE: phycoerythrin.

Figure 2.

Increased CMCs in patients with sentinel lymph node metastases. Patients with clinical stage I or II disease graphed according to pathologic stage after surgical treatment, including sentinel lymph node biopsy and lymphadenectomy where appropriate.

Table 1

Clinicopathologic characteristics of patients undergoing surgery for recurrent or metastatic GIST

Clinical factor	Total
N	87
Gender, n (%)	
Male	47 (54)
Female	40 (46)
Age, median (range), y	55 (22–75)
55, n (%)	43 (49)
>55, n (%)	44 (51)
Location, n (%)	
Stomach	33 (38)
Duodenum/Small Bowel	38 (43)
Colon/Rectum	10 (12)
Other	6 (7)
Stage at Initial Diagnosis, n (%)	
Localized	54 (62)
Metastatic	33 (38)
Disease State, n (%)	
Local Recurrence	10 (12)
Synchronous metastases, prior resection of primary	19 (22)
Synchronous metastases, simultaneous resection	15 (17)
Metachronous metastases	43 (49)
Extension of Disease, n (%)	
Single Site	44 (51)
<i>Peritoneum only</i>	19
<i>Liver only</i>	15
<i>Local</i>	10
Multiple sites	43 (49)
Primary & metastasis	13
<i>Liver</i>	8
<i>Peritoneum</i>	2
<i>Liver & Peritoneum</i>	3
Local Recurrence & Metastasis	7
<i>Liver</i>	4
<i>Peritoneum</i>	3
Liver & Peritoneum	19
Other	4
Disease Spread, n (%)	
Unifocal	24 (28)

Clinical factor	Total
Multifocal	63 (72)
"Treated" Liver metastasis not resected, n (%)	
Yes	15 (17)
<i>Liver only</i>	2
<i>Liver & Peritoneum</i>	8
<i>Primary & Liver</i>	5
No	72 (83)
Duration of pre-operative TKI therapy	
Median, days (range)	669 (43 – 3257)
365 days	26 (30)
>365 days	61 (70)
Response to TKI therapy	
Stable	9 (10)
Progression only	17 (20)
Regression only	22 (25)
Regression & progression	36 (41)

Table 2

Univariate analysis of factors associated with Time to Recurrence and GIST-Specific Survival

	Time to Recurrence		GIST-Specific Survival	
	Median Time to Recurrence; months (95% CI)	p-value	Median GIST-Specific Survival; months (95 % CI)	p-value
Gender		0.491		0.162
Male	25.1 (12.7–33.7)		90.8 (39.9–NR)	
Female	17.1 (7.8–30.8)		73.6 (34.5–90.9)	
Primary Tumor Location		0.152		0.378
Stomach	18.2 (7.2–40.5)		82.9 (25.2–NR)	
Small Bowel	17.9 (11.2–28.6)		59.6 (34.7–93.53)	
Colorectal	30.1 (5.07–NR)		NR	
Other	7.8 (3.83–NR)		38.9 (22.3–NR)	
Stage at Diagnosis		0.723		0.854
Localized	23.7 (12.7–32.5)		88.6 (39.9–105.6)	
Metastatic	17.9 (7.8–27.3)		48.8 (34.47–)	
Disease spread		0.019		0.021
Unifocal, single site	40.5 (17.3–90.5)		105.6 (90.8–NR)	
All others	14.7 (7.8–23.7)		50.8 (34.6–82.9)	
Duration of pre-operative TKI		0.028		0.012
365 days	33.7 (17.9–72.1)		NR	
>365 days	14.2 (7.8–23.7)		59.6 (34.7–90.8)	
Radiographic Response to TKI		<0.001		<0.001
No Progression	62.4 (28.6–136.7)		NR	
Any Progression	7.8 (5.7–17.2)		34.7 (25.2–61.4)	
Liver metastasis left <i>in situ</i>		<0.001		0.037
Yes	7.8 (4.1–17.9)		48.8 (20.7–73.6)	
No	25.1 (14.7–33.1)		90.8 (42.7–130.1)	

Table 3

Multivariate Competing Risk Regression evaluating preoperative factors associated with Time to Recurrence and GIST-Specific Survival

	Time to Recurrence		GIST-Specific Survival	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age (per 1-year increase)	1.01 (0.99–10.3)	0.305	1.34 (1.01–1.07)	0.007
Disease extent				
Unifocal, single site	1	0.055	1	0.027
All others	1.89 (0.99–3.63)		2.61 (1.12–6.06)	
Radiographic Response to TKI				
No Progression	1	<0.001	1	0.008
Any Progression	3.33 (1.91–5.82)		2.53 (1.27–5.06)	
TKI Time				
365 days	1	0.696	1	0.209
>365	1.12 (0.63–1.99)		1.68 (0.75–3.79)	
“Treated” Liver metastasis left <i>in situ</i>				
No	1	0.765	1	0.302
Yes	1.11 (0.55–2.23)		0.69 (0.33–1.4)	