

Circulating tumor cells in melanoma: a review of the literature and description of a novel technique

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Melanoma is a prevalent and deadly disease with limited therapeutic options. Current prognostic factors are unable to adequately guide treatment. Circulating tumor cells are a disease-specific factor that can be used as a prognostic variable to guide therapy. Most research to date has focused on identification of circulating tumor cells using various methods, including polymerase chain reaction. These techniques, however, have poor sensitivity and variable specificity and predictive significance. A recently developed technology to identify circulating tumor cells is the CellSearch system. This system uses immunomagnetic cell labeling and digital microscopy. This technology may provide an alternative method to identify circulating tumor cells in patients with advanced-stage melanoma and function as a prognostic factor. We review the literature on circulating tumor cells in melanoma and present data collected at our institution using the CellSearch system in nine patients with stage III or IV melanoma.

The incidence of melanoma in the United States has increased faster than that of any other cancer over the past 20 years (1). Once melanoma has metastasized, average survival is a dismal 6 to 9 months, with a 5-year survival rate of <40% (2, 3). A great deal of information has been compiled in an attempt to correlate prognostic factors with clinical outcomes in patients with melanoma. The American Joint Committee on Cancer (AJCC) has devised a system to categorize melanoma that incorporates tumor thickness (T), nodal status (N), and metastatic disease (M) (Table 1). Other prognostic factors, such as sex, age, anatomic location of melanoma, and residual disease after resection of melanoma, can be used in clinical decision making by a multidisciplinary team.

Resection of the primary melanoma is directed primarily by tumor thickness. Currently, resection of melanoma in situ is done with 0.5 cm margins; melanoma of <1 mm thickness, with 1 cm margins; 1 to 4 mm thickness, with 2 cm margins; and >4 mm thickness, with at least a 2 cm margin (1, 4). If the primary melanoma is ulcerated or >0.75 mm in thickness, many institutions incorporate a sentinel lymph node excision as part of the primary resection as a marker for possible regional or systemic spread. During surgery, the sentinel lymph node is identified with radiolabeled lymphoscintigraphy with or without isosulfan blue dye. Immunohistochemical stains and polymerase chain reaction (PCR) are also used to detect markers, such as S100,

MART1, HMB45, and tyrosinase, that are unique to melanoma cells. The use of these additional markers allows identification of melanoma cells in 12% to 20% of lymph nodes that would have been negative by hematoxylin and eosin stains alone (2). If the sentinel lymph node is negative for melanoma, the remaining lymph nodes are free of involvement at least 96% of the time (4). If metastatic melanoma cells are found in the sentinel lymph node, or if regional lymph nodes are palpable on examination or suspicious on radiographic studies, a full dissection of the lymph node basin is recommended (1). This is referred to as a therapeutic lymph node dissection.

Adjuvant treatment of melanoma is an important consideration in patients with stage III or IV disease. Interferon-alpha is currently approved by the Food and Drug Administration (FDA) for patients at high risk of recurrence of melanoma, including those with T4 and/or N1 disease (5). Multiple trials performed with interferon-alpha have shown an approximate 10% reduction in risk of recurrence and a statistically borderline improvement in survival of 3% (5). The benefits are weighed against the significant side effects of interferon therapy (6). For the very high risk patient, other adjuvant therapies, including radiation, observation, and participation in clinical trials, should be discussed (5).

Other new adjuvant treatments have utilized the burgeoning field of immunotherapy. Interleukin (IL)-2 and antitumor-reactive cells have been used to treat melanoma with some success; these approaches are referred to as adoptive immunotherapy. IL-2 has been approved by the FDA for the treatment of stage IV metastatic melanoma (1). Dendritic cells, which are potent antigen-presenting cells of the immune system, have been used to aid in regression of established tumor metastasis, and their use is currently under investigation in metastatic melanoma (7). Gene therapy as an adjunct to immunotherapy is also undergoing evaluation for use in melanoma (5). A multidisciplinary team is important to fully explore treatment options, given such a rapidly expanding and changing field.

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Table 1. The American Joint Commission on Cancer staging system for melanoma

Category	Stage	Description
Primary tumor (T)	TX	Primary tumor cannot be assessed (e.g., shave biopsy or regressed melanoma)
	T0	No evidence of primary tumor
	Tis	Melanoma in situ
	T1	Tumor 1.0 mm or less in thickness with or without ulceration
	T1a	Tumor 1.0 mm or less in thickness and Clark's level II or III with no ulceration
	T1b	Tumor 1.0 mm or less in thickness and Clark's level IV or V or with ulceration
	T2	Tumor more than 1.0 mm but 2.0 mm or less in thickness with or without ulceration
	T2a	Tumor more than 1.0 mm but 2.0 mm or less in thickness with no ulceration
	T2b	Tumor more than 1.0 mm but 2.0 mm or less in thickness with ulceration
	T3	Tumor more than 2.0 mm but 4.0 mm or less in thickness with or without ulceration
	T3a	Tumor more than 2.0 mm but 4.0 mm or less in thickness without ulceration
	T3b	Tumor more than 2.0 mm but 4.0 mm or less in thickness with ulceration
	T4	Tumor more than 4.0 mm in thickness with or without ulceration
	T4a	Tumor more than 4.0 mm in thickness without ulceration
T4b	Tumor more than 4.0 mm in thickness with ulceration	
Regional lymph nodes (N)	NX	Regional lymph nodes cannot be assessed
	N0	No regional lymph node metastasis
	N1	Metastasis to one lymph node
	N1a	Clinically occult (microscopic) metastasis
	N1b	Clinically apparent (macroscopic) metastasis
	N2	Metastasis to two or three regional nodes or intralymphatic regional metastasis without nodal metastases
	N2a	Clinically occult (microscopic) metastasis
	N2b	Clinically apparent (macroscopic) metastasis
N2c	Satellite or in-transit metastasis without nodal metastasis	
N3	Metastasis in more than four regional nodes, or matted lymph nodes, or in-transit metastasis or satellite(s) with metastatic regional node(s)	
Distant metastasis (M)	MX	Distant metastasis cannot be assessed
	M0	No distant metastasis
	M1	Distant metastasis
	M1a	Metastasis to skin, subcutaneous tissues, or distant lymph nodes
	M1b	Metastasis to lung
	M1c	Metastasis to all other visceral sites or distant metastasis at any site associated with an elevated serum lactic dehydrogenase

*Based on these definitions, the stage groupings are as follows:

- O: Tis, N0, M0
- 1A: T1a, N0, M0
- 1B: T1b, N0, M0; T2a, N0, M0
- IIA: T2b, N0, M0; T3a, N0, M0
- IIB: T3b, N0, M0; T4a, N0, M0
- IIC: T4b, N0, M0
- III: Any T, N1, M0; Any T, N2, M0; Any T, N3, M0
- IV: Any T, any N, M1

Currently available prognostic factors are not adequate to identify many of the patients who need adjuvant therapy (8). Fifteen percent of patients whose lymph nodes are negative for tumor cells at the time of surgical intervention eventually develop

metastatic disease (1, 9). Several theories have attempted to explain this. The parallel evolution theory of cancer development alludes to the ability of cancer cells to disseminate at an early genomic state prior to overt tumor formation (9). In addition, tumor cells may not actually be disseminated through lymphatics, and the circulatory system may play a larger role than we currently know (1).

Circulating tumor cells (CTCs) in the bloodstream of patients with melanoma can be identified. Such information could be used to (a) correlate with original tumor burden, (b) predict long-term prognosis, (c) identify patients likely to respond or currently responding to adjuvant therapy, (d) identify patients in need of neoadjuvant therapy, (e) follow patients for recurrence, and (f) guide the type of biochemical therapy.

The CellSearch system (Veridex LLC), which was recently approved by the FDA, uses a combination of immunomagnetic labeling and automated digital microscopy to detect CTCs. The utility of the CellSearch system for identifying CTCs in metastatic breast cancer was recently described in the *New England Journal of Medicine* (10). The number of CTCs before treatment was an independent predictor of progression-free and overall survival. This study provided evidence that CTCs appear to be a significant prognostic tool in patients with metastatic breast cancer. Further studies are ongoing regarding the alteration of systemic therapy based on progression of CTCs in breast cancer.

If a similar application of CTCs to malignant melanoma were possible, this method could prove useful in guiding treatment of this devastating disease. The use of the CellSearch system to identify CTCs in melanoma has not been extensively studied to date. We review the literature on CTCs in melanoma and present preliminary data on the use of the CellSearch system for melanoma CTCs at our institution.

METHODS

The CellSearch system uses ferrofluids coated with epithelial cell-specific EpCAM antibodies to immunomagnetically enrich epithelial cells in a peripheral blood draw sample (10, 11). Melanoma CTCs are defined as being positive for EpCAM and cytokeratin 8, 18, and 19. They must be CD45 negative to rule out lymphocytes.

The exact volume of blood needed to identify CTCs in melanoma patients is unknown. Currently, the CellSearch system can analyze a 7.5-cc tube of blood. Prior data on CTCs in breast cancer have used 20 to 30 cc of blood (10). For the

Table 2. Correlation of stage and survival with the number of CTCs, as determined by the CellSearch assay, in nine melanoma patients

Patient	Age	Sex	Date/location of primary	Date of CTC assay	Stage at time of CTC	Number of CTCs*	Survival at follow-up
1	52	F	1998/anal	1/05	IV	4	DOD at 10 mo
2	71	M	2001/back	3/05	III	0	DOD at 16 mo
3	54	M	1999/foot	5/05	IV	69	DOD at 3 mo
4	64	M	2001/back	6/05	IV	1	AWD at 18 mo
5	42	M	1996/back	7/05	IV	39	DOD at 4 mo
6	62	F	1988/calf	7/05	II	0	AWD at 3 mo
7	65	M	2005/groin	8/05	IV	0	DOD at 10 mo
8	28	F	2002/back	12/05	III	0	NED at 2 mo
9	48	M	2003/scalp	9/06	III	0	AWD at 2 mo

*The number is per 22.5 cc blood sample.

CTCs indicates circulating tumor cells; DOD, died of disease; AWD, alive with disease; NED, no evidence of disease.

current study, three tubes of blood at 7.5 cc per tube were drawn to run each assay. CTC results were reported as aggregate totals for each of the three 7.5-cc samples combined, giving a total number of CTCs per 22.5-cc blood sample.

Data were analyzed from patients with a diagnosis of melanoma that metastasized to at least one regional lymph node. The CellSearch assay was completed at the Mary Crowley Medical Research Center in Dallas, Texas, in all patients. Disease recurrence and survival data were collected during patients' routine follow-up visits. The institutional review board approved analysis of the blood and data.

RESULTS

All assays were run between January 2005 and September 2006. The follow-up period ranged from 2 to 18 months. Nine patients with stage III and IV melanoma were analyzed, including six men and three women, with an average age of 54 years (range, 28–71). Five patients had stage IV disease, and four had stage III disease, as determined by surgical, radiologic, and clinical criteria at the time CTCs were drawn. The CellSearch assay identified CTCs in four of nine patients, with a range of 1 to 69 CTCs identified. CTCs were more likely to be found with an increasing stage of disease. All four patients with positive CTCs were stage IV. Metastatic sites included brain (1), bilateral axilla (1), abdominal wall (1), and liver (1). No patients with stages I to III disease had positive CTCs with the CellSearch assay (Table 2).

Survival appeared to correlate with the extent of CTC positivity. Of the stage IV patients in whom CTCs were found, three died. The patient with 4 CTCs survived 10 months, the patient with 39 CTCs survived 4 months, and the patient with 69 CTCs survived 3 months. The only stage IV patient who is still alive has since undergone IL-2 therapy and is currently without evidence of disease.

DISCUSSION

Identification of CTCs in the blood of patients with a variety of primary tumors was reported as early as the 1960s (12).

The rationale for measuring CTCs is based on studies showing that tumor cells can be present in the circulation of patients with cancer. The detection of CTCs has been proposed as a tool in selecting patients at higher risk of relapse even when physical examination and radiographic tests cannot identify residual disease (12). A major challenge for oncologists is to be able to determine which patients who are currently disease free are at increased risk of recurrence and would benefit from aggressive treatment and follow up (3, 8). Meanwhile, there are numerous possible treatment options in melanoma, and there is an increasing need for more informative surrogate markers of disease progression.

It is estimated that cells shed from a tumor circulate in the bloodstream at extremely low concentrations, estimated to be on the order of one tumor cell in the background of 10^6 to 10^7

normal blood cells (13). Consequently, a technique that can accurately detect the circulation of tumor cells of the order of 1 in 10^6 blood constituent cells is necessary. Over the past 40 years, several methods to detect CTCs have been developed, and attempts have been made to verify their reproducibility and significance to the treatment of cancer patients. These techniques include PCR, automated digital microscopy, fiber-optic array scanning technology, microscopic cell labeling, and photoacoustics (13). Automated digital microscopy and fiber-optic array scanning technology rely on advanced optics to detect labeled CTCs. The problem with both systems is that 1×10^6 cells must be scanned before a single CTC is found. This can take upwards of 18 hours with available systems. Enrichment refers to techniques whereby CTCs are concentrated out of the background of normal hematopoietic cells to decrease the amount of time needed for digital microscopy to scan and recognize them. One technique for enriching the cell samples is the use of immunomagnetic beads (13). This technology is used in the CellSearch system.

PCR is the method most widely used and most extensively studied thus far for CTCs in melanoma (8, 14). For melanoma-derived cell lines, PCR amplifies genes specific for melanocytes. The most common target is the gene for tyrosinase, a tissue-specific gene for melanocytes that plays a role in the initial stages of the melanogenesis pathway (15). Since normal melanocytes are not thought to circulate in peripheral blood, detection of tyrosinase transcription should correlate to identification of CTCs (16, 17). Some investigators believe that the sensitivity and specificity of PCR can be increased by using multiple target markers (16). MART-1 (melanoma antigen recognized by T cells 1), GalNAc-T (beta 1 to 4-N-acetylgalactosaminyltransferase), GP-100, Muc-18, P97, and MAGE-A3 (melanoma antigen gene-A3) are some of the other markers used to identify CTCs in melanoma patients (12). There is no consensus, however, that multimarker PCR differs in diagnostic relevance from assessment of tyrosinase only (16).

The PCR technique itself is hampered by technical difficulties and high rates of false-negatives. RNA, the substrate for

Table 3. Studies using polymerase chain reaction to determine the presence of circulating tumor cells

Study	Stage	n	Positive for CTCs
Hoon et al, 2000 (3)	I-II	7	
	III	29	
	IV	10	
	Overall	46	93%*
Palmieri et al, 2003 (15)	0	9	56%
	I	93	75%
	II	51	84%
	III	24	92%
	IV	23	100%
	Overall	200	81%*
Reynolds et al, 2003 (12)	IIb/IIIa	59	54%
	IIIb	48	38%
	IV	11	8%
	Overall	118	23%
Tsao et al, 2001 (14) Meta-analysis of 23 studies of tyrosinase single-marker reverse transcriptase polymerase chain reaction	I	291	18%
	II	296	28%
	III	492	30%
	IV	501	45%
	Unspecified	219	
Overall	1799	32%	
Koyanagi et al, 2005 (8)	Overall	63	75%*
Keilholz et al, 2004 (16)	Overall	45	49%*
Mocellin et al, 2004 (6)	II or III	40	70%*
Carrillo et al, 2002 (21)	T1		23%
	T2-3		74%
	≥T4		83%
	Overall	58	
Quereux et al, 2001 (22)	IV	32	66%
Brownbridge et al, 2001 (23)	I	135	34%
	II	196	51%
	III	423	50%
	IV	156	65%
	Overall	910	

*Used multimarker polymerase chain reaction.

PCR, is present only during active transcription from nuclear DNA. Melanomas are highly heterogenous for tumor-related protein expression (18). If a gene is not being actively transcribed to RNA, PCR will not detect it. Some studies have shown that tyrosinase RNA expression levels can vary considerably from different tumor biopsies, even from a single patient, and amelanotic tumors can also have significantly less tyrosinase RNA (18). Tumor cell shedding may be intermittent and unpredictable as well (19). Some studies have shown that the same patient can oscillate between being PCR positive and PCR negative for CTCs depending of the timing of blood draws within the same day (16). Cancer cells also have genomic instability that could lead to a high rate of false-negative PCR results (3). Furthermore, nucleic acids are fragile and susceptible to degradation. Even if RNA is present in a sample, the degradation of nucleic acids before the completion of PCR analysis can influence results (18).

One method used in an attempt to standardize sample quality is the concurrent identification of certain “housekeeping

genes” (3). These housekeeping genes are constitutively expressed in all cells of the body—including CTCs. If certain levels of a housekeeping gene are detected, one can assume that the sample is of sufficient caliber and the PCR results are accurate. Some of the housekeeping genes used for melanoma PCR studies include porphobilinogen deaminase (*PBGD*) and beta-globulin (8, 15, 16).

False-positive results also plague the use of PCR in identifying CTCs. Tumor cell-specific markers must be absent in the blood of healthy patients in order for the test to be specific (20). The expression of RNA for tumor cell-specific markers in the blood of noncancer patients would be a source of false-positive results (18). False-positives can also occur if samples are contaminated. With the characterization of epithelial cancers, contamination with epithelial cells can happen easily and could be devastating to the statistical significance of data (6). The first several milliliters of peripheral blood drawn must be wasted to prevent epithelial cell contamination, and then epithelial cells must be kept out of the preparations until the end of data analysis (6). A meta-analysis of 50 different studies of PCR used for CTCs in melanoma patients estimated a false-positive rate of 0.4% (14). Characterization of CTCs using PCR requires careful scrutiny of the process and resultant data. Until these issues are resolved, PCR remains susceptible to misinterpretation and statistical inaccuracy.

The sensitivity of PCR to identify CTCs is <50% in many studies—even in patients with known stage IV melanoma (6). Stage I disease rarely has detectable CTCs, demonstrating the low likelihood of systemic disease and subsequent low recurrence rate (14). For example, a recent meta-analysis of PCR detection of CTCs in melanoma patients by Tsao analyzed 1799 patients. This study found an overall CTC positivity rate of 18% for stage I, 28% for stage II, 30% for stage III, and 45% for stage IV disease (14). Similar numbers have been presented by multiple other articles in the literature using PCR to analyze melanoma CTCs (*Table 3*).

The purpose of detecting CTCs is to hopefully evaluate tumor progression, predict prognosis, and alter therapy. Hoon et al at the John Wayne Cancer Institute have shown that detection of melanoma CTCs with PCR can correlate to AJCC stage, predict disease recurrence, and correlate with survival (3). Forty-six patients without evidence of clinically detectable disease at the time of CTC draw were analyzed using multimarker reverse transcriptase PCR. The majority of patients had a high stage of melanoma: 29 patients (63%) were stage III, and 10 (22%) were stage IV. Only 3 (6%) had no CTCs on PCR, and the number of positive markers in the blood showed a significant correlation to AJCC stage. The probability of having disease recurrence over a 60-month follow-up increased from 25% to 56% in patients that had 0 to 2 positive markers versus 3 to 4 positive markers, respectively. Survival over the follow-up period decreased from 82% to 61% in patients with 0 to 2 positive markers and 3 to 4 positive markers, respectively (3).

Some studies have shown that a change in CTCs can occur with treatment and could act as an indirect marker of therapy

effectiveness (6, 8, 24). Reynolds et al at New York University have shown that treatment with a melanoma vaccine can decrease the percentage of CTC-positive patients (12). They studied 95 patients with resected stage IIb through IV melanoma with reverse transcriptase PCR before and after immunotherapy with a melanoma vaccine. Overall, 23% were positive for CTCs using PCR at baseline, and they did not find any difference in CTC positivity between various stages of disease. After 5 months of vaccine treatment, only 11% had CTCs using PCR, showing a statistically significant decrease of 55% (12). These studies suggest that CTCs may correlate to patient response to various treatment paradigms.

There may be benefits to using a system such as CellSearch over existing assays such as PCR. PCR results are heterogeneous in regards to technique, type of PCR, results, and sensitivity (11). The heterogeneity of series reporting results on PCR studies of CTCs also prevents direct comparisons between published results (6). If data from multiple centers could be pooled into a single set of reproducible and accurate data points, statistical analysis would be easier and widespread clinical application could be faster. The detection of CTCs using CellSearch technology in patients with known malignancies, including prostate, breast, ovarian, colorectal, and lung cancers, has ranged from 20% to 57% (10, 25). As mentioned previously, CTC positivity in PCR studies has ranged anywhere from 20% to 100%. Before CTCs can have useful clinical application, the sensitivity, reproducibility, and specificity for detection of CTCs in patients must be elucidated (11).

Our data thus far show that CTCs can be detected in patients with melanoma. The sensitivity of the assay seems reliant on the AJCC stage of disease, with currently only stage IV patients showing positive results. Of the nine patients with stage III or IV disease, four had positive assays. Further analysis with more patients is necessary to elucidate what percentage of patients will have CTCs with less than stage IV disease. Our results also suggest that increasing numbers of CTCs may correlate with disease-specific survival. Continued data will be needed to reach statistically significant values.

We plan to continue running CellSearch assays on patients with stage III and IV melanoma both before and after surgical resection. Improvement of several technical aspects would be useful, including determining the optimal volume of blood to detect CTCs in melanoma patients. Standardization of techniques would allow larger and more statistically significant patient numbers to be studied in our and other laboratories. We hope that CTCs prove to be useful in clinical decision-making by a multidisciplinary team by predicting tumor burden, prognosis and recurrence, and guiding existing and future therapies.

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