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Melanoma and Cutaneous Malignancies

Circulating Tumor Cells, DNA, and mRNA: Potential for Clinical Utility in Patients With Melanoma

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Circulating tumor cells • Circulating tumor DNA • Circulating mRNA • Melanoma • Clinical utility

Abstract

Circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and messenger RNA (mRNA), collectively termed circulating tumor products (CTPs), represent areas of immense interest from scientists' and clinicians' perspectives. In melanoma, CTP analysis may have clinical utility in many areas, from screening and diagnosis to clinical decision-making aids, as surveillance biomarkers or sources of real-time genetic or molecular characterization. In addition, CTP analysis can be useful in the discovery of new biomarkers, patterns of treatment resistance, and mechanisms of metastasis development. Here, we compare and contrast CTCs, ctDNA, and mRNA, review the extent of translational evidence to date, and discuss how future studies involving both scientists and clinicians can help to further develop this tool for the benefit of melanoma patients. ***The Oncologist*** 2016;21:84–94

Implications for Practice: Scientific advancement has enabled the rapid development of tools to analyze circulating tumor cells, tumor DNA, and messenger RNA, collectively termed circulating tumor products (CTPs). A variety of techniques have emerged to detect and characterize melanoma CTPs; however, only a fraction has been applied to human subjects. This review summarizes the available human data that investigate clinical utility of CTP in cancer screening, melanoma diagnosis, prognosis, prediction, and genetic or molecular characterization. It provides a rationale for how CTPs may be useful for future research and discusses how clinicians can be involved in developing this exciting new technology.

Introduction

Circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and circulating messenger RNA (mRNA), collectively termed circulating tumor products (CTPs), have attracted great interest and investment. In 2014 alone, more than 600 publications searchable on PubMed described the diversity of CTP-related isolation techniques, proof-of-principle findings, and implications for human disease management and clinical study design. In melanoma, several reviews have been written to summarize the state of the art in CTP detection, isolation, and genetic characterization, yet its potential contributions to precision medicine remain uncertain [1–7].

Several important questions have yet to be answered. What is the relative potential clinical utility of CTCs, ctDNA, and mRNA? Can measuring these CTPs be used to detect new or recurrent disease, or monitor response to therapy? What clinical protocols need to be designed to demonstrate the utility of these assays? Even if these trials are positive, will these data lead to changes in current practice paradigms?

Melanoma serves as an important clinical and scientific model for considering these issues. Melanoma is one of the most frequently diagnosed cancers in men and women living in developed countries [8]. In contrast to the stable or declining trends for most malignancies, incidence of melanoma has significantly increased in the U.S. over the past decade [9]. Melanoma, therefore, represents a significantly increasing case load in clinical oncology. Additionally, the management of melanoma lies at the forefront of the precision medicine revolution [10–14]. Variation in prognosis and the availability of powerful targeted therapies demands tools to better define risk stratification, inform the optimal timing of therapy initiation, and detect drug resistance. Melanoma's variability in surface marker expression poses a unique

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biological challenge to reliable detection, thus inspiring great variation in CTC detection methodologies [1–3, 5–7]. On the other hand, well-described, melanoma-specific DNA mutations, such as *BRAF* (B-Raf proto-oncogene, serine/threonine kinase) mutations, allow for ease in conducting proof-of-concept studies. Therefore, the diversity of CTP detection techniques and rapidly changing treatment paradigms make melanoma an ideal model disease for discussing the potential benefit and challenges of CTP assays in the clinic.

Here, we review existing data on CTP studies in patients with melanoma and outline the key laboratory, clinical trial, and commercialization considerations that may pave the way toward a practice-changing technology. Although we highlight the utility of CTCs, ctDNA, and mRNA in various clinical applications, we do not directly compare the three, as they are not mutually exclusive technologies and their emergence in clinical medicine may very well overlap.

Overview of CTCs, ctDNA, and mRNA

We briefly review the techniques involved in isolating each CTP (Fig. 1), focusing on their potential for clinical application (Table 1). Ideally, CTPs must demonstrate strong test characteristics of high sensitivity and specificity, high negative or positive predictive value, robustness and reliability, reproducibility, and cost-effectiveness. While detailed comparisons between methodologies are outside the scope of this review, this has been excellently analyzed by Rodic et al. in 2014 [2], Nezos et al. in 2011 [5], and Medic et al. in 2007 [4].

Circulating Tumor Cells

CTCs are intact cells shed from the primary tumor and detected within peripheral blood samples. In patients with melanoma, the number of detected CTCs range from 0 to more than 10,000 CTCs per 10 mL of blood [3, 15, 16]. In the same blood sample, there may be approximately 100 million leukocytes and 50 billion erythrocytes. Therefore, CTC assays face technical challenges of removing the overwhelming population of white and red blood cells while positively selecting for CTCs.

Rodic et al. recently published a systematic review of CTC detection in melanoma, categorizing isolation strategies into marker-dependent and marker-independent techniques (Fig. 1A, 1B) [2]. Marker-dependent strategies use melanoma-specific surface antigens and immunomagnetic beads to positively select for melanoma CTCs in blood. Surface markers such as high molecular weight melanoma-associated antigen (HMW-MAA), also known as melanoma-associated chondroitin sulfate proteoglycan (MCSP), CD146 or melanoma cell adhesion molecule (MCAM), and ATP-binding cassette subfamily B member 5 (ABCB5) are used either in isolation or combination to facilitate CTC capture [16–22]. In contrast, marker-independent strategies capitalize on CTCs' physical properties of size and density. Size-based isolation techniques, such as isolation by size of epithelial tumor cells (ISET), use a porous filter to trap large cells (larger than 8 μm) regardless of surface marker expression. The cells can be evaluated for mRNA or DNA mutations or transferred onto a slide for immunohistochemistry (IHC) evaluation [23–25]. Density-based techniques use Ficoll-hypaque or Oncoquick centrifugation separation media to enrich for a layer of cells containing CTCs, suitable for further isolation [15, 26, 27].

What are the particular features of CTCs that may be most useful in the clinic (Table 1)? First, CTCs are intact cells. Among all CTPs, intact CTCs are the closest representation of "human tissue" compatible with IHC and traditional pathology protocols. Furthermore, CTCs may be pooled or analyzed as single cells to facilitate further understanding of CTC genomics, transcriptomics, and even proteomics. These characterizations of single CTCs, however, are prone to the pitfalls of tumor heterogeneity and sampling bias [28]. Clinical protocols such as tracking non-small cell lung cancer evolution through therapy (TRACERx) may help define expectations for heterogeneity in CTC analyses [29]. The study prospectively follows patients with lung cancer through multiregion and longitudinal tumor sampling, seeking to evaluate concordance between CTCs and the genetic composition of sampled metastases. Given the longitudinal nature of the study, the results may help inform the prevalence of CTC heterogeneity as a function of time, treatment, and treatment resistance.

CTCs are also thought to have the potential to seed metastases and, therefore, are valuable for metastasis research and identification of new therapeutic targets [3, 15]. Evidence demonstrating this causal relationship in melanoma and other human malignancies is limited, in part because of slowly maturing technologies in CTC identification and isolation [30]. In breast cancer, human CTCs have been found to give rise to bone, lung, and liver metastases in mice [31]. Small cell lung cancer CTCs have also been shown to produce CTC-derived explants in nude mice [32]. However, more research is needed to establish the putative causal relationship between CTCs and metastases. An important step toward this end is to differentiate live, dead, or dying cells, and identify subpopulations of CTCs relevant in metastasis research [3].

Circulating Tumor DNA

ctDNA refers to circulating DNA fragments containing cancer-specific mutations that are detectable in peripheral blood samples (Fig. 1) [33–35]. The major challenges in identifying ctDNA are in detecting low levels of ctDNA, accurate quantification, and differentiating tumor DNA from normal cell-free DNA circulating in the blood stream.

Diaz and Bardelli recently reviewed the state of the art in ctDNA detection [35]. They describe an evolution of DNA analysis technologies to accurately interrogate small fragments of DNA [35–38]. Approaches include massively parallel paired-end sequencing [39–44] and recently published techniques such as tagged-amplicon deep sequencing [45], cancer personalized profiling by deep sequencing [33], and droplet digital polymerase chain reactions (PCRs) [34, 46]. These strategies allow the extraction of a variety of personalized data about genomic alterations, including copy number variations, point mutations, rearrangements, and methylation patterns.

Compared with CTCs, ctDNA is easier to isolate using existing clinical protocols (Table 1). Many common blood tests, such as serum cholesterol or glucose levels, are already performed via commercially available serum separation tubes. Therefore, ctDNA analysis may integrate easily into the clinical laboratory workflow. Although ctDNA detection precludes single cell analysis, it may reveal important information about tumor heterogeneity. For example, serial sampling of ctDNA from patients with metastatic cancer may provide a means to

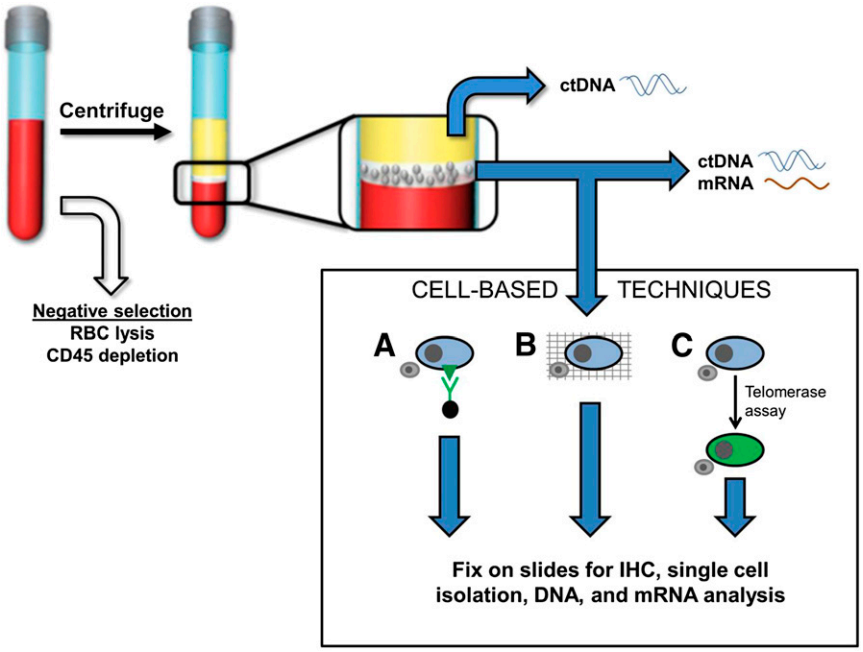

Figure 1. Overview of circulating tumor cell (CTC), ctDNA, and mRNA isolation techniques. Negative selection applied to whole blood removes RBCs and CD-45-expressing leukocytes. After separation of blood components, ctDNA can be extracted from plasma (yellow layer). The mononuclear layer (white layer) can be used for ctDNA, mRNA, or CTC extraction (illustrated in the box labeled Cell-Based Techniques). CTC detection and isolation can result in downstream IHC analysis, single cell isolation, and DNA and mRNA analysis. Lane A: Surface-marker dependent detection methods use antibodies against melanoma-specific surface marker antigens. Antibodies may be linked to ferrous beads for magnetic pull-down or fluorescent proteins for visualization. White blood cells (WBCs) are represented in the illustration by a small gray cell. Lane B: Surface-marker independent detection methods whereby CTCs may be captured along with WBCs on a fine porous barrier. This is termed the ISET, or isolation by size of epithelial tumor cells, method. Lane C: A telomerase live-cell assay applied to CTCs and WBCs causes cells with elevated telomerase promoter activity to produce high levels of green fluorescent protein.

Abbreviations: ctDNA, circulating tumor DNA; IHC, immunohistochemistry; RBC, red blood cell.

Table 1. Comparison of CTCs, ctDNA, and mRNA

Characteristic	CTCs	ctDNA	mRNA
Origin	CTCs	Primary tumor, metastatic tumors, or CTCs	Primary tumor, metastatic tumors, or CTCs
Prognostic or predictive potential	Yes	Yes	Yes
Ability for genetic characterization	Yes	Yes	Yes
Ability for transcriptional characterization	Yes	No	Yes
Ability for molecular characterization	Yes	No	No
Unique features	Intact cells, closest representation to metastases	Ease of acquisition and durability of DNA	Ease of acquisition and scalability

Abbreviations: CTC, circulating tumor cells; ctDNA, circulating tumor DNA.

survey the overall genetic composition of multiple metastases without requiring multiple biopsies [47, 48]. This may lead to downstream identification of candidate treatment resistance genes to facilitate research in drug resistance.

A major limitation of ctDNA analysis is its requirement for a priori knowledge of abnormal DNA sequences. The potential for discovery of new genetic abnormalities with therapeutic or prognostic implications is, therefore, restricted. While ctDNA is thought to be primarily derived from the solid tumor with a small fraction, if any, from CTCs, it is also possible that ctDNA may derive from noncancerous dysplastic tissues or any number of metastatic deposits. Therefore, conclusions made from ctDNA analysis hinge upon the reliability with which the primary tumor is the main source of the ctDNA and reflects changes in overall disease burden, drug sensitivity, or drug resistance.

mRNA

mRNA analysis is predicated on extracting RNA from the mononuclear layer of peripheral blood samples, composed of white blood cells and CTCs (Fig. 1) [49]. Sensitivity for melanoma, as opposed to circulating melanocytes, standardization of the choice of mRNA biomarkers, and agreement on clinically relevant thresholds remain consistent challenges for this approach [50, 51].

Since this technique was first described by Smith et al. in 1991, using tyrosinase mRNA PCR analysis, several additional candidate mRNA biomarkers have been proposed, including

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melanoma antigen recognized by T cells (MART-1), gp-100, melanoma-associated antigen 3 (MAGE-A3), paired box 3 transcription factor (PAX3), and β-1,4 *N*-acetylgalactosaminyltransferase (GalNAc-T) in various combinations [2, 4, 20, 21, 50, 52–57]. Even within the John Wayne Cancer Institute Group, who used this technique with two international multicenter trials published in the same year, the choice of biomarker cocktail has not been consistent [56, 57]. However, research groups tend to agree on the basic principles of the technique. Red blood cells in peripheral blood samples are lysed and RNA is extracted from the remaining intact cells, consisting presumably of white blood cells and CTCs. The RNA is converted to complementary DNA (cDNA) using reverse transcription. A cocktail of PCR primers for any of the abovementioned biomarkers is then added to cDNA and subsequently analyzed in quantitative PCR amplification.

The primary strength of mRNA analysis is its ease of acquisition and scalability (Table 1). reverse transcriptase PCR studies have already been successfully embedded in international multicenter clinical trials involving more than 1,000 patients in total [54, 56, 57]. However, mRNA analysis has not yet been used to identify key genetic abnormalities in melanoma, such as *BRAF*, *c-Kit*, and *PTEN* (phosphatase and tensin homolog). Unlike ctDNA isolation, RNA analysis has not provided clues as to differential protein amplification or expression profiles present among individuals with melanoma. A concerning study using tyrosinase mRNA as a biomarker found positive signal in a patient with a benign congenital nevus, bringing into question the specificity of tyrosinase and other melanocytic markers that serve as surrogates for CTCs [51]. Because of the long-term instability of RNA and cDNA when stored in freezers, the potential for using extracted products for downstream analysis and future applications is limited to approximately 1 year [49]. In addition, it is unclear to what extent these RNA products are derived from CTCs and not solid tumor. Even if they are CTC derivatives, whether these RNA products are derived from live or dying cells remains a further important question.

Applications Toward Melanoma Diagnosis

Screening

Cancer screening, especially through the use of a noninvasive blood test, is of great interest to translational researchers, public health workers, policy makers, clinicians, and patients alike. CTPs can have high sensitivity for early-stage disease, and studies have successfully described the ability to detect ctDNA or mRNA in early-stage melanoma and other cancers [44, 53]. The implications are enormous, as many cancers such as ovarian and pancreatic carcinomas tend to present clinically in later stages and may serve to benefit the most from early detection.

Considering current and past candidate cancer-screening tests, we learn that the barriers to implementation are quite high because of intense scrutiny over the cost-benefit ratio for these tests. The prostate-specific antigen test is the subject of unyielding controversy between the potential survival benefit of detecting prostate cancer and the harm of overdiagnosis and overtreatment [58]. Other important and well-known biomarkers, such as CA-125, CA19-9, and carcinoembryonic antigen are expressly not recommended for use in cancer screening because they fail to meet specificity requirements. To meet criteria for a screening biomarker, large-scale studies need to be conducted to demonstrate cost-effectiveness, improvement in disease outcomes as a result of early detection, reliable test performance, and technology scalability.

Melanoma, however, is already detected at an early stage because of increased public awareness and use of regular skin checks. Melanoma is less likely to benefit from an additional biochemical screening test compared with other malignancies that present more frequently at an advanced stage, such as ovarian and lung cancers. Therefore, the consideration for CTPs as screening biomarkers relies not only on the test performance and scalability, but also on the chosen disease sites for which cost-effectiveness analysis is performed.

Diagnostics

Another topic generating great enthusiasm is the potential for CTPs to aid in cancer diagnostics. Importantly, cancer diagnostics have been traditionally performed using tissue samples obtained after visual or radiographic observation of abnormal masses. Without the ability to obtain tissue and stain for tumor-specific markers, the diagnosis of cancer is typically limited to radiologic appearance, location of disease, and clinical experience alone. In current clinical practice among oncologists, pathologists, and radiologists, there remains a strong preference for tissue and the visualization of tumor-specific staining as necessary criteria for cancer diagnoses and subsequent treatment.

Among CTPs, only intact CTCs fixed to slides are able to satisfy the minimal requirements for cancer diagnosis—the presence of tissue that can be subjected to staining for tumor-specific antigens (Fig. 2). However, no studies have yet investigated CTCs in melanoma diagnosis. While circulating mRNA can be found in patients with early-stage disease [53], the potential of mRNA or ctDNA as a diagnostic tool seems limited because of the incompatibility of this technology with commonly used methods for melanoma cancer diagnosis.

An important and emerging alternative application of CTPs to diagnostics is the ability to corroborate radiologic findings in the subset of patients for whom tissue biopsy is unsafe.

An important and emerging alternative application of CTPs to diagnostics is the ability to corroborate radiologic findings in the subset of patients for whom tissue biopsy is unsafe. For instance, consider the frail patient with prior history of melanoma resection who presents with new development of multiple subcentimeter radiographic findings thought to represent lung or brain metastases. In this scenario, if an alternative method such as CTCs, ctDNA, or mRNA can reliably, sensitively, and specifically confirm the presence of cancer, there may be an important role for CTPs in diagnostics.

Directing Clinical Decision-Making

In light of the increased complexity of treatment options for patients with melanoma, particularly the advent of novel immune and checkpoint antibody treatments, the need for new biomarkers to direct clinical decision making in melanoma is great [59].

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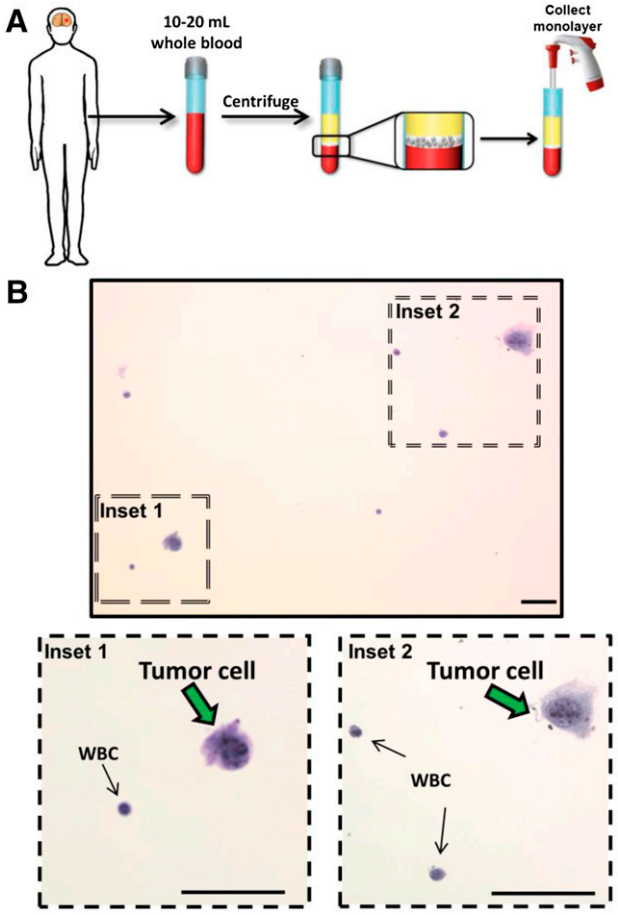

Figure 2. Schematic of a potential cancer diagnosis strategy using circulating tumor cells (CTCs) as stainable tissue. **(A):** Patients' blood samples are centrifuged and the monolayer of CTCs and WBCs are collected. **(B):** After ThinPrep transfer onto a microscope slide, H&E stain is applied. In this proof-of-concept example, cultured cancer cells are spiked into control blood samples and identified after H&E staining. Inset 1 demonstrates a tumor cell adjacent to a benign and small WBC. Inset 2 demonstrates a tumor cell adjacent to 2 WBCs. Scale bars = 50 μm.

Abbreviation: WBC, white blood cell.

CTPs as Prognostic Biomarkers

CTCs are already U.S. Food and Drug Administration approved as overall survival (OS) and progression-free survival (PFS) prognostic biomarkers in prostate, breast, and colon cancer [60–62]. In melanoma, the majority of studies analyzing patient samples also seek to make conclusions about the prognostic value of CTCs, ctDNA, and mRNA (Table 2).

Many groups have already shown statistically significant associations between the detection of CTPs and poor clinical outcomes in OS and PFS (Table 2). Sample sizes for these studies tend to be small, so findings should be considered cautiously optimistic and still investigational. Even in studies with large sample sizes ($n > 200$), results should be interpreted with caution, as these studies pooled patients from both arms of multicenter therapeutic clinical trials. Thus, the OS and PFS rates may not be reflective of prognoses expected with standard treatment [55–57].

The added value of CTP prognostic information is an important consideration for application in clinical melanoma management. Melanoma already has a number of validated histological, clinical, and blood-based prognostic biomarkers. CTP prognostic claims, therefore, must compete with existing metrics for physician adoption and, importantly, insurance coverage. This is illustrated in the example of the CellSearch CTC platform (Janssen Diagnostics, Raritan, NJ, https://www.cellsearchctc.com) in metastatic breast cancer. Despite validation of prognostic utility, the added clinical value is limited [60, 63, 64]. This has been directly expressed in Medicare local coverage determinations not to cover CTC analyses and is indirectly evident in differential coverage decisions by private insurance companies across the U.S. [65, 66].

Another important application of CTPs as prognostic biomarkers lies in the correlation between CTPs and traditional prognosticators such as stage and disease volume [22, 53–55]. Of particular use to clinical melanoma management is the ability to further risk stratify patients with stage II (sentinel lymph node negative) disease into groups of low and high risk for recurrence. While studies have shown direct mRNA analysis of sentinel lymph nodes can lead to upstaging of disease and offer prognostic value, this finding has not been corroborated using CTP analysis in peripheral blood draws [67, 68]. CTP trends have also been used as markers of disease burden, response to therapy, and predictors of clinical outcomes (Fig. 3A) [17, 20]. These trends may be especially helpful in diseases for which radiographic imaging is difficult to interpret in the postoperative or postradiation setting (i.e., pseudoprogression vs. true progression after immunotherapy in patients with melanoma). Figure 3B illustrates the benefit of CTP analysis in evaluating pseudoprogression in glioblastoma.

CTPs as Predictive Biomarkers

CTP predictive potential for therapeutic outcome is perhaps its most compelling clinical application. This has been most recently modeled by the Oncotype DX assay for breast cancer (Genomic Health, Redwood City, CA, http://www.oncotypedx.com) [69–71]. Initially marketed as a prognostic biomarker [72], its transition to a predictive biomarker [73–75] for response to chemotherapy has contributed significantly to its increasing use among clinicians [76]. Thus, to appeal more strongly to clinicians, CTP studies should seek to progress from prognostic to prediction studies.

Few predictive studies have been reported in melanoma CTP-related publications. The presence of ctDNA in the form of methylated *RASSF1A* (Ras association domain family 1) or loss of heterozygosity at microsatellite regions has shown potential utility in predicting response to chemotherapy and/or immune modulating therapy [77, 78]. In another study of CTC trends in patients with melanoma who were treated with vemurafenib ($n = 8$), a decrease in CTC counts was associated good treatment response [17]. Such studies have been limited by small sample size and more patients are needed to elucidate the predictive value of CTP assays.

Among other disease sites, such as breast cancer, several international interventional clinical trials have been initiated to determine the predictive value of the CellSearch CTC enumeration platform (Table 3) [64]. The SWOG500 study, which opened for accrual in 2006, was the first clinical trial incorporating CTCs as a biomarker to inform treatment-arm stratification. This randomized phase III trial for metastatic

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Table 2. Evidence suggesting prognostic significance of CTCs, ctDNA, and mRNA in patients with melanoma

CTP and detection method	Findings	Ref.
CTC		
Marker dependent: MCSP, MCAM, ABCB5, CD271	Decreasing CTC trend associated with response to treatment and prolonged OS in patients treated with vemurafenib. Baseline CTC numbers not prognostic in OS/PFS for patients treated by surgery, vemurafenib, ipilimumab, or dacarbazine	[17]
Marker dependent: HMW-MAA via CellSearch	Baseline CTC level ≥2 CTCs/7.5 mL associated with shorter median OS in univariate and multivariate analysis [16, 20]	[16, 20, 22]
	≥1 CTC/7.5 mL shortens PFS and OS in univariate analysis, not significant in multivariate analysis [22]	
Marker dependent: Negative selection (CD45 and RBC depletion), cytospin, and IHC	Near statistically significant association between increased CTC counts and decreased OS ($p = .12$)	[85]
Marker independent: Oncoquick plus telomerase probe	Trend toward statistically significant association between increased CTC counts and disease progression ($p = .21$)	[27]
ctDNA		
Bidirectional PAP	Low levels of ctDNA associated with longer PFS and OS in univariate and multivariate analysis	[22]
Methylation-specific PCR	Hypermethylation of *RASSF1A* associated with shorter OS in univariate and multivariate analysis	[77]
PCR	Loss of heterozygosity at DNA microsatellites associated with disease progression and OS	[78, 82, 84]
mRNA		
MART-1, MAGEA3, PAX3	>0 biomarker detected at pretreatment significantly associated with decreased DFS and OS in multivariate analysis	[56]
	Serial presence of CTCs (>0 biomarker detected) significantly associated with decreased DFS and OS	
MART-1, MAGEA3, GalNAc-T	Baseline CTC level (≥2 biomarkers detected) significantly associated with decreased DFS, recurrence-free survival, and melanoma-specific survival in multivariate analysis	[62]
MART-1, GalNAc-T, MAGEA3, PAX3	Increased number of biomarkers significantly associated with decreased relapse-free survival and OS	[54]
Tyrosinase, p97, MUC-18, MAGEA3	Number of markers correlated with disease stage. Increased number of positive markers significantly associated with disease recurrence.	[98]

Abbreviations: ABCB5, ATP-binding cassette subfamily B member 5; CTC, CTC, circulating tumor cells; ctDNA, circulating tumor DNA; CTP, circulating tumor product; DFS, disease-free survival; GalNAc-T, β-1,4 *N*-acetylgalactosaminyltransferase; HMW-MAA, high molecular weight melanoma-associated antigen; IHC, immunohistochemistry; MAGEA3, melanoma-associated antigen 3; MART-1, melanoma antigen recognized by T cells; MCAM, melanoma cell adhesion molecule; MCSP, melanoma-associated chondroitin sulfate proteoglycan; OS, overall survival; PAP, pyrophosphorolysis-activated polymerization; PAX3, paired box 3 transcription factor; PCR, polymerase chain reaction; PFS, progression-free survival; *RASSF1A* = Ras association domain family 1; RBC, red blood cell; Ref., reference.

breast cancer was designed to determine whether persistently high CTC levels (≥5 CTCs per 7.5 mL) after the first cycle of chemotherapy could indicate disease progression, and to determine whether an early switch to alternative chemotherapy would result in improved prognostic outcomes [63]. Early reported results redemonstrated the prognostic value of baseline CTCs but did not show improvement in overall survival from switching chemotherapies early. This study's weaknesses include the heterogeneity of chemotherapy regimens allowed on the trial, poor outcomes of salvage chemotherapy regardless of switching treatment regimens, and lack of prior evidence that CTC values 3 weeks after treatment initiation can reliably assess treatment response. More studies are needed to determine how CTCs may offer the most predictive value. Three ongoing international clinical trials continue to investigate the association between CTC counts and surface marker expression profiles (i.e., human epidermal growth receptor 2 [HER2] expression) that may be associated with treatment response. Similarly designed predictive trials represent important next steps for melanoma CTP studies.

CTPs May Provide Genetic and Molecular Characterization

The ability to characterize a tumor's changing genetic and molecular features offers valuable implications in precision medicine from clinical trial design to risk stratification and treatment response prediction [80]. In breast cancer, for example, CTC clinical trials are currently prescribing HER2 inhibition therapy to otherwise HER2-negative breast cancer patients on the basis of CTC analyses identifying HER2 positivity (Table 3) [79]. In melanoma management, the utility of genetic and molecular characterization is equally important, if not more so, because of the increased availability of targeted agents against BRAF and mitogen-activated protein kinase kinase (MEK), as well as other immunotherapies [10–14].

Genetic characterization has been widely achieved in melanoma CTP studies (Table 4). A subset of known melanoma-associated mutations with therapeutic relevance, such as *BRAF* V600E and Kirsten rat sarcoma viral oncogene homolog (*KRAS*), has been identified via CTC isolation and in ctDNA studies [18, 21, 27, 44, 46, 81]. Additional genetic

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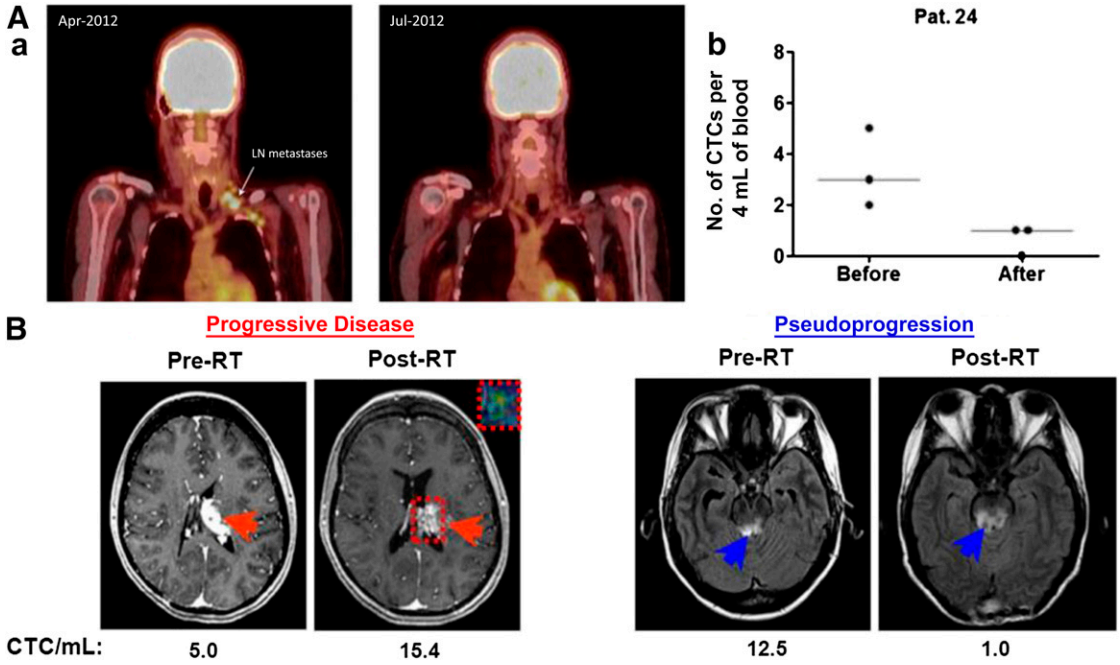

Figure 3. CTC levels may correspond to disease status. **(Aa):** Illustration of therapeutic response in a patient with metastatic melanoma who was treated with vemurafenib (reproduced from Klinac et al. [17], original vertical display edited to horizontal). Left panel: Representative images of the positron emission tomography scans before and during vemurafenib treatment. The arrow indicates lymph node metastasis detected prior to treatment and a complete metabolic response 2 months after treatment. Right panel: Reduction in the number of CTCs in 4 mL of whole blood in the same patient. A total of 12 mL of blood was collected at each time point (three 4-mL tubes). **(Ab):** The graph illustrates the number of CTCs found in each of the three blood samples and the median for each time point. **(B):** CTC trends differentiating between progressive disease and pseudoprogression in two patients with glioblastoma (reproduced with permission from MacArthur et al. [95]). Magnetic resonance imaging (MRI) was performed within 2 weeks prior to initiation of RT and approximately 1 month following completion of treatment. CTC results (given as number of CTCs per mL) are below the axial MR image at the respective time points. Red arrows indicate a left thalamic lesion prior to and following RT (left panels). The inset box delineated by the dotted red line in the post-RT image demonstrates the tumor area of interest and the associated advanced MRI relative cerebral blood volume map, which confirmed active tumor progression. Blue arrows indicate MR signal abnormality in the midbrain lesion and surrounding area on axial view prior to and following RT (right panels).

Abbreviations: CTC, circulating tumor cell; LN, lymph node; Pat. 24, patient 24; RT, radiation therapy.

Table 3. Clinical trials investigating the predictive value of CTCs in breast cancers

Trial	Hypothesis	Finding
SWOGS0500	Persistent CTC elevation predicts disease progression and justifies switching chemotherapy regimens	CTCs were prognostic but not predictive for salvage chemotherapy outcomes
CirCe01	CTC trends predict treatment resistance	Recruiting
DETECT III	HER2+ CTCs in patients with HER2-metastatic breast cancer predict treatment response to HER2 inhibition	Recruiting
Treat CTC	HER2+ CTCs in patients with HER2-nonmetastatic breast cancer predict response to HER2 inhibition	Recruiting

Abbreviations: CirCe01, Circulating Tumor Cells to Guide Chemotherapy for Metastatic Breast Cancer trial; CTC, circulating tumor cell.

studies have identified genomic changes with potential relevance as biomarkers, such as hypermethylated DNA and loss of heterozygosity at DNA microsatellite regions [77, 78, 82–84]. However, many CTC studies do not visually confirm the presence of CTCs prior to DNA extraction and analysis [18, 21, 85]. This may be motivated by ease of harvesting genetic material, but it also compromises the assurance that the detected mutations are derived from intact circulating cells. Currently, the only mechanism of isolating intact melanoma CTCs for DNA extraction and analysis is through microcapillary dissection [19, 27].

The identification of melanoma-specific genetic and molecular mutations has primarily served as proof-of-concept findings in many CTC and ctDNA studies, but these changes may also have potential prognostic and predictive significance [77, 78, 82–84]. For example, a CTP analysis that detects mutations (e.g., epidermal growth factor receptor [*EGFR*] T790M) or molecular changes (e.g., downregulation of programmed death-ligand 1 [PD-L1]) related to drug resistance may motivate earlier initiation of next-line therapy. Or, CTP analysis may identify patients who are eligible for targeted therapies when previous biopsies demonstrated they were ineligible (e.g., biopsy to CTP conversion from HER2− to HER2+ or PD-L1− to PD-L1+) [79]. Serving as a noninvasive biopsy of genetic and molecular changes, CTPs may, therefore, be helpful in illuminating new drug sensitivities or resistances.

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Table 4. Genetic data obtained from CTCs and ctDNA in patients with melanoma

CTP and detection method	Finding	Ref.
CTC		
Marker dependent: MCSP, ABCB5, MAGEA3, RANK by digital droplet PCR	Detect cancer mutations (e.g., *BRAF*) with high sensitivity (77% concordance)	[18]
Marker dependent: 7 antibodies against CSPG4	CTCs isolated via capillary-based micromanipulator. CNV analysis identified known and new genomic changes: deletions of *CDKN2A* and *PTEN*, amplifications of *TERT*, *BRAF*, *KRAS*, *MDM2*. Novel chromosomal amplifications of chromosomes 12, 17, and 19 were also found.	[19]
Marker dependent: HMW-MAA via CellSearch	Captured cells were first confirmed as CTCs using qRT-PCR, then a second sample was used to detect *BRAF* mutation in 81% of patients	[21]
Marker dependent: Negative selection (CD45 and RBC depletion), cytospin, and IHC	Performed microRNA analysis of CTCs using qRT-PCR, which enriched microRNA 106a, 20a, and 21	[85]
Marker independent: ISET plus IHC	No genetic testing done, but molecular phenotype assessed through IHC, which found intrapatient and interpatient heterogeneity in S100, Melan-A, MITF, MCAM, HMW-MAA, CD271, and MAGEC1 expression	[25]
Marker independent: Oncoquick plus telomerase assay	CTCs isolated using microcapillary-assisted device, WGA, and PCR for *BRAF* V600E mutation	[27]
ctDNA		
Digital droplet PCR	Circulating free *BRAF* V600E detected	[46]
Massively parallel sequencing or PCR/ligation method	Circulating free *BRAF* V600E, *NRAS*, and *ALK* mutations detected	[44]
Serum PCR	Circulating serum *BRAF* V600E detected	[81]
Methylation-specific PCR	Hypermethylation of *RASSF1A*, *RAR2*, *MGMT* detected	[77, 83]
PCR	Loss of heterozygosity at DNA microsatellites detected	[78, 82, 84]

Abbreviations: ABCB5, ATP-binding cassette subfamily B member 5; *ALK*, anaplastic lymphoma receptor tyrosine kinase; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; CNV, copy number variation; CSPG4, chondroitin sulfate proteoglycan 4; CTC, circulating tumor cell; ctDNA, circulating tumor DNA; CTP, circulating tumor product; HMW-MAA, high molecular weight melanoma-associated antigen; IHC, immunohistochemistry; ISET, isolation by size of epithelial tumor cells; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; MAGEA3, melanoma-associated antigen 3; MAGEC1, melanoma antigen family C1; MCAM, melanoma cell adhesion molecule; MCSP, melanoma-associated chondroitin sulfate proteoglycan; *MDM2*, *MDM2* proto-oncogene, E3 ubiquitin protein ligase; *MGMT*, O6-methylguanine DNA methyltransferase; MITF, microphthalmia-associated transcription factor; *NRAS*, neuroblastoma RAS viral (v-ras) oncogene homolog; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; *PTEN*, phosphatase and tensin homolog; RANK, receptor activator of nuclear factor κ B; RAR-2, retinoic acid receptor-2; *RASSF1A*, Ras association domain family 1; RBC, red blood cell; Ref., reference; *TERT*, telomerase reverse transcriptase; WGA, whole-genome amplification.

Tumor Material Available for Future Discovery

CTPs may unlock new understanding of melanoma progression mechanisms that lead to the development of novel therapies and decision-making tools. For example, CTCs may be used to establish cell lines for long-term experimentation. Although no melanoma CTC cultures have been reported in the literature to date, CTCs have been successfully isolated and cultured in colon, gastric, and pancreatic cancers [86–88]. These are important recent developments resulting from maturing CTC isolation technologies. In the way that cultured human cancer cell lines revolutionized cancer research, cultured CTCs may have a profound impact on our understanding of cancer dynamics, metastasis, and drug resistance, and may enable identification of new drug targets.

In the absence of cell lines, emerging capabilities in single-cell and small-sample analysis enables genetic, transcriptional, and molecular discovery in CTPs. Melanoma ctDNA studies have already identified hypermethylation and loss of heterozygosity mutations with prognostic significance.

In the absence of cell lines, emerging capabilities in single-cell and small-sample analysis enables genetic, transcriptional, and molecular discovery in CTPs. Melanoma ctDNA studies have already identified hypermethylation and loss of heterozygosity mutations with prognostic significance [77, 78, 82, 84]. Array-based analyses incorporating the most common ctDNA mutations among all cancers may be used to identify new associations between known mutations and cancer sites [33]. A growing body of evidence is also evolving to define changes that initiate survival in circulation and metastasis development [30]. For example, several groups have detected increased expression of mRNA regulating epithelial-mesenchymal transition in CTCs from breast, colon, and head and neck cancers [89–92]. Other studies in lung cancer suggest survival in circulation and resistance to apoptotic stimuli may be mediated by CTC clustering [93, 94]. Distinguishing among live, dead, and dying cells may further facilitate ongoing research in CTCs and metastases [3]. Importantly, a methodology has been previously described to isolate live CTCs and has been effective for melanoma, glioma, bladder cancer, and non-small cell lung cancer [27, 95–97]. The continued development of this technique, along with other CTP analysis methods, enables better understanding of tumor changes that accumulate over the course of treatment.

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The Role of the Clinical Researcher

Clinician input into the development of CTP technologies is critical. With such a diverse array of potential applications, insight into the types of CTP outcomes that will address the unmet needs in cancer management is extremely valuable. Furthermore, incorporating CTPs as secondary or exploratory outcomes in clinical trials may contribute significantly to the literature around predictive and prognostic value of these novel biomarkers. Clinicians are also likely able to foresee paradigm hurdles, such as how the importance of tissue staining in cancer diagnosis may pose barriers for the adoption of ctDNA technologies in diagnostics. Therefore, clinicians can offer valuable insight to the development of this technology and should feel empowered to further investigate how CTPs can benefit their patients in the future.

Conclusion

CTPs have strong potential to change the practice of melanoma management. Several studies have established their prognostic value, and future clinical protocols should be designed to further elucidate the predictive or diagnostic values of CTPs. As liquid biopsy specimens, CTPs offer the exciting potential to evaluate real-time changes in tumor genetics that confer new drug resistance or sensitivity. CTPs also enable the discovery of novel biomarkers, drug targets, and insight into the biology of melanoma metastasis. Given the variety of practice-changing CTP applications, involving clinical researchers to forecast and participate in the development of this technology is critical.

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Disclosures

Giorgos Karakousis: AMGEN, Castle Biosciences (H); **Erica L. Carpenter:** Janssen, Sysmex/Inostics (RF). The other authors indicated no financial relationships.

(C/A) Consulting/advisory relationship; (RF) Research funding; (E) Employment; (ET) Expert testimony; (H) Honoraria received; (OI) Ownership interests; (IP) Intellectual property rights/inventor/patent holder; (SAB) Scientific advisory board

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