Mini-review

Melanoma circulating tumor cells: Benefits and challenges required for clinical application

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Abstract

The implementation of novel therapeutic interventions has improved the survival rates of melanoma patients with metastatic disease. Nonetheless, only 33% of treated cases exhibit long term responses. Circulating tumor cell (CTC) measurements are currently of clinical value in breast, prostate and colorectal cancers. However, the clinical utility of melanoma CTCs (MelCTCs) is still unclear due to challenges that appear intrinsic to MelCTCs (i.e. rarity, heterogeneity) and a lack of standardization in their isolation, across research laboratories. Here, we review the latest developments, pinpoint the challenges in MelCTC isolation and address their potential role in melanoma management.

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Introduction

Although melanoma is potentially curable when detected in its earliest stages, it can metastasize to other tissues, drastically reducing survival rates [5]. Recent advances in immune- and targeted therapies have improved survival for metastatic cutaneous melanoma [35]. However, immunotherapies are highly toxic and effective in only a proportion of patients [40,48,57], and the majority of patients undergoing targeted therapies with MAPK inhibitors rapidly develop drug resistance [47,49,54]. In order to overcome these challenges, biomarkers that can guide treatment decisions, monitor response to treatment and identify resistance, are urgently required in the clinical setting.

During the last decade, circulating tumor cells (CTCs) have received widespread attention as prognostic biomarkers [1,18] (see Table 1 for the full list of terms). These cells are derived from primary and/or metastatic tumors and can be assessed at any point during disease course. Thus, their presence in peripheral blood can serve as a “liquid biopsy” of solid tumors, particularly when a biopsy cannot be undertaken due to inaccessibility of the tumor, or when multiple metastases are present in a patient. For increased clinical benefit, the analysis of these tumor-derived cells needs to assist with (a) disease prognosis, (b) prediction of clinical outcome to specific treatment, (c) patient-tailored, real time monitoring of response, (d) early detection of treatment resistance or recurrence and progression, and (e) discovery of new therapeutic targets and mechanisms of resistance. Additionally, the molecular characterization of these rare cells harbors significant information about cancer dissemination.

The enrichment and detection of CTCs from patients is critically challenging, mainly due to the limited amount of blood sample available and the very low concentration of these cells in peripheral blood. For melanoma, the difficulties are magnified because common CTC markers, such as EpCAM, used in CTC enrichment of epithelial cancers including breast and prostate cancers, are not commonly expressed by MelCTCs, since melanocytes originate from the neural crest and not the epithelium [19]. In addition, MelCTCs are a very heterogeneous population of cells [21,25,29], yet current techniques used to enrich melanoma cells from blood do not commonly consider this factor, as their principle for CTC detection relies on the expression of only one or two markers. The isolation of MelCTCs usually follows two common steps: first, CTCs are enriched from the background of millions of blood cells and second, CTCs are detected and characterized in the enriched fraction.
Here we detail the progress of MelCTC isolation techniques, from the use of single surface markers to novel methodologies that rely on physical characteristics of MelCTCs. We also describe the clinical significance of current MelCTC studies, addressing the issues that in our informed opinion, hamper the progress of this research field.

**Melanoma CTC enrichment techniques**

Despite MelCCTC heterogeneity [25,29,53], methods for their capture and enrichment have relied predominantly on the expression of one or two known cell surface markers (Fig. 1). For example, immunomagnetic enrichment with magnetic beads coupled to antibodies against known melanoma-specific antigens has been used to enrich CTCs (positive selection). Alternate methods that deplete white blood cells (WBCs) using beads targeting common leukocyte (CD45 or CD34) antigens (negative selection) are also widely used [28,34,44,52].

The CellSearch™ system (Veridex LCC) involves immunomagnetic capture of CTCs followed by cancer-specific marker staining for CTC detection. This system is the only FDA-approved CTC enumeration platform for breast cancer, where EpCAM is used to capture CTCs followed by immunostaining with cytokeratin [2,10–12]. This adhesion molecule has been described as important in tumor growth, EMT and metastasis [37,38]; EpCAM is however, expressed exclusively in epithelial-derived neoplasms. A different CellSearch™ kit was therefore developed for MelCTC isolation, which captures CTCs expressing melanoma cell adhesion molecule (MCAM) from whole blood and detects CTCs by immunostaining with MCSP/CSGP4/HMW-MAA (melanoma-associated chondroitin sulphate proteoglycan).

Khoja and colleagues [28] used this melanoma specific CellSearch™ kit to detect CTCs in 101 metastatic melanoma patients, and found 0–36 CTCs/7.5 mL of blood prior to treatment, with 40% of patients having at least one CTC. Similarly, Rao and colleagues, found 0 to 8042 CTCs/7.5 mL of blood in 23% of the patients (n = 44), with greater than 10 CTCs detected in only three patients (4%) [44]. Given the low frequency of CTCs detected by targeting only one marker for enrichment, other approaches increased the number of antigens targeted in order to isolate a larger number of MelCTCs.

Freeman and colleagues [21] found that using a combination of MCSP, MCAM, ATP-binding cassette sub-family B member 5 (ABCB5), and cluster of differentiation 271 (CD271) targeting antibodies, captured MelCTCs in a significantly higher proportion of metastatic melanoma patients than did the use of MCSP or MCAM alone. This finding demonstrated for the first time the high diversity of MelCTCs and improved the sensitivity of bead-based CTC enrichment compared with experiments that targeted single markers. In this study, patients from all stages showed significantly higher numbers of CTCs than controls (n = 15), detecting at least 1 CTC/mL of blood in 73.9% of patients (range: 0–2.5 CTCs/mL of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>List of terms.</th>
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<tr>
<td>ABCB5</td>
<td>ATP binding cassette subfamily B member 5</td>
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<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
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<td>BRAF</td>
<td>proto-oncogene B-Raf; v-Raf murine sarcoma viral oncogene homolog B</td>
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<tr>
<td>CD144</td>
<td>VE-Cadherin</td>
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<tr>
<td>CD271</td>
<td>LINGR (low-affinity nerve growth factor receptor)</td>
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<td>CD34</td>
<td>p75 NTR (neurotrophin receptor)</td>
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<td>CD45</td>
<td>cluster of differentiation 45</td>
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<td>CDH12A</td>
<td>cyclin-dependent kinase Inhibitor 2A</td>
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<td>CDX</td>
<td>Cancer or CTC derived xenografts</td>
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<td>CNV</td>
<td>copy number variation</td>
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<td>CTC</td>
<td>circulating tumor cell</td>
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<td>CTM</td>
<td>circulating tumor microemboli</td>
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<td>ddPCR</td>
<td>epithelial cell adhesion molecule</td>
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<tr>
<td>EpcAM</td>
<td>US Food and Drug Administration</td>
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<td>FDA</td>
<td>glycoprotein 100; melanocyte protein PMEL</td>
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<td>gp100</td>
<td>herringbone-chip</td>
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<td>HB-chip</td>
<td>immunocytochemistry</td>
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<td>ICC</td>
<td>isolation by size of epithelial tumor cells</td>
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<td>ISET</td>
<td>proto-oncogene K-Ras; Kirsten rat sarcoma virus</td>
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<td>KRAS</td>
<td>Cytokeratin 18</td>
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<td>KRT18</td>
<td>Cytokeratin 8</td>
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<td>KRT8</td>
<td>mitogen-activated protein kinase</td>
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<td>MAPK</td>
<td>melanoma cell adhesion molecule</td>
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<td>MCAM</td>
<td>melanoma-associated chondroitin sulphate proteoglycan</td>
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<td>MCSP/CSGP4/HMW-MAA</td>
<td>proto-oncogene MDM2</td>
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<td>MDM2</td>
<td>melanoma circulating tumor cells</td>
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<td>MelCTC</td>
<td>macrophage migration inhibitory factor</td>
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<td>MIF</td>
<td>microphthalmia-associated transcription factor</td>
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<td>MITF</td>
<td>melanoma antigen recognized by T cells 1</td>
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<td>MLANA/MART-1</td>
<td>NOD scid gamma mice</td>
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<td>NSG</td>
<td>paired box gene 3</td>
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<td>PAX3</td>
<td>programmed cell death protein 1</td>
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<td>PD-1</td>
<td>programmed Death-ligand 1</td>
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<td>PD-L1</td>
<td>patient derived xenografts</td>
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<tr>
<td>PDX</td>
<td>progression free survival</td>
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<td>PFS</td>
<td>phosphatase and tensin homolog</td>
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<td>PTEN</td>
<td>receptor activator of NF-κB</td>
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<td>RANK</td>
<td>red blood cells</td>
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<td>RBCs</td>
<td>telomerase reverse transcriptase</td>
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<td>TERT</td>
<td>white blood cells</td>
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However, despite this multimarker approach aimed at improving the sensitivity of CTC capture, it still yielded low capture efficiency (34%) in spiking experiments [21], suggesting that only a few MelCTCs were being isolated.

To improve capture efficiency, the herringbone-chip (HB-chip) technology has been developed. This device uses micro-vortices generated by herringbone-shaped grooves to direct cells toward channel walls coated with a combination of antibodies targeting melanoma-specific antigens. The combination of HB-chip technology with a pool of 12 melanoma-specific antibodies for detection, allowed capture of CTCs (on average 8 CTCs/2.5 mL) by immunostaining in 32/41 (79%) metastatic melanoma patients at various stages of treatment [36]. These results underscore the need for multiple markers to identify MelCTCs given their remarkable heterogeneity. Nevertheless, these methods are not able to capture all CTCs present within a patient, as those that do not express the antigen of interest are missed.

To avoid capture bias, other studies have used negative selection procedures which capture leukocytes with anti-CD45 antibody coated beads followed by WBC depletion using magnetic separation. Systems such as EasySep™ or RosetteSep™ use this approach. Fusi and colleagues, using the EasySep™ method and detecting CTCs using gp100 and MLANA (melanoma antigen recognized by T cells 1) by flow cytometry, found 28/32 (87.5%) metastatic patients had CTCs with a median of 53 CTCs/10 mL of blood [22]. Using RosetteSep™, Girotti and colleagues successfully enriched CTCs and injected them into NSG mice to generate CDX models [23]. While the CTC quantities used to generate the models are unknown, it is likely that relatively large numbers of cells were isolated for successful tumor uptake.

Although negative selection is advantageous for removing cells that do not express the most common melanoma markers, the purity of CTCs obtained in the enriched fraction is low, hampering their quantification and downstream analysis. In fact, a spiking experiment comparing the recovery and purity of CD45 depletion with positive enrichment, or a combination of both methods, showed that the greatest recovery was found by using negative selection (58% recovery rate). However, the greatest purity of the CTC fraction was obtained by using the combination method (background reduced from 3 × 10^7 to 1.5 × 10^5 of WBCs) [34].

To further improve CTC capture, alternative techniques have been developed recently that exploit the larger cell size of MelCTCs compared to WBCs. Although it has been shown that MelCTCs can have a diverse range of cell sizes [4,41], most of the CTCs are thought to be larger (10–20 μm) than other blood components, such as RBCs (6–8 μm), leukocytes (7–12 μm) or platelets (2–3 μm). Taking advantage of this perceived difference in cell size, the “enrichment by size of epithelial tumor cells” (ISET™) technique was developed [58]. The ISET™ system uses polycarbonate filters with 8 μm diameter circular pores for CTC enrichment and detection of cells trapped in filters. De Giorgi and colleagues detected CTCs in 29% and 62.5% of patients with primary invasive and metastatic melanoma respectively, using qPCR to detect Tyrosinase transcripts after ISET™ filtration; the limit of detection was 1 CTC/ml of blood [14]. However, this approach also detected benign circulating nevus cells [15], suggesting the inability of this assay to distinguish between benign nevus cells and melanoma cells. Alternatively, when using the same ISET enrichment technique, with CTCs defined by positive immunohistochemistry expression of S100 and negative expression for CD45 or CD144 (leukocyte and endothelial cell markers, respectively), 51/90 (57%) metastatic melanoma patients had detectable CTCs (1–44 CTCs/ml of blood) [25]. The low percentage of metastatic patients with high-burden disease found with CTCs, shows the unsuitability of this method for detecting all CTCs present in patients. This drove the combination of technologies that rely on physical properties of the MelCTCs with those detecting expression of specific surface markers.

The CTC-iChip separates cells based on size using deterministic lateral displacement and inertial focusing followed by negative depletion. Using this chip, CTCs from two metastatic melanoma patients were successfully enriched and detected as positive by staining for the melanoma antigen recognized by T cells 1 (MART-1/MLANA) [41].

The OncoQuick™ system is another size-based technique that incorporates a filter for CTC separation in conjunction with density-
based centrifugation [50]. Spiking experiments showed a ≥60% recovery rate of 4, 20, 100 and 500 spiked SkMel28 cells when assessed by qPCR amplification of cytookeratin 8 (KRT8) and 18 (KRT18) RNA. The SkMel28 cell line strongly expresses these intermediate filament proteins and KRT18 expression has been previously identified as an adverse prognostic factor in melanoma [8]. For melanoma CTCs, when transcript levels of MLANA, MIF (Macrophage Migration Inhibitory Factor), TYR, and MITF (Melanogenesis Associated Transcription Factor) were assessed by qPCR after OncoQuick® enrichment, results showed that about 1/3 of patients (mostly early-stage) expressed elevated levels of MIF and MLANA transcripts, in comparison with healthy controls (p < 0.0001 and p < 0.001, respectively) [9]. The authors suggest that identification of early-stage patients with CTCs may be used to delineate those that would benefit from a more aggressive therapy at an earlier stage.

Previous studies have found circulating tumor microemboli (CTM) or CTC clusters in the blood of melanoma patients, raising the idea that cells enter the bloodstream via collective cell migration, allowing them to survive shear stress and anoikis forces [29,42]. Recently, the Cluster-Chip was developed to specifically isolate CTC clusters of two or more cells from 4 mL of blood, independently of tumor-specific marker expression. This microchip technology relies on the strength of the cluster union and on their behavior when a flow speed is applied through a set of triangular pillars. Captured CTC clusters were identified and detected by immunostaining in 30% (~0.15 CTCs/mL) of 20 tested metastatic melanoma patients. Interestingly, no correlation was found between the number of CTC clusters and the number of single CTCs isolated (n = 19) [53].

While these methods have proven the ability to capture CTCs, clinical validation of their prognostic value in large clinical samples is still needed.

Melanoma CTC detection methods

While the above techniques have been developed to improve isolation of CTCs, optimization of detection methods is also required. Detection of MelCTCs without a previous enrichment step has been reported by Ruiz and colleagues [51], where the Epic Sciences platform detected MelCTCs from whole blood, using a panel of seven anti-MCSP monoclonal antibodies. MCSP is a cell surface protein involved in melanoma proliferation, spreading and migration of cells and it is overexpressed in more than 90% of melanoma tumor tissue samples [6,13]. Using this marker for detection, 1–250 CTCs were detected in 8 mL of blood (0.5–371.5 CTCs/mL of blood) from 22/40 metastatic melanoma patients (55%). Interestingly, this method also enabled whole genome amplification and copy number variation (CNV) analyses of single MelCTCs, which revealed deletions of CDKN2A and PTEN, and amplifications of melanoma related genes, TERT, BRAF, KRAS and MDM2 amongst others [51].

Most commonly, once CTCs are enriched by the techniques discussed above they are detected by methods that assess their morphology and/or protein expression using immunocytochemistry (ICC) or flow cytometry. In these techniques a cocktail of antibodies against cell surface or intracellular markers associated with melanocyte biology or melanoma pathogenesis [21,25,30,55] are used to recognize the cells. Alternately, molecular approaches that detect RNA or DNA from enriched MelCTCs, by quantitative real-time PCR (qRT-PCR) [4,59] or droplet digital PCR (ddPCR) [45], respectively, have been used for CTC detection and characterization. A new and promising method based on the presence of elevated telomerase activity commonly found in melanoma cells, is being trialed for CTC detection [61].

Based on our previous identification of heterogeneous MelCTCs [21], we recently developed a flow-cytometry multimarker approach to detect and analyze CTCs for the presence of melanoma-associated markers, such as MCSP and MCM, in combination with melanoma stem cell markers, such as ABCB5, RANK (receptor activator of NF-κβ) and CD271 [25]. Using this approach we provided for the first time, a detailed insight into the diversity of MelCTCs within each patient, and showed that the prognostic utility of MelCTCs may not rely on the total count of CTCs but on the CTC subpopulations circulating within an individual. This study indicated that a high number of MelCTCs express melanoma-initiating or stem cell markers (ABCBS and RANK) while only very low numbers of CTCs express melanoma markers MCSP and MCM [25]. Importantly, the common expression of these melanoma-initiating markers by MelCTCs did not correlate with the expression of these markers in patient-matched tumors, where a low frequency of melanoma tumor cells positive for these markers was observed. This finding provides evidence that most CTCs, at least in melanoma, are derived from rare subpopulations of tumor cells which may have the ability to seed new metastases, and not from the bulk melanoma cells shaping the tumor [25].

Aya-Bonilla and colleagues [4] more recently interrogated for the first time, the enrichment of MelCTCs using spiral microfluidic technology [39,60]. With this device, recovery rates of greater than 55% and a 2.5–3 log depletion of WBCs were observed in spiking experiments using melanoma cell lines with different cell sizes which represents an improvement to depletion rates similar to those obtained by the CTC-iChip [41]. After microfluidic enrichment of blood from 20 metastatic melanoma patients, MelCTCs were identified by flow cytometry, gene expression analysis and immunostaining, in 40%, 54% and 43% of cases, respectively. As found previously [21,25,29], MelCTCs showed diversity in their marker population with CTCs analyzed by flow cytometry most commonly expressing ABCBS alone or in combination with RANK, a marker of treatment resistance. Gene expression analysis of the CTC-enriched fractions also detected transcripts of PAX3, alone or in combination with ABCBS expression in 6 out of 7 metastatic melanoma patients positive for melanoma transcripts; transcripts of the melanocytic gene, MLANA, were detected in the remaining patient. MLANA, PAX3 and ABCBS are highly expressed in melanoma tumors and have been described to play an important role in melanoma pathogenesis and resistance [17,20,43]. In this study, isolated CTCs were also characterized by multimarker immunostaining for intracellular melanocytic proteins gp100, S100 and MLANA (1–4 CTCs/8 mL of blood), which indicated that MelCTCs are also diverse in cell size (range: 13–21 μm) [4]. This study unmistakably confirmed the phenotypic and molecular heterogeneity of MelCTCs.

Although great advances have been made in MelCTC isolation (Fig. 1; Table 2), their quantification remains challenging given the low numbers of CTCs identified even when a variety of multimarker assays are used for their detection. This is presumably due to our limited knowledge of the spectrum of diverse MelCTCs. Studies are needed to investigate MelCTC phenotypes, their role in melanoma biology and prognosis as well as their differential pharmacodynamic responses to treatment [31].

Are CTCs of clinical utility in melanoma?

Studies to date show CTCs are a suitable biomarker of disease status. Furthermore, monitoring the levels of CTCs before and during melanoma treatment has, in limited studies, been shown to be informative with respect to prognosis and therapy response in melanoma [25,28,31,36,46].

Using RT-PCR to detect transcripts in blood, Reid and colleagues showed that in 230 patients, the presence of MLANA and ABCBS
transcripts were associated with disease recurrence and the expression of MCAM was significantly more common in patients with a poor treatment outcome [46]. Also, the presence of multiple melanoma markers in patient blood significantly correlated with their AJCC stage [32], and the detection of more than one marker at baseline and at any time during treatment administration was a negative prognostic factor for disease-free survival (DFS) and for overall survival (OS) [26].

Several studies using immunomagnetic enrichment have also shown that the number of MelCTCs is higher in the blood of patients with advanced disease [21,36,62]. Moreover, the number of CTCs was also shown to be associated with treatment failure and time that patients are receiving treatment [28,44]. By contrast, a low CTC count at baseline (\(\leq 2\) CTCs per 7.5 mL) are found during the first 72 h of treatment. GIrotti and coworkers [23] have been successful in generating CDXs in 6 out of 21 cases (28.6%) and showed that CDX models established from advanced stage patients could aid in the prediction of patient responses to treatments [23]. While the isolation of only a few CTCs capable of developing xenografts may underestimate the tumor heterogeneity, these are excellent first steps, addressing several of the issues surrounding the clinical benefit of CTC characterization, including the fact that CTCs that develop xenografts are capable of seeding metastases and therefore harbor significant information about the metastatic process. Additionally, further studies characterizing CTC subpopulations prior to or concurrently with injecting them into mice will provide crucial information about CTC phenotypes that are most likely to develop CDXs.

Although CTCs quantification shows great potential, the role of MelCTCs in melanoma management is still under investigation. For example, the continued presence or an increase of MelCTCs after therapy initiation may suggest disease progression. Contrarily, a decrease or a continued value of zero MelCTCs might suggest response to treatment. Additionally, changes in CTC phenotype after treatment initiation may be an early indicator of the emergence of resistance, leading to an early change of therapy. Although these changes are difficult to assess when low numbers of MelCTCs are being isolated, these measurements could offer unique prognostic information. Furthermore, MelCTCs could be tested for molecular evolution of tumors prior to therapy, to identify markers of intrinsic resistance; and during therapy, to identify the development of drug resistance to targeted therapies. There is also a need to evaluate the ability of CTC analysis to inform treatment decision in patients with AJCC stage III and resected stage IV melanomas, as there are now more clinical trials and FDA approved therapeutics for melanoma in stage III and stage IV [3,35]. The lack of standardization and the variety of methodologies used for their isolation has hampered the ability to implement the analysis of MelCTCs into large clinical studies. Therefore, there is an urgent need to standardized protocols for MelCTC enrichment, detection, and quantification across different laboratories.

**What additional isolation steps are required to identify the full spectrum of melanoma CTC subtypes and their prognostic potential?**

Melanoma CTCs are rare and very heterogeneous. The comprehension of their aggressiveness and their application in clinical settings is still limited by the capacity to successfully and routinely...
isolate viable heterogeneous CTCs from the majority of patients.

New platforms that enable effective and repetitive isolation of MelCTCs should be unbiased, which means that CTCs should be enriched and detected without relying on known expression of CTC markers, rather, methods should be based on broader traits of CTCs, such as physical properties (cell size, morphology, rigidity, nuclear/cytoplasm ratio). In addition, isolation methods should be highly efficient in enriching the vast majority of CTCs present in the blood at high purity (i.e. low WBC background). Moreover, methods should allow isolation of viable and intact CTCs for “omic” characterization and, ultimately, the establishment of CTC-derived cell cultures and xenografts (CDX). Furthermore, methods are required that allow high throughput, are low cost and accessible in both research and clinical environments.

Integration of genomic and transcriptomic data from bulk tumors [7] together with single-cell RNA-seq of melanoma tumors [56] have reinforced the abundant diversity between and within melanoma tumors. Thus CTCs derived from such tumors would similarly carry heterogeneous features. However, the challenges in isolating all CTCs have significantly flawed the interrogation of the real genomic, transcriptomic and proteomic diversity of MelCTCs.

Finding a system able to capture and detect CTCs independently of their cell marker characteristics is urgently needed in melanoma, and a few studies trialing this isolation approach have been reported [4,14,29,41]. Advances remain hampered however by the uncertain biology of these cells, and the lack of optimal technologies along with robust standardization and validation of these technologies.

Although challenging, studying the gene expression and mutational landscape of single MelCTCs, their relationship with tumor tissue cells and their connection with treatment response and resistance, will significantly increase the clinical value of this biomarker. Improvements along these lines will dramatically advance CTC use in the clinic. The recent implementation of devices capable of isolating viable and label-free MelCTCs paves the way for studies aimed at dissecting their real heterogeneity and the mechanisms underlying their role in melanoma spreading. Moreover, the isolation and study of MelCTC clusters will provide an

Fig. 2. Potential applications and clinical benefits of melanoma circulating tumor cells.
insight into their role in melanoma progression and metastasis and in the tumor-immune interactions.

Conclusion

In conclusion, a variety of isolation methods have been developed in order to study the prognostic and predictive applications of CTCs in melanoma. Moving forward, the implementation of optimal isolation techniques allowing phenotypic, genomic and transcriptomic approaches is critical in order to unveil the diversity of MelCTCs and provide new insights into their clinical opportunities (Fig. 2). The latest studies suggest that examining CTC subpopulations instead of quantifying CTCs, could significantly impact their clinical utility. However, the variety of subpopulations needs to be identified and clinical trials assessing the biomarker utility of these subpopulations needs to be undertaken to draw meaningful conclusions. Also, critical factors such as the time of blood collection (i.e., different time points), site of collection of blood sample [24], sample handling, transport and storage must not be overlooked and remains to be standardized. Although promising, MelCIC isolation and study still holds technological limitations that ought to be considered by MelCIC specialists worldwide to maximize their potential applications in clinical practice.

Conflict of interest

The authors declare no conflicts of interest.

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