Perspective

Cancer Research

Perspective on Circulating Tumor Cell Clusters: Why It Takes a Village to Metastasize

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Abstract

Circulating tumor cell (CTC) clusters may represent one of the key mechanisms initiating the metastasis process. However, the series of pathophysiologic events by which CTC clusters originate, enter the circulation, and reach the distant sites remain to be identified. The cellular and molecular mechanisms that provide survival advantage for CTC clusters during the transit in the blood stream are also still largely unknown. Understanding the biology of CTC clusters is critical to assess this unified scheme employed by cancer and to device strategies to overcome key pathways responsible for their improved metastatic potential. CTC clusters remain an underdeveloped area of research begging the attention of multidisciplinary cancer research teams. Here, we provide insight on existing preclinical evidence on the potential mechanisms leading to CTC cluster formation and dissemination and on processes that may offer survival advantage. We also offer our perspective on future directions to delineate the role of CTC clusters in metastatic cascade and discuss their clinical significance. *Cancer Res; 78(4); 845–52. ©2018 AACR.*

Introduction

Solid tumors can release a surprisingly high number of circulating tumor cells (CTC) everyday into the circulation (1). Although CTCs originating from primary tumors are considered transitional in the search for a new home, most of these cells are fated to die in circulation owing to mechanical and environmental trauma such as shear forces, oxidative stress, and attack by the immune system. In fact, only a small fraction of CTCs are capable of surviving, seeding distant organs, and eventually giving rise to overt metastatic disease. Most CTCs have a short half-life of less than 2.5 hours in circulation (2) and are apoptotic (3, 4). Therefore, only the CTCs with a survival advantage during their transit in the blood stream and a better potential for colonization in the distant sites can likely contribute to metastasis. To discover better prognostic and predictive markers of early metastatic recurrence and novel targets for its prevention and treatment, it is critical to identify and characterize the CTC population with highest metastatic potential. Recent preclinical and clinical studies suggest a link between CTC clusters and worse clinical outcomes (3, 5). CTC clusters are defined as groups of two or more aggregated CTCs found in the blood of patients with solid tumors (6). Despite the prognostic implications for CTC clusters, the molecular mechanisms responsible for their formation or dissemination and the pathways conferring their survival advantage and metastatic potential remain largely unknown. Here, we examine preclinical evidence on the sources of CTC clusters, potential mechanisms of CTC clusters formation (genesis), transit to distant sites (dissemination), their survival advantage, and increased metastatic potential. We also deliberate open questions (Table 1), unmet research needs, and future directions (in italics) to delineate the clinical significance of CTC clusters in the metastatic cascade. We finally discuss methods that are most common and clinically relevant or novel and promising for isolating CTC clusters and describe clinical evidence for their prognostic value.

Sources of CTC clusters

Both primary and metastatic tumors may constitute the source of CTC clusters forming multidirectional transit routes (Fig. 1A). CTC clusters originating from a primary tumor could "self-seed" the original site or travel to distant sites of metastasis. CTC clusters arising from a (micro)metastatic site could return to the primary tumor site or the original (micro)metastatic site or could travel to another distant site of metastasis. To support this hypothesis, tumor self-seeding has been a well-accepted concept for CTCs in general (7). The self-seeding potential (7) and the oligoclonality of CTC clusters (5) were both demonstrated in the same mouse xenograft model of MDA-MB-231 LM2 cell line. Although CTC clusters were found to be a minority (2.6%) in the overall CTC population in this model, their calculated probability of metastatic formation was 50 times higher, as suggested by formation of dual-color metastasis from dual-color primary tumors (5). Although the study evaluated self-seeding concept only for the primary tumors, cross-seeding of primary and (micro)metastatic tumors can also be envisioned. This multisite exchange of CTC

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Table 1. Open questions in various areas of CTC cluster research

Area	Open questions
Genesis	• Does breast density regulate "cell jamming" and CTC cluster formation? • Which tissue development/regeneration pathways support CTC cluster formation? • What multiple passive and/or active modalities of extracellular matrix, tumor microenvironment, and/or tumor cells support CTC cluster formation?
Dissemination	• Are invadopodia-based and/or macrophage-dependent pathways involved in intravasation of CTC clusters into circulation? • Can disruption of CTC clusters in circulation prevent metastatic disease? • Are there any "druggable" and FDA-approved drugs that can dissociate CTC clusters? • What factors influence the structural plasticity (linear versus spherical) of CTC clusters and therefore their site of colonization?
Survival advantage	• How does cellular plasticity confer survival advantage for CTC clusters? What are the molecular mechanism(s) that facilitate cellular plasticity within CTC clusters? • What are paracrine interactions between stromal/immune cells and tumor cells in heterotypic clusters? How are these interactions regulated? • What is the difference in cell metabolism between single CTCs and CTCs in clusters?
Metastatic potential	• What is the expression of stem cell markers on CTCs within clusters? Where within CTC clusters are these potential tumor-initiating cells located? • What are the nontumor cell types (e.g. immune cells) within heterotypic CTC clusters? How do their interactions with tumor cells promote metastases? • Does the dormancy status of CTC clusters predict outcomes in patients/survivors? • How the CTC cluster–host interactions at the distant site facilitate metastasis?

clusters may allow communication between each tumor site to collectively acquire capability of surviving the tremendous treatment pressures, and eventually promoting the tumor growth and progression.

Genesis of CTC clusters

The physiological events by which CTC clusters originate are still largely unknown. The concept that CTC clusters could form by intravascular grouping of single CTCs has been disproven by a

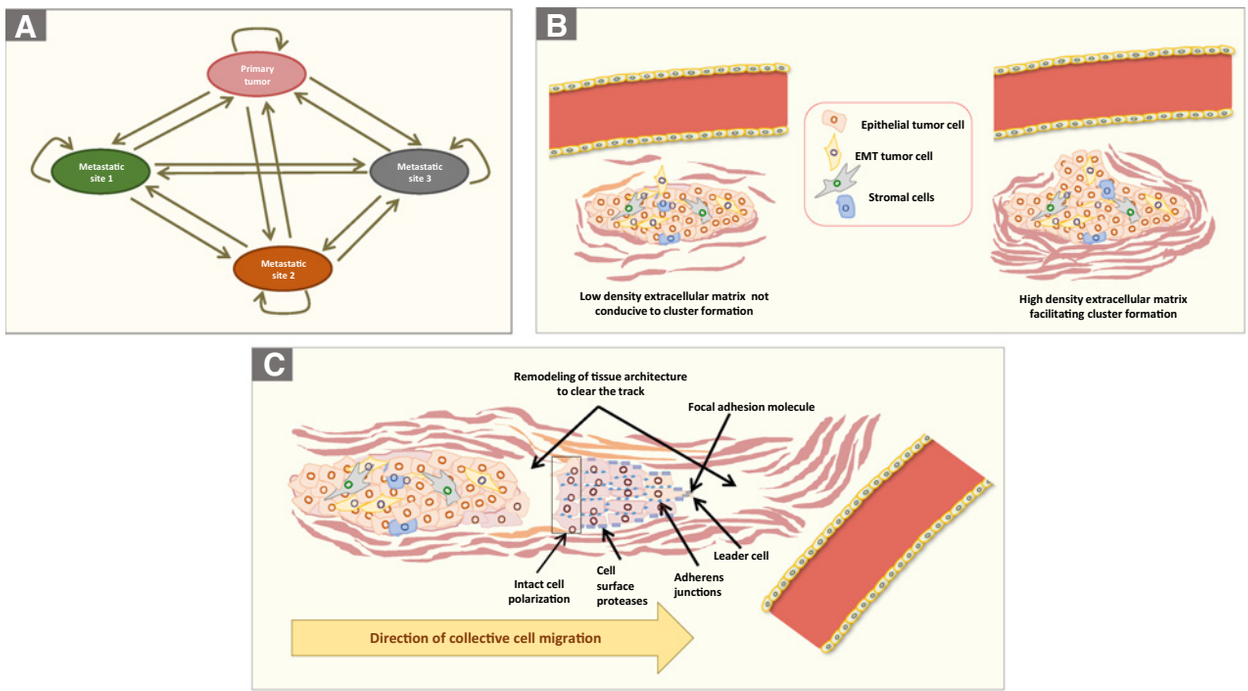

Figure 1.
Sources and potential mechanisms of CTC clusters origin. **A,** Multidirectional transit routes of CTC clusters. CTC clusters originating from a primary tumor could "self-seed" the original site or travel to distant sites of metastasis. CTC clusters arising from a (micro)metastatic site could return to the primary tumor site or the original (micro)metastatic site or could travel to another distant site of metastasis. This multisite exchange of CTC clusters may allow communication between each tumor site to collectively acquire capability to survive the tremendous treatment pressures, eventually promoting the tumor growth and progression. **B,** Origin of CTC clusters due to "cell jamming." The "cell jamming" principle proposes that increasing confinement from the growing mass of tumor or higher density of extracellular matrix may promote grouping of the cells. In this context, higher mammographic density may facilitate CTC cluster formation. **C,** Orchestrated origin of CTC clusters through activation of tissue development and regeneration pathways. These pathways include (i) intact cell polarization (not graphically represented in the figure), (ii) acquired expression of cell surface proteases and various cell adhesion molecules, (iii) the presence of adherens junctions for which plakoglobin is an important mediator, and (iv) remodeling of tissue/tumor architecture to clear the track, which is facilitated by a keratin-14–positive leader cell. These mechanisms may in turn facilitate tumor cell cooperativity and their collective cell migration as CTC clusters.

recent study (5). However, that still does not address the question about the exact steps leading to CTC cluster formation. An emerging hypothesis is that CTC clusters are formed due to "cell jamming" (Fig. 1B). This principle proposes that increasing confinement from the growing mass of tumor or higher density of extracellular matrix (ECM) may control the mode of tumor cell dissemination. Higher ECM density was shown to shift the preference of mesenchymal tumor cells to collective invasion whereas lower ECM density was associated with single cell invasion in an *in vitro* model (8). This principle may also apply to mammographic breast density, which is an important prognostic factor for locoregional recurrence in early-stage breast cancer and of progression-free survival in metastatic breast cancer at initial diagnosis (9, 10). However, the influence of breast density on "cell jamming" and preference for CTC cluster formation is an open question, which can be investigated in animal models with collagen I defects, LOX-mediated collagen crosslinking, or CD36 repression (11–13).

A more strategic preparation for the tumor cells to form a cluster may involve activation of pathways involved in tissue development and regeneration. These molecular mechanisms may in turn facilitate tumor cell cooperativity and collective cell migration (14). A few decades ago preclinical studies focusing on wound healing and morphogenesis have shown that epithelial cell aggregates are capable of spreading movements *in vitro* and *in vivo* (14). These aggregates are able to migrate while maintaining cell–cell interactions (15). Some of the mechanisms proposed for this collective cell migration include (i) intact cell polarization, (ii) acquired expression of cell surface proteases and various cell adhesion molecules, (iii) presence of adherens junctions, and (iv) remodeling of tissue/tumor architecture to clear the track (Fig. 1C). Interestingly, some of these pathways seem to be also important for ECM-induced collective invasion (8). If present in CTC clusters, these processes may allow cell–cell coupling and multicellular organization and ultimately facilitate the formation of CTC clusters. More research is needed to address the exact cellular events leading to CTC cluster formation, with a possibility of multiple passive and active modalities supported by ECM, tumor microenvironment, and/or tumor cells themselves.

Similar to morphogenetic movements, collective movement occurs in many cancers in which cells are not completely dedifferentiated (16). In this regard, collective cell migration may be led by a keratin 14-positive leader cell, which may create a path for the other tumor cells in its group through the surrounding tissue, in the blood stream, and potentially in the invaded site (Fig. 1C; ref. 17). Interestingly, ECM-induced "cell jamming" is shown to also require track clearance by leader cells (8). Plakoglobin, which is involved in cell–cell junction and is highly expressed in CTC clusters compared to single CTCs, may also provide a preference for CTC clusters formation and integrity throughout their transit in the blood (5) (Fig. 1C). *Keratin 14*, *plakoglobin*, *E-cadherin*, and other epithelial cytoskeletal and adhesion proteins form the core of the machinery necessary for formation and dissemination of CTC clusters. Here, the unexplored research topics include the molecular processes by which the epithelial framework facilitates CTC cluster formation and its dependence on various mechanisms of cell invasiveness and migration.

Dissemination of CTC clusters

The access of CTC clusters into the blood stream could be made possible by the porous and leaky blood vessels formed within rapidly growing tumor masses, via hasty neoangiogenesis (Fig. 2A; ref. 18). This supports the tumor self-seeding hypothesis to allow the entry of CTC clusters back to the original site. Conversely, a choreographed entry through invadopodia and macrophage-dependent transendothelial migration (19) may also be possible for CTC clusters to gain the access to circulation (Fig. 2A). The invadopodia are the protrusive and adhesive structures of cancer cells thought to arise in response to a range of signals primarily from tumor microenvironment. The proteolytic function of invadopodia through localized activity of matrix metalloproteases and their role in transendothelial migration of individual tumor cells are particularly important for metastasis. However, whether similar invadopodia-based and/or macrophage-dependent pathways are involved in intravasation of CTC clusters for dissemination remains to be explored. Future studies may also include capturing the live events of CTC clusters in transit by utilizing recent advances in the three-dimension real-time microscopy imaging *in vivo* (20).

Once in circulation, CTC clusters have slower flow rate than single CTCs within the blood vessels (21), which suggests its embolus/thrombus-like behavior. In support of this, administration of a thrombolytic agent called urokinase-type plasminogen activator (uPA) is effective at breaking down CTC clusters into single cells as well as modestly reducing the numbers of metastatic lesions and improving survival (21). Contrariwise, uPA expression is associated with enhanced tumor migration and invasion and higher rates of tumor progression and metastasis (22, 23), suggesting a differential role of uPA in early versus metastatic stage. Disaggregation of CTC cluster has also been reported with genetic knockdown of plakoglobin, which leads to their compromised metastatic efficiency in the animal models (5). The observation of reduced metastasis and improved survival by disrupting CTC clusters directly, either by systemic uPA administration or by genetic knockdown of plakoglobin (Fig. 2B), in animal models raises interesting questions: Can disaggregation of CTC clusters in circulation provide an advantage in preventing metastatic disease? What are the "druggable" targets that can dissociate CTC clusters? Are there any FDA-approved drugs that can disaggregate CTC clusters? In this regard, CTC clusters are often found to be associated with platelets (24, 25). It is not clear if platelets play any role in maintaining their integrity during the transit or their dissemination ability. Whether currently available anti-platelet agents can dissociate CTC clusters may be an interesting question to evaluate in preclinical models.

Slowly moving CTC clusters if arrested in small veins or capillaries may find residence and give rise to overt metastases from within the vessel (Fig. 2A). Because lung is the first organ encountered by these CTC clusters released from various organs, this potentially could be one of the mechanisms by which they are responsible for lung metastases. Indeed, a preclinical study has demonstrated that lung metastasis originates from the intravascular proliferation of endothelium-attached tumor cells rather than from CTCs that were able to extravasate and invade lung parenchyma (26). This phenomenon might also explain the formation of brain metastases despite the presence of an intact blood brain barrier. However, a deliberate movement of a group of tumor cells through microvessels is also possible due to CTC clusters organized in a linear arrangement of single-cell files (27, 28), which may also explain the trans-pulmonary passage of CTC clusters into other organs and rise of metastases in other distant site (Fig. 2A). What factors influence the structure of CTC clusters

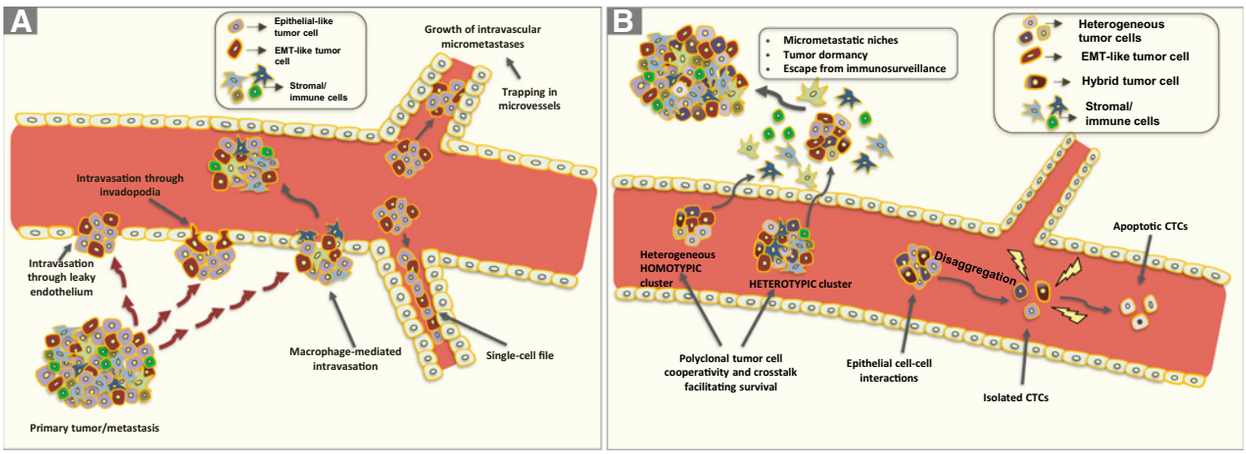

Figure 2.

Dissemination, survival advantage, and increased metastatic potential of CTC clusters. **A,** Dissemination of CTC clusters. CTC clusters may enter the circulation either by porous and leaky blood vessels formed by hasty neoangiogenesis required by the growing mass of tumor or by a choreographed entry through invadopodia and macrophage-dependent transendothelial migration, which has been demonstrated for single CTCs. When in circulation, a CTC cluster may act like a thrombus and get arrested in the small veins or capillaries. Here, they may find residence and give rise to overt metastasis. An (re)arrangement of the CTCs within a cluster in a linear fashion as a single-cell file may allow the grouped cells to pass through microvessels to reach more distant sites. **B,** Survival advantage and increased metastatic potential of CTC clusters. Persistent adhesion-dependent survival signals in CTC clusters can provide survival stimuli and thus contribute to effective metastatic spreading. Although epithelial cell–cell interactions may be important, cellular plasticity of CTCs within clusters have also been seen as in form of expression of EMT markers and presence of hybrid cells with both epithelial and EMT characteristics. Disaggregation of CTC clusters by uPA or plakoglobin knockdown or any other method may give rise to single CTCs. Although single CTCs may experience many survival challenges such as shear forces, environmental or oxidative stresses, and immune assault leading to apoptosis, CTCs within clusters may be shielded from them. This does not rule out the possibility that a few single CTCs may still be able to colonize a distant site. Cellular heterogeneity [such as undifferentiated vs. differentiated and epithelial vs. EMT] for homotypic clusters made up of only tumor cells and interaction between tumor cells and other nontumor cells (such as stromal or immune cells) for heterotypic clusters may offer competitive advantage for colonization at distant sites. Furthermore, activation of tumor dormancy program could favor formation of micrometastatic niche and escape from immunosurveillance at the distant sites.

and possibly the site of colonization for CTC cluster is another important area of research.

Survival advantage for CTC clusters

CTC death may be in part due to the loss of adhesion-dependent survival signals leading to anoikis (29), which might explain apoptotic CTCs of epithelial phenotype (Fig. 2B; refs. 30, 31). This supports the hypothesis that persistent epithelial cell–cell interactions in the form of clusters can provide survival stimuli and thus contribute to effective metastatic spreading (16). However, CTC clusters also express more mesenchymal versus epithelial markers compared to single CTCs (31). How would that provide additional survival advantage from anoikis during the transit is not known. Although epithelial-to-mesenchymal transition (EMT) in CTC clusters is counterintuitive because it is expected to result in high propensity of single cells, the cellular plasticity and cooperativity within a cluster may confer resistance to various stresses within the circulation (32). In support of this, the hybrid epithelial/mesenchymal state of CTC clusters has recently been described (Fig. 2B; ref. 25); however, its direct association to increased survival advantage is less clear. The molecular mechanism(s) allowing the cells to have the plasticity is a highly important area of investigation as they may provide additional pharmacological targets for the prevention of metastasis.

Other conceivable mechanisms for the survival advantage of clusters include the cooperation between cells within CTC clusters shielding from shear forces, environmental or oxidative stresses, and immune assault (Fig. 2B). Indeed, tumor fragments are found to survive and grow better after they are transplanted (33) or injected (34, 35) into a new host. In this context, heterotypic clusters containing more durable stromal or immune cells aggregated with CTCs may provide additional advantage (36, 37). It can also be hypothesized that paracrine interactions between cells of various origin in heterotypic clusters may play a pivotal role in seeding of tumor clusters and in the evasion of immunosurveillance at the distant site, further providing a survival advantage (Fig. 2B). The mechanisms that regulate these interactions and their influence on survival advantage and colonization potential of CTC clusters also remains unknown.

A predominantly glycolysis-driven cell metabolism of a cancer cell allows the cells to survive in hypoxic conditions while being maintained in the tumor microenvironment. While in circulation, the oxygen deprivation may be even more severely restricted so that only the toughest cells survive. Significant work has been done to understand how cancer cell metabolism affects the tumor cell growth as well as its migratory or invasive capability (38, 39). The role of EMT in rewiring of the cancer cell metabolic network and, vice versa, the importance of metabolic reprogramming on EMT are also starting to be deciphered (40). On one hand, EMT controls the expression of genes involved in metabolic pathways such as glycolysis, lipid metabolism, mitochondrial metabolism, and glutaminolysis. However, deregulated expression of metabolic enzymes in these pathways promotes EMT. Although these pathways are shown to be relevant in migrating single CTC with mesenchymal characteristics, the differences in cancer cell metabolism between single CTCs and CTC clusters need to be investigated.

Enhanced metastatic potential of CTC clusters

The polyclonal tumor cell cooperativity and crosstalk within the network of migrating homotypic (made of only CTCs) or heterotypic clusters (made of CTCs and other stromal/immune cells) may facilitate stabilizing and initiating metastatic growth (27). For homotypic clusters, cellular heterogeneity (such as undifferentiated vs. differentiated and epithelial vs. EMT) may offer competitive advantage for colonization at distant sites (Fig. 2B). While expression of stem cell markers within some CTCs has been described, this remains an important area of future investigation for CTC clusters. Single cell analysis of stem cell marker expression and the localization of tumor-initiating cells within the cluster may be an important factor to examine. For heterotypic clusters, interaction between tumor cells and other nontumor cells (such as stromal or immune cells) may also be important for colonization and escaping immunosurveillance (Fig. 2B). Given the recent findings of the various tumor-suppressive/promoting roles played by different tumor-associated immune cells (41), identifying the nontumor cell types, especially immune cells, and their interactions within clusters will shed light on their biological functions and clinical significance in metastasis. With the latest advances in single-cell molecular profiling technologies, analysis of each cell within clusters may reveal mechanisms of cooperativity and define the roles of nontumor cells within CTC clusters.

The observation that CTC clusters are nonproliferative (42), which may allow their escape from the pressures of cytotoxic treatments during the transit and at the distant site, is of interest. This may reflect activation of tumor dormancy programs (43), which, in turn, could favor formation of micrometastatic niches and escape from immunosurveillance at the distant sites (Fig. 2B). The clinical relevance of this nonproliferative status of CTC clusters is an area of active research (3). Although CTCs have been reported in cancer survivors 7 to 22 years after their initial treatment (2), whether CTC clusters are present in these survivors and if their presence predicts imminent recurrence is unknown. Because the "nondormant" state of CTCs as assessed by the proliferation index predicts relapse in breast cancer patients (44), the assessment of this dynamic in CTC clusters may provide additional insight into the mechanisms of tumor progression. Recently, it was proposed that cancer of unknown primary may be explained by the presence of dormant CTCs forming a premetastatic niche and giving rise to a metastatic disease before the tumors at the primary site can be detected (45). It will also be important to determine the role of CTC clusters in this process. Furthermore, the mechanisms by which cluster-host cell interaction at the distant site can intervene and facilitate metastatic cascade also need further investigation.

Isolation and detection of CTC clusters

The major challenges in isolating CTC clusters are related to (i) their paucity as they are found in numbers as low as one cluster per over 10^7 leukocytes and 10^{10} red blood cells, (ii) the potential for their dissociation during blood processing, and (iii) the variations in their physical, cellular, and molecular characteristics. A desired platform to isolate CTC clusters would be able to isolate live and intact CTC clusters of different size, shape, and composition independently of tumor-specific cell surface markers with reduced processing time, robust clinical feasibility, and demonstrated clinical validity in predicting prognosis in patients. While considerable numbers of platforms have been developed for CTC isolation in general, only some have shown the capacity to detect clusters of CTCs, with only a handful demonstrating the prognostic significance of CTC clusters in patients, as described in the section below. These include (i) immunomagnetic-based isolation methods, such as the CellSearch system, which is currently the only FDA-approved platform for the detection of CTCs as a prognostic marker in metastatic cancer patients (46); (ii) size-based filtration methods, such as the Isolation by SizE of Tumor cells (ISET; refs. 47, 48); and (iii) microfluidic devices or chips, operating on various passive or active separation principles (49). These platforms are compared in Table 2 for their desired features for CTC clusters research, which highlights the need to develop a platform specifically for CTC cluster research.

To date, microfluidic devices seem to be the most promising platform for isolating CTC clusters, as they offer several advantages such as (i) ability to process whole blood without the need for red blood cells (RBC) removal, which results in less potential of cluster dissociation from shear or centrifugation forces and faster processing time and (ii) collection of live CTC clusters. The main drawback of this platform has been the need for cell surface marker-based capture. To overcome this limitation, size-based isolation methods using spiral microfluidics have been optimized (50). These spiral systems have shown excellent recovery rates and very efficient depletion of white blood cells. Importantly, these devices can be produced at low cost and be easily operated, making them available for a widespread use. To customize selective capture of CTC clusters, a first generation of platform named Cluster-Chip was developed. Cluster-Chip used specialized bifurcating triangular micropillars acting as traps under low-shear stress to preserve CTC cluster integrity and demonstrated high efficiency capture of clusters in patients with metastatic breast or prostate cancer and melanoma (51). To overcome the physical limitation of Cluster-Chip platform that impact viability of CTC clusters, the same group has recently adapted a two-stage deterministic lateral displacement (DLD) approach in a continuous flow microfluidic device to sort clusters based on size and asymmetry from whole blood (52). Here, the first stage is designed to extract clusters based on size such that larger ones will be moved laterally, while smaller clusters and single cells will follow the streamlines through the device to arrive at the

Table 2. Main features of common methods for the detection and study of CTC clusters

	CellSearch	Microfluidic chips	Filtration devices
Robust clinical feasibility	X		
Reduced processing time/ability to process whole blood		X	X
Cell surface marker-independent isolation of CTC clusters			X
Size-independent isolation of CTC clusters	X	X	
Reduced risk of dissociation or omission of CTC clusters due density gradient		X	X
Ability to capture live CTC clusters		X	

Table 3. Association of CTC clusters and clinical outcomes in cancer patients

Association between		
Features of CTC clusters	**Clinical outcomes**	**Cancer type [Detection method]**
Presence and higher number of CTC clusters at baseline	Shorter PFS and/or OS	Lung cancer (3) [CellSearch]
		Breast cancer (55, 56) [CellSearch]
		Melanoma (54) [ISET]
		Gastric cancer (58) [ISET]
		Colorectal cancer (57) [ISET]
		Liver cancer (60) [ISET]
		Pancreatic ductal adenocarcinoma (59) [Microfluidics-CMx]
	Platinum resistance	Ovarian cancer (61) [Microfluidics-EC]
Persistence of CTC clusters after treatment initiation	Shorter PFS and/or OS	Breast cancer (30, 56) [CellSearch]
		Head and neck squamous cell carcinoma (62) [ISET]
Bigger CTC cluster size ($\geq$3 cells per cluster)	Shorter OS	Breast cancer (56) [CellSearch]

Abbreviations: CMx, biomimetic supported lipid bilayer surface-coated microfluidic chip conjugated with anti-epithelial cell adhesion molecule (EpCAM) antibodies; EC, electrically conductive chip incorporating a nanoroughened microfluidic platform utilizing microchannels conjugated with streptavidin and then exposed to biotinylated antibodies against EpCAM, TROP-2, EGFR, vimentin, and N-cadherin; ISET, isolation by size of tumor cell method.

second stage, which captures smaller clusters based on asymmetry. Another microfluidic device purposely developed to isolate clusters of CTCs is a three-dimensional (3D) scaffold chip, which can efficiently capture clusters by combining specific antibody-dependent recognition and physical barricade effect of the 3D scaffold structure (53). Here, the scaffold is uniformly coated with thermosensitive gelatin hydrogel, which dissolves at 37°C, allowing gentle release of the captured cells, and thus assuring high viability for downstream applications, including cell culturing.

The future innovation in microfluidic approach to capture CTC clusters requires integration of multiple separation principles to cover the wide physical variations seen in this rare population and shortening of the processing time. An ideal platform will also have demonstrated clinical feasibility as well as validity by confirming the prognostic value of CTC clusters in cancer patients. Commercialization of this platform will also be critical to undertake multitude of future studies described above to uncover the biological and clinical roles of CTC clusters.

Clinical relevance of CTC clusters

Although it is still unclear which tumor or patient characteristics can predict the presence of CTC clusters, recent clinical studies have demonstrated the prognostic value of CTC clusters (Table 3). The presence and high numbers of CTC clusters at baseline have shown to be associated with shorter progression-free survival (PFS) and overall survival (OS) in patients with various types of solid tumors (3, 54–60). Moreover, platinum resistance has been observed in patients with primary or recurrent ovarian cancer with CTC clusters (61). Finally, cancer patients with persistence of CTC clusters after treatment initiation and with bigger CTC cluster size are shown to have shorter survival (PFS and/or OS; refs. 30, 56, 62). These clinical findings not only suggest the prognostic value of CTC clusters but also emphasize their biological significance in tumor progression and treatment outcome.

Summary

Evidence so far supports a functional role of CTC clusters in surviving pressures of travelling through the bloodstream, such as anoikis, shear forces, and immune attack, as well as colonizing distant organs. The advantage may be offered by the composition and cooperativity among the CTCs within a cluster, compared to the single CTCs. However, much is still unknown about their genesis, transit, and settlement. The clinical data so far also indicate the prognostic value of CTC cluster analysis in predicting treatment resistance and survival outcomes in cancer patients. Nevertheless, the precise cellular and molecular mechanisms enhancing the metastatic ability of clusters remain unclear. A combination of microfluidic and computational simulation of cluster movement with real-time *in vivo* microscopy may help us understand the early events of CTC cluster formation and dissemination. In addition, comparing molecular profiling of single versus clustered CTCs may reveal the pathways responsible for their extended survival and drug resistance and define the roles of nontumor cells associated with CTCs. The biological studies to answer the open questions related to CTC clusters presented here will allow a deeper understanding of the role of CTC clusters in tumor progression, identify novel therapies, and eventually guide clinical studies for personalization of therapeutic decision-making.

Disclosure of Potential Conflicts of Interest

M. Cristofanilli has received speakers bureau honoraria from Pfizer and is a consultant/advisory board member of Vortex. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: M. Giuliano, S. De Placido, M. Cristofanilli, R. Schiff, M.V. Trivedi

Development of methodology: M. Giuliano, R. Schiff, M.V. Trivedi

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Giuliano, A. Shaikh, G. Arpino, R. Schiff, M.V. Trivedi

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