Circulating tumor cell clusters: What we know and what we expect (Review)

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Abstract. The major cause of cancer-associated mortality is tumor metastasis, a disease that is far from understood. Many studies have observed circulating tumor cells (CTCs) in patients' circulation systems, and a few latest investigations showed that CTC clusters have a potentially high capacity of metastasis. The capture and analysis of CTC clusters offer new insights into tumor metastasis and can facilitate the development of cancer treatments. We reviewed the research history of the CTC clusters, as well as the technologies used for detecting and isolating CTC clusters. In addition, we discuss the characteristics of CTC clusters and their roles in tumor dissemination. Clinical relevance of CTC clusters was also implicated in currently limited data. Moving forward, the next frontier in this field is to develop more efficient capture methods and decipher conundrums of characterization of CTC clusters. This will ultimately identify the clinical value of CTC clusters as a biomarker and therapeutic target.

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1. Introduction

The vast majority of cancer-related deaths are caused by metastasis, the dissemination of tumor cells from their original sites to distant organs mainly through the blood circulation system (1,2). Unfortunately, after decades of exploration, our understanding of tumor metastasis is still far from complete, let alone enough for prevention and cure. Circulating tumor cells (CTCs) have recently been attracting great attention due to their key role in tumor metastasis, even though they were first discovered almost 150 years ago (3,4). Given the value of prognosis prediction of CTCs, the US Food and Drug Administration (FDA) has approved their clinical use in metastatic breast, colorectal and prostate cancers (5). CTCs can be frequently and conveniently detected and are valuable for personalized treatment. Increasing studies have proved the value of CTC detection in a number of different types of cancer (5-9); however, great efforts are needed to decipher the clinical implication underlying CTCs.

Additionally, referred to as circulating tumor microemboli, circulating micrometastases or circulating tumor aggregates, CTC clusters are defined as groups of tumor cells (more than two or three cells, varied among studies) that travel together in the bloodstream (10). Early in the 1970s, a series of preclinical studies demonstrated that CTC clusters had a greater predisposition of forming distal metastasis than single CTCs (11-15). However, further studies were stagnant for decades due to limitations of CTC cluster isolation in human. In spite of great advances on CTCs recently, little progress has been achieved. For instance, investigators have already recognized that CTCs are heterogeneous, with certain subgroups of CTCs harboring higher metastatic potential (16-18).

The prevalence and amount of CTC clusters can be underestimated due to their short detection window and lack of appropriate detection methods. On one hand, the life span of CTC clusters in circulation is extremely short [much shorter than individual CTCs, which can also exist in the circulation for only several hours (19)] due to their interception by small vessels. On the other hand, current technologies are responsible for the underrating of CTC clusters because few specialized devices exist for the detection of CTC clusters.

The relative rarity and incompetence of existing capture methods limit our knowledge of CTC clusters, thus many questions are awaiting to be answered, including where CTC clusters come from, what they consist of, and how they take advantage to form metastases. To resolve these problems, acquirement of enough viable CTC clusters is the key, which makes subsequent molecular, genetic and functional experiments possible. In this review, we discuss the valuable knowledge of CTC clusters from all relevant aspects including discovery history, detection and isolation methods, pathophysiological characteristics as well as their clinical implications.

2. The discovery history of CTC clusters

Similar to the long history of CTCs, the first recognition of CTC clusters can be dated back to 1950s (Fig. 1). In 1954, Watanabe highlighted the role of CTC clusters in tumor metastases formation by injecting bronchogenic carcinoma cells from jugular veins of mice, showing that viable tumor cells in clusters formed metastases, unlike individual cells (20). Studies in the following two decades confirmed this result on both melanoma-derived lung metastases and colon cancer-derived liver metastases models (11,15,21,22) and also illustrated that the metastases formation partially depended on the size and concentration of CTC clusters (13,23). Although cancer cells intercepted by vessels were seen in animal models decades ago, the tumor cell emboli entrapped in vessels in human were proved most recently in the microvasculature of the lungs in the 3 out of 8 patients with metastatic breast and cervical carcinomas (24).

Pioneers in the field also studied the relationship between CTC clusters and the sites of metastases formation. Liotta *et al* revealed that the size distribution of vessels was an important determinant of the distribution and survival of CTC clusters in the circulation system (14). In addition, some investigators found that CTC clusters of different tumor cells harbored different metastasis proclivity (25).

An attempt was also made to explore the mechanisms of how CTC clusters possess survival and metastasis advantages. Recent studies imply that CTC clusters have their specialized microenvironments and are not simply an aggregation of tumor cells (26). Interaction between tumor cells and accessory cells was found to provide tumor cells with survival advantages via different ways, although the detailed mechanisms required in-depth investigation (16,27,28). Nowadays, with the improvement of CTC cluster isolation technology, other physical properties of CTC clusters such as density and electromechanical characteristics have been under assessment and we can soon expect deeper understanding of these aspects.

Despite the long history in this field, information surrounding CTC clusters remain largely unknown. Increased efforts are urgently required to characterize CTC clusters and fully understand their roles in tumor metastasis, both clinically and mechanically.

3. Methods for CTC cluster isolation, capture and identification

Currently, very few methods have been developed for specialized detection of CTC clusters. In most cases, CTC clusters were incidentally observed when detecting individual CTCs. The devices used for CTC isolation and capture are based on the differences in physical properties (e.g., density, size, deformability, electric charges), and biological properties (e.g., antigen expression) between CTCs and non-tumor cells. Currently, limited data of CTC clusters in patients vary greatly according to tumor type, disease stage, detection platform, and other factors (Table I). However, these existing platforms are not ideal for CTC cluster isolation since they usually underestimate the amount of CTC clusters. Thus, it is important to approach with caution when interpreting the results of CTC clusters derived from single CTC specialized isolation platforms.

Antibody-based methods. Antibody-based methods are the most widely used capture techniques for CTC clusters. The antibodies are mainly pertained to epithelial cell surface markers that are absent from blood or stroma cells. Among these antibodies, the epithelial cell adhesion molecule (EpCAM) is the most commonly used. In CellSearch®, the first and only commercial CTC isolation platform approved by the US FDA, a CTC cluster is defined as a group of CTCs containing three or more cells expressing EpCAM and cytokeratins (CKs) without expression of CD45 and has (4',6-diamidino-2-phenylindole)-stained nuclei (29). Blood-spiking experiments with HeLa cells expressing histone H2B-GFP confirmed that CTC clusters were not artifacts caused by sample manipulation (10). Although CTC clusters were rarely captured by CellSearch in a majority of solid tumors, they were frequently detected (25/97) in small cell lung cancer (SCLC) and indicated poor prognosis (10). As a representative of high-throughput microfluidic devices, herringbone (HB)-chip was developed to improve capture efficiency by using microvortices to increase the interaction between tumor cells and antibodies (26). The number of tumor cells in a CTC cluster captured by HB-chip ranged from 4 to 12; however, CTC clusters were only found in 3 out of 19 patients with metastatic prostate or lung cancer (26). In order to further identify invasive CTC clusters, a platform was developed to detect CTC clusters that uptake cell-adhesion matrix, and 17% of samples were found positive in patients with metastatic castration-resistant prostate cancer (30).

Antibody-conjugated magnetic microbeads or nanoparticles binding to a specific surface antigen such as CD45 were also used for isolating CTCs and CTC clusters (31-34). After incubation, the blood sample was exposed to a non-uniform magnetic field to isolate the labeled cells. In a study using CK and prostate specific antigen as conjugated biomarkers in prostate cancer, the prevalence of CTC clusters was as high as 80% (32). In another study for colorectal cancer, the immunomagnetic labeled CK antibodies could separate CTC clusters from the peripheral blood in 68.8% of patients (33). However, certain investigators used a negative selection strategy to remove non-tumor cells from CTCs and CTC clusters, and found that the prevalence of CTC clusters was rare (31). In general, the efficiency of immunomagnetic methods mainly depends on two factors: i) the expression and specificity of the target antigen and the affinity between antigens and antibodies; and ii) the efficiency of immunomagnetic labeling process and magnetic particles.
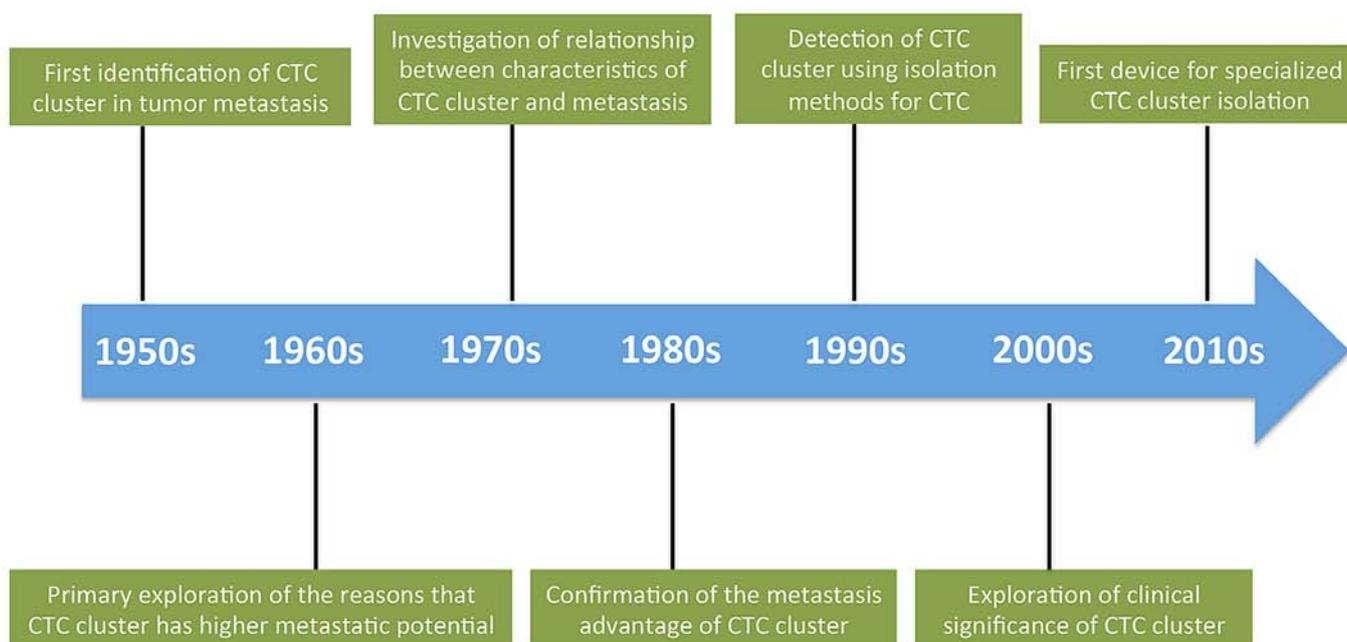
Figure 1. The milestones of CTC cluster discovery and isolation.

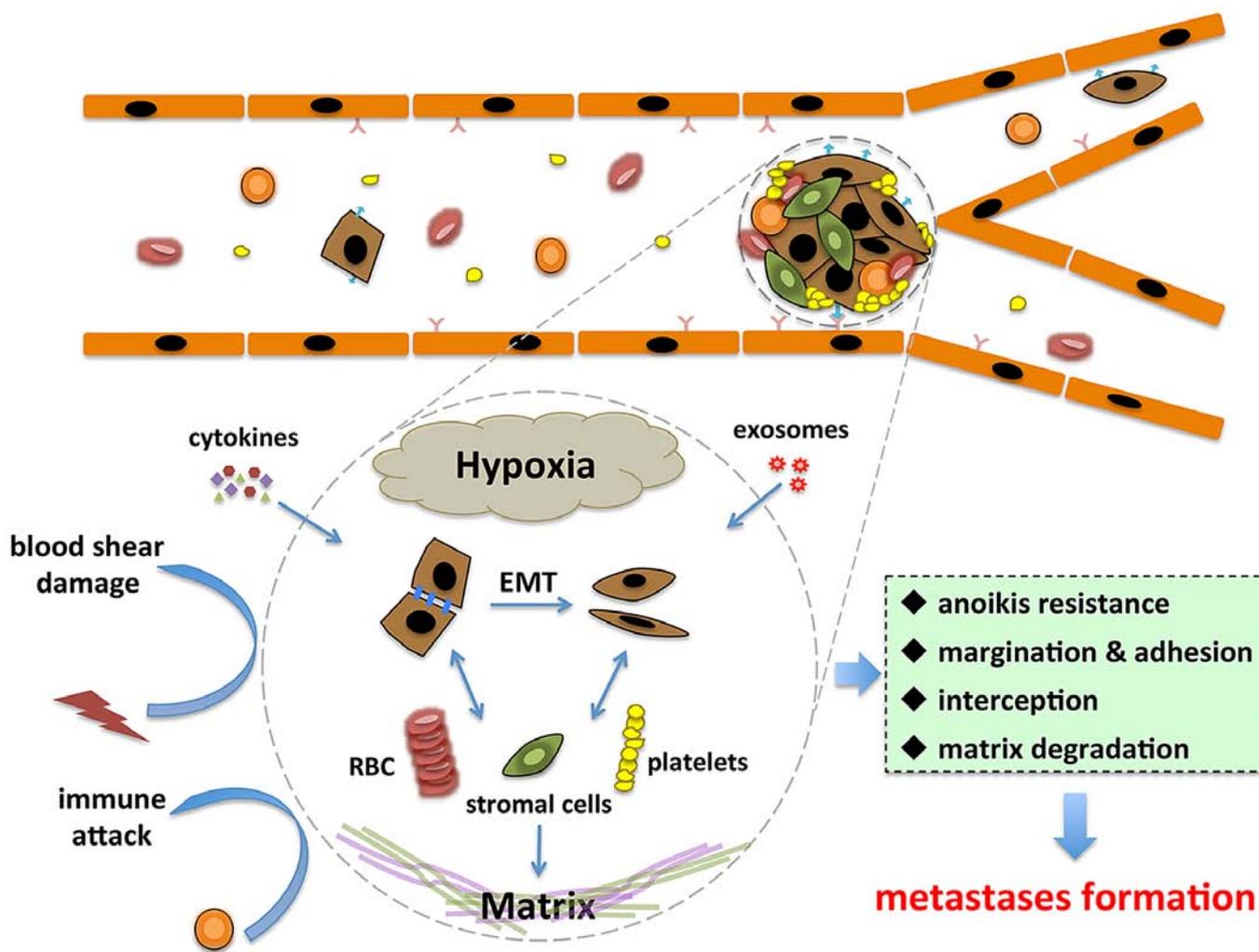Figure 2. The CTC clusters merit higher potential of metastasis. In the circulation system, the hypoxic microenvironment of CTC cluster comprises of CTC cells, mesenchymal cells, epithelial cells, pericytes, immune cells, platelets, and cancer-associated fibroblasts would have some interactions with cytokines and exosomes. Such microenvironment protects CTC clusters from blood shear force damage and immune attack.

Table I. Prevalence and size of CTC cluster detected in different types of cancer.

Type of cancer	Disease, stage	Isolation/identification method	Sample	Prevalence	No. of cluster	No. of cells per cluster	Refs.
Lung cancer	NSCLC, stage III-IV	ISET/ICC	1 ml, peripheral blood	0/5 (IIIA) 15/35 (IIIB/IV)	2-39	3-45	(38)
	NSCLC, stage I-IV	Subtraction method/IF	Venous blood, volume unknown	40/78	1-72/ml[d]	NA	(83)
	NSCLC, stage IV	NA/IF	NA	7/14	4.8%[a]	NA	(47)
	SCLC, extensive; NSCLC, stage IIIB-IV	ISET (8-μm filter)/ICC	3 ml, peripheral blood	7/7	16-320[e]	NA (averagely 3.5-12[f])	(10)
	Adenocarcinoma, squamous cell, bronchioloalveolar carcinoma	Subtraction method/IF	3 ml, venous blood	8/55	NA	1-3	(49)
	Adenocarcinoma, squamous cell, small cell, carcinoid; stage IA-IV	EpCAM-based microfluidic chip/IF	5 ml for pulmonary vein, 6-8 ml for peripheral vein	7/32	NA	NA (a 6-cell cluster was shown)	(84)
	Metastatic	HB-Chip/IF	~4 ml, blood	1/4 or 2/4[d]	NA	2-12[f]	(26)
	Metastatic	Filter/trypan blue	0.6 ml, blood	3/8[b]	4-18	NA	(76)
	NSCLC, stage III-IV	ScreenCell Cyto filter/ICC	3 ml, peripheral blood	15/26	1-12	3-30	(81)
Breast cancer	NA	Subtraction method/IF	3 ml, venous blood	0/3 or 1/3[c]	NA	NA	(49)
	Metastatic	Filter/trypan blue	0.6 ml, blood	5/8[b]	4-20	NA	(76)
	Stage IV	NA/IF	NA	13/21	3.4%[a]	NA	(47)
	Metastatic	Cluster-Chip/IF	4 ml, blood	11/27	~0.5/ml	2-19	(35)
	Stages III and IV	CellSearch system/IF	7.5 ml, blood	20/115	NA	NA (4-cell clusters were reported)	(78)
	Metastatic	CellSearch system/IF	7.5 ml, blood	9/52	1-18	NA (a 7-cell cluster was shown)	(79)
Glioblastoma	NA	Subtraction method/IF	3 ml, venous blood	0/12	NA	NA	(49)
Colorectal cancer	Dukes stage B-D	Cytocentrifuge/ICC	20 ml, antecubital venous blood	22/32	1-8	3-10 (excluding doublets)	(33)
	Metastatic	Filter/trypan blue	0.6 ml, blood	4/8[b]	4-27	NA	(76)

Table I. Continued.

Type of cancer	Disease, stage	Isolation/identification method	Sample	Prevalence	No. of cluster	No. of cells per cluster	Refs.
Liver cancer	Non-metastatic	ISET/ICC	3 ml, peripheral blood	2/44	NA	NA (2-cell and 4-cell clusters were shown)	(41)
Renal cancer	Clear cell and non-clear cell carcinomas, with or without metastases	ISET (75-μm microfilter)/IHC	Renal venous outflow, and 500-750 ml perfundate of kidney	14/42	NA	NA	(85)
Prostate cancer	Non-metastatic	Immunomagnetic method/IF	5 ml, peripheral blood	8/10	1-17	NA	(32)
	Stage IV	NA/IF	NA	11/15	5.3%[a]	NA	(47)
	Metastatic	HB-Chip/IF	~4 ml, blood	1/15 or 2/15[d]	NA	4-12	(29)
	Metastatic	Filter/trypan blue	0.6 ml, blood	1/4[b]	142	NA	(76)
	High-risk, non-metastatic	CellSearch/IF	7.5 ml, peripheral blood	1/36	1	4	(86)
	Metastatic castration-resistant	Vitatex CAM platform/IF	2 ml, blood	4/23	4-200/ml	NA (a 7-cell cluster was shown)	(30)
	Metastatic	Cluster-Chip/IF	4 ml, blood	4/13	~0.28/ml	2-8	(35)
Pancreatic cancer	Stage IV	NA/IF	NA	4/18	6.2%[a]	NA	(47)
	Metastatic	Filter/trypan blue	0.6 ml, blood	1/3[a]	3	NA	(74)
	Stage I-IV	CMx platform/IF	2 ml, peripheral blood	51/63	29.7	NA	87
Melanoma	Metastatic	Cluster-Chip/IF	4 ml, blood	6/20	~0.15/ml	2-6	(35)
	Metastatic	ISET/cytopathology	10 ml, blood	44/128	2-6	NA (a cluster with at least 45 cells was shown)	(88)

[a]Percentage of total number of CTCs. [b]Indicated as 'increased' or 'positive', namely, more than 3 clusters detected. [c]Based on different methods. [d]Valid speculation because of not clearly reported by the authors. [e]Data derived from a part of patients. CAM, cell-adhesion matrix; EpCAM, epithelial cell adhesion molecule; HB, herringbone; IF, immunofluorescence; ISET, isolation by size of epithelial tumor cells; NA, not available; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

As a by-product of CTC detection, CTC clusters are insufficiently detected by antibody-based methods for several reasons. Compared to single CTCs, CTC clusters have smaller surface-area-to-volume ratios, which reduce the efficiency of antibody capture. This situation can be more obvious in larger CTC clusters. In addition, the integrity of CTC clusters is prone to be impaired in devices with turbulent flow, which can result in an anamorphic number and size of CTC clusters (35). Other limitations of antibody-based methods for single CTC detection are also applicable to CTC clusters, such as the limited expression of target antigens (35).

Physical property-based methods. Physical property differences in cell density, size, dielectric properties and mechanical plasticity, can all be utilized to isolate CTCs and CTC clusters. For instance, size-based isolation relies on the larger size of CTCs compared to that of blood cells. This has been achieved by using track-etched polycarbonate filters, which are porous membranes containing numerous randomly distributed 8-μm-diameter, cylindrical pores (36). Isolation by size of epithelial tumor cells (ISET) platform seems more reasonable for CTC cluster isolation since the difference between CTC clusters and non-tumor cells is more significant. One study reported that 2 out of 23 patients with primary liver cancer were positive for CTC clusters using an ISET platform (37). Another study using a similar ISET platform isolated CTC clusters from all patients with lung cancer (29). As an unbiased method, ISET was believed to be more sensitive than antibody-based methods. For example, CTC clusters were observed in 43% of patients with non-small cell lung cancer (NSCLC) using ISET but were completely undetectable by CellSearch (38). Generally, the ISET platform holds a few advantages: i) it retains the natural status of CTC clusters without the binding of antibodies or nanoparticles; ii) it allows direct filtration of peripheral blood without preprocessing; iii) it can maintain the integrity of CTC clusters; iv) it is cost-friendly compared to antibody-based methods.

Physical property-based methods of CTC cluster isolation can be also combined with and strengthened by microfluidic technology (39-44). By taking advantage of centrifugal forces which can facilitate CTC and CTC cluster separation, a two-inlet, two-outlet spiral microchannel with a total length of 10 cm was designed (45). This device is able to continuously collect viable CTCs and CTC clusters allowing simple coupling with a convectional 96-well plate for subsequent biological assays, and CTC clusters with size of 50-100 μm in patients were successfully detected (45). Harouaka *et al* developed a new flexible micro spring array (FMSA) device for enrichment of viable CTC clusters according to their sizes. The FMSA device was based on flexible structures at micro scale that minimized cell damage and could preserve cell viability while maximizing throughput to allow for rapid enrichment directly from blood samples without sample preprocessing. CTC clusters with 2-20 tumor cells were detected in patients with breast, lung, and colorectal cancer using the FMSA device (46). Extraordinarily, the first attempt for specific isolation of CTC clusters was achieved in 2015. The Cluster-Chip, based on microfluidic and antigen-independent technologies, is able to isolate CTC clusters through specialized bifurcating traps under low-shear stress conditions that preserve their integrity.

Even two-cell clusters can be efficiently captured using this technique (35). The chip comprises of a set of triangular pillars and captures CTC clusters by relying on the strength of cell-to-cell junctions as clusters flow through the pillars at physiological speed. This model is designed to exclude two-cell clumps with a loosened combination, which may occur in incidentally attached cells. Cluster-Chip was able to find CTC clusters in 30-40% of patients with metastatic breast, prostate cancer or melanoma (35).

Additional innovative detection strategies for CTC clusters. Some additional approaches have been developed to detect CTC clusters by taking advantage of the physical and biological properties of epithelial cells. High-resolution imaging combined with enrichment methods was used to isolate CTC clusters. A group of investigators separated CK-positive, CD45-negative CTC clusters, which were then analyzed by a hematopathologist. In their report, CTC clusters were detected in 93, 54, 50 and 22% of patients with prostate cancer, breast cancer, NSCLC, and pancreatic cancer, respectively (47). Another study reported a novel integrated cellular and molecular approach of subtraction enrichment and immunostaining-fluorescencen *in situ* hybridization (48). The integrated platform depleted white blood cells and red blood cells and established an expeditious detection of non-hypotonic damaged and non-hematopoietic CTC clusters, regardless of CKs or EpCAM expression or size variation. This platform was able to efficiently detect, isolate, and characterize CTC clusters from various types of cancer including lung cancer, glioma and melanoma (48).

Special methods for CTC cluster identification. Theoretically, it is difficult to judge whether an individual cell is a tumor cell or not. This dilemma also exists in the identification of CTC clusters. Most researchers prefer to use CKs as tumor markers. Some investigators adapted fluorescence *in situ* hybridization with the centromere of chromosome 8 (CEP8), since more than 2 hybridization signals of CEP8 indicates chromosomal variation and the cell is expected to be malignant (49). Aptamers specifically selected from postoperative tumor tissue were also used for tumor cell identification, and were able to detect more CTC clusters than CKs (50).

4. Biological significance of CTC cluster and its role in tumor metastasis

Beyond the enumeration of CTC clusters, their molecular characterization offers insights into their origins and metastatic potential. It may also provide clues to their evolution during the course of cancer treatment and the mechanisms of treatment resistance relevant to CTC clusters. However, CTC clusters with different sizes or components have distinct biological and physical characteristics. Specifically, King *et al* systematically studied the physical features of CTC clusters that consisted of 2-5 tumor cells, which provided a straightforward view of the enhanced metastatic potential of CTC clusters (51).

Origins of CTC clusters. The origins of CTC clusters and the way their integrity is preserved are both interesting to note. CTC clusters can directly be derived from primary tumors or

from the aggregation/proliferation of single CTCs. Current evidence acquired from breast cancer remains limited, and excludes intravascular aggregation of CTCs as a main cause of CTC clusters (16). In parallel with this, we designed an *in vitro* platform to mimic blood stream, and the results showed that the aggregation/proliferation of single CTCs was impossible because of the sheer force of blood (unpublished data). However, the aggregation and proliferation were still possible when CTCs were located in inflammatory sites or when small vessels intercepted CTCs.

Differently originated CTC clusters may have different formation mechanisms and possess different biological characteristics. The molecules responsible for tumor cell aggregation within a CTC cluster are currently under investigation and could be good targets for treatment. At least in breast cancer, plakoglobin and keratin 14 that are both associated with desmosomes and hemidesmosomes have been found to be critical for CTC cluster formation. Inhibition of these proteins reduced CTC cluster formation and distal metastases (16,52). Interactions between Thomsen-Friedenreich glycoantigen and galectin-3 also take part in breast cancer cell aggregation (53). In addition, some pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α could also promote tumor cell growth as clusters and induce adhesive recruitment in the circulation system (54). For patients of metastatic stage, the existence of CTC clusters from metastases is possible and makes things more complicated. The dynamic change of CTC clusters in the circulation system is also mysterious because it is difficult to monitor CTC clusters *in vivo*. In the future, more investigations are warranted to solve these specific problems, and should then be directed towards finding targeted therapeutic approaches that can be performed to lower the risk of CTC clusters and improve cancer treatments.

Internal characteristics of CTC clusters. Some studies have demonstrated that CTC clusters are comprised of a number of tumor cells with or without non-tumor cells such as mesenchymal cells, epithelial cells, pericytes, immune cells, platelets, and cancer-associated fibroblasts (16,27,55-62). These non-malignant components contribute to the survival and metastatic advantages of CTC clusters in various ways. For instance, the presence of heterotypic tumor-derived stromal cells (e.g., fibroblasts, endothelial or tumor-infiltrated myeloid cells) increased the viability of tumor cells within CTC clusters, and facilitated metastases formation (27). However, when the cancer-associated fibroblasts were partially depleted, a significantly decreased number of metastases was observed (27). Another study using tumor cell and endothelial cell co-culture in a mouse model found that tumor cells were more potent in promoting angiogenesis in the presence of endothelial cells and resulted in increased numbers and larger size of metastases (28). Promotion of metastasis was also associated with the interplay between tumor cells and non-tumor cells such as platelets and leukocytes within CTC clusters (56,57,59,63). Platelets in the CTC clusters are believed to protect tumor cells from blood shear damage and immune attacks by physically shielding other complex influences via paracrine signaling and direct contact (64). Furthermore, some undefined cells such as CK-positive dendritic-like cells were also found in CTC clusters, but the nature and significance were unknown (33).

CTC clusters also show mesenchymal traits (63). The epithelial-mesenchymal transition (EMT) status of tumor cells within a CTC cluster is convincing, and is more obvious than that of single CTC (29). For example, at least one third of tumor cells in the clusters were CK negative in colorectal cancer (33). EpCAM and CK were both heterogeneously expressed in CTC clusters derived from NSCLC patients (29). Specifically, a majority of isolated CTC clusters derived from metastatic NSCLC patients harbored dual epithelial-mesenchymal phenotype, suggesting that EMT is a relevant process for metastasis caused by CTC clusters (65). Several hypotheses have been proposed to explain the EMT status in CTC clusters. For instance, the expression of mesenchymal markers in a CTC cluster could result from proliferation of a single cell that had undergone EMT, or from the mesenchymal transformation of a pre-existing CTC cluster in the circulation system (63).

Whether CTC clusters are of oligoclonal/polyclonal or monoclonal origin is also under investigation. In breast and pancreatic cancers, oligoclonal/polyclonal rather than monoclonal CTC clusters were observed by assessing lung metastases in a mouse model (16,66). Although CTC clusters have up to 100-fold increased metastatic potential compared to individual CTCs (67), it remains uncertain whether the tumor cells within a CTC cluster have identical metastatic potentials. Intravenous injections of CTC clusters comprised of two cell lines with distinct metastatic potentials resulted in lung metastases with the metastatic unique cell line karyotype (68). This finding suggested that the presence of metastatic cells did not change the inability of non-metastatic cells to form a distant organ, and implied that different tumor cells within the same cluster maintained their own metastatic potential (68). Accordingly, the metastatic potential of a CTC cluster may be dependent on the most malignant tumor cells. However, contradictory results were provided by another study, in which two cell lines that harbored different metastatic potential were mixed and injected into the flank of nude mice (58). Intriguingly, in addition to the polyclonal primary tumor at the inoculation site, the CTC clusters and almost 90% of the lung metastases were also found to be polyclonal (58). When the cell lines were injected respectively, merely 10% of the metastases arose from the less metastatic cell line, suggesting that tumor cells with lower metastatic potential acquired higher metastatic capability when cooperating with other cell lines in the CTC clusters (58).

In summary, clear evidence has shown eminent heterogeneity in CTC clusters and their marked complex compositions. Future studies should pay close attention to the dynamic change of the composition of CTC clusters, crosstalk between different compositions, and their roles in metastasis.

Metastasis-associated features of CTC clusters. Current studies have partially elucidated the reasons for CTC clusters to have higher potential of metastasis (Fig. 2). First, tumor cells within CTC clusters showed prolonged survival and decreased apoptosis (29). Mechanically, CTC clusters prevent tumor cells from anoikis through interaction between circulating galectin-3 and cancer-associated mucin1 (MUC1), also promoting the formation and survival of CTC clusters in the circulation system (69). The elevated galectin-3 in the circulation also facilitate tumor cancer adhesion to endothelial

Table II. Association between CTC cluster and clinical characteristics.

Type of cancer	Clinical relevance	Refs.
Prostate cancer	Antioxidant gene expression level in CTC clusters could be used for tumor diagnosis and recurrence prediction	(77)
Breast cancer	The presence of CTC cluster was associated with worse PFS	(78)
	The presence of CTC cluster impaired prognosis	(79
Renal cancer	The presence of CTC cluster was correlated with larger primary tumor, with higher frequency of lung metastasis, and with micronodular phenotype in the primary tumor	(85)
SCLC	The presence of CTC cluster was associated with shorter PFS and OS	(10)
NSCLC	The presence of CTC cluster was not associated with histological subtype	(38)
	The presence of CTC cluster was not associated with tumor type or stage	(80)
	No significant difference between numbers of CTC cluster and disease stage	(83)
Liver cancer	The presence of CTC cluster was association with tumor diffusion, portal tumor thrombosis, and shorter survival	(41)
Colorectal cancer	Elevation of CTC cluster number was correlated with macroscopic progression, and the numbers of cancer cells in the CTC clusters were significantly higher in non-responder after chemotherapy	(60)
Pancreatic cancer	PFS and OS were significantly shorter in unfavorable group (>30/2 ml) compared to those of favorable group (<30/2 ml). CTC cluster was an independent prognostic factor for PFS and OS	(87)
Melanoma	OS was significantly decreased in patients with CTC clusters, independent of treatment	(88)

CTC, circulating tumor cell; NSCLC, non-small cell lung cancer; OS, overall survival; PFS, progression-free survival; SCLC, small cell lung cancer.

cells by interacting with MUC1 on tumor cell surface (70,71). These findings deepen our understanding of the molecular mechanisms of CTC cluster dissemination and suggests that interference of this interaction may provide a novel therapeutic approach for preventing metastasis. Moreover, accessory cells such as fibroblasts, endothelial cells, platelets, and immune cells can form a niche (27,72,73), which is beneficial for tumor cells in CTC clusters when facilitating metastasis (27,73,74). For instance, platelets induce tumor cells to undergo EMT via TGF-β/Smad and NF-κB pathways by secretion of TGF-β and direct interaction with tumor cells (72).

Second, the physical specialty of CTC clusters allows for a greater likelihood of it residing in distant organs. Microvasculature of viscera can retain large CTCs, thus it can retain CTC clusters more easily (24). Compared to single CTCs, CTC clusters have larger sizes and a slower travelling velocity, making them easier to be intercepted by small vessels (75). In traditional concepts, interaction of tumor cells with vascular endothelial cells makes up the premise of the extravasation of tumor cells. From a physical biology standpoint, it has been reported that CTC clusters tended to undergo margination, rotation, and adherence to endothelium via interaction with E-selectin (51). CTC clusters have a much lower rolling velocity than individual CTCs, and are susceptible for margination and attachment to vascular wall even in vessels with diameters not small enough to intercept clusters

(51). In addition to sphere-like clusters, various appearances of CTC clusters associated with the collective migration of tumor cells were observed (29). Most noticeably, CTC clusters with linear or triangular geometry showed no difference in velocity along the vessel (51). These clusters also provide a special microenvironment for the tumor cells within them, and this microenvironment varies according to different sizes and constitutions of the clusters. CTC clusters with bigger sizes have significant hypoxic microenvironments compared to those with smaller sizes and single CTCs (76).

In addition, cytokines such as interleukin-6 and tumor necrosis factor-α can induce a positive feedback loop, leading to the aggregation of subsequent tumor cells and adhesion of tumor cells to endothelium (54). Alternatively, intravascular metastatic formation has also been proposed in certain cases in order to better understand tumor metastasis (49,53,77). CTC clusters seem more likely to take this strategy due to their large size and survival advantage in vessels. However, this assumption is challenged by a recent study. By using microfluidic devices and zebrafish models, Au *et al* discovered that CTC clusters containing ≤20 cells were able to pass through capillary-sized vessels by reorganizing into single-file chain-like geometries (67) Astonishingly, the shape of these CTC clusters is highly plastic, and the cells are able to easily reorganize again into a sphere-like cluster after having traversed the capillaries (67). From another view,

these findings provide us with a better understanding of CTC cluster-based metastasis.

5. Clinical application of CTC clusters

As a minimally invasive technique with potential roles in diagnosis, decision-making, treatment assessment, and relapse monitoring in patients, CTCs have been involved in many preclinical studies and clinical trials. Particularly, investigators have explored the clinical implications of CTC clusters (Table II). The presence of CTC clusters in patients is a harbinger of bad prognosis, and certain studies have even identified an association between CTC clusters and survival. One such study enrolled 97 patients with SCLC and found that 32% of patients were positive for CTC clusters, and the presence of CTC clusters at baseline indicated shorter progression-free survival and overall survival (11). An additional prognostic value of CTC clusters in patients with elevated CTCs were reported in two prospective cohorts of advanced stage or metastatic breast cancer (78,79). Furthermore, information regarding CTC clusters were indicative of the failure of chemotherapy treatments in colorectal cancer (60). Similarly, CTC clusters also had predictive values in NSCLC patients (38). It was demonstrated that the prevalence of CTC clusters increased in NSCLC of higher stages (10/23 in stage IV, 5/12 in stage IIIB, and 0/5 in stage IIIA) (38). Unfortunately, there was no association found between histological subtypes and the presence of CTC clusters (38). Likewise, CTC clusters can be detected in more than half of 28 consecutive patients with stage III-IV lung cancer, but no correlation between the number of CTC clusters and tumor type or stage was observed (80). Moreover, the association between abundance of CTC clusters and disease stage also failed to be detected in colorectal cancer, though a trend of CTC cluster elevation was noted in patients with higher stage (33).

Gene expression and changes of CTC clusters during treatment were also studied. In patients with prostate cancer, the antioxidant gene expression level had excellent prognostic and predictive value, and could be used for evaluation of therapy and monitoring of tumor recurrence (81). Although treatments may influence the stability of CTC clusters, chemotherapy was incapable of destroying all CTC clusters. A higher reproducible rate of CTC clusters than that of single CTCs was found during the follow-up of patients after adjuvant chemotherapy (33). Similarly, the reduction of the proportion of CTC clusters was not significant even though the total number of CTCs was dramatically reduced (75), suggesting that CTC clusters may have different clinical relevance with single CTCs.

Although the importance of CTC clusters is gradually accepted, few effects have been made in cancer treatment by targeting CTC clusters. Recently, a Korean group succeeded in prolonging survival in a mouse model of breast cancer by dissociating CTC clusters using urokinase (82), making a first step in CTC cluster-relevant treatment. However, because the dissociated CTCs can still form metastases, the effects of such strategy may be mild and needs further evaluation.

In conclusion, the clinical value of CTC clusters currently remains elusive. Further diligent study is necessary in order to exploit the full potential of CTC clusters in clinical applications.

6. Perspectives

The molecular characterization of CTC clusters may revolutionize our interpretation of cancer metastasis. However, many questions need to be answered before CTC clusters can begin making sense for biological understanding and clinical use. First, methods optimized for specific isolation of CTC clusters preserving their original status are required due to the lack of an efficient or widely approved detection platform that does not hinder in-depth study of CTC clusters. Second, further identification is needed for the adhesion molecules responsible for CTC cluster formation, as well as the genetic and biological specialty of the tumor cells harboring these molecules. Third, the biological details of creation, travelling in the circulation, and metastases formation in distant organs of CTC clusters also demand further investigation. Finally, the clinical significance of CTC clusters is awaiting confirmation, and treatments based on CTC clusters may be promising to reduce metastasis events. Finding viable solutions to these problems will open up further fields of study for CTC clusters, and affirm its value in clinical practice spanning from early diagnosis of cancer to relapse monitoring.

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