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Review

Circulating tumor cells in pancreatic cancer: more than liquid biopsy

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***Abstract*:** Circulating tumor cells (CTCs) are tumor cells that slough off the primary lesions and extravasate into the bloodstream. By forming CTC clusters and interacting with other circulating cells (platelets, NK cells, macrophage, etc.), CTCs are able to survive in the circulatory system of tumor patients and colonize to metastatic organs. In recent years, the potential of CTCs in diagnosis, prognostic assessment, and individualized therapy of various types of tumors has been gradually explored, while advances in biotechnology have made it possible to extract CTCs from patient blood samples. These biological features of CTCs provide us with new insights into cancer vulnerabilities. With the advent of new immunotherapies and personalized medicines, disrupting the heterotypical interaction between CTCs and circulatory cells as well as direct CTCs targeting hold great promise. Pancreatic cancer (PC) is one of the most malignant cancers, in part because of early metastasis, difficult diagnosis, and limited treatment options. Although there is significant potential for CTCs as a biomarker to impact PC from diagnosis to therapy, there still remain a number of challenges to the routine implementation of CTCs in the clinical management of PC. In this review, we summed up the progress made in understanding biological characteristics and exceptional technological advances of CTCs and provided insight into exploiting these developments to design future clinical tools for improving the diagnosis and treatment of PC.

Keywords: circulating tumor cells, diagnosis, metastasis, pancreatic cancer, personal therapy

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Introduction

Pancreatic cancer (PC) is one of the leading causes of cancer death worldwide, resulting in nearly identical morbidity and mortality rates. Over the past 25 years, the global burden of PC has more than doubled. As a gastrointestinal carcinoma, early symptoms of PC are insidious and atypical, and clinical features are unclear, leading to a low surgical resection rate, frequent postoperative recurrence, and early metastatic dissemination. As such, numerous PC patients suffer from locally advanced or even metastatic disease, and only 20% of PC patients have the opportunity for early detection and surgical intervention.[1,2]

Circulating tumor cells (CTCs) are tumor cells that slough off the primary lesions and extravasate into the bloodstream.[3] CTCs were first described in the middle 1800s by a resident physician named Thomas Ashworth. Ashworth discovered these cells in the blood of a patient with numerous subcutaneous tumors and described them as "exactly in shape, size, and appearance to those seen in the primary lesion." Since the hypothesis of Ashworth in 1869, the relationship of CTCs in the bloodstream and cancer metastasis caused wide attention.[4] Most CTCs undergo apoptosis or phagocytosis after entering peripheral blood, and few of them are able to escape and anchor to develop into metastatic lesions.[5] Numerous studies have shown that CTCs exist in peripheral blood in various forms, both as free individual CTCs and aggregated cell clusters (circulating tumor microemboli, CTM).[6,7] Epithelial mesenchymal transition (EMT) will

occur during tumor cells entry into blood circulation, so there are different types of CTCs, including epithelial, mesenchymal, and mixed epithelial and mesenchymal phenotypes.[8] CTM and CTCs with mesenchymal phenotypes are considered to have stronger metastatic potential.

As a non-invasive method, CTCs are regarded as surrogate biomarkers for many solid tumors in clinical research. A lot of studies have been carried out, including breast cancer,[9,10] prostate cancer,[11] lung cancer,[12,13] liver cancer,[14,15] PC,[16–18] and melanoma. Although the use of CTCs is not included in clinical guidelines, many studies have predicted the great potential of CTCs in clinical applications, including early diagnosis of cancer, evaluation of the cancer prognosis, and monitoring of the therapeutic response.

In the past two decades, emerging exploration for CTCs has mentioned mainly in early diagnosis and technologies for CTC isolation. In this review, the role of CTCs in liquid biopsy in PC, as well as their biology, is fully reviewed. In addition, the potential of CTCs in targeting therapy will be summarized.

Biological characteristics of CTCs

CTCs have the characteristics of epithelial-mesenchymal transition

Metastasis is one of the main factors in providing unfavorable prognosis and short survival of PC patients. Cancer cells detach from the primary site and disseminate into distal and surrounding organs. Some tumor cells may undergo plastic alterations, and they may demonstrate features of EMT during the process of metastasis.[19] In the EMT process, intercellular adhesion of epithelial cancer cells will be reduced and acquire mesenchymal and invasive properties. Activation of EMT results in cancer cells detaching from the basement membrane and entering the circulatory system, serving as CTCs and traveling to distant sites.[20] A reverse process termed mesenchymal-epithelial transition (MET) occurs when CTCs extravasate, and CTCs proliferate to from macrometastases. Herein, the property of EMT-MET was also proposed to play an important role in the metastatic process of CTCs.[19] Tumor cells can invade proximal blood vessels by passive shedding or active invasion. Hypoxia is an important factor that induces active invasion of tumor cells, which is crucial for the formation of CTCs.[21] Hypoxia will activate the ubiquitous transcription factor hypoxia inducible factor (HIF), which results in the activation of a series of target genes such as Stromal Cell Derived Factor 1 (SDF-1) and CXC-chemokine receptor 4 (CXCR4). Activation of SDF-1/CXCR4 axis leads to transendothelial migration. The secretion of SDF-1 by endothelial cells (also by tumor and stroma cells) and CXCR4 expressed by tumor cells will promote tumor cells entry into the blood vessels, which contribute to tumor cell migration, adhesion to endothelial cells and intravascular infiltration of CTCs.[22] Moreover, hypoxia promotes the formation of CTC clusters by upregulating cell–cell adhesion molecules and making CTCs survive stably in blood circulation. The oxygen-sensing prolyl hydroxylase domain protein 2 (PHD2) serves as an oxygen sensor and may regulate oxygen level. Endothelial haplodeficiency of PHD2 may shift tumors to a less malignant, metastasizing phenotype. This transition is based on HIF-driven upregulation of vascular endothelial growth factor receptor-1 (VEGFR-1) and VE-cadherin.[23] Orphan nuclear receptor nuclear receptor subfamily 2 group F member 1 (NR2F1)/COUP-TF1 was found to serve as a barrier to early dissemination in breast cancer. The expression of NR2F1 was decreased in patients with ductal carcinoma in situ (DCIS). Loss of NR2F1 increased dissemination and was accompanied by low E-cadherin expression, activation of WNT-dependent β-catenin signaling, disorganized laminin 5 deposition, and increased expression of EMT genes such as TWIST1, ZEB1, and PRRX1,[24] as well as the formation of end feet through neural Wiskott–Aldrich syndrome protein (N-WASP)-directed cytoskeleton reorganization.[25]

The relationship between CTCs and EMT has also been highlighted in the extraction of CTCs and the clinical application in PC. One study included 272 blood samples from 74 PC patients (41 local, 33 metastatic) and isolated CTCs. The analysis revealed that there were four distinct subpopulations of CTCs: epithelial (E-CTC), mesenchymal (M-CTC), partial epithelial mesenchymal transition (pEMT-CTC), and stem cell like (SC-CTC). The total CTC count was not associated with any clinicopathological variables. However, the proportion of pEMT-CTC was associated with advanced disease, poor progression free and overall survival (OS) in all patients, and early recurrence after resection.[26]

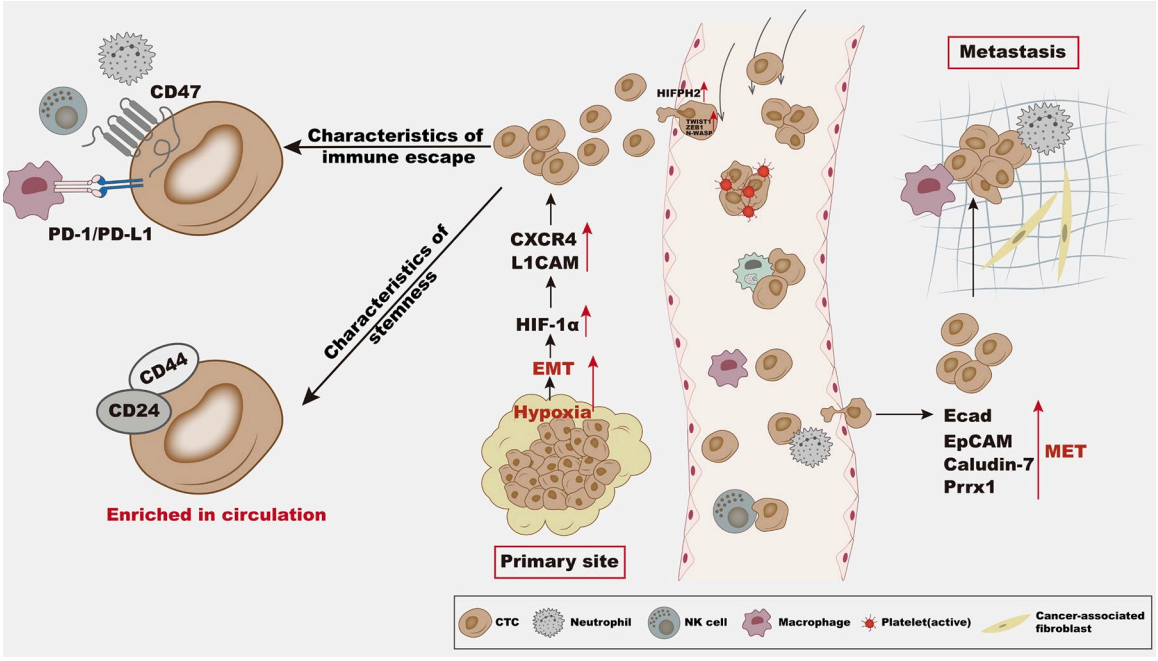

Figure 1. Overview of CTCs-blood cells interactions during disseminating into the circulatory system.
Enhanced EMT signaling at the primary tumor site causes tumor cells to detach from the tissue and invade the adjacent blood vessels, where CTCs can be present in the blood circulation as single cells, doublets or clusters of CTCs and express heterotypical surface receptors. In addition, CTCs constantly interact with circulating immune cells and other components of the blood. Upon reaching the distal metastatic site, MET occurs and CTCs colonize the metastatic site, aided and protected by immune cell-rich stroma. Finally, CTCs and CTC clusters multiply and eventually develop into metastatic tumors.
CTC, circulating tumor cells.

In the study of tumor metastasis and CTCs, several genes related to EMT are usually detected. Vimentin is undoubtedly one of them, this mesenchymal type III intermediate filament is considered a typical biomarker of EMT, and vimentin is also functionally associated with pro metastatic functions, including tumor cell migration or CTC survival.[27] In addition, EMT can regulate the expression of several epithelial adhesion molecules, thereby altering cell-cell interactions, such as reduced expression of E-cadherin and increased expression of N-cadherin. The adhesion molecule EpCAM (*epithelial cell adhesion molecule*) has been used in pioneering studies to enrich CTCs and has also been identified as an EMT target gene examined in many CTC studies.[28] The core transcription factors related to EMT[29,30] are also commonly evaluated in CTCs, particularly the ZEB (zinc finger E-box binding homeobox) (ZEB1 and ZEB2) and Snail (Snail and Slug) families, as well as Twist. EMT induces the expression of stem cell properties,[31,32] and certain EMT and CSC markers are commonly co-expressed in tumor cells. Therefore, stem cell markers, including CD44, ALDH1, or CD133, are often evaluated in CTCs and are typically associated with typical EMT markers. Altogether, enhanced EMT of tumor cells is an important factor leading to the formation of CTCs (Figure 1).

CTCs exhibit characteristics of immune escape

In addition to EMT markers, CTCs have been found to express "immune decoy receptors," which aid in CTC T-cell immune evasion within the circulation.[4,33] To evade the clearance of immune cells, CTCs can express programmed cell death protein 1 (PD-1) receptor and its ligand (PD-L1) or CD47.[33,34] PD-1 and PD-L1 are critical checkpoint proteins for the regulation of anti-tumor immune response. PD-L1 positive CTCs are more likely to connect with PD-L1, immune cells (Treg regulatory T cells, myeloid-derived suppressor cells (MDSC)), and cytokines (CK), which can limit T cell function and proliferation to promote immune tolerance.[35] The expression of PD-L1 in CTCs is associated with poor prognosis in advanced lung cancer and gastrointestinal cancer. The utilization of PD-L1 as a means of immune evasion by CTCs holds promise as a

therapeutic target for metastatic disease. Compared with single-agent therapy, dual targeting of EpCAM or HER2 in combination with an immune checkpoint inhibitor (PD1, PD-L1, CTLA4) will produce a more satisfied cell killing rate. The EGF (epidermal growth factor)-like domain I within the extracellular domain of EpCAM (EpEX) binds to EGFR (epidermal growth factor receptor), activating AKT and MAPK (mitogen-activated protein kinase) signaling pathways, and inhibiting the function of forkhead transcription factor O3a (FOXO3a) and stabilizing PD-L1 protein, respectively. EpAb2-6, an EpCAM neutralizing antibody, can inhibit AKT and FOXO3a phosphorylation, increase FOXO3a nuclear translocation, and upregulate expression of high temperature demand A2 (HtrA2) to promote apoptosis, thereby reducing PD-L1 protein levels to enhance the cytotoxic activity of $CD8^+$T cells.[36] However, its application in solid tumors that undergo hematogenous metastasis remains in its early stages.

CD47 is a protein that inhibits the cytotoxicity and phagocytic activity of activated immune cells and can release the "don't eat me" signal. CTCs exhibit a distinct non-immune phenotype through overexpression of CD47, which is associated with their ligand signaling regulatory proteins α binding, expressed on macrophages and dendritic cells, inhibits phagocytosis, and changes in the expression of apoptotic proteins FAS (fas cell surface death receptor) and/or FASL (fas cell surface death receptor ligand). Previous studies on cancer patients have shown the presence of CD47 positive CTC in the bone marrow, which may be a cause of tumor recurrence. Other groups have shown that upregulation of constitutive CD47 is a key requirement for immune tolerance and transmission in non-Hodgkin's lymphoma. Inhibition of dissemination by anti-CD47 antibodies was dependent on blockade of phagocyte SIRPα (signal regulatory protein alpha) and required macrophage effector cells.[37,38] In addition to PD-L1 and CD47 expressed by CTCs themselves, CTCs can also interact with other cells or platelets to evade immune surveillance.

Stemness of CTCs

Previous studies have shown that there are invasive CTC subpopulations with "stemness" characteristics in different cancers, which refers to their self-renewal and tumor growth inducing properties. OCT, a crucial stemness maintenance protein, was reported overexpression in bladder cancer.[39] Theodoropulos et al. found that 35.2% of CTCs in metastatic breast cancer patients were $CD44^+/CD24^{-/low}$, and 17.7% of CTCs were $ALDH1^{high}/CD24^{-/low}$, providing evidence that CTCs have stem cell characteristics.[40] In hepatocellular carcinoma (HCC), CTCs from 71.4% of HCC patients showed CD44 positivity.[41] CTCs in PC have also been found to have stem cell properties. In PKCY mice (*Pdx1-Cre; Kras*^{G12D}; *p53*$^{fl/+}$; *Rosa*^{YFP}), CTCs with $CD24^+CD44+$ phenotype were significantly enriched in the circulation compared with pancreatic orthotopic tumor cells. Therefore, CTCs exhibited increased viability and self-renewal ability under low attachment conditions in PC.[42] According to cell stemness genes (Aldh1a1, Aldh1a2, Cd24a, Cd44, and Klf4), PC CTCs are divided into two subpopulations: CTC-S and CTC-N. Both epithelial and mesenchymal markers are expressed on CTC-S. CTC-S significantly overexpressed Klf4, and Klf4 forms a protein complex with β-catenin. Klf4/β-catenin complex is necessary for the self-renewal ability of stem cancer cells.[43]

CTCs survival via cell-cell interaction

When transported into the circulatory system, most of CTCs are restricted by detrimental shear stress or die from anoikis, a programmed cell death occurring upon cell detaching from the extracellular matrix. High shear stress causes deformation, anoikis, fragmentation, and cell death.[44,45] Anoikis prevents CTCs from colonizing elsewhere and is thus essential for the prevention of cancer metastasis. Only a small portion of CTCs can escape from anoikis and immune system by interacting with platelets, neutrophils, macrophages, MDSCs, or cancer-associated fibroblasts (CAFs). The interaction between these cells and CTCs ensures the survival of CTCs in the circulatory system and promotes tumor metastasis[46] (Table 1).

CTCs gather into CTC clusters and survive from anoikis

CTCs in the blood occur as both single cells and CTC clusters (multicellular CTC aggregates). CTCs can form clusters among each other, which are known to acquire an increased metastatic potential and are more resistant to anoikis compared with single CTCs.[47,48] Two kinds of CTC clusters with different formation mechanisms have been reported, tumor cell-tumor

Table 1. Summary of CTCs-blood cells interactions.

Cell type	Interacting molecule	Downstream signal	Consequence on CTCs	Reference
CTC clusters	ICAM1-ICAM1	CDK6	Formation of CTC clusters Mediating CTCs aggregation Promoting CTCs proliferation Enhancing stemness of CTCs	49
	CD44-PAK2	FAK pathway	Formation of CTC clusters	48
CTCs-platelets	CD97-LPAR	Particle secretion	Protecting CTCs from anoikis	53
	αIIbb3	—		55
	P-selectin	—		
	PSGL-1, GPIb/V/IX	—		
	PECAM-1	—		
	MHC class I	—	Immune escape	58
	RGS18	AKT pathway		59
CTCs-Macrophages	CD47-SIRPα	pITIM/SHP-1/2	Evade phagocytic signals	61
CTCs-MDSCs	—	ROS/NRF2/ARE/Notch	Formation of CTC-MDSC clusters Promoting CTCs proliferation	63
CTCs-NK cells	FASL, TRAIL	—	CTCs apoptosis	66
CTCs-Neutrophils	Mac-1/ICAM-1	—	Formation of CTC-neutrophil clusters Facilitating extravasation	69

cell homotypic clusters and tumor cell-blood cell heterotypic clusters, and homotypic CTC clusters are a default term in most of the literatures. The mechanism of CTC aggregation might be enhanced cohesive interactions between tumor cells.

ICAM1 is a cell surface glycoprotein typically expressed on endothelial cells and certain leukocytes, which was found ~20-fold higher expression in lung metastatic cells, compared to that of primary tumor cells in triple negative breast cancer (TNBC) models. Combining cutting-edge single-cell RNA sequencing analysis and molecular functional studies of micrometastasis, this study elucidates a novel ICAM1 mediated pathway that is independent of CD44 and plays a role in initiating CTC cluster formation and driving TNBC lung metastasis. ICAM1 was found playing a key role in the initiation of metastasis in the following ways: (i) mediating tumor cell aggregation through homologous ICAM1-ICAM1 interaction to form CTC clusters, and enhancing tumor cell-endothelial cell crosstalk by promoting heterotypic ICAM1-ICAM1 interaction of trans-endothelial migration (TEM); (ii) enhancing stemness by upregulating stemness-related genes by interacts with CDK6, OCT3/4, NOTCH1, MCM3, ZEB1, Sec23a, and HIF1A. In addition, the blood and lung microenvironment not only facilitate the selection of rare ICAM1$^+$ tumor cells with aggregation, migration, and metastasis tendencies, but also provide niche specific signals to enhance the stemness/plasticity of ICAM1 expressing tumor cells in lung metastasis; and (iii) promoting tumor cell proliferation by maintaining or activating downstream CDK6.[49]

Previous studies have shown that CD44 mediates intercellular interactions by binding to the ligand hyaluronic acid in lymphocytes. However, CD44-mediated CTC aggregation is not dependent on hyaluronic acid, but is mediated by its intercellular homotypic interactions. CD44-mediated cell aggregation to form tumor cell clusters occurs through a unique pathway that involves direct

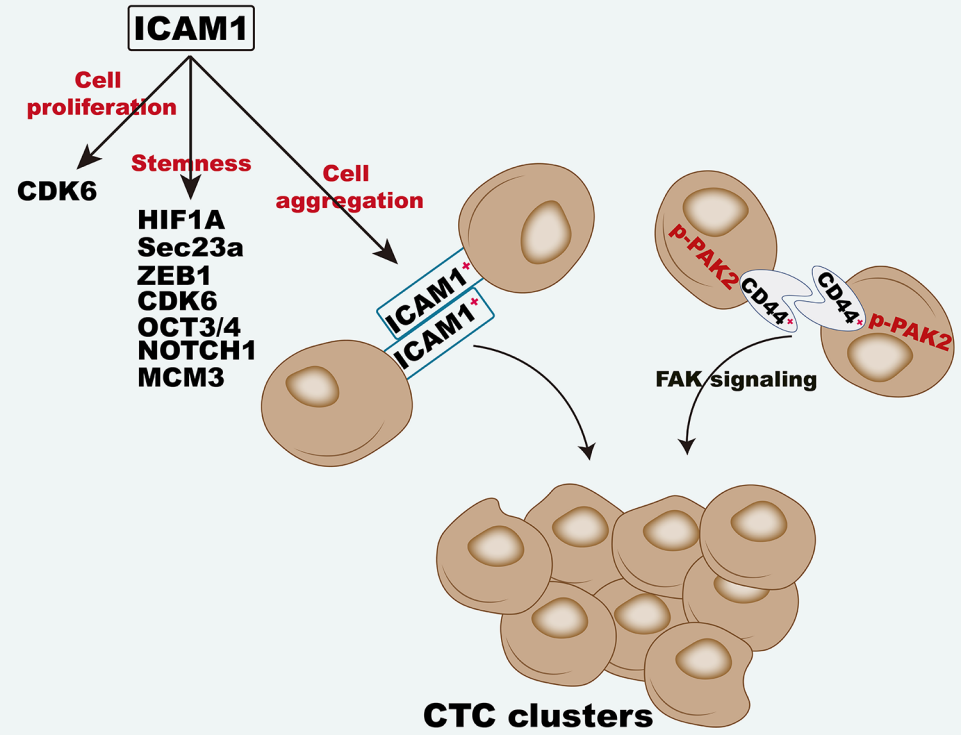

Figure 2. Formation of CTC clusters.
ICAM1 promote CTCs proliferation, stemness and aggregation through CDK6, OCT3/4, NOTCH1, MCM3, ZEB1, Sec23a, and HIF1A. ICAM1-ICAM1 interaction promotes the formation of CTC clusters.
CTC, circulating tumor cells.

interaction with PAK2 kinase and subsequent activation of focal adhesion kinase (FAK) signaling. Through mass spectrometry analysis, the authors found that p21 protein (Cdc42/Rac) activated kinase 2 (PAK2) was identified as a key component of CD44 regulation in proteins regulated by CD44, such as FAK signaling, paxillin signaling, actin cytoskeleton signaling, and TNFR1 signaling. Knocking out PAK2 in breast tumor cells revealed that siPAK2 mimicked siCD44 transfection in reducing FAK protein levels and FAK activation and phosphorylation. Surprisingly, the knockout of PAK2 also reduced the levels of CD44 protein in tumor cells, indicating a positive feedback and promoting effect between CD44 and PAK2, which may be mediated by protein complexes to stabilize the two proteins[48] (Figure 2).

In PC, the presence of CTC clusters was found positively correlated with poor prognosis. A study detected the central venous catheter (CVC) and portal blood (PV) of 35 patients with early PC. The results showed that patients with less than 15 clusters of PV showed longer OS than those with more than 15 clusters (19 months vs 10 months; $p = 0.004$).[50] Another study included 16 patients with unresectable PC; the authors separated CTC clusters and followed up survival period. The results showed a positive correlation between CTC clusters and poor prognosis (PFS, progression-free-survival, $p = 0.0159$; OS, $p = 0.0186$). There is also a positive correlation between the increase of CTC clusters and rapid disease progression during follow-up.[51] Overall, efficient CTC cluster separation and analysis technology will enhance the understanding of PC metastasis process and contribute to personalized disease management.

Platelets interact with CTCs and support them avoid from anoikis and immune capture

It has been reported that CD97 expressed on CTCs may lead to granule secretion of platelets, which promotes evasion of CTCs in the blood stream and that consequently promotes metastasis.[52,53] CD97 activates platelets and mediates particle secretion. Platelet-derived lysophosphatidic acid (LPA) induces tumor metastasis through proximal CD97-LPAR heterodimer signaling, which combines with tumor cell migration and

vascular permeability to promote trans-endothelial migration.[53] Furthermore, many adhesion molecules including integrins (aIIbb3), selectins (P-selectin), leucine-rich glycoproteins (PSGL-1 and GPIb/V/IX), and immunoglobulin superfamily proteins (PECAM-1). These molecules form a "shield" around CTCs and protect them from physical stress in the blood.[54,55] It has been demonstrated that the platelet-CTC aggregation has the effect of protecting the CTCs from anoikis. The detachment of tumor cells from extracellular matrix and adhesion by platelets will change the cell structure, focal adhesion formation and cytoskeleton arrangement, and activate expression of RhoA (ras homolog family member A). Activated RhoA-MYPT1-PP1 mediates dephosphorylation of YAP1 and promotes its nuclear translocation which induces a pro-survival gene expression signature and inhibits apoptosis. The specific mechanism is that high expression of YAP1 plays an important role in both in vitro and in vivo metastasis. The interaction between platelets and cancer cells leads to the activation of RhoA and YAP1 dephosphorylation through PP1-YPT1 phosphatase, thereby inducing protein nuclear localization. Once in the nucleus, YAP1 may bind to the TEAD transcription factor family, which enhances platelet-induced resistance to anoikis.[56]

In addition to the role of platelets as a shield for tumor cells in the blood and the protection against anoikis, they can also help CTCs escape from major histocompatibility complex class I (MHC class I MHC-I)-mediated recognition by natural killer (NK) cells. NK cells can preferentially eliminate targets with low or absent expression of MHC class I and play an important role in tumor immunosurveillance. Tumor cells will be coated rapidly in the presence of platelets in vitro, and CTCs of cancer patients show expression of platelets markers. Platelets, through this encapsulation, may lead to the transfer of MHC class I to the surface of CTCs. Studying the high expression of MHC class I at the ultrastructural level revealed that platelets, in addition to coating tumor cells, also exhibited a degranulation phenotype, that is, activated platelets released a variety of soluble factors after interacting with tumor cells.[57] Tumor cells and platelets showed close membrane contact, and tumor cell pseudopodia were formed around platelets. MHC class I has significant levels in these tumor cell pseudopodia regions. This indicates that platelet coverage of tumor cells leads to the activation and degranulation of platelets and further leads to the transfer of platelet specific molecules to tumor cells. These events result in a high level of expression of platelet-derived normal MHC class I. By conferring a "pseudo-normal" phenotype on CTCs, metastatic tumor cells are able to evade T-cell-mediated immunity without inducing an NK-cell response.[58] In 2023, a study included six PC patients with liver metastasis who had not undergone any treatment. Three types of samples were obtained from the patients: primary tissue, liver metastasis tissue, and CTCs. Using single-cell transcriptome technology, the researchers depicted the transcriptome profile of CTC, primary and metastatic lesions of human pancreatic ductal adenocarcinoma (PDAC) with single-cell precision. In vitro and in vivo cell interaction analysis and functional experimental studies have shown that CTCs and NK cells mediate immune escape through immune checkpoint molecule interactions with HLA-E:CD94-NKG2A. Disrupting this interaction by blocking NKG2A or inhibiting HLA-E expression can enhance NK-cell-mediated tumor cell killing in vitro and prevent tumor metastasis in vivo. Mechanistic studies have shown that platelet-derived RGS18 promotes HLA-E expression through the AKT-GSK3β-CREB signaling pathway, and RGS18 overexpression promotes pancreatic tumor liver metastasis. In summary, platelet-derived RGS18 assists CTCs in evading NK-mediated immune surveillance by participating in the immune checkpoint HLA-E:CD94-NKG2A. Blockade of immune checkpoint HLA-E:CD94-NKG2A signaling can prevent tumor metastasis in vivo by eliminating CTCs by immune killing[59] (Figure 3).

CTCs resist the killing of immune cells in the circulatory system

Besides shear force and anoikis, CTCs will face hostile conditions after entering the circulatory system, one of which is the phagocytosis of the immune system, which leads to CTCs' death. In order to escape immune surveillance, CTCs not only express various surface proteins, but also interact with immune cells to ensure their survival in the circulatory system[60] (Figure 4).

Macrophages. CD47/SIRPα axis is a kind of myeloid-specific immune checkpoint that suppresses macrophage removal of hematopoietic stem cells (HSCs), but can be exploited by hematologic and solid malignancies. It has been shown that CTCs interact directly with macrophages via

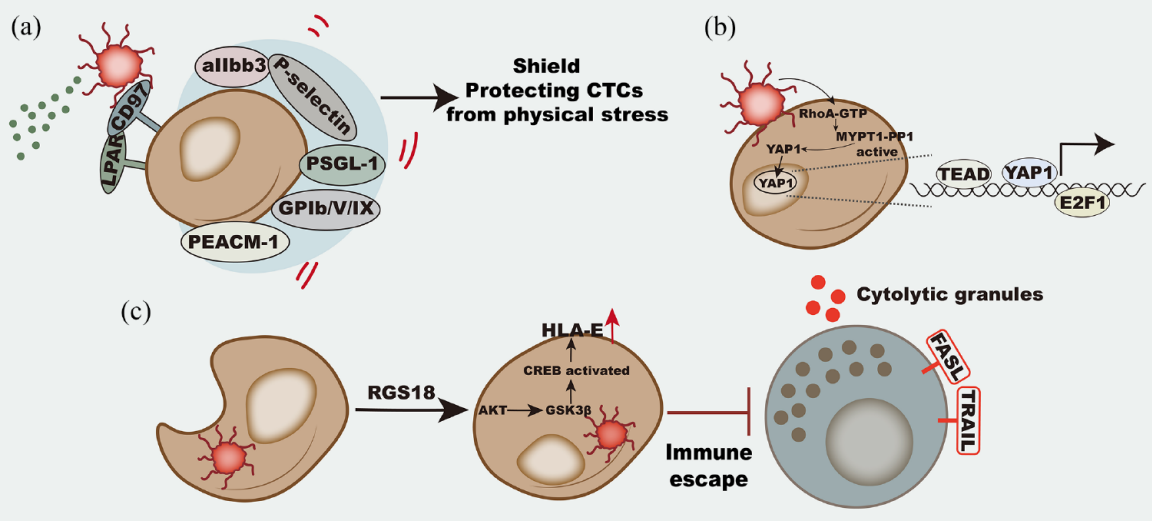

Figure 3. Interactions between platelet and CTCs. (a) Platelet-derived LPA induces CD97-LPAR heterodimer signaling, which combines with tumor cell migration and vascular permeability to promote trans-endothelial migration. Furthermore, allbb3, P-selectin, PSGL-1 and GPIb/V/IX, and PECAM-1 form a "shield" around CTCs and protect them from physical stress in the blood. (b) Platelets improve anoikis resistance of cancer cells and increase metastasis by activating Yap through a RhoA/MYPT-PP1 pathway. (c) CTCs upregulate immune checkpoint molecule HLA-E by engaging platelet-derived RGS18.
CTC, circulating tumor cells.

CD47, which can bind to the macrophage fusion receptor SIRPα. The immune checkpoint function of CD47-SIRPα is mainly achieved by blocking the formation of macrophage pseudopodia. CD47-SIRPα binding can cause the phosphorylation of ITIM tyrosine. The phosphorylated ITIM subsequently recruits and activates tyrosine phosphatase SHP-1/2 protein, and the activated tyrosine phospholipase further dephosphorylates a series of proteins in the cell, losing relevant biological functions and finally inhibiting macrophage phagocytosis.[61] However, the interaction between macrophages and CTCs remains further study.

Myeloid-derived suppressor cells. MDSCs are a group of heterogeneous immune cells that arise from the bone marrow myeloid stem cell lineage and possess potent immunosuppressive capabilities. The presence of MDSCs in the circulation is significantly elevated in breast cancer patients and is correlated with an increased number of CTCs.[62] In addition, CTC-MDSC clusters were found in the blood of tumor patients, which induce a pro-tumorigenic differentiation of MDSCs, and promote CTC survival and immune escape through the reactive oxygen species (ROS) and Notch signaling pathways. CTCs form physical clusters with polymorphonuclear MDSCs (PMN-MDSCs) and induce their pro-tumor differentiation through paracrine Nodal signals, increasing the production of ROS by PMN-MDSCs. These findings were validated by the significantly higher levels of nodal and ROS detected in the blood of metastatic melanoma and breast cancer patients with naïve, atypical CTC/PMN-MDSC clusters. Enhanced PMN-MDSC ROS upregulates the expression of Notch1 receptor in CTCs through the ROS-NRF2-ARE axis, thereby initiating CTCs to respond to ligand mediated (Jagged1) Notch activation. The bidirectional paracrine interaction and signal transduction interaction between CTC/PMN-MDSC were functionally validated in inhibitor-based analysis, indicating that the combined inhibition of Nodal and ROS eliminated the CTC/PMN-MDSC interaction, leading to reduced CTC survival and proliferation.[63] The portal vein blood of patients with PDAC also showed similar results, in which the ex vivo cultures of portal vein MDSCs and CTC spontaneously formed clusters and promoted CTC proliferation and migration.[64]

NK cells. NK cells mature and differentiate within the bone marrow and secondary lymphoid organs, and are a key component of the innate lymphoid immune system, providing rapid responses to pathogens and tumor formation. NK

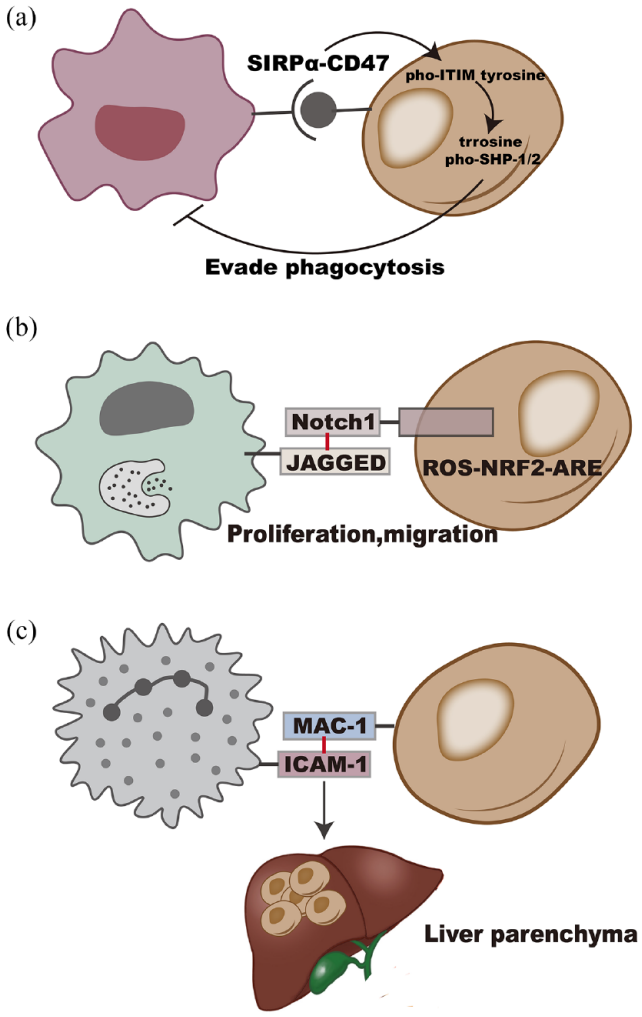

Figure 4. Interactions between immune cells and CTCs. (a) The interaction between Macrophages and CTCs. CD47-SIRPα binding causes the phosphorylation of ITIM tyrosine. Phosphorylated ITIM subsequently recruits and activates tyrosine phosphatase SHP-1/2 protein, finally inhibiting macrophage phagocytosis. (b) The interaction between MDSC and CTCs. PMN-MDSC ROS upregulates the expression of Notch1 receptor in CTCs through the ROS-NRF2-ARE axis, and initiating CTCs to respond to Jagged1-Notch activation. (c) The interaction between neutrophils and CTCs. Neutrophils adhere to CTCs via the Mac-1/ICAM-1 interaction, thereby serving as a bridge between the tumor cells and liver parenchyma.
CTC, circulating tumor cells.

cells have limited accessibility to solid tissues, so they mainly eliminate CTCs in the circulatory system. The immune response mechanism of NK cells consists of three main pathways: (1) extracellular release of cytolytic granules, inducing target cell apoptosis. (2) NK cell-mediated release of IFN-g, activating further immune cells and enhancing tumor-suppressing immune responses. (3) Activated NK cells express death-inducing ligands, such as FASL and TRAIL (tumor becrosis factor related apoptosis inducing ligand), on their cell surface.[65] NK cells are the main threat to the survival of CTCs in the bloodstream and are, therefore, considered to be an important inhibitor of metastasis formation. However, a positive correlation between NK cell counts in the bloodstream and CTCs has also been described, and a specific subset of NK cells ($CD56^{high}CD16^{low}$) is associated with a shorter OS rate.[66]

Neutrophils. Neutrophils are the most prevalent leukocytes in circulation, and an elevation in their count is correlated with a poor prognosis in numerous types of cancer. After the discovery of CTC-leukocyte clusters, it was found that there

was a significant correlation between CTCs and neutrophils in breast cancer. Compared to CTC alone, CTC-neutrophil clusters exhibited a higher metastatic potential. The binding between CTCs and neutrophils is facilitated through cell–cell junctions. $Ly6G^+$ neutrophils can increase metastatic inoculation in at least two different ways: (i) by inhibiting the cytotoxic activity of NK cells, neutrophils can protect tumor cells captured in the lumen from rapid clearance by these innate immune cells, and (ii) by accelerating extravasation through the action of MMP-9.[67] A series of cell adhesion proteins, including cadherins, integrins, and surface glycoproteins, among others, play a pivotal role in mediating the anchoring of CTC clusters with neutrophils on the vascular endothelial surface. This anchoring process enables the extravasation of CTC clusters while simultaneously resisting shear stress. Neutrophils can indirectly promote metastasis. Neutrophil extracellular traps (NETs) are networks composed of DNA-histone complexes and proteins released from activated neutrophils. NETs have the ability to seize CTCs within the cycle, thus facilitating metastasis. Furthermore, the formation of NETs by circulating neutrophils can impede the activation of peripheral leukocytes, the performance of NK cells, the anti-tumor response of effector T cells, and even the anti-tumor impact of other immune cells, including IL17-producing γδ T cells, leading to the evasion of CTCs from immune surveillance.[68–70]

Development in CTC enrichment and isolation

In the past few years, numerous methods have been proposed to capture CTCs. However, due to the extremely low proportion of CTCs in human blood, accurately separating CTCs from a large number of blood cells, especially developing an applicable method that can effectively detect live CTCs for subsequent in-depth analysis, remains a great challenge.

CTC technology generally has three core points, including capture and enrichment, detection and identification, and release of CTCs. The principle of capture and enrichment is to use specific materials to adsorb CTCs through physical interaction or antibody-antigen interaction. Identifying CTCs refers to utilization of fluorescence microscopy, fluorescence spectrophotometry, flow cytometry, surface-enhanced Raman scattering, or electrical impedance techniques to identify and screen captured CTCs. Finally, the obtained CTCs are released, mainly for multiomics analysis and CTCs culture.[71]

Physical methods

The physical separation and enrichment method of CTCs is based on the differences in size, density, deformability, and electrical properties between CTCs and blood cells. The development of this method includes an 8-μm diameter polycarbonate TRACK-ETCH-type membrane, pressure regulating system and a flexible micro spring array device, the Oncoquick system (a density-dependent technique), and Apostream (using dielectric electrophoresis techniques in the microfluidic chamber).[72,73] However, after repeated improvements, the above methods are generally inefficient, with poor purity and lack of specificity, although they have good vitality and relatively low cost.

Biological methods

Another important method for extracting CTCs is based on their biological characteristics. Based on the interaction between antibody and antigen, epithelial (EpCAM) and mesenchymal (vimentin) markers are commonly used for positive enrichment of CTCs, while CD45 is used for negative enrichment to deplete unwanted leukocytes. EpCAM-related technology is the most commonly used technique by researchers. The CellSearch (CS) system is the only FDA-approved clinical device in the United States that uses EpCAM antibody-coated ferromagnetic beads to enrich $CK^+/CD45^-/DAPI^+$ CTCs and remove $CK^+/CD45^+/DAPI^+$ WBCs.[74] The MagsWeeper system uses magnetic rods to enrich CTCs and eliminate cells that do not bind to magnetic beads, thus improving the CS system's difficulty in releasing CTCs. CanpatrolTM is another representative of EpCAM-dependent technology, which provides morphological, cytological, and genetic characteristics of individual CTCs.[75] However, due to the high heterogeneity of CTC surface antigens, CTCs with low EpCAM expression may not be enriched.

Microfluidic-based and nanotechnology-based techniques

With the development of nanomaterials and microfluidics, some newer technologies including microfluidic-based and nanotechnology-based

methods have been applied to CTCs isolation and enrichment.

Microfluidic-based cell sorting strategies use fluid dynamic forces versus external forces (mainly magnetic, electric field, acoustic, and optical forces) to separate cells, and then select target cells from heterogeneous cell samples based on their distinct physical and biological characteristics.[76] Based on this principle, a silicon microfluidics platform, the CTC chip, was developed. CTCs are captured on glass slides coated with molecular markers. In recent years, the invention of the NP-^{HB} CTC-Chip and the monolithic CTC-iChip has made tremendous contributions to the development of CTC detection.[77,78] However, due to their prolonged setup time, elevated initial cost, unwieldy size, and restricted capacity for single-cell molecular analysis, these tools have not been extensively employed in clinical application. Notably, the self-powered microcavity array chip can perform cell loading, lysis, isothermal amplification, and signal readout on a single chip. This novel chip can perform genetic analysis at the single-cell level and shows great potential for personalized diagnostic protocols and monitoring of treatment effects. Another automated microfluidic system proposed by Wang and his colleagues can capture and identify CTCs within 90 m, which has the advantages of automation, stability, cost-effectiveness, and ease of operation, and holds great promise for cancer screening and prognosis.[79] In addition, Lee and colleagues invented an integrated microfluidic system that uses magnetic field gradients and immunofluorescence differences to enable simultaneous on-chip isolation and characterization of circulating tumors. On-chip post-processing combines microfluidics with in situ molecular mapping technology to enable the on-chip platform to molecularly analyze individual CTCs from metastatic breast cancer cancers and other metastatic cancers in patients, thereby eliminating the off-chip processing needs. This suggests the potential for early molecular detection of cancer metastasis.[80] In conclusion, cost-effective automated and integrated microfluidic systems offer significant clinical value for easy detection of CTCs and cellular analysis.

Nanotechnology-based methods

With the advancement of nanotechnology, methods based on nanomaterials are becoming potential tools for CTC detection and cancer development monitoring. Nanomaterials can adsorb a large number of targeted ligands due to their large surface area ratio, which can bind to specific molecules on cancer cells, allowing for the highly specific separation of CTCs and the highly sensitive detection of CTCs. At present, studies have reported many types of nanomaterials for CTC detection, including tannic-acid-functionalized magnetic nanoparticles, Two-dimensional nanozyme with gold nanoparticles.[81–83] However, there are still significant limitations and challenges in the clinical application of nanotechnology. First, many factors (e.g., nanoparticle probes combined with potential off-target effects) can affect CTC adsorption and detection, resulting in reduced reliability and reproducibility. Second, nanoparticles may be toxic and currently only applicable for experimental research.

Relying on the enrichment and identification strategies described above, dozens of different commercial CTC detection systems have been developed. Unfortunately, however, all of these methods have some degree of shortcomings and have not been massively popularized in clinical practice. In order to better meet clinical needs, CTC detection technologies must face the following challenges and find appropriate responses. First, only by capturing as much of the complete population of CTCs as possible, will the information obtained subsequently be more comprehensive and credible. However, a significant proportion of late-stage patients have undetectable CTCs or insufficient numbers of CTCs to complete downstream analyses, suggesting that the enrichment efficiency of the assay could be improved. There may be several reasons affecting the enrichment efficiency, including limitations of the enrichment means, heterogeneity of the CTCs themselves, unnecessary cell loss, and low blood volume of the assay. Second, the large number of leukocytes in the peripheral blood, even after CTC enrichment, still leaves a large number of residuals, so CTCs must be identified by specific methods. Among the existing methods, phenotypic identification is an immediate test, but false-positive and false-negative results are inevitable; functional identification is more specific, but time-consuming, and is not suitable for routine clinical practice. In future clinical practice research, some key issues need to be addressed: (1) Develop clinically feasible strategies for separating CTCs from various body fluids based on

specific requirements for tumor liquid biopsy. (2) More efficient and simpler methods are needed to identify CTCs without reducing their cell viability. (3) Develop more advanced CTC in vitro culture technology, calibrate the isolation technique by collecting CTCs from cancer patients, and conduct in-depth biological mechanism research. The potential drawback of CTC capture and detection systems is that there are limitations in using multiple washings to process small amounts of samples to improve sensitivity and capture cells. Nanoprobes are only suitable for small sample screening, and in clinical practice, it is necessary to develop devices that can filter high-throughput and large amounts of blood samples, and combine them with CTC separation, release, and molecular analysis synergy. In addition, there is a lack of recognized interpretation standards for real-time companion diagnostics using CTC, which will need to be established and continually revised in the future in conjunction with large-sample clinical studies. These multicenter clinical studies are a very difficult and long process, which is a huge burden for any CTC technology R&D company, but also a challenge that has to be faced for the development of the industry as a whole, because only in this way can sufficient reliable evidence-based medical evidence be accumulated, and ultimately gain access to the clinic.

The role of CTCs in diagnosis and prognosis of PC

Diagnosis

Enriching and recovering CTCs from peripheral blood samples is the main technical challenge for successfully implementing CTCs as a liquid biopsy method. There are significant differences in the efficiency and specificity of CTC separation techniques. Most CTC separation platforms are still hindered by the low number of CTCs detected in blood, complex procedures, high costs, and low purity. Currently, some studies have explored the diagnostic efficacy of CTCs in PC.

A meta-analysis including 44 studies showed that the overall positive rate of single or multiple CTCs detected in PC was 65%. However, due to the limitations of CTC detection technology mentioned above, the detection rate of CTCs in these studies varied greatly, ranging from 5% to 100%. The detection rate of CS system in PC was only 26%, and the methods using magnetic beads and physical filtration platforms were 73% and 86%, respectively.[84] Ankeny et al. used EpCAM coated nano media to detect CTCs in 72 patients with PC at different stages. The detection rate of CTCs in the first stage was 0%, in the second stage it was 60.7%, in the third stage, it was 78.6%, and in the fourth stage, it was 96.3%. The sensitivity of CTC detection methods based on different platforms varies greatly, but these studies have shown that the specificity of CTC detection is very satisfied (99.7%–100%).[85]

In early diagnosis of PC, CTCs also showed high application value. Rhim et al. found that some epithelial cells undergone EMT and emerged in the circulatory systems of both low-grade PanIN and high-grade PanIN mice, and these cells are considered at the early stage of malignant transformation. More importantly, epithelial cells that have undergone EMT have also been detected in the peripheral blood of patients with intraductal papillary mucinous neoplasm (IPMN), and this discovery extends the observation of mice to humans.[42]

Although the current research shows that CTCs may form earlier than in situ tumors, the determination of this conclusion still needs to detect the blood of a large number of patients with early PC or precancerous lesions. For the potential of early diagnosis of CTCs, large-scale clinical trials are also needed in the future. Currently, two related studies are ongoing: one from Rouen, France (NCT02072616), which explores the improvement of CTCs for diagnostic testing, another study (NCT03551951) from Missouri and Columbia Province explored liquid biopsy markers, including CTCs in various solid tumors[86] (Table 2).

Prognosis

Traditional prognostic factors, such as tumor size, lymph node status, and neural invasion, can only be evaluated after tumor resection. CTCs provide clinicians with a window to understand cancer development and progression through blood samples. At present, studies have found that the disease-free survival rate and OS rate of PC patients with positive CTC detection are significantly shorter than those of patients with negative CTCs. In addition, the positive rate of CTCs was associated with postoperative recurrence of PC. After neoadjuvant chemotherapy or

Table 2. Diagnosis and prognosis value of CTCs in PC.

Number of PC patients	Stage	Isolation/ enrichment technology	Positive rate	Main results	Reference
100	All stages	Nano-Velcro	78%	CTCs demonstrate potential as a preoperative biomarker for identifying patients at high risk of occult metastatic disease.	93
69	All stages	Immunomagnetic (CK and EpCAM, using MACS system) versus CellSearch	33%	CTCs affect the outcome of patients with pancreatic cancer independent from other risk factors, including patients receiving (adjuvant) cytotoxic therapy.	114
72	Pre-treatment	Nano-Velcro	75%	CTCs as a diagnostic marker: Sensitivity = 75.0%, Specificity = 96.4%, ROC = 0.867, 95% CI = 0.798–0.935, $p < 0.001$. A cut-off of ⩾3 CTCs in 4 mL VB was able to discriminate between local/regional and metastatic disease (ROC = 0.885; 95% CI = 0.800–0.969; and $p < 0.001$).	85
22	Pre-treatment	Immunomagnetic (CD45 depletion)	68%	A cut-off of 2 cells/3.75 mL, the sensitivity and specificity in the diagnosis of PC was 68.18 and 94.87%, respectively. CTCs positive patients showed metastasis and worse survival rate.	87
60	Pre-treatment	ISET-size based	78%	77% with $CK^+/ALDH^+$ phenotype correlated with worse OS and DFS, 57% with $CK^+/CD133^+/CD44^+$ phenotype, correlated with worse DFS.	90
46	Pre-treatment	TU-chip™	83%	A validated multivariable model consisting of disjunctively combined CTC phenotypes: "H-CTC ⩾ 15.0 CTCs/2 mL OR E-CTC ⩾ 11.0 CTCs/2 mL" generated an optimal prediction of metastasis with a sensitivity of 1.000 (95% CI 0.889–1.000) and specificity of 0.886 (95% CI 0.765–0.972).	91

primary tumor resection, the CTC burden significantly decreases, and preoperative CTC positivity is a risk factor for postoperative recurrence. The molecular characteristics of CTCs in PC patients have also been clarified.

A total of 61 samples were identified by combining CK, CD45, DAPI, and CEP8, including 22 cases of PC, 3 cases of borderline solid pancreatic pseudopapilloma, 6 cases of benign pancreatic tumors, and 30 healthy people. The results showed that enriched cells were divided into five patterns: CK+CD45-DAPI+CEP8=2 (2 hybridization signals), CK+CD45-DAPI+CEP8>2 (>2 hybridization signals), CK-CD45-DAPI+CEP8>2, CK-CD45-DAPI+CEP8=2, and CK+/-CD45+DAPI+CEP8=2 or >2. Among them, CK+CD45-DAPI+CEP8=2, CK+CD45-DAPI+CEP8>2, and CK-CD45-DAPI+CEP8>2 were considered as CTCs, while CK-CD45-DAPI+CEP 8=2 and CK+/-CD45+DAPI+CEP8=2 or >2 were considered as uncertain cells. When the cut-off value was set at 2 cells/3.75 mL based on the ROC curve, the sensitivity and specificity for cancer diagnosis were 68.18% and 94.87%, respectively. CTC profile analysis showed that CTCs from different patients or different CTCs from the same patient exhibited various karyotypic abnormalities. Dynamic monitoring of changes in CTCs in patients with PC before and after surgery showed that most patients had a decrease or insignificant decrease in CTC counts 3 days after surgery, while CTC counts dynamically increased 10 days

after surgery. This dynamic characteristic of CTCs may be related to the time required for tumor cells to spread in the circulation, for example, after surgical resection of primary tumors, dormant and damaged tumor cells can be reactivated and released into the circulation. Patients with positive CTCs had a poorer 1.5-year survival rate after surgery. According to ASCO's recommendations in 2006, a decrease in postoperative CA19-9 levels indicates that surgical treatment is effective. However, the number of CTCs in these patients still increases, which may be due to stress response to surgery. This suggests that CTC counting may help assess the disease status of patients with normal CA19-9 levels. However, the application of CTCs in clinical diagnosis still needs more exploration.[87]

In a study of 50 patients, the label-free ISET® device was used to isolate and evaluate CTCs stained with cytokeratin and the mesenchymal-marker vimentin.[88] After considering other prognostic factors, the presence of cytokeratin-positive CTCs was shown to be an independent predictor of survival by univariate and multivariate analysis. CTCs that express both cytokeratin and vimentin were shown to be predictive for tumor relapse. This study also evaluated the marker for tumor-initiating cells (TICs) and cell stemness in CTCs of PDAC patients. They found that ALDH-positive CTCs and triple-positive CTCs were closely related to low survival rates.[89,90]

In recent years, some studies have classified PC patients by defining different CTC subtypes and evaluating the prognosis of patients with different CTC subtypes. Sun et al. prospectively collected venous blood samples from 46 patients with pathologically diagnosed PC in their study, and classified the captured CTC into epithelial (E), mesenchymal, and hybrid (H) phenotypes by TU chip™ immunolabeling. By combining different CTCs phenotypes, they established a prognostic evaluation model and validated it: "H-CTC$\geqslant$15.0 CTCs/2ml OR E-CTC$\geqslant$11.0 CTCs/2ml," with a sensitivity of 1.000 (95% CI 0.889–1.000) and specificity of 0.886 (95% CI 0.765–0.972). The median OS of patients whose E-CTC $\geqslant$ 11.0 CTCs/2 mL was 5.5 months and the patients whose E-CTC $<$ 11.0 CTCs/2 mL was 16.5 months.[91]

A study introduced diagnostic leukocytosis (DLA) into CTC separation strategy to improve CTC enrichment efficiency. 60 PC patients were subjected to DLA, and approximately 5% of the resulting DLA products were analyzed using CS system. They found that DLA significantly increased CS-CTC detection rate, yielding a 60-fold increase in CS-CTC enrichment. More importantly, DLA CS-CTCs exhibited a pronounced negative prognostic impact on OS, evidenced by a reduction in OS from 28.6 to 8.5 months (univariate: $p = 0.002$; multivariable: $p = 0.043$). In addition, the sensitivity of CTCs was also enhanced compared to peripheral blood. For further validation, 228 PC patients were included (CS-CTCs from PB-samples), and the established association between CTCs and poor OS (8.5 vs 19.0 months, $p < 0.001$) was consolidated.[92]

The consistency of KRAS mutation and CTCs in PC has also been verified. NanoVelcro platform in conjunction with high-resolution fluorescent microscopy and a multicolor immunocytochemistry (ICC) were used to isolate and detect CTCs.[93] Then, KRAS mutation of single CTCs was analyzed, and all the five samples had KRAS mutation (4 $KRAS^{G12D}$ and 1 $KRAS^{G12V}$). In addition, CTCs were found in 0.0%, 60.7%, 78.6%, and 96.3% of AJCC (The American Joint Committee on Cancer) stage I, II, III, and IV patients, respectively. Therefore, future studies are expected to not only confirm the utility of CTCS as biomarkers, but also prove their potential to provide feasible information about tumor biology, which has great prospects in achieving "personalized" treatment of PC (Table 2).

CTCs and mesenchymal-epithelial transition

EMT and MET are currently the standard models for the metastatic process proposed in tumor research. Epithelial cells undergo EMT to transform into cells with a mesenchymal phenotype and increase their mobility, during which a large number of tumor cells shed into the circulatory system. Tumor cells in the metastatic foci exhibit epithelial-like morphology and molecular characteristics, which can be used to determine their origin from the primary tumor. Therefore, mesenchymal cells need to be re-transformed into epithelial-like cells to complete secondary organ invasion in a reversal of EMT, called (MET or reverse EMT (rEMT)).[19,94]

The re-expression of Ecad in the metastatic site is considered as possible evidence for MET

occurrence. MET is an indispensable process in the metastasis of PC. In the spontaneous metastatic model of PC, the expression of EpCAM in CTCs was observed, and the size of liver metastatic area was positively correlated with the number of exfoliated epithelioid cells. The expression of Ecad and Caludin-7 increases as disseminated CTCs evolved from isolated tumor cells to micrometastatic and macrometastatic clusters. Conversely, mesenchymal markers, such as FSP1 and ZEB1, were reduced. Paired-related homeodomain transcription factors (Prrx1, Prrx1a, and Prrx1b) are involved in pancreatic development, pancreatitis, and carcinogenesis. Prrx1b enhances invasion, de-differentiation of tumors, and EMT. Conversely, Prrx1a stimulates liver metastasis growth, tumor differentiation, and MET. Activation of Prrx1a or suppression of Prrx1b in cancer cells promoted metastatic outgrowth in the liver, and 30% of disseminated CTCs that acquire mesenchymal traits are Prrx1a positive.[95]

David et al. used RNA-seq to detect CTCs purified from the blood of patients with early localized PDAC (locPDAC) and metastatic PDAC (metPDAC) to gain insight into the promoting factors of CTCs biology. RNA-binding protein LIN28B significantly overexpresses in CTCs from metPDAC and promotes PDAC metastasis through the LIN2B/let-7 pathway. Using LIN28B small molecule inhibitors (*N*-methyl-*N*-[3-(3-methyl-1,2,4-triazolo[4,3-*b*]pyridazin-6-yl)phenyl]acetamide) can disrupt the metastasis efficiency of CTCs.[96]

Current clinical application of CTC

Prognosis

The clinical value of CTC in liquid biopsy and prognostic evaluation cannot be ignored. The important characteristic of CTC over other liquid biopsy markers is that it comes from the proliferating tumor area, and they contain a full repertoire all at once and available for interrogation, that is, RNA, DNA, proteins, sugars, and lipids.[97] The application of CTC in prognosis in PCa has been discussed before (see part 'The role of CTCs in diagnosis and prognosis of PC, Prognosis'). The CS system has been used in several trials to investigate whether CTC enumeration carried out at baseline and during chemotherapy might be predictive of cancer patient's response to treatment. The STIC CTC trial is the first attempt to demonstrate the clinical utility of a biomarker to guide the choice of first-line treatment in hormone receptor-positive, ERBB2-negative MBC. When choosing between endocrine therapy (ET) or chemotherapy, the first-line treatment plan is determined based on the number of CTCs (chemotherapy if $\geqslant$5 CTC/7.5 mL; ET if CTC count is low). After a median follow-up of 4.7 years, 382 out of 755 patients died (50.6%). The median OS of the CTC group was 51.3 months (95% CI, 46.8–55.1), while the standard group was 45.5 months (95% CI, 40.9–51.1) (hazard ratio for mortality [HR], 0.85; 95% CI, 0.69–1.03; $p = 0.11$). Among 189 clinically recommended ET patients (25.0%), CTC counts were higher and chemotherapy was superior to ET (hazard ratio for mortality, 0.53; 95% confidence interval, 0.36–0.78; $p = 0.001$). This clinical trial demonstrated the clinical utility of CTC counts.[98]

The phase I trial of the PARP inhibitor niraparib used changes in CTC count and nuclear gamma H2AX expression as endpoints for anti-tumor activity in the mCRPC patient cohort (The poly(ADP-ribose) polymerase inhibitor niraparib (MK4827) in BRCA mutation carriers and patients with sporadic cancer: a phase I dose-escalation trial). A phase I clinical trial of ARQ197, a selective inhibitor of liver growth factor receptor c-MET, evaluated changes in CTC counts in patients with different types of tumors and found that CTC count can predict patient treatment efficacy.[99] EZN-4176 is a second-generation antisense oligonucleotide of androgen receptor exon 4, and its phase I trial uses changes in CTC count to evaluate drug efficacy in prostate cancer patients.[100] In summary, there is evidence to suggest that CTC inquiry through molecular analysis can provide important clues to support clinical decision-making, involving not only the differences between high-risk and low-risk patients, but also the molecular characteristics of cancer.

CTCs culture and drug screening

The concept of "precision medicine" relies on the association between genotype and drug sensitivity. However, in the late stages of the cancer and after multiple failed treatment courses, tumors exhibit highly complex genetic makeup (such as multiple genetic variations and driver factor mutations in different pathways), leading to unclear treatment plans and rapid disease progression. In this case, a small-scale, personalized drug screening of the

patient's CTC is conducted for further medication guidance. Multiple CTC strains were obtained from patients with advanced metastatic breast cancer, and drugs approved by PDA were screened based on CTC strains,[101] which can identify compounds (and combinations) with enhanced tumor killing activity. However, obstacles still need to be overcome in transforming CTC personalized drug screening into clinical practice. First, in vitro CTC amplification is currently a lengthy process that typically takes several months to obtain stable cell lines from freshly isolated cells. Second, we need to understand whether drug screening of cell populations from a small number of CTCs is truly meaningful.

Diagnosis

At present, the clinical translation of CTC mainly focuses on prognosis assessment, and it is difficult to detect CTC in the early stages of tumors. These limitations currently hinder the implementation of CTC based early detection and screening in the population. However, the high specificity of CTCs makes them a valuable diagnostic tool as they are almost undetectable in healthy individuals.[102]

A study conducted by Ankeny et al. covered nearly half of early PDAC patients (43.1% in early stage I/II), with a sensitivity of 75.0% and specificity of 96.4% for CTC diagnosis of PDAC.[85] There was a significant difference in CTC count between PDAC and non-adenocarcinoma diseases. Compared to CTCs that are typically detectable in late stage cancer, circulating epithelial cells (CECs) may have the potential to identify patients earlier in the disease process, as hematogenous spread may occur before tumor formation.[103] A prospective blinded trial found that among 19 control individuals, only 3 individuals (15.8%) exhibited detectable CECs, with a maximum count of 3 CEC/mL. On the contrary, in a cohort of nine PDAC patients, 77.8% (seven individuals) showed detectable CECs, while in 20 pancreatic cystic disease patients, 40% (eight individuals) showed detectable CECs.[42] Several other clinical studies have also confirmed the detectability of CECs in patients with benign, precancerous, and malignant pancreatic lesions, particularly in patients with highly atypical precancerous lesions.[104,105] In addition, pancreatic-derived CTCs may play a crucial role in tumor metastasis and provide preliminary evidence for the diagnostic value of PDAC, although further validation is needed in a larger patient population.

Individualized therapy and immunotherapy

In recent years, the development of CTC in tumor immunotherapy and personalized treatment has achieved gratifying results, paving the way for the application of CTC in the field of tumor treatment. The most important biomarker for immunotherapy decision-making is the expression of PD-L1, which is currently commonly evaluated in tumor specimens through immunohistochemistry. However, intra-and inter-tumor heterogeneity, as well as the differential expression of PD-L1, make accurate measurement of PD-L1 expression in tumor tissues complex. As mentioned earlier, CTCs often express PD-L1, so using the CS system to measure the possibility of PD-L1 positive CTCs is a breakthrough in personalized therapy, which constitutes a clinically useful and non-invasive method for real-time assessment of PD-L1 status.[33,106]

Previous studies have shown that in NSCLC, the presence of CTCs and high expression of PD-L1 on their surface are associated with poor prognosis in patients treated with the programmed cell death protein (PD-1) inhibitor nivolumab at baseline and 3 months of treatment. In addition, after 6 months of treatment, patients carrying PD-L1 negative CTCs had a better prognosis, while patients with PD-L1 positive CTCs entered the disease progression stage, indicating that the persistent presence of PD-L1$^+$CTCs may reflect the mechanism of treatment escape.[107] In a study involving 155 patients with different advanced cancers, it was found that PD-L1-positive CTCs reflect patients' response to PD-1/PD-L1 inhibitors. In this study, the PFS (4.9 months vs 2.2 months, $p < 0.0001$) and OS (16.1 months vs 9.0 months, $p = 0.0235$) of patients with PD-L1 high CTC were significantly longer than those without PD-L1 high CTC.[108]

CTCs constitute an intermediate stage of metastasis. They exist in the bloodstream as single cells or CTC clusters that are oligoclonal precursors of cancer metastasis.[76] The development of targeted therapy for CTCs is expected to inhibit tumor metastasis and prolong patient survival. CTCs can enter a dormant state by upregulating the immune escape pathway through high expression of CD47.[109,110] Blocking CD274 (PD-1, PD-L1, B7-H1) and CD47 checkpoint using corresponding antibodies can inhibit tumor cell growth.[111] In addition to targeting CTCs themselves, immune cells that interact with CTCs have also been

found to be potential targets for tumor therapy. A single center prospective study demonstrated the short-term safety and efficacy of irreversible electrolysis (IRE) combined with allogeneic NK cell immunotherapy in the treatment of unresectable primary liver cancer (PLC). The combination therapy of IRE and NK cell immunotherapy significantly reduced CTC improved immune function, and Karnofsky's performance status. In addition, the PFS and OS of the IRE-NK group were significantly improved, demonstrating the synergistic effect of these two therapies.[112] Kolostova et al. co-cultured mature monocyte derived dendritic cells (mMo-DCs) and autologous non-target blood cells (NTBCs) and found that the patient's own CTCs could activate monocyte derived dendritic cells (Mo-DCs). Using multimarker gene expression profiling monitoring, it was found that mature Mo-DCs have a promoting effect on T cell activation. In addition, due to the observed increase in PD-L1 gene expression, mMo-DCs may play an important role in the PD-L1/PD1 regulatory axis.[113]

Discussion

CTCs are a key component of the metastatic cascade. Studying CTCs may reveal the fundamental process of tumor metastasis, including the mechanism of extravasation of primary tumor CTCs, how CTCs interact with blood components and immune system to survive in the circulatory system, and how CTCs colonize distant organs and grow into new metastases. The molecular characteristics of CTCs with strong metastatic capability are of great significance for anti-metastatic targeted therapy. Therefore, focusing on the study of CTCS in the process of metastasis can provide more in-depth insights for related therapeutic targets.

At present, various CTC detection technologies are developing rapidly, but the sensitivity and specificity of these technologies need to be improved. CTC detection technology based on epithelial markers has opened up a new era for CTC analysis and clinical application. However, EMT is a key characteristic of metastatic cells, suggesting that CTC detection technology based on epithelial cell markers may have the disadvantage of low capture efficiency. In recent years, CTC detection technology based on microfluidics and nanotechnology has also become popular, but the efficiency of these technologies still needs to be validated in large-scale cohorts. High specificity and high-order capture capability are still the two key challenges for CTC detection technology.

Molecular characterization is a key point in tumor mechanism research and clinical translation. The limited content of genomic DNA, RNA, and protein in CTCs is a bottleneck in exploring their molecular biological characteristics. Single-cell sequencing of CTCs has attracted much attention in recent years, but exploring the protein expression profile of CTCs at the single-cell level is still not feasible. In addition, CTCs should not be studied as a separate component, and the role of the blood microenvironment and immune environment surrounding CTCs in their survival and colonization cannot be ignored. For example, CTC clusters in the circulatory system are likely to have stronger adaptability than individual CTCs. Therefore, in future research, we should improve the clinical applicability of CTC by comprehensively assessing the tumor burden and patient immune status, while improving CTC extraction techniques.

Understanding the biological characteristics of CTC clusters and single CTCs in various aspects will help us better understand the dynamics, heterogeneity, and metabolic adaptability of CTCs during their survival and distant colonization in the blood. The genome, transcriptome, and proteome of CTCs will be key components of precision medicine in the future, as their phenotypic, genotypic, and functional characteristics can provide a large number of targets for anti-metastatic targeted drugs. There are still many problems to be overcome, such as the low efficiency of CTC isolation methods and the difficulty in simulating the real survival environment of CTCs in the blood. However, with the development of 3D organoid culture technology, single-cell technology, and nanoscience, it is feasible to isolate CTCs in the future, and we are confident in the promising potential of CTCs in the field of cancer.

In summary, CTC has been included in the World Health Organization Cancer Classification Fifth Edition. The term "cM0 (i+)" indicates that there is no obvious metastasis, but tumor cells are detected in the blood, bone marrow, or lymph nodes. However, CTC has not yet been included in the clinical practice guidelines of major cancer societies. The true advantage of CTCs lies in their potential to represent highly metastatic tumor subclones, as well as their

richness as a source of the latest biomarkers for molecular and functional research. CTCs, as living cells, can be cultured in vitro and analyzed for drug phenotype in principle, providing information for treatment decision-making. However, such a workflow must be significantly improved in terms of technology to achieve clinical application. In future clinical translation work, we should prioritize the potential of CTC in diagnosis and prognostic evaluation.

At present, we still need to further understand the relationship between tumor clonality and CTC, such as whether CTC is a decisive factor in tumor metastasis, whether CTCs from tumor patients have sufficient individualized characteristics, and how many CTCs need to be analyzed to provide such information. In this regard, a spontaneous metastasis in vivo model using barcode tracking of tumor clones and subsequent analysis of their molecular characteristics will play an important role.

The priority for addressing the clinical application of CTC should focus on improving capture efficiency and enhancing in vitro culture conditions. In the context of early cancer, positive and negative predictive values can be improved by benchmarking CTCs alongside other circulating analytes such as circulating tumor DNA and extracellular vesicles. At present, CTC has been validated as an independent prognostic marker, thus expanding the traditional TNM system. In addition to counting, CTC's potential as a detection-based therapeutic target or resistance mechanism in the future requires extensive clinical trials for testing and validation.

Conclusion

CTCs are tumor cells that detach from primary lesions and infiltrate into the bloodstream. Due to the biological characteristics of CTCs, its role in tumor progression, especially metastasis, cannot be ignored. On the one hand, CTCs evade immune killing (formation of CTC clusters, expression of surface molecules, etc.), resist physical forces (that is, shear stress), and achieve anoikis-free outcomes in the circulatory system through various mechanisms. These characteristics endow CTCs with strong survival and migration capabilities in the circulatory system, while also increasing the likelihood of successful liquid biopsy and in vitro culture based on CTCs capture. On the other hand, early observation of CTC clusters and recent analysis of their biological characteristics have also exposed their vulnerability, thus exposing opportunities for them as therapeutic targets. In PC, there are many observational studies to verify the role of CTCs in metastasis prediction and prognosis evaluation, but there are few intervention studies aimed at treatment. To unleash the potential of CTCs in tumor patient management, it is necessary to move from simple CTC enrichment and counting to highly controllable molecular characterization to develop more accurate liquid biopsy markers. To achieve the above goals, technological progress and innovation are required. Therefore, multidisciplinary collaborative research in materials science, oncology, pharmacy, and biology will pave the way for promoting the clinical application of CTCs.

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Consent for publication

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Author contributions

Zeru Li: Conceptualization; Writing – original draft; Writing – review & editing.

Cheng Qin: Conceptualization; Writing – review & editing.

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Tianyu Li: Visualization.

Yutong Zhao: Writing – review & editing.

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Competing interests

The authors declare that there is no conflict of interest.

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