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Interrelationships of Circulating Tumor Cells with Metastasis and Thrombosis: Role of MicroRNAs

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Abstract

Metastasis and thrombosis are serious threats to cancer patients and generally associated with poor prognosis. The elusive mechanisms underlying the pathogenesis of metastasis and thrombosis have been subjects of extensive investigations. The presence of circulating tumor cells (CTCs) is closely related to tumor metastasis, and these cells play an important role in thrombosis in cancer patients. In this review, we describe the latest findings on the role of CTCs in tumor metastasis and cancer-related thrombosis and the regulatory role of microRNAs in CTCs and thrombosis. Additionally, we discuss anticoagulant-based strategies for the prevention of thrombosis and reduction of cancer metastasis and the potential to translate current knowledge on these strategies to the treatment of cancer.

Keywords

Circulating tumor cells; metastasis; thrombosis; microRNA; anticoagulation

Introduction

The incidence of cancer is increasing annually, and cancer has become the leading cause of death globally. In most cases, the mortality of cancer results from metastasis and thrombosis. Specifically, metastasis and thrombosis are the first and second most common causes of cancer-related death, respectively [1,2].

Metastasis is the consequence of tumor cells spreading from the primary tumor to targeted organs. Metastatic processes include growth of the primary tumor, dissemination of primary tumor cells into the circulation, survival of tumor cells in the circulation, extravasation of tumor cells into targeted organs, and formation of distant metastasis [3]. It has been reported that about 90% of patients with epithelial cancers, accounting for 80% of all cancers worldwide, die of metastatic disease [1]. However, routine diagnostic methods, including histopathology, high-resolution imaging, and application of serum tumor markers, are not capable of providing enough information about ongoing metastasis. Recent studies revealed that formation of circulating tumor cells (CTCs) is a key step in metastasis and that detection of CTCs in cancer patients' blood using immunocytochemical and molecular assays may reflect ongoing metastasis [1,4-6]. Thus, to detect early micrometastasis and cut off the metastatic cascade at its initiation, determining the roles of CTCs in cancer biology and developing effective CTC detection techniques are critical requirements.

As stated above, thrombosis, which is a circulating coagulation disorder, is another key cause of cancer-related death [7]. Cancer was associated with a fourfold increase in the incidence of venous thromboembolism and that chemotherapy increases this risk to sixfold to sevenfold over that in the general population [8]. New antitumor agents have more pronounced prothrombotic activity than do traditional chemotherapeutics [9]. However, the exact mechanism of coagulation deregulation and cancer-associated thrombosis are largely unknown. Recent studies demonstrated that CTCs may play an important role in coagulation and thrombosis.

In this review, we summarize the roles of CTCs in early detection of micrometastasis and cancer-related thrombosis and describe the regulatory effect of microRNAs (miRNAs) on CTCs and thrombosis and anticoagulant-based strategies for the prevention of thrombosis and inhibition of cancer metastasis.

1. The Key Roles of CTCs in Cancer Metastasis and Early Detection of Micrometastasis

1.1. Key Roles of CTCs in Cancer Metastasis

CTCs were first described in 1869 and defined as cancer cells in the circulation. However, progress in CTC-related research has been hindered until recently by the identification and molecular characterization of CTCs [10]. The roles of CTCs have been investigated in many cancers, including head and neck, breast, lung, colorectal, gastric, prostate, bladder, renal, pancreatic, and liver cancer (Table 1).

Formation of CTCs is widely accepted to be the key step in the metastatic process [1,6]. With the growth of a primary tumor, angiogenesis is activated, and subsequently, primary tumor cells enter the bloodstream, leading to the formation of CTCs. Most of the CTCs are destroyed or undergo apoptosis in the circulation. However, a few survive, leave the bloodstream, and seed metastatic lesions. Metastatic tumor as well as CTCs from the primary tumor may exhibit characteristics different from those of cells in the primary tumor. Metastatic tumor can also release CTCs, which can in turn affect the primary tumor growth;

these cells in peripheral blood may find their repositories (such as bone marrow) and form new metastatic lesions as a result of immunity deterioration (Fig. 1).

The common characteristics of CTCs determine their roles in ongoing metastasis, prognosis, and assessment of the efficacy of adjuvant therapy for cancer [13,16,26,31]. Alix-Panabières and Pantel advocated using CTC analysis as a real-time “liquid biopsy” for patients with cancer [32]. Cancer therapeutics that target CTCs and the methods for real-time monitoring of CTC's response to therapies may be developed given the molecular characteristics of CTCs [33]. A critical requirement for the development of effective CTC-based diagnostics and therapeutics for cancer will be improvement in CTC detection technology.

1.2. Conventional CTC Detection Systems

The conventional CTC detection assays are based primarily on the characteristics of CTCs, which are commonly accepted to be nonleukocytic, nucleated, typically of epithelial origin in the peripheral blood, larger than normal blood cells, and having integral membrane surface areas. In addition, CTCs are positive in adhesion molecule (EpCAM)⁺ and cytokeratin (CK)⁺ expression and negative in CD45 expression [34,35]. Researchers developed most of CTC detection assays in enrichment steps according to the physical and biological properties of CTCs, including morphologically based isolation techniques such as isolation by size of epithelial tumor cells, the Ficoll-Hypaque technique, OncoQuick, and RosetteSep and immunological approaches such as the CellSearch CTC test (Veridex). Some of CTC identification processes involve cytometric and nucleic acid-based techniques [4,5,32]. These identification processes are based on the enrichment step, which is a technical challenge owing to the large background of normal blood cells and low concentration of CTCs. The CellSearch CTC test is the only CTC detection method approved by the U.S. Food and Drug Administration for clinical use to separate CTCs from blood cells in patients with breast, colorectal or, prostate cancer [36]. However, the morphological features of CTCs have not been clearly defined until now, and these features vary according to cancer type, clinical stage, and state. Thus, further studies are required to improve the specificity and sensitivity of CTC detection.

Recently, investigators have developed many new CTC detection strategies. For example, detection of CTCs in combination with a panel of mRNA markers, including human telomerase reverse transcriptase, CK-19, carcinoembryonic antigen, and mucin 1 mRNA, and CTCs expressing survivin is sensitive in predicting the prognosis for gastric cancer [37,38]. Also, enumeration of CTCs expressing tumor biomarkers such as CA-153 is an effective way to predict prognosis for breast cancer [39]. In addition, researchers have applied photoacoustic and photothermal techniques to the detection of CTCs and have identified early rare CTCs [40]. Furthermore, CTCs can be enumerated in areas besides the peripheral blood. For example, By detecting CTCs in the cerebrospinal fluid of breast cancer patients using the CellSearch CTC test, leptomeningeal metastasis may be diagnosed much earlier, and the efficacy of chemotherapy may be evaluated [41].

Researchers have made great progress in the detection of CTCs, and many clinical trials have demonstrated the roles of CTCs as new biomarkers for cancer. However, a large percentage of CTC⁺ cancer patients have had overt metastasis. Further studies demonstrated

that only populations of CTCs with clonal capacity could initiate metastatic growth in distant organs; these cells are termed metastasis-initiating cells (MICs) [42]. Owing to the limits of current CTC detection technology and to epithelial-mesenchymal transition (EMT), cancer patients with metastasis may have CTCs negative for those molecular markers. Some CTCs may go undetected using current detection methods, and few CTCs are detected in the majority of cancer patients with overt metastasis (especially in the brain). Recent studies demonstrated that expression of epithelial markers in CTCs was downregulated during EMT, causing the present detection methods to fail in providing enough information on MICs with EMT, which may be the most relevant metastatic targets [4]. Thus, development of new technologies and devices that enable identification of viable and retrievable CTCs and improve the diagnostic and predictive value of these cells should be a new research direction.

1.3. New CTC Detection Technologies

The main limitations of current CTC detection systems are low yield, purity, and throughput, which result from changes in the expression of CTC surface markers during cancer treatment [33-35]. Thus, improvement of the yield, specificity, and sensitivity of CTC detection is a new research area.

Increasing the blood sample volume in analysis is a natural method of enriching CTCs for detection, for example, using the GILUPI CellCollector nanodetector. This device is inserted into a peripheral arm vein, and up to 1.5 Liter of blood is collected, allowing for a large quantity of CTCs to bind to anti-EpCAM antibodies [32]. Researchers in many studies have proposed that an individual's entire blood volume may be filtered to increase the CTC yield. In this scenario, every CTC in the blood could be captured, whereas the CellSearch CTC test analyzes only 7.5 ml of blood, with a detection rate of 0.1-0.2% [33]. Investigators are developing other CTC isolation technologies to increase CTC yield. For example, the microfluidic platform-based CTC-Chip is an immunological isolation technology used to isolate rare CTCs that can improve the efficiency of CTC capture, separation, and enrichment [43,44].

Besides increasing the CTC yield, new technologies are focused on increasing the specificity and sensitivity of CTC detection. Nucleic acid-based technologies, which are related mainly to reverse transcriptase-polymerase chain reaction, are effective in specifically isolating peripheral CTCs. By analyzing special epithelial or organ markers, these are the most sensitive methods for detection of CTCs [45,46]. Also, cytometrically based methods are combinations of conventional cytometry and flow cytometry. Recently, researchers greatly improved cytometrically based techniques, including imaging cytometry (ImageStream [Amnis]) and photoacoustic flow cytometry, so that they can effectively detect CTCs [47,48]. Furthermore, cytometric techniques in combination with molecular biomarkers expressed on the surface of CTCs can sensitively detect rare CTCs [49].

These technological and research developments have greatly improved the yield, sensitivity, and specificity of CTC detection. However, more research and clinical trials are needed to increase the sensitivity and specialty of CTC detection and clarify the predictive roles of CTCs in cancer patients.

1.4. Detection of Circulating Tumor Microemboli

Circulating tumor microemboli (CTM) are defined as clusters of CTCs containing three or more distinct nuclei that have increased potential for metastasis when injected into mice [18,20,50]. Recent studies revealed that CTM are not simply groups of cells in the blood but rather cell clusters that break off from the primary tumor and that these clusters may represent a potential mechanism of preventing anoikis and enhancing the metastatic potential of tumor cells [51,52].

Detecting CTM is difficult owing to their complexity and heterogeneity. Authors reported that CTC detection assays may be applied to the collection and detection of CTM. These assays include cytometric methods, immunomagnetic isolation (CellSearch), size-based filtration (ISET), microsieving chips, and microvortex-generating herringbone-chips. However, because of the large size, deficiency in epithelial markers, and dissociation of CTM, determining the CTC detection efficacy of these assays requires further study [53]. Recently, researchers investigated a slide-based high-definition CTC imaging platform and strongly recommended its use for the detection of CTM [33]. Also, cytopathological and cytochemical examinations, with their reliable, accurate standardization, are highly recommended for the identification of CTM after CTC enrichment [53].

1.5. EMT and CTC Detection

Tumor cells can undergo EMT during their journey to metastatic sites. EMT, in which tumor cells lose their epithelial traits and acquire mesenchymal properties, is a very important process during cancer metastasis and is frequently associated with cancer aggressiveness, invasion, angiogenesis, and survival. The traits of epithelial cells include cell-cell adhesion, apical-basal polarity, lack of motility, and expression of epithelial surface markers such as EpCAM and CKs. In comparison, their mesenchymal properties are related to motility, invasiveness, expression of vimentin and N-cadherin, and resistance to apoptosis. Thus, EMT increases the ability of tumor cells to migrate [34,54-56].

Owing to changes in epithelial cell surface markers such as EpCAM and CKs during EMT, CTCs in the bloodstream may not be detected using detection assays based on antibodies against EpCAM and CK, especially the most aggressive CTCs [55,57]. For example, Gorges *et al.* [58] reported that EpCAM-based CTC enrichment techniques failed to detect CTC populations that had undergone EMT. Thus, CTC enrichment techniques using new markers of CTCs should be developed to detect mesenchymal-like subpopulations of CTCs.

Recently, Yu *et al.* [59] used a dual colorimetric RNA *in situ* hybridization assay to detect mesenchymal CTCs (M^+ CTCs) and epithelial CTCs (E^+ CTCs) according to expression of different markers in CTCs in patients with different subtypes of breast cancer, including ductal carcinoma *in situ* and estrogen receptor/progesterone receptor⁺, HER2⁺, and triple-negative (estrogen receptor⁻/progesterone receptor⁻/HER2⁻) breast cancer. Their results demonstrated that benign breast lesions (ductal carcinoma *in situ*) were exclusively epithelial, whereas all of the other three subtypes of invasive breast cancer contained both E^+ and M^+ CTCs, especially in patients with primary triple-negative breast cancer, and M^+ CTCs were associated with disease progression and treatment response. This study

implicated different subtypes of CTCs as potential biomarkers of therapy resistance of and prognosis for breast cancer. Similarly, using fluorescent immunocytochemistry, Yokobori *et al.* [60] demonstrated that PLS3 was expressed in EMT-induced CTCs in CTCs whose formation was induced by EMT in the peripheral blood of colorectal cancer patients with distant metastasis. Multivariate analysis demonstrated that PLS3⁺ CTCs was independently associated with prognosis for colorectal cancer, especially in patients with Dukes class B cancer, making PLS3 a novel biomarker for metastatic colorectal cancer cells during EMT. Thus, development of optimized CTC detection techniques, especially with a focus on optimization of EMT-related markers, is urgently needed. These techniques should include more markers than those whose expression is repressed during EMT, which may distinguish CTCs from the surrounding blood cells [6,32].

Current CTC enrichment methods can eliminate a large proportion of leukocytes from blood. However, considerable numbers of leukocytes remain after enrichment [61]. This leukocyte contamination of CTCs, together with low yield of CTCs, is a major challenge to CTC detection and characterization. Thus, identification of more sensitive and specific novel biomarkers of CTCs and development of new technologies and devices that facilitate the detection and characterization of CTCs are urgently needed.

2. The Roles of CTCs in Cancer-Related Coagulation and Thrombosis

In the 1860s, Armand Trousseau reported that malignant tumors were closely associated with venous thromboembolism [62]. Researchers later defined this as Trousseau syndrome, which included spontaneous blood coagulation events in cancer patients [63]. Abnormal coagulation occurs in more than 90% of cancer patients, and local and systemic venous and arterial thrombosis are often the first clinical manifestations of tumors [64]. Several studies have demonstrated that abnormal coagulation facilitates the growth and metastasis of tumors [65]. However, the mechanisms of thrombosis and abnormal coagulation in cancer patients are largely unknown. Recent studies demonstrated that CTCs mediate coagulation and thrombosis via expression of tissue factor (TF) on their surface and in activating platelets and that CTCs play important roles in the association between cancer and thrombosis [66].

2.1. CTCs and TF Expression in Cancer-Related Thrombosis

A transmembrane glycoprotein, TF is the physiological initiator of coagulation and is essential for hemostasis [66]. Furthermore, cell-mediated coagulation requires exposure of blood to TF-expressing cells [8,66,67]. TF is expressed in many types of leukemia and solid cancer cells, including non-small-cell lung cancer, colorectal cancer, pancreatic cancer, glioma, and renal cell cancer cells, and expression of TF in cancer cells is related to metastatic potential [68,69].

The most extreme example of CTCs is leukemia, which are composed of CTCs. Tormoen *et al.* [66] reported that leukemia patients are apt to experience thrombosis, especially those with specific types of leukemia. For instance, acute myelogenous leukemia subtype M3 and acute promyelocytic leukemia are associated with a high risk of thrombosis and bleeding. These findings regarding the TF activity on the leukemia cell surface in patients with such subtypes demonstrate that the leukemia cells are procoagulant. Solid malignant tumors are

also abundant sources of circulating TF, and TF-expressing CTCs take part in thrombosis in solid tumor patients. By modeling and simulating procoagulant CTCs in blood flow, researchers showed that activation of blood coagulation by TF-expressing CTCs was dependent on TF's spatiotemporal relationship with the blood and the number of CTCs added to plasma [70]. Berny-Lang *et al.* [71] revealed that breast cancer cell lines added to plasma could promote coagulation *in vitro*. Specifically, the effect of cancer cells on clotting time was cell number-dependent and inhibited by a neutralizing antibody against TF as well as inhibitors of activated factor X and thrombin. This study demonstrated that the prothrombotic potential of CTCs may partially be a consequence of TF expression. Furthermore, in a study of three different types of tumor cells, Tormoen *et al.* [72] demonstrated that all three of them hastened plasma clotting over that with control cell lines and that the clotting time depended on the number of cancer cells added to plasma. Meanwhile, a monoclonal anti-TF antibody abrogated the ability of the tumor cells to clot plasma or prolonged the clotting times for these cells. Similarly, pretreating tumor cells with annexin V abrogated the cells' ability to clot plasma or prolonged clotting times in a concentration-dependent manner. In an *in vivo* study, Amirkhosravi *et al.* [73] injected tumor cells into the lateral tail veins of mice and observed tumor cell-induced coagulation within 30 minutes. However, changes in coagulation were almost completely abolished in anticoagulated animals; simultaneously, lung tumor formation was markedly lower in the systemic anticoagulation group than in the controls. All of this evidence proves that malignant tumor-related hypercoagulation and thrombosis can be caused by cancer cell-expressed TF or exposure of cancer cell surfaces to acidic phospholipids.

TF⁺ microparticles (MPs) represent another important source of circulating TF. Kasthuri *et al.* [8] suggested that tumor cells release TF⁺ procoagulant MPs into the circulation, which may trigger the venous thromboembolic process. Furthermore, they pointed out that *in vitro*, the addition of cancer cells to blood and plasma-promoting coagulation occurred in a TF- and phosphatidylserine-dependent manner, and the coagulation kinetics were strongly dependent on the number of cancer cells.

Other coagulation factors may interact with TF and take part in tumor-related coagulation. For example, Gay and Felding-Habermann [74] reported that coagulation factor VII (FVII) was activated in cancer patients and that complex interactions took place among tumor cell-expressed TF, FVII, and coagulation factor X, which generate thrombin and promote tumor progression.

All of the studies described above demonstrate that blood coagulation is rapidly activated by procoagulant (TF-expressing) tumor cells entering the circulation, particularly at sites of tumor cell arrest, and that anticoagulation factors can abolish tumor cell-induced coagulation. Further studies are needed to develop novel anticoagulants and investigate the safety and efficacy of anticoagulants for long-term use in cancer patients [73].

2.2. CTCs and Platelets in Cancer-Related Thrombosis

After entering the circulation, tumor cells activate platelets and induce platelet aggregation, which protects tumor cells from immune surveillance, promotes tumor cell arrest within the vasculature, and enhances tumor cell survival, thereby supporting the formation of

secondary lesions. Platelets are activated locally within the tumor vasculature or systemically, and the extent of platelet count elevation is inversely correlated with tumor cell survival in most cancer patients, including those with prostate, breast, lung, colon, or gastric cancer [75]. The critical role of platelet aggregation in cancer progression makes thrombocytosis a marker of poor prognosis [76].

Researchers have demonstrated that metastatic tumor cells express glycoconjugate ligands, including P-selectin glycoprotein ligand 1, mucins, and CD44 variants, as well as key integrins, such as $\alpha v \beta 3$. These factors mediate the complex interaction between platelets and tumor cells and facilitate hypercoagulation and metastasis in cancer patients, whereas treatment with antiplatelet drugs can reduce metastasis [74]. Also, platelet-initiated coagulation can activate receptors on tumor cells, such as PAR1 and PAR2, and promote the release of TF, which further enhances procoagulant activity [66,74]. In addition, activated platelets can release MPs, which consist of surface membrane particles (30-100 nm) and exosomes (100 nm-1 μ m) [77]. These MPs are derived from α -granules fused with the plasma membrane and express several surface receptors (α Ib β 3 [or GPIIb/IIIa], the GPIb α -IX-V receptor complex, P-selectin, and CXCR4), bioactive lipids (LPA and S1P), and cytoplasmic and secreted proteins, all of which are related to coagulation. Investigators also demonstrated that platelet MPs provide a procoagulant membrane surface for the activation of thrombin and promote thrombosis in cancer patients [78]. These studies suggested that by interacting with platelets and platelet MPs, CTCs promote coagulation in cancer patients.

Accordingly, CTCs mediate coagulation and thrombosis by expressing TF on their surface and activating platelets and play important roles in the association between cancer and thrombosis [66]. Still, the relationship of CTM with coagulation deserves further study. Hou *et al.* proposed that CTM originate in a coagulation-driven manner and interact with tumor-related thrombosis in an anfractuous manner. However, studies focusing on the relationship between CTM and coagulation are limited, and whether CTM interact with platelets and other procoagulant factors such as CTCs requires further investigation.

3. The Regulatory Role of MiRNAs in CTCs and Thrombosis

MiRNAs may function as both oncogenes and tumor suppressor genes, and each miRNA targets an average of 200 mRNA transcripts [79]. Thus, miRNAs execute widespread control of the expression of proteins and play essential roles in the initiation and progression of cancer [80]. Researchers have revealed that miRNAs can regulate cellular differentiation and migration, are associated with cancer metastasis, and predict poor prognosis and therapy responses in cancer patients (Table 2) [79,81,82]. Recently, investigators widely studied and reviewed the functions and mechanisms of miRNAs in regulating cancer metastasis and found that miRNA-based gene therapy for cancer is quite appealing effective. The roles of miRNAs in CTCs and thrombosis, which are closely related to metastasis, are frequent topics of study but rarely summarized. Therefore, we discuss below the roles of miRNAs in CTCs and thrombosis in cancer patients.

3.1. MiRNA Biology

MiRNAs are small noncoding RNAs (21-22 nt) that play a fundamental role in posttranscriptional gene regulation in both animals and plants by binding to the 3'-untranslated regions of their target mRNA [101,104]. In the nucleus, the miRNA gene is transcribed into primary miRNA (pri-miRNA) by RNA polymerase II, and the pri-miRNA is cleaved into precursor miRNA (pre-miRNA) by the complex composed of the RNase II enzyme Drosha and the binding partner DiGeorge syndrome critical region 8 (or Pasha). Subsequently, the pre-miRNA with a stem loop hairpin structure is transported from the nucleus to the cytoplasm by exportin 5. The premiRNA is cleaved further by another complex composed of Dicer (RNase III enzyme) and its binding partner TARBP2 (human immunodeficiency virus-1-transactivating response RNA-binding protein), and double-stranded RNA (dsRNA) is generated. The dsRNA contains mature miRNA, which is recognized by the RNA-induced silencing complex and is carried to the target mRNA complementary sites. Mature miRNA will then exert its function as an mRNA inhibitor by inducing mRNA degradation or translational repression (Fig. 2) [79,105,106].

3.2. MiRNAs in CTCs

Researchers have detected and characterized CTCs according to their physical properties, expression of proteins and mRNAs, gene amplification, and genetic aberrations. However, their sensitivity and specificity of biomarkers are low. Recent studies demonstrated the feasibility of using miRNAs as novel markers to detect and characterize CTCs and that quantifying miRNAs in CTCs to correlate with miRNA signals is better than only CTC counts, which aids in the identification of CTCs [105].

MiRNAs expressed in the peripheral circulation are either cell-free circulating or CTC-associated molecules. Numerous studies have demonstrated the feasibility and clinical relevance of cell-free circulating miRNAs as prognostic factors, drug targets, and predictors of therapy response in cancer patients [105]. However, many cell-free circulating miRNAs are found in the blood of healthy individuals, which may yield false-positive results of quantification of cell-free circulating miRNAs [107]. Thus, quantifying CTC-associated miRNAs is preferable, as CTCs are shed from primary and metastatic tumors. MiRNA levels are aberrant in cancer cells, and miRNAs have become important biomarkers for different cancers. Also, the miRNA expression profile in CTCs is apparently different from that in blood samples without detectable CTCs. Thus, CTC-associated miRNAs are effective biomarkers for the detection of CTCs on a high leukocyte background [108]. Zhou *et al.* [101] revealed that the level of miRNA-421 expression in mononuclear cells was markedly higher in gastric cancer patients than that in normal subjects and can be used as a biomarker for monitoring CTCs. In another study of gastric cancer, miRNA-21 expression was much high in peripheral blood, which indicated that miRNA-21 also may be used as a tool for monitoring CTCs [100]. Although investigators have made many attempts to use miRNAs as biomarkers to detect CTCs and predict prognosis for cancer, the number of miRNAs that can be used in detection of CTCs is limited, and more research should be carried out.

3.3. MiRNA and Thrombosis

Alteration of miRNA expression levels in activated platelets indicates that miRNAs may play an important role in thrombosis [104]. The activity of miRNAs in platelets is related to a variety of pathologies, including thrombotic disorders, cancer, and inflammation. Many investigations have demonstrated that platelet miRNAs (e.g., miRNA-223, miRNA-96, let-7c) are aberrant in cancer patients and influence platelet function [109]. Landry *et al.* [110] revealed that miRNAs took part in thrombosis by regulating the expression of P2Y₁₂ on the surface of platelets and modulated their function. Also, Liu *et al.* [84] found that the expression of miRNA-135a was highly elevated in hepatocellular carcinoma patients with portal vein tumor thrombosis and that treatment with pLKO-antimiRNA-135a greatly reduced the severity of PVTT as compared with that in the control group. Furthermore, miRNA-96 has controlled platelet activity and thrombosis in cancer patients by regulating the expression of VAMP8 [111].

According to Tayler's system, MPs function as delivery systems for miRNAs. By shuttling a wide variety of premature and mature miRNAs, MPs play a critical role in the communication among different cells and tissues and facilitate the genetic exchange among different tissues. A number of studies suggested that MPs are involved in activation of the coagulation system [112]. In those studies, MP shedding from platelets had procoagulant activity, and the number of MPs in cancer patients was highly correlated with the risk of thrombosis. Meanwhile, TF was expressed on the surface of MPs, which also took part in thrombosis. Based on the results of these studies, we concluded that both miRNAs and their MP-based delivery system have an important function in thrombosis.

MiRNA expression varies in CTCs, and miRNAs also play a critical regulatory role in thrombosis. MiRNAs can alter recipient cell gene expression by transferring miRNAs to these cells and act as essential regulators of tumor microenvironmental evolution, which influences tumor progression [79,113]. In addition, expression of miR-223 and miR let-7 family members is abnormal in both platelets and CTCs [109,110,114]. All of these results prompted us to speculate that miRNAs regulate the relationship between CTCs and thrombosis. However, further studies are needed to demonstrate the role of miRNAs in this relationship.

4. Anticoagulation and Tumor Metastasis

In 1968, Gasic *et al.* [115] reported that neuraminidase, which induces thrombocytopenia *in vivo*, reduced the number of lung metastases and that the decrease in metastasis was closely related to neuraminidase levels in platelets. A further study demonstrated that the antimetastatic effect of neuraminidase was mediated by its effect on platelets. Later, the studies focused on anticoagulation and tumor metastasis are widely performed. Today, with recent developments in cancer biology and pharmacy, anticoagulation strategies consist mainly of inhibition of the activity of TF/VIIa, platelets, and fibrinogen.

Amirkhosravi *et al.* [73] showed that the incidence of lung metastasis could be reduced by inhibition of human TF/VIIa-dependent factor X activation with the antibody H36. Also, Kasthuri *et al.* [8] demonstrated that inhibition of the TF/FVIIa complex with either an anti-

TF antibody or a small-molecule inhibitor of FVIIa could reduce the incidence of cancer metastasis. In addition, Bambace and Holmes [76] analyzed the roles of platelets in cancer metastasis and suggested that antiplatelet therapy can suppress the formation of metastasis and decrease the risk of cancer recurrence and death.

Inhibiting blood coagulation by blocking fibrin formation may reduce metastasis [116]. By inhibiting blood coagulation with the thrombin antagonist hirudin, researchers found that plasma fibronectin (pFN) was necessary for the prometastatic activity of this coagulation. Furthermore, they injected tumor cells into the tail veins of pFN-deficient mice and found that lung metastasis in the mice was greatly reduced, indicating that pFN supported metastasis by activating blood coagulation and promoting tumor cell retention in the lungs. MPs also play essential roles in coagulation, and metastasis and therapeutic strategies targeting MPs have sparked extensive interest among researchers. In addition, studies demonstrated that treatment with diannexin, an antithrombotic agent that directly targets phosphatidylserine, can inhibit epidermal growth factor receptor-driven tumor growth, reduce tumor microvascular density, and exert an antimetastatic effect [112].

All of these investigations demonstrated that anticoagulation strategies can reduce the progression and metastasis of cancer. However, to translate these results to the clinic, further investigations should be carried out to illustrate the efficiency and safety of the combination of anticoagulation drug administration and tumor-targeted therapy.

5. Conclusion and Perspectives

Metastasis and thrombosis are two major problems in cancer patients and may be determinants of prognosis in these patients. In this review, we summarize studies of the role of CTCs in cancer metastasis and demonstrate that CTC formation is the key step in the metastatic process and that CTC analysis can be termed real-time liquid biopsy for cancer patients. Use of the CellSearch CTC test for the detection of CTCs has been ubiquitous, and further studies of CTC detection in cancer patients have focused primarily on the development of new methods to improve the sensitivity and specialty of this detection. In particular, accurate identification of CTCs in patients with various cancers is highly meaningful, as it may accurately foretell and prevent early metastasis. New technologies are urgently needed to verify the predictability of CTCs, the meaningfulness of CTCs, and the CTCs that undergo EMT, which are characteristics of distinct subtypes of cancer.

Thrombosis, in which CTCs have an important role by shuttling certain cell surface factors and interacting with platelets, is another threat that cancer patients must face. *In vitro* and *in vivo* studies have demonstrated that use of coagulation treatment can reduce the incidence of cancer metastasis. However, whether anticoagulation drugs can be used to control and prevent cancer metastasis as they do in cardiovascular disease requires further study, and the mechanism of anticoagulation's effect on cancer metastasis should be verified. Furthermore, the efficiency and safety of the combination of anticoagulation drugs and tumor-targeted therapy should be demonstrated before translation to the clinic.

MiRNAs are key regulators of the initiation and progression of cancer and can be used to predict poor prognosis and therapy response. According to some studies, miRNAs

participate in the regulation of both metastasis and thrombosis and can be used as novel biomarkers for the detection of CTCs. Further investigations should be carried out to demonstrate the molecular mechanisms of miRNAs in regulating metastasis and thrombosis, and therapeutic agents targeting miRNAs must be exploited for the treatment and prevention of metastasis and thrombosis in cancer patients.

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Fig. 1.

CTCs and metastasis. When a primary tumor grows, CTCs are shed from it and enter the circulation. Most CTCs are destroyed or undergo apoptosis, leaving only a few CTCs to survive and arrive at the targeted organ. The metastatic tumors can also release CTCs, which may enter the circulation and form new metastases.

Fig. 2.

Biogenesis of miRNA. In the nucleus, the miRNA gene is transcribed by RNA polymerase II (POL II) into pri-miRNA. The pri-miRNA is then cleaved into pre-miRNA by Drosha. After being transported from the nucleus to the cytoplasm by exportin 5, the pre-miRNA is cleaved further into dsRNA by Dicer in the cytoplasm. The mature miRNA is recognized by the RNA-induced silencing complex (RISC) and degrades the mRNA with perfect complementarity or leads to translational repression with imperfect alignment.

Table 1
CTC Detection and its Clinical Significance in Patients with Different Types of Cancer

Cancer Type	Detection Method	Cutoff Value	Significance	Blood Volume	Reference
HNSCC	CellSearch CTC test	1	CTCs are useful markers of poor prognosis and predict local or metastatic recurrence	7.5 ml	[11]
HNSCC	Flow cytometric analysis, nested RT-PCR	1	CTCs are potential prognostic tools for inoperable HNSCC	7.5 ml	[12]
Breast cancer	CellSearch CTC test	1	CTCs are useful prognostic tools for HER2 ⁺ MBC	7.5 ml	[13]
Breast cancer	CellSearch CTC test	3	A threshold of 3 CTCs/7.5 ml of blood was a significant predictor of MBC treated with first-line chemotherapeutics combined with bevacizumab	7.5 ml	[14]
MBC	CellSearch CTC test	5	CTCs were early, strong predictors of MBC after the first cycle of treatment	7.5 ml	[15]
SCLC	CellSearch CTC test	2	CTCs were the strongest predictor for SCLC patient overall survival after the first cycle of chemotherapy	7.5 ml	[16,17]
SCLC	CellSearch CTC test	50	CTCs are independent prognostic factors for SCLC	7.5 ml	[18]
NSCLC	CellSearch CTC test	5	CTC formation may be predictive of response to targeted therapy in patients with advanced NSCLC	7.5 ml	[19]
NSCLC	CellSearch CTC test ISET	2	CTC number is an independent prognostic biomarker for NSCLC	7.5 ml	[20]
CRC	CellSearch CTC test	1	CTCs are prognostic factors for colorectal cancer in patients at high risk for recurrence	7.5 ml	[21-23]
CRC	CellSearch CTC test	3	CTC formation and KRAS status were independent prognostic factors for metastatic CRC treated with bevacizumab with or without chemotherapy	7.5 ml	[24]
Gastric cancer	CellSearch CTC test	4	CTCs may determine response to S-1-based or paclitaxel-based regimens in patients with advanced gastric cancer	7.5 ml	[25]
Gastric cancer	CellSearch CTC test	2	CTC formation predicts survival, and CTCs can be used to monitor response to therapy	7.5 ml	[26]
Prostate cancer	CellSearch CTC test	5	CTCs are informative biomarkers for and reflect disease progression	7.5 ml	[13]
Prostate cancer	CellSearch CTC test	3	CTC counts in combination with short PSA-DT, AKP and LDH levels, and low Hb levels are indicative of high risk of disease progression	7.5 ml	[27]
Renal cancer	CellSearch CTC test	1	CTCs can be used to assess the benefit of treatment of renal cancer with sunitinib	7.5 ml	[28]
Renal cancer	autoMACS and immunocytochemical staining	1	CTCs are independent prognostic markers for renal cancer	16 ml	[29]
Pancreatic cancer	CellSearch CTC test	1	CTCs may be useful for prognosis for, assessment of, or stratification of LAPC	7.5 ml	[30]

Note: HNSCC, head and neck squamous cell carcinoma; RT-PCR, reverse transcriptase-polymerase chain reaction; MBC, metastatic breast cancer; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; ISET, isolation by size of epithelial tumor cells; CRC, colorectal cancer; PSA-DT, prostate-specific antigen doubling time; AKP, alkaline phosphatase; LDH, lactate dehydrogenase; Hb, hemoglobin; LAPC, locally advanced pancreatic adenocarcinoma.

Table 2
New Findings Regarding miRNA Expression in Patients with Different Types of Cancer

miRNA	Cancer Type	Function	Target	Mechanism	Reference
miR-148	HCC	Tumor suppressor	Met signaling	Repression of the migration and pulmonary metastasis of hepatoma cells	[83]
Let-7c	HCC	Tumor suppressor	Ras oncogene	Regulation of cellular differentiation	[81]
miR-135a	HCC	Oncogene	MTSS1 and GAS7	Enhancement of tumor cell invasion and metastasis	[84]
miR-192	Breast cancer	Tumor suppressor	BMP-6	Regulation of cellular proliferation	[85]
miR-190a	Breast cancer	Tumor suppressor	PAR1	Inhibition of cancer metastasis	[86]
miR-1288	Colon cancer	Tumor suppressor	-	Regulation of cellular proliferation	[87]
miR-34a	Colorectal carcinoma	Tumor suppressor	PAR2 and cyclin D1	Mediation of PAR2-induced cyclin D1 upregulation	[88]
miR-106b	Endometrial carcinoma	Tumor suppressor	TWIST1	Modulation of EMT and suppression of endometrial carcinoma cell invasion	[89]
miR-23b		Tumor suppressor	Rho family	Remodeling of the cytoskeleton	[90]
miR-429	Esophageal carcinoma	Tumor suppressor	Bcl-2 and SP1	Inhibition of invasion and promotion of apoptosis	[91]
miR-16	Bladder cancer	Tumor suppressor	Cyclin D1	Inhibition of cellular proliferation	[92]
miR-198	Pancreatic cancer	Tumor suppressor	MSLN, PBX-1, and VCP	Inhibition of tumor growth and metastasis	[93]
miR-30c	NSCLC	Tumor suppressor	MTA1	Inhibition of tumor invasion	[94]
miR-31	Prostate cancer and OSCC	Oncogene	ITGA5 and RDX	-	[82,95]
miR-181, miR-24, and miR-211	OSCC	Oncogene	-	Enhancement of cellular migration and invasion	[82]
miR-21	Lung cancer, OTSCC, PDAC, and gastric cancer	Oncogene	Increased VEGF, DKK2, PIAS3, and PTEN	Induction of tumor angiogenesis and promotion of cellular invasion via the Wnt/ β -catenin pathway	[96-100]
miR-421, miR-106a, and miR-17	Gastric cancer	Oncogene	CBX7, RBMXL1	Enhancement of cancer cell proliferation	[101,102]
miR-200c	Gastric cancer	Oncogene	-	-	[103]

Note: HCC, hepatocellular carcinoma; NSCLC, non-small-cell lung cancer; OSCC, oral squamous cell carcinoma; OTSCC, oral tongue squamous cell carcinoma; PDAC, pancreatic ductal adenocarcinoma; VEGF, vascular endothelial growth factor; DKK2, dickkopf-related protein 2; PIAS3, protein inhibitor of activated signal transducer and activator of transcription 3; PTEN, phosphatase and tensin homolog.