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The Mechanism of Circulating Tumor Cell (CTC) Detection

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Abstract:

The high lethality of cancer is mainly attributed to its metastatic ability. Circulating tumor cells (CTCs), as important mediators driving tumor metastasis, have significant research value in tumor progression and prognosis assessment. In recent years, CTC detection methods have been continuously evolving, mainly including detection based on cell surface proteins and detection based on intracellular miRNA molecular markers. Surface protein detection techniques are mature and high-throughput, but prone to missed detections when epithelial-mesenchymal transition (EMT) causes phenotypic changes; miRNA detection, on the other hand, can dynamically reflect the status of tumor cells, with high sensitivity, yet the process is complex and difficult to apply on a large scale. Currently, capturing the phenotypically heterogeneous and rare CTCs accurately remains a challenge in the field of detection. This paper systematically analyzes the principles, methods, current applications, and respective issues of the two major classes of CTC detection technologies: miRNA and surface protein detection. Furthermore, it discusses the positive role of combined detection in enhancing detection sensitivity and accuracy. Studies indicate that multi-marker combined detection can more comprehensively identify CTC heterogeneity, aiding in early tumor diagnosis, dynamic monitoring, and precise treatment. This paper provides a reference for the optimization of subsequent CTC detection technologies and in-depth research on tumor metastasis mechanisms, while pointing out current deficiencies in standardization, automation, and functional status analysis. Future research may focus on directions such as multi-omics integration and intelligent analysis.

Keywords:-Component; Circulating Tumor Cells (CTCs); Surface Protein Detection; miRNA-based Detection; Combined Multi-Marker Detection

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I. Introduction

Cancer has always been a major medical concern, with its lethality mainly manifested in its metastatic and invasive characteristics. Among these, epithelial-mesenchymal transition (EMT) is considered a crucial mechanism in cancer progression and metastasis. EMT, regulated rigorously by various internal and external factors, coordinates the transition of cells from an epithelial state to a mesenchymal state, allowing tumor cells to detach from the primary tissue and spread throughout the body. Circulating tumor cells (CTCs) are a type of tumor cell that detach from the primary tumor and enter the circulatory system, thus initiating the metastasis process [1]. In almost all types of tumors, CTCs exhibit abnormal expression, but their numbers are extremely scarce, typically ranging from 1 to 10 CTCs per milliliter of blood [2]. Despite this scarcity, CTCs, as important biological markers for studying cancer metastasis, are highly accessible. Thus, understanding the methods of CTC detection is an important step toward understanding the biology of cancer metastasis.

There are currently two predominant categories of CTC detection methods: methods based on surface proteins, and methods based on intracellular molecular marker miRNA. The CTC detection methods based on surface proteins are more developed, and the most commonly used methods are immunofluorescence labeling [3], immunomagnetic enrichment [4], and imaging flow cytometry (IFC) [5]. The underlying principles of these methods are CTC identification and separation through specific antibodies that recognize specific proteins on the CTC, such as EpCAM, CK, etc. The methods accomplished identification based on the specific binding of antibodies to the antigens, leading to efficient capture and detection of CTCs. These methods have the advantages of high-throughput and ease of use. Based on their previous applications, the aforementioned CTC detection methods have application in the clinical translation. However, the limitation of detection is that during the EMT process, CTCs exhibit down-regulated or completely absent surface marker proteins, resulting in decreased detection sensitivity and an increased heterogeneous populations of CTCs, a major limitation to detection.

On the other hand, detection methods based on intracellular molecular marker miRNA have attracted more attention in recent years. miRNA, an important molecule that regulates gene expression, shows specific changes of expression when a tumor occurs and progresses. miRNA detection technologies include real-time quantitative PCR (qRT-PCR) [6], nanodigital multiplexed flow cytometry (Nano-DMFC technology) [7], and the CRISPR/Cas13 system [8]. These methods typically have high sensitivity, capable of detecting very low levels of miRNA molecules. The expression of miRNA can dynamically reflect the biological status of tumor cells, helping to overcome the issue of CTC phenotypic heterogeneity caused by EMT. However, miRNA detection still faces challenges in clinical applications, such as complex sample pre-processing, insufficient standardization, and specificity analysis. Furthermore, surface protein detection still holds certain advantages in high-throughput screening and automation.

II. Detecting CTCs using Intracellular Marker miRNA

A. Principles of miRNA Detection in CTCs

The principle of miRNA detection in CTCs is mainly based on the expression changes of specific miRNA molecules within tumor cells, especially miRNAs closely associated with tumor metastasis, such as the miR-200 family [9]. Before tumor cells undergo metastasis, they typically go through EMT, a process that causes tumor cells to lose their original epithelial characteristics and acquire enhanced migration and invasion abilities. During the EMT process, the expression of surface markers on tumor cells is downregulated, leading to a decrease in sensitivity of traditional CTC detection methods that rely on surface proteins. In contrast, miRNA, as an intracellular molecular marker, can more directly reflect the biological status of tumor cells through its expression changes.

Represented by the miR-200 family, this type of miRNA plays a crucial role in maintaining epithelial phenotype and inhibiting the EMT process. The miR-200 family (including miR-200a, miR-200b, miR-200c, miR-141, and miR-429) mainly targets and inhibits the EMT-related transcription factors ZEB1 and ZEB2, preventing tumor cells from undergoing mesenchymal transition. When the expression of miR-200 family members is downregulated, ZEB1/2 expression is upregulated, activating EMT, and enhancing the metastatic ability of tumor cells. Studies show that changes in expression of the mir-200 family are correlated with development, progression, and prognosis of many cancers [10].

The method to detect miRNA-based CTCs follows these steps: isolate CTCs in blood samples from patients, measure a specified amount of specific miRNAs, detect if tumor cell has undergone EMT, and measure the metastatic potential of the tumor cell. This molecular detection strategy circumvents a significant barrier to CTC detection, which is the heterogeneity among CTC phenotypes, providing increased sensitivity and specificity, with possible

early warning and ongoing monitoring of tumor transient metastasis.

B. Methods for Detecting CTCs using miRNA

What I am currently aware of, methods that are mostly used to detect CTCs are generally regarded as qRT-PCR, nanodigital multiplexed flow cytometry technology (Nano-DMFC technology), and CRISPR/Cas13 system. Each has its own pros and cons. qRT-PCR is referred to as the gold standard method for miRNA quantification for its high sensitivity, specificity, and accurate quantification [11]. qRT-PCR for the detection of miR-21 has been extensively used. Advantages of the quantitation of miR-21 via qRT-PCR uses its mature operational process, widespread equipment, and its ability to accurately quantify low-abundance miRNAs.

In qRT-PCR, the process starts by converting the miRNA in the sample into complementary DNA (cDNA) through a reverse transcription reaction. Subsequently, during the PCR amplification process, specific primers and fluorescent probes are used to amplify and monitor the target miRNA in real-time. The accumulation of fluorescent signals dynamically reflects the quantity of amplified products, enabling highly sensitive and quantitative detection of miRNA expression levels. RT-qPCR demonstrates extremely high sensitivity and specificity, capable of detecting very low levels of miRNA molecules, making it particularly suitable for analyzing rare miRNAs in complex biological samples like blood. The technology process is mature, equipment is widely available, operation is relatively straightforward, and the results exhibit good reproducibility and comparability [12].

C. Current Applications of miRNA Detection in CTCs

Currently, qRT-PCR has been widely used for the detection and analysis of miRNA in CTCs, demonstrating significant value in early cancer diagnosis, treatment assessment, and prognosis monitoring. Numerous studies have shown that quantitatively detecting miRNAs closely associated with tumor metastasis and invasion in CTCs (such as miR-21, miR-200 family, etc.) through qRT-PCR can effectively assist in assessing the biological behavior and developmental trends of tumors.

Taking breast cancer as an example, researchers have used qRT-PCR technology to detect the miR-200 family members in CTCs from patients' blood samples. They found that the expression level of miR-200c was significantly downregulated in the blood of breast cancer patients, while the expression level of miR-141 was higher in the blood of stage I-III breast cancer patients. These abnormal expressions are closely related to the invasiveness and metastasis of breast cancer [13].

In other types of cancers such as lung cancer and colorectal cancer, detecting the expression changes of specific miRNAs using qRT-PCR can also reflect the early occurrence of tumors, their tendency to metastasize, and their sensitivity to treatment. For example, miR-92a has been confirmed to be overexpressed in CTCs of colorectal cancer with poor outcomes in patients [14]. Meanwhile, the downregulation of miR-34a in CTCs derived from non-small cell lung cancer suggests the tumor has a greater invasive and metastatic capacity [15].

Detection technologies and sample processing methods are advancing, thus the use of qRT-PCR for miRNA detection in CTCs has normalised and is consistently being used. Its sensitivity, speed, and reproducibility make it useful for basic research and potentially for clinical translational practices. Future combined detection of additional types of miRNA markers will enable more precise personalized diagnosis and treatment of cancer individuals.

D. Challenges of miRNA Detection in CTCs

Although qRT-PCR is the gold standard method of detecting miRNA in CTCs, it also has several challenges to practical application. First, the low content of miRNA in blood and the limited number of CTCs, means that the preprocessing is very important to optimizing the detection results and any loss or degradation could yield false-negative results [16]. Second, the Ct (cycle threshold) of qRT-PCR can also be affected by numerous variables, including the time, location, and method of sample collection as pre-analytical factors, plus the target sequences of the PCR reactions, inhibitory factors of the PCR, and efficiency of the primers as analytical factors. There may be large differences in the comparability of detection results from different laboratories that use different methods of qRT-PCR as well as different kits for qRT-PCR [17]. Furthermore, the presence of abundant free miRNA and exosomal miRNA components in the blood poses a challenge in effectively distinguishing miRNA originating from CTCs and preventing background interference, a problem that has not been fully resolved. Lastly, qRT-PCR does not have the capacity for high-throughput or multiplex detection. Therefore, a number of simultaneous and efficient detections of multiple miRNAs is difficult. In summary, despite the dependable and accrued qRT-PCR use in the field of CTC miRNA detection, thorough optimization and breakthroughs in sensitivity, standardization, and high-throughput methods of detection are still warranted.

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III. Extracellular Marker Surface Protein Detection in CTCs

A. Principles of Surface Protein Detection in CTCs

CTCs arise via the regulation of cell surface proteins, which are a process that can be influenced by abnormal miRNA expression, specifically by the miR-200 family. miR-200 is considered the major inhibitor of the EMT process because without the expression of miR-200, the EMT transcription factors, ZEB1 and ZEB2, can increase expression by being relieved from inhibition. Expression of ZEB1 and ZEB2 can inhibit expression of the epithelial marker, E-cadherin (an important cell adhesion protein) and increase expression of mesenchymal markers like N-cadherin and vimentin. The reduction in expression of E-cadherin will weaken adhesion at the attachments between the tumor cells and primary tissue, essentially allowing devitalized cells to detach from the primary tissue, in the blood, leading to CTCs [18].

The premise of surface protein detection in CTCs is based on dynamic changes of these molecules. Most commonly, epithelial markers such as E-cadherin, EpCAM, and CK are used to target CTCs as a recognition and separation vehicles. These proteins can be labeled by antibodies that find and capture CTCs. However, as surface epithelial markers are downregulated and mesenchymal markers upregulated through the EMT mode, the phenotype of CTCs change. Some tumor found even lose traditional surface antigens precluding detection. Thus, the relevant expression changes of the miR-200 family affect not only the molecular phenotype of CTCs, but also their detectability in the circulation.

In conclusion, the principles depend on surface antigen expression changes of tumors cells associated with epithelial mesenchymal transition EMT, specifically the change in E-cadherin downregulation and relevant mesenchymal markers upregulation; this provides insights of molecular mechanisms of CTC formation, as well as critically important molecular targets for tumor metastasis diagnosis and intervention.

B. Methods of Surface Protein Detection in CTCs

Detection of surface proteins on CTCs can be performed using immunofluorescence labeling, immunomagnetic enrichment, and IFC, three common tools in CTC research. In immunofluorescence labeling, specific surface proteins are visualized by the binding of antibodies and signal by fluorescent markers. Immunofluorescence provides qualitative and quantitative analysis of CTCs and is easy to use as it primarily relies on the user to observe under a microscope; however, relying primarily on visual observation makes it difficult to conduct high-throughput studies, automated analyses of multi-parameters, and also makes it susceptible to non-specific staining from a complex sample background [3]. Immunomagnetic enrichment employs magnetic beads conjugated to antibodies to isolate and enrich CTCs specifically from blood samples, which markedly increases the detection of rare CTCs. However, despite the purifying CTCs by magnetic isolation, it does not provide morphology and multi-marker expression which usually requires an additional round of fluorescence staining or an alternative detection method [4]. The immunomagnetic process relies specifically on antigen expression, and therefore it also runs the risk of excluding CTCs based on cellular morphological transformation or downregulation of surface antigen expression.

In contrast, IFC combines the high-throughput, multi-parameter automated analysis capabilities of traditional flow cytometry with the high-resolution imaging properties, which facilitates the concurrent acquisition of multiple fluorescence signals and cellular morphological information at the single-cell level [19]. Firstly, IFC can analyze a large number of cells in a short period and support multiple fluorescence labeling, enabling synchronous detection of various surface proteins to comprehensively capture the diverse phenotypes of CTCs. Secondly, with high-resolution imaging, IFC can not only quantitatively analyze the expression levels of target proteins but also finely capture the spatial distribution and localization of proteins on the cell surface, greatly enhancing the ability to identify CTC heterogeneity. Additionally, the automation and software analysis functions of IFC provide significant advantages in data objectivity, reproducibility, and large-scale sample processing, reducing human errors and subjective judgments. Overall, IFC can compensate for the shortcomings of traditional methods in high-throughput analysis, information richness, and identification of complex cellular heterogeneity, making it the preferred technical solution in the field of CTC surface protein detection.

In the specific methodological process, IFC typically involves several key steps: Firstly, using immunomagnetic bead enrichment techniques (such as MACS) to enrich CTCs from blood, specific antibodies against target surface proteins (such as E-cadherin, EpCAM, etc.) are coupled with magnetic beads to separate target cells from a large number of blood components. Subsequently, the isolated CTCs are fluorescently labeled, commonly using fluorophores like FITC, PE, etc., to simultaneously label multiple surface proteins based on experimental design

[20]. The stained cell suspension is then introduced into the Imaging Flow Cytometer, where the instrument performs multi-channel fluorescence collection for each detected cell and synchronously records high-resolution cell images. Finally, specialized analysis software comprehensively analyzes multiple parameters such as fluorescence intensity, protein localization, cell morphology, etc., to achieve accurate identification, classification, and functional status assessment of CTCs. This method not only allows for the expeditious and high-throughput detection of CTCs, but also provides a robust experimental platform for future explorations of tumor heterogeneity and the mechanisms underlying metastasis.

C. The Current Application Status of Surface Protein Detection in CTCs

Over the past few years, IFC has received a great deal of attention and has many applications in CTC surface protein detection. Due to its technical advantages of high-throughput, multi-parameter, and single-cell resolution, IFC is a powerful tool allowing further insight into the phenotypic heterogeneity of CTCs and for understanding tumor metastasis. IFC provides multiple benefits over traditional detection methods due to its ability to capture cell fluorescence during capture, as well as the morphological features of cells, and therefore can enable multidimensional and comprehensive analysis of CTCs.

In the example of breast cancer, IFC shows unique benefits, and possibly broad application for detection of CTC surface proteins. The high-throughput screening and multi-parameter analysis of CTCs, is well suited using IFC. Breast cancer researcher often characterize CTCs harvested from peripheral blood from breast cancer patients with IFC. For example, breast cancer metastasis study can achieve multiple fluorescence labeling for E-cadherin (epithelial marker) and N-cadherin and vimentin mesenchymal markers to simultaneously detect expressions and spatial specificity of multiple proteins from within a single cell [21]. Previous studies show that IFC can synchronously recover E-cadherin and EpCAM (surface proteins) from CTCs in a single tube and quantitively describe the expression of surface proteins and differentiate CTC subtype morphology. In patients with breast cancer, the decrease in E-cadherin expression has been associated with tumor invasiveness and thus metastatic risk, so interestingly we found that some CTCs expressed epithelial and mesenchymal markers indicate the high heterogeneity of tumor cell phenotypes, which gives novel insight into the understanding and mechanisms for tumor metastasis development.

IFC detection of surface proteins for breast cancer CTCs not only helps in early tumor screening but also to monitor patient response to treatment and predict recurrence risk in patient treatment plans [20]. The merit of multiple markers, single-cell resolution, and morphology makes IFC highly valuable with a strong technical advantage for accurately detecting breast cancer CTCs and to study the mechanisms of tumor metastasis. Additionally, IFC provides possibilities for individual treatment and prognosis assessments in each case of breast cancer.

D. Challenges in Surface Protein Detection of CTCs

While IFC offers technical benefits for detecting surface proteins in CTCs; it is high throughput, multi-parametric, and single-cell resolution; there are some key issues that need to be addressed for its use. First, CTCs are extremely rare in blood. Thus, there is the potential for cell loss while going through the initial enrichment and separation steps, which would affect the accuracy and sensitivity of the remaining analyses. Second, CTC surface proteins vary tremendously based on heterogeneity of tumor types, and all CTCs that have gone through an EMT, may downregulate the expression of conventional epithelial markers (e.g., EpCAM, E-cadherin) that affect detection sensitivity and build in false-negative findings [18]. Lastly, non-specific background cells (like white blood cells) can sometimes be misidentified as CTCs because of non-specific staining/similar morphology, distorting the accuracy of detection [5]. In conclusion, while innovative the advances associated with IFC for expression detection of surface proteins in CTCs is significant, there is still needed improvement and optimization involved in particular values of cell enrichment and marker selection.

IV. The Combined Detection of miRNA and Surface Proteins in CTCs

The abnormal miRNA expression and changes in surface protein expression can be very valuable for CTC identification. miRNA has been identified as an emergent biomarker for early cancer diagnosis and similar classification. It has high stability and reflects dynamic changes in tumors very sensitively. When using high sensitivity and specificity techniques (real-time qRT-PCR technology) miRNA is a good biomarker. High-throughput detection of surface proteins has a significant advantage to efficiently isolating, enriching, and classifying CTCs. Combining miRNA and surface protein detection will allow for a deeper level of interrogation of heterogeneity and biological characteristics of CTCs both at the molecular and phenotypic level, reflecting dynamic changes in the tumors

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and offering efficiency in CTC isolation and detection accuracy.

In recent years, several researchers have designed new technologies that combine miRNA detection with surface protein detection. Zhang et al. identify surface proteins and miRNA of CTC and developed a removable magnetic nano-device (MS-RI) that both efficiently capture CTCs and accurately characterize CTC subtypes [22]. The system consists of two modules (simultaneously), a magnetic separation module is included and an identification imaging module. The magnetic separation module works with magnetic beads modified with streptavidin, combined with a modified MUC1 aptamer; this combination allows the specific capture and separation of CTCs. The imaging module uses imaging probes to detect intracellular miRNA in CTCs (miR-21 and miR-141), thus allowing a more detailed molecular breakdown of CTC subtypes. The specific process is the following: first, the MUC1 aptamer is used to capture and collect the surface protein MUC1 of CTC (isolate breast cancer cells, ie., MDA-MB-231 and MCF-7). By using magnetic separation it isolates CTCs from blood or mixed cell systems. Then the magnetic separation module and imaging module is separated by introducing a denaturing agent. This allows for the imaging probes to be released into the cells and specific miRNA expression to now be identified.

The imaging probes, through molecular design, only produce a clear fluorescent signal when bound to the target miRNA (such as miR-141 or miR-21). For example, in the case of MDA-MB-231 and MCF-7 breast cancer cells, miR-141 and miR-21 are abundant in MDA-MB-231, efficiently binding to the probes and generating a strong green fluorescent signal, while miR-141 expression is lower in MCF-7, resulting in a weaker green fluorescent signal, and normal cells (such as L02 cells) show almost no fluorescent signal. This method not only enables efficient CTC isolation but also allows subtype analysis of heterogeneous CTCs based on miRNA expression patterns, facilitating dynamic monitoring of CTC functional status and tumor progression potential.

In conclusion, the combined detection of miRNA and surface proteins helps overcome the limitations of single biomarker detection, enhancing the accuracy of early tumor diagnosis, classification, and treatment evaluation. It provides a solid technical foundation and new research perspectives for personalized cancer diagnosis and treatment, as well as dynamic monitoring.

V. Conclusion

This study primarily explores the two main technological approaches for detecting CTCs: detection based on intracellular miRNA molecular biomarkers and detection based on extracellular surface proteins. MiRNA detection can sensitively reflect the molecular status and dynamic changes of tumor cells, suitable for revealing the biological characteristics of CTCs, but it involves a complex operational process, high preprocessing requirements, and faces challenges in achieving high-throughput screening. On the other hand, surface protein detection methods are mature, convenient for automation and high-throughput analysis, but are susceptible to influences like EMT processes, leading to potential missed detections due to phenotypic transformations in some CTCs. Single biomarker detection struggles to comprehensively represent the complexity of CTCs. The combination of both approaches allows for the consideration of both molecular and phenotypic information, complementing each other's strengths and enabling a more comprehensive and accurate identification and classification of CTCs. By summarizing the advantages and disadvantages of existing technologies, this study provides important references for optimizing and innovating CTC detection methods in the future, laying the foundation for a deeper understanding of tumor metastasis mechanisms and achieving dynamic monitoring. However, there are still some limitations in this study. For instance, the analysis of the functional status of CTCs, their interactions with the microenvironment, and the identification of novel molecular biomarkers have not been deeply explored. In the future, with the continuous emergence of novel molecular biomarkers and cutting-edge detection technologies, multi-omics combined analysis and AI-assisted interpretation are expected to further enhance the clinical utility of CTCs, providing more powerful tools for early cancer diagnosis, dynamic monitoring, and personalized treatment.

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