Molecular Assays for the Detection and Characterization of CTCs

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Abstract

Molecular characterization for circulating tumor cells (CTCs) can be used to better understand the biology of metastasis, to improve patient management and help to identify novel targets for biological therapies aimed to prevent metastatic relapse. New areas of research are directed towards developing novel sensitive assays for CTC molecular characterization. Towards this direction, molecular detection technologies that take advantage of the extreme sensitivity and specificity of PCR, offer many advantages, such as high sensitivity, specificity, and significant flexibility in the clinical lab setting, in terms of high-throughput analysis, multiplexing, and quality control issues. Using molecular assays, a variety of molecular markers such as multiple gene expression, DNA methylation markers, DNA mutations, and miRNAs have been detected and quantified in CTCs in various cancer types, enabling their molecular characterization. Here, we present the main molecular detection technologies currently used for CTC analysis and molecular characterization.

Contents

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1 Introduction

Circulating tumor cell (CTC) analysis is a promising new diagnostic field toward the estimation of risk for metastatic relapse or metastatic progression [1–3]. Especially in breast cancer, CTC detection and enumeration have been established in several clinical studies, showing a correlation with decreased progression-free survival and overall survival in operable [4–9] and metastatic breast cancer [10].

Different analytical systems for CTC isolation and detection have been developed as immunocytochemistry and molecular assays, most including separation steps by size or biological characteristics such as expression of epithelial or cancer-specific markers. Recent technical advancements in CTC detection and characterization include multiplex quantitative reverse transcription-PCR (RT-qPCR) based methods, image-based approaches, and isolation technologies like microfilter and microchip devices [11]. New areas of research are directed toward developing novel sensitive assays for CTC molecular characterization. The molecular characterization of CTC can provide important information about the molecular and biologic nature of these cells. This is very important for the identification of therapeutic targets and resistance mechanisms in CTC as well as for the stratification of patients and real-time monitoring of systemic therapies [11, 12].

Here, we present the main molecular detection technologies currently used for CTC analysis and molecular characterization.

2 Molecular Detection Technologies for CTC Analysis

Molecular assays are based on the analysis of nucleic acids and take advantage of the extreme sensitivity and specificity of PCR. They are high-throughput and easy to perform since they are based on the isolation of total RNA from viable CTC, and subsequent RT-PCR amplification of tumor or epithelial specific targets. Especially, RT-qPCR assays can be *in silico* (through the use of specific software programs) designed, easily automated, and subjected to internal and external quality control systems [13].

The major advantages of molecular assays are the following: (a) they give information only for living cells since RNA is very sensitive, (b) a variety of molecular markers (gene expression, DNA methylation markers, DNA mutations, etc.) can be detected and quantified in CTC, enabling their molecular characterization, (c) offer extreme sensitivity; through this technology a small number of

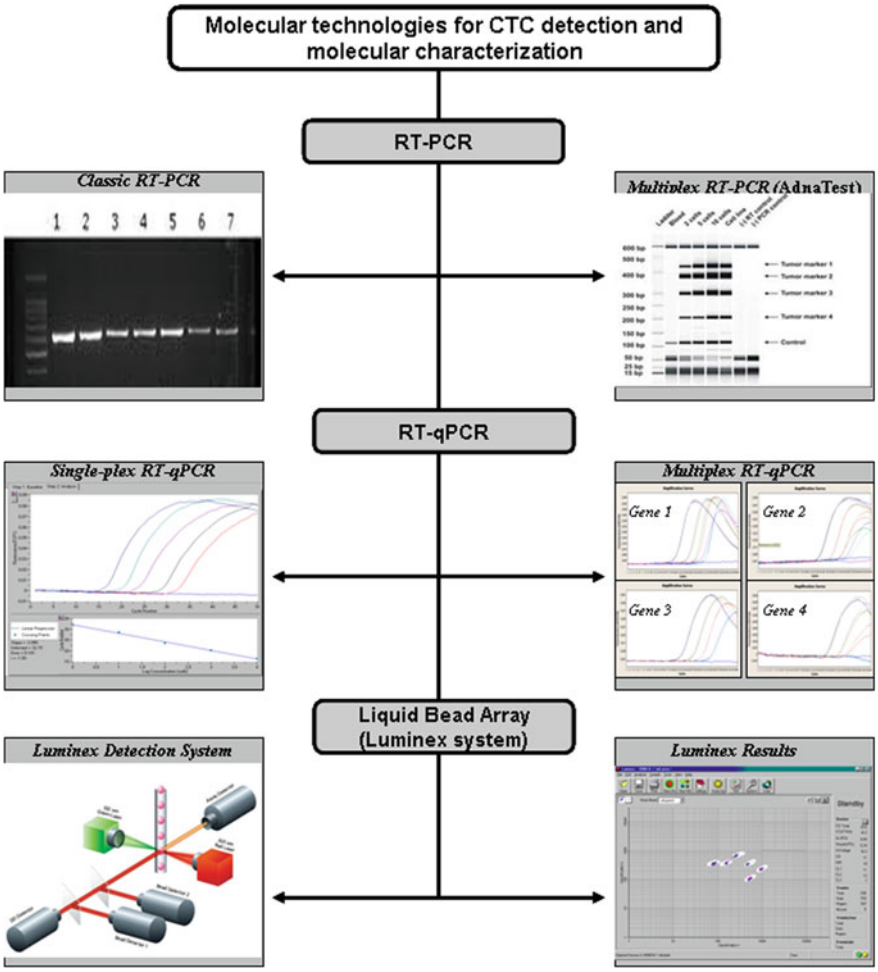

Fig. 1 Outline of the main approaches for CTC molecular detection technologies

CTCs can be detected through the highly sensitive detection of epithelial markers in the presence of millions of peripheral blood mononuclear cells, (d) offer a significant flexibility in the clinical lab setting, in terms of high-throughput analysis, multiplexing, (thus reducing the amount of sample required), time and cost of analysis and quality control issues. The main disadvantage of molecular assays by this approach is the fact that unless combined with imaging systems it is not possible to obtain any morphological information about CTC. Moreover, we cannot estimate accurately the number of CTCs present in a sample, since a different number of transcripts can be expressed from different cells. An outline of the main approaches for CTC molecular detection technologies is presented in Fig. 1.

2.1 RT-qPCR

RT-PCR methodology for the detection of micrometastases in patients with breast cancer has been based initially on the estimation of the number of *CK*-19 transcripts in blood and bone marrow samples [14]. Our group has developed for the first time a highly sensitive and specific real-time RT-qPCR assay for *CK*19-mRNA [15, 16] and evaluated its sensitivity, specificity and clinical potential for the molecular detection of occult carcinoma cells in peripheral blood of breast cancer patients [6–9]. In parallel, many different molecular assays based on RT-PCR, specifically designed for different gene transcripts in CTCs have been developed, such as mammaglobin [17], and EGFR [18–20]. By using a multimarker RT-PCR assay for CTC in early breast cancer, we have shown that *CK*-19, *Mammaglobin* and *HER*2- positive CTC are associated with shorter disease-free survival [7].

2.2 Multiplex RT-qPCR

Several mRNA markers have been already used for RT-PCR-based detection of CTCs. Quantification of these mRNAs is essential to distinguish normal expression in blood from that due to the presence of CTCs. Few markers provide adequate sensitivity individually, but combinations of markers may produce good sensitivity for CTC detection. An important limitation of most available methodologies for CTC analysis is the small number of gene targets that can be analyzed, due to the limited amount of available samples. However, identification of specific subtypes of CTC based on the expression of an increasing number of cancer important genes can provide information about the biology of metastasis and improve patient management. To be effective, the method used to identify CTC must detect all tumor cell types. However, the fact that CTCs are very rare and the amount of available samples is very limited presents a tremendous analytical and technical challenge [21, 22]. Multiplex RT-PCR assays for CTC analysis can overcome these problems.

By using RT-qPCR Obermayr et al. have shown that a panel of six genes was found superior to EpCAM and mammaglobin for the detection of CTC in breast cancer, and they may serve as potential markers for CTC derived from endometrial, cervical, and ovarian cancers as well [23]. Reinholz et al. have shown that molecular characterization of circulating epithelial cells using mammaglobin and B305D-C offers potential for early detection of invasive breast cancer [24].

By using the commercially available AdnaTest BreastCancer (AdnaGen AG, Germany) kit, (based on the enrichment of CTCs from peripheral blood of breast cancer patients followed by identification of CTC-associated marker transcripts by reverse transcription and multiplex PCR), Aktas et al., have detected EpCAM, MUC-1 and HER2 transcripts in CTC and found that a major proportion of CTC in metastatic breast cancer patients showed Epithelial Mesenchymal Transition (EMT) and tumor stem cell characteristics [25]. Interestingly, when the expression of the ER and PR was

assessed in CTCs by RT-PCR, the spread of CTCs was mostly found in triple-negative tumors and CTCs in general were mostly found to be triple-negative regardless of the ER, PR, and HER2 status of the primary tumor [26].

A quantitative gene expression profiling methodology based on RT-qPCR, specific and sensitive to detect one CTC was performed by using a set of genes with no or minor expression by leukocytes [27]. We have recently developed a highly sensitive and specific multiplexed quantitative RT-qPCR to detect the expression of six genes, (*CK*-19*, MAGE-A*3*, HER*-2*, TWIST*1*, hTERT* α+β+, and *mammaglobin)* and validated it in CTC of early and metastatic breast cancer patients [28].

2.3 Liquid Bead Array

A highly sensitive and specific multiplexed PCR-coupled liquid bead array to detect the expression of multiple genes in CTC has been recently developed [29]. By using this approach, six established CTC gene targets; *HER*-2, mammaglobin *(hMAM), CK*-19*, MAGE-A, TWIST*-1, and *PBGD* were simultaneously amplified and detected in the same reaction, in a very limited amount of CTC samples thereby saving precious samples and reducing the costs and time of analysis This novel assay forms the efficient basis for a multiplex approach to study the expression of up to 100 genes in CTC.

3 Molecular Characterization of CTCs

Molecular characterization for CTCs can be used to better understand the biology of metastasis, to improve patient management and help to identify novel targets for biological therapies aimed to prevent metastatic relapse. The role of CTCs in treatment failure and disease progression could be explained by their relation to biological processes, such as epithelial-to-mesenchymal transition and tumor dormancy [30]. Identifying metastatic stem cells through molecular characterization approaches in the CTC population might result in the development of new therapeutic concepts. CTCs are highly heterogeneous [1, 25–29, 31, 32] and this is highly important especially in the case that therapeutic targets are expressed in CTCs and not in the primary tumor. We present the main findings of the application of molecular detection technologies for the molecular characterization of CTC (summarized in Table 1).

3.1 Gene Expression Studies

CK-19. As already mentioned above, CK-19 is the most widely used epithelial marker in molecular assays for CTC detection and enumeration [6–9, 14–20].

Table 1 Molecular characterization of CTCs based on molecular detection technologies

Markers expressed in CTCs	Cancer type	Analytical methodology	Refs
Gene expression			
CK-19	Breast cancer/early: before adjuvant chemotherapy	Nested RT-PCR	[4]
CK-19	Breast cancer/early: after adjuvant chemotherapy	Nested RT-PCR	[5]
CK-19	Breast cancer/early: node negative patients	Real-time RT-qPCR	[6]
CK-19	Breast cancer/early: after adjuvant chemotherapy	Real-time RT-qPCR	[9]
CK-19, hMAM, HER-2	Breast cancer/early	Multimarker RT-PCR	[7]
HER-2	Breast cancer/ operable: after adjuvant chemotherapy	Nested RT-PCR	[40]
hMAM	Breast cancer/ operable	Nested RT-PCR	[17]
ER/PR	Breast cancer/early	RT-PCR	[26]
CK-19, HER-2, MAGE-A3, hMAM, TWIST-1, hTERT $a+\beta+$	Breast cancer/early and metastatic	Multiplex RT-qPCR	[28]
CK-19, HER-2, MAGE-A3, hMAM, TWIST-1	Breast cancer/early and metastatic	Liquid bead array	[29]
CK-19, HER-2	Breast cancer/patients prior chemotherapy	RT-PCR	[37]
EpCAM, MUC1, HER-2, ER, PR	Breast cancer/ metastatic	AdnaTest	[35]
DNA mutations			
EGFR	Non small cell lung cancer	DNA extraction from CTC, SARMS assay (Real-time PCR for mutation detection)	[45]
BRAF V600E	Melanoma	DNA extraction from CTC, peptide nucleic acid-clamping PCR assay	[46]
Androgen receptor	Castration resistant prostate cancer	DNA extraction from CTC	[47]
KRAS	Colorectal cancer	DNA extraction from CTC, digital PCR mutation detection assay	[48]
MiRNAs			
10 MiRNAS identified as differentially expressed in CTC	Breast cancer	Real-time RT-PCR	[49]

HER2. HER2 analysis in CTC may have clinical significance for HER2-targeted therapy. There is now a growing body of evidence that HER2 status can change during disease recurrence or progression in breast cancer patients [33]. Based on this, re-evaluation of HER2 status by assessment of HER2 expression on CTC is a strategy with potential clinical application [34–41]. Therapy-resistant CK-19 mRNA-positive cells in peripheral blood could be effectively targeted by trastuzumab administration [37]. Moreover, the detection of HER2 mRNA-positive CTC after the completion of adjuvant chemotherapy may provide clinically useful information concerning the efficacy of treatment and the prognosis of patients with operable breast cancer [40]. Changes of HER2 status in CTC compared with the primary tumor during treatment for advanced breast cancer have also been reported [40, 41]. Detection of HER2 mRNA-positive CTC after the completion of adjuvant chemotherapy may provide clinically useful information concerning the efficacy of treatment and the prognosis of patients with operable breast cancer [42].

ER/PR. The expression of estrogen receptor (ER) and progesterone receptor (PR) was assessed in CTC by RT-PCR by Fehm et al. [26]. According to their findings, the spread of CTCs was mostly found in triple-negative tumors and CTCs in general were mostly found to be triple-negative regardless of the ER, PR, and HER2 status of the primary tumor [26].

Mammaglobin. Mammaglobin expression has also been reported in CTCs and has been correlated with prognosis in patients with breast cancer [7, 17, 24].

EGFR. Payne et al. have shown that measurements of EGFR expression on circulating tumor cells are reproducible over time in metastatic breast cancer patients [18], while Liu et al. have shown that eradication of EGFR-positive circulating tumor cells and objective tumor response with lapatinib and capecitabine [19].

Cancer stem cell markers and EMT markers in CTCs. The expression of stemness and EMT markers in CTCs was associated with resistance to conventional anti-cancer therapies and treatment failure, highlighting the urgency of improving tools for detecting and eliminating minimal residual disease [43]. Although the relationships between EMT and CTCs remains largely unexplored, data validating the implication of EMT processes in CTC formation and animal models with transplantable human breast tumor cells to help characterizing EMT/CTC relationships have been recently reviewed. Indeed, through many different studies it has been shown that subsets of CTCs have a putative breast cancer stem-cell phenotype, and express EMT markers. The expression of cancer stem cell markers such as CD44, CD24, or ALDH1 by molecular assays [25] has also been shown in CTC.

3.1.1 DNA Methylation

In a recent study, Chimonidou et al. [44] analyzed DNA extracted from EpCAM-positive immunomagnetically selected CTC fraction for the presence of methylated and unmethylated *CST6*, *BRMS1*, and *SOX17* promoter sequences by methylation-specific PCR (MSP). According to this study, these tumor suppressor and metastasis

suppressor genes are epigenetically silenced in CTCs isolated from peripheral blood of breast cancer patients. These findings add a new dimension to the molecular characterization of CTC.

3.1.2 Mutations

Moreover, the molecular characterization of CTC may provide a strategy for noninvasive serial monitoring of tumor genotypes during treatment. Few studies till date have evaluated the presence of specific DNA mutations in CTC. Maheswaran et al. have captured highly purified CTC from the blood of patients with non-small-cell lung cancer (NSCLC) using a microfluidic device containing microposts coated with antibodies against epithelial cells and performed EGFR mutational analysis on DNA recovered from CTC using allele-specific PCR amplification. In this way they identified EGFR activating mutation in CTC for the first time [45]. Kitago et al. have tested for the expression of a melanoma-associated gene panel (MLANA, MAGEA3, and MITF) with RT-qPCR and for the presence of BRAFmt (a BRAF gene variant encoding the V600E mutant protein) in immunomagnetic beads—isolated CTCs from melanoma patients. By using a sensitive peptide nucleic acid-clamping PCR assay for BRAFmt analysis, they detected BRAFmt in 81% of the 21 assessed stage IV melanoma patients [46]. Moreover, Jiang Y et al. have recently detected coding mutations in the AR (androgen receptor) gene in CTC isolated from patients with castration-resistant prostate cancer, by using the CellSearch system for CTC isolation and subsequent molecular analysis in DNA isolated from CTC [47]. Punnoose et al. have shown that nucleic acids prepared from CTC captured using the CellSearch RUO profile kit were also amenable to biomarker assays including an RT-qPCR gene expression assay for breast cancer molecular subtype and a PCR assay for KRAS mutations [48].

3.1.3 miRNAS

Sieuwerts et al. used RT-PCR to study the expression of microRNAs (miRNAS) in CTC isolated with the EpCAM based CellSearch profile kit. They identified ten miRNAs that were more abundantly expressed in samples from patients with at least five CTCs in 7.5 ml of blood compared with samples from nine patients without detectable CTCs and healthy donors [49].

4 Quality Control in CTC Detection Systems: Comparison of Different Methodologies

The lack of standardization and harmonization of different technology hampers the implementation of CTC measurement in clinical routine practice. Clinical results of CTC analysis largely depend on the detection technology used. Despite the fact that most of these methods are highly specific and sensitive, there are not so far extensive studies especially designed to compare their efficacy when using the same clinical samples. This is an important issue for their clinical use, since especially in early disease, differences in analytical sensitivity between these methods plays a very critical role. Thus, standardization of micrometastatic cell detection and characterization is important for the

incorporation of CTCs into prospective clinical trials testing their clinical utility. Numerous single-institutional studies suggest that CTCs play an important role for risk stratification and monitoring of therapeutic efficacy. These findings need to be evaluated in trials to verify the principle of this concept in the clinical setting.

RT-qPCR based molecular methods can be used for routine clinical laboratory use since they can be standardized according to recently clearly described required quality issues such as Cq values, limit of detection, precision and accuracy, and recovery experiments [13]. A direct comparison of DTC detection rates in a large cohort of 385 patients using both standardized ICC and RT-PCR protocols has shown a significant correlation between ICC and RT-PCR ($P < 0.01$) and the results of both methods agreed in 73% of cases (280/385) [50]. Another recent study was designed to directly compare three techniques for detecting CTCs in blood samples taken from 76 patients with metastatic breast cancer (MBC) and from 20 healthy controls: the CellSearch CTC System, the AdnaTest Breast Cancer Select/Detect and a previously developed qRT-PCR assay for the detection of CK-19 and mammaglobin transcripts [51]. A substantial variation in the detection rates of CTCs in blood from breast cancer patients using three different techniques was observed. A higher rate of positive samples was observed using a combined qRT-PCR approach for CK-19 and mammaglobin, which suggests that this is currently the most sensitive technique for detecting CTCs. Standardization of the AdnaTest BreastCancer kit and direct comparison with other established breast cancer CTC enrichment and detection techniques is still lacking, but highly needed.

What is also very important is the fact that especially in early disease, CTCs are extremely rare as rare events follow the Poisson distribution [52]. To detect these cells occurring at these low frequencies reliably, a high assay efficiency and highly standardized preparation protocol are an absolute necessity. The limit of detection in the case of CTC is not limited by addition of extra CTC identifiers or instrument improvement but by the amount of blood that can be examined for the presence of CTC. This has to be taken into account seriously prior to starting any analysis, especially in the case of early disease [52].

Various studies address quality control issues in CTC, by comparing different methodologies, as outlined in a recent review [11].

5 Conclusions: Future Perspectives

Molecular characterization of CTCs will provide important information for identification of therapeutic targets and understanding resistance to therapies. Further research on the molecular characterization of CTCs will contribute to a better understanding of the biology of metastatic development in cancer patients. Toward this direction the combination of modern powerful technologies such as advanced imaging systems, molecular detection technologies, and next generation sequencing will enable the elucidation of molecular pathways in CTCs and lead to the design of novel molecular therapies specifically targeting CTCs.

The major advantages of molecular assays for CTC analysis are the following: (a) they give information only for living cells, (b) enable the analysis of a variety of molecular markers, such as gene expression, DNA methylation, DNA mutations in CTC, (c) offer extreme sensitivity, (d) offer a significant flexibility in the clinical lab setting, in terms of high-throughput analysis, multiplexing, (thus reducing the amount of sample required), time and cost of analysis, and quality control issues.

Molecular detection technologies enable the molecular characterization of CTC, and offer a significant flexibility in the clinical lab setting, in terms of high-throughput and cost-effective analysis, multiplexing, and quality control issues.

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