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REVIEW

Role of Circulating Tumor Cells and Cell-Free Tumor Deoxyribonucleic Acid Fragments as Liquid Biopsy Materials in Breast Oncology

Pelin TELKOPARAN AKILLILAR,[1] Gökhan YILDIZ[2]

[1]Department of Medical Biology, Yuksek Ihtisas University Faculty of Medicine, Ankara-*Turkey*
[2]Department of Medical Biology, Erzincan University Faculty of Medicine, Erzincan-*Turkey*

SUMMARY

Breast cancer is the most commonly diagnosed type of cancer among females worldwide. It is also the leading cause of female cancer-related death in developing countries. Though novel biomarkers and new strategies for diagnosis, monitoring course of disease, and treatment of breast cancer have been found, these methods are invasive, costly, labor-intensive, and inadequate to fully gauge treatment response and disease recurrence. Therefore, novel biomarkers with greater sensitivity and specificity, and which are easy to perform are needed in breast cancer oncology. There is growing interest in the potential use of circulating tumor cells and circulating tumor deoxyribonucleic acid fragments in liquid biopsy as non-invasive biopsy materials for early detection of breast cancer, monitoring disease progression, and understanding reasons for treatment resistance. This review is a discussion of current status of utilization of these liquid biopsy materials in breast oncology.

Keywords: Breast cancer; liquid biopsy; circulating tumor cells; cell-free tumor DNA fragments; biomarker.

Introduction

Breast cancer (BC) is the most common metastatic cancer type, which accounts for globally 25% of all cancer cases and 15% of all cancer deaths among females.[1] It is estimated that 1.7 million new cases and more than 500.000 BC related deaths occurred in 2012.[1] The incidence of BC has increased in Turkey during the last decades.[2] The increasing costs of BC treatment and screening are among growing public health problems of Turkey.[2] With the advent of next generation sequencing (NGS) and high-throughput gene expression profiling technologies, new biomarkers were identified and novel diagnosis methods were developed in recent decades for several cancer types, including BC.[3] However, BC related mortality rates are still high due to resistance to current drug therapies and cancer metastasis.[4] The overall sensitivity and specificity of current BC therapy approaches are between 60–70%.[5] In addition, histopathological analysis procedures of cancer tissues are invasive, cost inefficient, time consuming, and potentially risky for patients.[6] Hence, there is a need for discovery of novel biomarkers with greater sensitivity and specificity to allow more personalized cancer management to determine disease prognosis and to monitor treatment response. Whereas, tumor tissue itself is the major source of tumor DNA, using biopsy to isolate tumor DNA is an invasive, costly, risky approach, and it is sometimes inappropriate to perform. Analysis of peripheral blood (PB) samples

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Dr. Pelin TELKOPARAN AKILLILAR
Department of Medical Biology,
Yuksek Ihtisas University Faculty of Medicine,
Ankara-Turkey
E-mail: pelinta@yiu.edu.tr

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Table 1 The advantages of liquid biopsy collection over standard biopsy

Standard biopsy	Liquid biopsy
Invasive	Minimally invasive
Not easy to obtain from some organs	Easy to obtain from patient blood
Expensive	Less expensive
Long processing time	Short processing time
Sometimes high failure rate (Due to tumor not identified or quantity not sufficient)	Low failure rate
Serial biopsies are difficult to tolerate throughout the disease process	Serial biopsies can be tolerated throughout the disease process
Sample can remain stable when processed for long periods of time under *ex vivo* conditions	Sample can remain stable for long periods of time under *ex vivo* conditions
The evaluation of tumor heterogeneity is limited to that of the analyzed biopsy	Can capture tumor heterogeneity
Not easy to follow treatment response	Easy to follow treatment response

of cancer patients is called 'liquid biopsy' (LB).[7] Recently, LB is becoming hotspot research topic mainly due to its remarkable advantages in comparison to tissue biopsy (Table 1). LB samples include circulating tumor cells (CTCs) and cell-free DNA fragments (cfDNA) of cancer cells, which are called circulating tumor DNA fragments (ctDNA), among many other biological materials in the patient blood (Figure 1).[7] In this review, we focus on CTCs and ctDNAs detection methods and practical utilization of these materials as diagnosis, prognosis and therapeutic response monitoring biomarkers in breast oncology.

Roles of CTCs in BC

Tumor cells that detach from their primary site and enter to blood stream or lymphatic vessels to metastasize are called CTCs.[8] It is estimated that, nearly 1 million tumor cells enter circulation everyday, but 85% of them disappear within 5 minutes. Therefore, only one CTC can be found in 1 ml of blood sample or 1 CTC per billion of nucleated hematopoietic cells in blood.[8] So, even though CTCs were first described in 1869,[9] recent advancements in single cell analysis techniques in the last decade rendered CTC research area as one of the hotspot research topics of cancer research, especially for BC.

CTC detection methods

Since CTCs are in less number than the other cell types in blood samples, several two-step approaches are used as breast CTC detection methods. At the first step, CTCs are selected in LB samples either by positive selection (enrichment) or negative selection (depletion) methods.[10] Immunomagnetic-/microfluidic-based methods and filtration (isolation by size) method are used as positive selection methods, while density-gradient centrifugation method is used as negative selection method at the first step. Epithelial cell adhesion molecule (EpCAM) is the most frequently used biomarker targeted by immunomagnetic enrichment methods.[10] ISET (isolation by size of epithelial tumor cells) filtration of CTCs method is used based on the fact that CTCs are slightly bigger than red and white blood cells.[11] Cells, other than CTCs in the blood are cross-linked to erythrocytes and removed for negative selection with density gradient centrifugation.[11] At the second step, the isolated cells are either subsequently immunostained to detect CTCs by fluorescence microscopy, flow cytometry or tumor-related messenger ribonucleic acid (mRNA) transcripts are detected by reverse transcription-polymerase chain reaction (RT-PCR) method.[11]

CellSearch system is currently the gold standard method for breast CTC detection by being the only Food and Drug Administration (FDA)-approved method.[10,11] This system targets EpCAM molecule on cell surface of CTCs for positive selection at the first step of CTC detection. EpCAM-positive CTCs are isolated from blood by immunomagnetic selection and then fluorescently labeled for DAPI, CD45 and CK19 to be sure about breast CTCs at the second step of the system.[10,11] Currently, CTCs isolated with CellSearch system are in utilization for prognosis evaluation of BC patients. Downstream high-throughput genomic, transcriptomic or proteomic characterization methods can also be used as alternatives of validation step of the system.[10,11] However, mRNA expression detection based RT-PCR or probe-based methods are currently

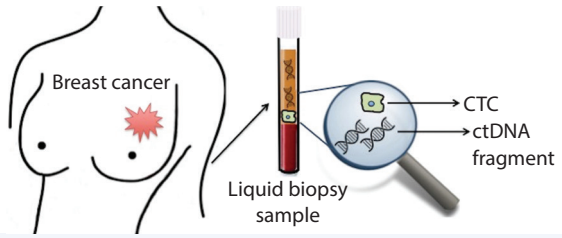

Fig. 1. Breast cancer related circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) fragments are released into blood circulation by tumor cells and they can be isolated from the blood of breast cancer patients.

not alternatives because these methods have less specificity compared to the other methods. Thus utilization of mRNA expression detection methods have been discontinued as part of the detection systems, but studies are ongoing.[10,11]

The other frequently used methods for CTC detection from LB of BC patients are AdnaTest, which is another immunomagnetic enrichment based method, EPISPOT-assay that detects CTCs via specific proteins such as CK19, fluorescence in situ hybridization (FISH) technique that detects chromosome aberrations in the CTCs, micro RNA (miRNA)-profiling that detects CTCs by profiling altered miRNA expression, 'CTC-chips' that detect EpCAM-expressing cells and mRNA-based PCR detection methods can be used.[12]

Clinical utilization of CTCs in breast oncology

Breast tumor cells encountered at secondary homing sites, such as bone marrow (BM) and PB, are currently seen as surrogate markers and precursors of distant metastasis.[13] At the late 90's, several studies investigated the role of BM disseminated tumor cells (DTC) in the micrometastatic process of BC. After that, several groups developed different techniques to detect DTCs in BM of early BC patients, mostly based on epithelial cell staining and cytological visual screening.[13,14] However, BM DTC detection methods have not been implemented in the routine clinical workup of early BC patients and only limited attempts were initiated to demonstrate clinical utility of these methods because these techniques were labor-intensive.[14] On the other hand, technological advancements on single cell research techniques and minimally invasive nature of LB, attracted attention on roles of CTCs in breast oncology and numerous clinical trials were performed in relative easy mostly using recently developed methods such as CellSearch and AdnaTest.

The SUCCESS trial, which is the largest clinical trial on the prognostic relevance of CTCs in early BC, observed CTC status of prechemotherapy and post-chemotherapy BC patients and determined that CTC-negative patients have higher overall survival (OS) and disease-free survival (DFS) values both before and after chemotherapy compared to CTC-positive patients. Thus, the SUCCESS trial demonstrated that CTC persistence correlates with shorter DFS and OS in early BC.[15] In addition, this trial determined that, women with at least five CTCs in 30 ml of blood samples had highest risk for relapse at early BC, and this cut off value is still in clinical use. Another study determined that at least one CTC presence in 7.5 ml blood of early BC patients is an independent predictor of shorter DFS and OS.[16] Although evidence from CTC-based clinical trials showed that persistence of CTCs in blood is an important predictor of worse survival in large trials, data supporting prognostic relevance of specific CTC subtypes in early BC is limited; because expression profiles of CTCs may not correlate with their corresponding primary tumor subtype.[17] However, clinical trials on possible use of CTC monitoring to decide therapy choices for early BC are still ongoing. The ongoing TREAT CTC trial is the first LB-based large study evaluating the concept of targeting chemoresistant early BC.[18] In this clinical study, HER2 status of the CTCs are assessed and effects of trastuzumab treatments are evaluated based on CTC counts.

Possible utilizations of CTCs as prognosis, therapy monitoring and therapy selection tool in metastatic BC are also evaluated with several clinical trials. In general, results of these trials indicate that, 40–80% of patients with metastatic BC have CTCs in PB. In addition, the study of Cristofanilli et al. demonstrated that, patients who have CTC counts above the cutoff value of at least 5 CTCs in 7.5 ml blood when they were diagnosed are associated with impaired clinical outcome.[19,20] The prognostic value of this cutoff value has been further verified by several studies and still in utilization.[21,22]

Besides the prognostic role of CTC status, alteration in CTC levels during treatment has also been shown to reflect therapy response in metastatic BC. The study of Hayes et al. indicated that, a decrease in CTC levels under the threshold of five-cells/7.5 ml PB predicted better PFS and OS in metastatic BC.[23] In addition, treatment efficacy assessment with CTC counting provided better prediction results compared to the standard radiological imaging in metastatic BC patients.[24]

Although prognostic significance of CTC counting has been proven for metastatic BC, studies on effects

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of specific CTC types on patient survival have contradictory results. For example, while one study determined that patients with HER2-positive CTCs had significantly longer PFS,[22] another study showed that patients with HER2-positive CTCs had significantly worse survival;[25] and another study determined that HER2 status of CTCs in metastatic BC had no correlation with clinical outcome.[26] An ongoing clinical trial called DETECT may provide results to resolve this enigma. Because in this study women who have HER2-negative metastatic BC with at least one HER2-positive CTC; and women who have HER2-negative BC (hormone receptor-positive or triple-negative) and exclusively HER2-negative CTCs are in study groups.

Roles of ctDNAs in BC

cfDNAs are short (160–180 bp), non-cellbound nucleic acid fragments in blood circulation. The discovery of cfDNA dates back to 1940s; Mandel and Metais reported presence of cfDNA in cell-free blood compartment in 1948.[27] They detected cfDNA in bloodstream of healthy individuals and patients.[27] In 1965, Bendich and colleagues became the first researchers hypothesized that cancer-induced cfDNA could be associated with metastasis.[28] Two different groups observed presence of same K-RAS and N-RAS mutations in tumor tissues and isolated cfDNA in blood samples of cancer patients in 1994.[29,30] At the following years, in addition to RAS mutations, other known cancer tissue specific mutations (such as TP53 mutations) were detected in ctDNA as part of the total cfDNA pool, which specifically derived from tumors in plasma isolated cfDNAs of the patients with several different cancers including breast, colon, lung, melanoma and hepatocellular carcinoma.[31] In addition to genetic alterations, cancer tissue specific epigenetic alterations such as hyper-methylation in promoter of suppressor genes were also identified in blood ctDNAs of cancer patients.[32] Recent advancements in genomics and bioinformatics research techniques cause increase in cfDNA detection method development and clinical utility investigation research studies.

cfDNA detection methods

cfDNAs can be detected in both from plasma and serum. Because the lower background concentration of cfDNAs, researchers mostly prefer to isolate them from plasma rather than serum.[33] The analysis of tumor specific cfDNA requires sensitive detection techniques to separate small fraction of tumor specific circulating DNA from others. There are mainly two approaches to analyze plasma-collected cfDNA; quantification of the presence of ctDNA from whole cfDNA in plasma, and identification of tumor specific genomic alterations including point mutations, chromosomal and microsatellite alterations and methylation changes. The sensitivity of traditional approaches to DNA analysis is insufficient for detection of somatic mutations in plasma ctDNA from patients with cancer. To overcome these limitations a variety of digital PCR (dPCR) methods have been developed with a high level of analytical sensitivity and specificity as alternative techniques to classical quantitative-RT-PCR (q-RT-PCR) for absolute quantification and detection of genetic alterations in ctDNA isolated from LB cancer patients.[34] dPCR has permitted to detection of fragmented and low abundant cell free nucleic acid targets from body liquids in a short time period.[34] It has been shown that dPCR identifies copy number variations that differ by only 1 copy and identifies allele frequencies lower than 0.1%.[35] Furthermore, dPCR detects point mutations, genetic alterations (loss of heterozygosity, aneuploidy), and copy number alterations in ctDNA.[34] dPCR technology improves ctDNA recovery and decreases the lower limit of detection to 0.01%.[34,36]

One of the successful dPCR molecular techniques is called BEAMing (Beads, Emulsion, Amplification and Magnetics) that consist of emulsion PCR with magnetic beads and flow cytometry for highly sensitive detection and quantification of ctDNA fragments.[37] The more recently developed technology of dPCR is droplet digital PCR (ddPCR).[38] In ddPCR method, a DNA sample is partitioned into 10.000 to 20.000 droplets to provide a digital counting of nucleic acid targets in the chip-based platform.[38]

Due to high efficiency and low-cost, high-throughput NGS technologies have started to be used frequently to identify genetic alterations in plasma ctDNA.[39] Many different targeted deep sequencing approaches (Tamseq, Safeseq, Ion-Ampliseq CAPP-seq) are in use to analyze known cancer-related mutations such as EGFR, BRAF, KRAS.[40] In addition, non-targeted genome-wide analyses enable the identification of tumor specific changes without prior knowledge about the aberrations present in the tumor. Furthermore, such approaches can be used to discover genetic changes underlying therapy resistance and to identify new feasible targets for cancer patients.

In future, dPCR and NGS methods will likely to be used as complementary methods for LB analyses fluid biopsy assays. The dynamic individual mutations can be detected by using former approach, but it requires

prior knowledge about the mutant allele. New generation methods make it possible to discover novel mutated variants, but have higher costs and cannot be easily applied to long-term patient follow up.

Use of cfDNAs in breast oncology

There are a number of studies that try to evaluate utility blood cfDNA level determination approach for distinguishment of benign and malignant breast tumors. These studies identified positive correlation with cfDNA counts and BC compared to healthy people.[41] However, more studies are required for clinical utility of this approach, because the defined ranges are wide and overlapping. Thus, currently cfDNA quantification approach for BC diagnosis and screening is not eligible to use in breast oncology.

The size and integrity of isolated ctDNAs of different stage BC patients and healthy donors were also compared in several studies to identify utility of this approach for early diagnosis and stage determination for BC. Umetani et al. identified that, mean serum DNA integrity was significantly higher in stage II-IV BC patients compared to healthy donors, but not statistically significantly different between normal and stage 0 or stage I individuals.[42] Iqbal et al. also found higher DNA integrity in BC patients, especially in stage IV patients, compared to healthy controls.[43] However, clinical utility of this approach is low because of its low ability on distinguishment of early BC patients and healthy women.

The utility of identification and quantification of BC specific alterations in ctDNAs of BC patients were also studied. Chimonidou et al. identified promoter methylation in CST6 gene from cfDNAs in 13–40% of BC patients but in none of healthy controls.[44] Dulaimi et al. determined hypermethylation of RASSF1A, APS, and DAP kinase gene promoters in the ctDNAs of 70% BC patients and none in serum DNA from healthy women.[45] Oshiro et al. found PIK3CA mutations in cfDNAs in 22% of BC patients who have PIK3CA mutations in their tumors, but none in healthy women or patients with non-PIK3CA mutated BC.[46] Investigation of BC specific genetic alterations in ctDNAs of BC patients seem promising for its clinical utility due to high specificity for BC, but sensitivity of this approach need to be increased with additional studies.

Several other studies have also conducted to determine prognosis and therapeutic response monitoring functions of breast ctDNAs. Studies in BC patients have determined that cfDNA concentrations decrease after surgery and chemotherapy,[47,48] and post-operative detection of ctDNAs was predictive of early relapse for BC.[49] Thus, breast ctDNAs can be potentially used for therapy response observation for BC patients. However, current studies indicate that ctDNA levels do not reflect BC prognosis, and further studies are required.[41]

Conclusion and Future Perspectives

Since CTCs and ctDNA fragments may be originated from a number of metastatic sites, these LB derived materials are potentially better representatives of the whole disease compared to single site biopsy. However, most of the current CTC detection methods are able to isolate and detect only epithelial type CTCs by targeting EpCAM-positive epithelial CTCs; yet it is known that mesenchymal-type of CTCs have also been observed as a result of epithelial-to-mesenchymal transition (EMT) in human BC patients.[12] For example, EpCAM-negative breast CTCs, which metastasize to brain have recently been identified.[50] Identification of these CTCs suggests that, EpCAM-negative CTC sub-populations may be present in LB samples of BC patients that need to be identified. In order to detect all heterogeneous types of breast CTCs novel antigen-independent CTC enrichment techniques need to be developed in the future.

As presented, CTCs are invaluable tools, which have clinical utilization during disease stage evaluation, disease progression monitoring and targeted personalized therapy development applications for breast oncology. The real-time monitoring and characterization of CTCs can provide administration of suitable and personalized targeted therapy for BC patients compared to other methods. For example, human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER) and progesterone receptor (PgR) status of breast cancer patients can be monitored with CTCs in real-time to evaluate stage and progression of the disease as well as to decide suitable targeted and personalized therapies for BC patients. However, more studies are needed.

The quantitative and qualitative analysis of ctDNA in BC demonstrate tumor-associated genetic and/or epigenetic alterations and treatment response in BC patients.[19,31,46,47] Before utilization of ctDNA analysis methods in clinical practice, more data should be obtained by using different methods, and more clinical studies are needed. Since the patient-derived ctDNAs only inform us about the dying tumor cell genomes, the obtained data may be misleading about the genetic alterations in resistant tumor cell populations, and this possibility should also be further investigated.

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In conclusion, CTCs and ctDNAs have potential to be major biomarkers of diagnosis, prognosis monitoring and therapy monitoring tools for personalized therapy in breast oncology, in the near future.

Disclosure Statement

The authors declare no conflicts of interest.

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