12 Potential and Challenges of Liquid Biopsies

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Abstract

Although tumor genotyping is still the most currently used method for categorizing tumors for clinical decisions, tumor tissues provide only a snapshot or are often difficult to obtain. To overcome these issues, methods are needed for a rapid, cost-effective, and noninvasive identification of biomarkers at various time points during the course of disease. The analysis of circulating tumor cells (CTCs), cell-free circulating tumor DNA (ctDNA), circulating RNAs, and exosomes, frequently referred to as liquid biopsy, has recently gained enormous momentum. Due to technological advances, novel circulating tumor biomarkers were shown to have a great potential to improve patient treatment in terms of estimation of prognosis, monitoring treatment response, early detection of resistance mechanisms, identification of actionable targets, and detection of minimal residual disease. However, despite all efforts, liquid biopsies are not yet routinely used mainly due to technological hurdles, lack of analytical and pre-analytical standards and conclusive evidence that patients indeed benefit from such analyses. In this chapter, the different entities with respect to state-of-the-art technologies, potential clinical applications, and their limitations are discussed.

12.1 Introduction

Liquid biopsies are noninvasive blood tests that detect and analyze circulating biomarkers, such as circulating tumor cells (CTCs), cell-free circulating tumor DNA (ctDNA), circulating RNAs, and exosomes, released from primary tumors and their metastatic deposits. In general, liquid biopsies are highly beneficial compared to tissue biopsy since repeated sampling during the entire disease course is easily achievable. Here we discuss the current impact and future directions of liquid biopsies, including the biology of the different entities, pros and cons, current state-of-the-art technologies, and their potential clinical applications.

12.2 Circulating Tumor Cell

12.2.1 Biology of Circulating Tumor Cells (CTCs)

The hematogenous dissemination of tumor cells, either as single cell or as tumor cell cluster, is an important step in metastasis formation. Cancer cells can leave their primary site already at early stage of disease [1]. As a first step, tumor cells intravasate into the vascular system followed by physical arrest at capillary walls of a distant organ and eventually extravasate from the vascular system into the parenchyma of target organs [2]. These steps of metastasis formation are shown to be very inefficient, as the number of CTCs greatly exceed the number of formed metastases [3]. Generally, the passage in blood is a very stressful event for CTCs which most of them do not survive [2]. Experimental data in mice have shown that CTCs which travel in clusters are more protected and more proliferative at the target site, eventually bringing their own tumor stroma [4]. Additionally, platelets which are coating the cell surface of CTCs have been shown to promote CTC survival [5]. To progress from a

CTC to a full-blown metastasis, several other factors are necessary. Those factors include the stemness-like features of CTCs [6], suppression of immune defense [7], and a supportive niche by the adjacent stroma to sustain tumor survival and growth [8]. In the metastatic cascade, a biological process called “epithelial to mesenchymal transition” (EMT) has been described as an important factor [9]. The EMT is linked to an upregulation of genes, such as vimentin, N-cadherin, Twist, Snail, and others, which are associated with mesenchymal cells [10]. Vice versa, epithelial markers, such as cytokeratins, are downregulated [10]. Cells which undergo EMT show a stemness-like behavior, being more aggressive in forming a successful metastatic lesion [11]. To address the different subtypes of CTCs, such as mesenchymal- or epithelial-like phenotypes, a broad array of technologies is in place. In the following section, we focus on a few promising technologies, which are described below.

12.2.2 Methodological Aspects for the Isolation and Analysis of CTCs

CTCs are a group of extremely rare cells, with 1–10 cells in 1 mL of peripheral blood which contains few millions of leukocytes and billions of erythrocytes [12]. Therefore, technologies for CTC enrichment are required to be highly sensitive and specific, which remains technically challenging. Up to now, more than 50 different platforms have been described, which are reviewed by Alix-Panabiers et al. [13]. In general, two different approaches are used for the detection and isolation of CTCs. The first approach is making use of biological properties of CTCs, such as expressed epithelial-derived antigens on the cell surface. The second approach is exploiting the physical properties that distinguish CTCs from most peripheral blood cells, including cell density, cell size, electrical charges on the surface of the cell membrane, and deformability of cells. However, since an efficient isolation of CTCs in a viable and intact state is preferable, isolation methods based on physical properties provide a compelling advantage over those relying on fixation and extra- or intracellular staining. A representative overview of currently available methods is shown in Table 1 and discussed in the next paragraph:

1. Exploiting biological properties

   Since CTCs express tumor-associated antigens on their surfaces—that are usually not found on blood cells—these biological features can be targeted by antibodies. Typically, a CTC is defined as a cell with an intact nucleus, being positive for the expression of epithelial cell adhesion molecule (EpCAM) and other epithelial markers, such as cytokeratin (CK), and negative for CD45 [27]. Therefore, EpCAM is a widely used epithelial marker for positive selection of CTCs. In order to obtain a higher specificity, many platforms use combined antibody staining of epithelial markers and the leukocyte-specific surface marker CD45. To date, the gold standard for CTC detection, the Veridex CellSearch system, which was first introduced in 2004, is the only Food and Drug Administration (FDA)-cleared CTC detection device for the enumeration of CTC in 7.5 mL of blood. Using the CellSearch system, numerous studies indicated the prognostic value of CTCs in metastatic breast, colon, prostate, and lung

Table 1 Representative overview of currently available methods

Enrichment device/method	Technique	Features and advantages	Limitations	Reference
Based on biological features				
CellSearch	Magnetic-activated cell sorting	FDA approved	Low flexibility for research applications	Allard et al. [14]
AdnaTest, AdnaGen	Immunomagnetic based	CTC detection and further transcript analysis	Only EpCAM-positive CTC is being detected	Antonarakis et al. [15]
CTC-chip HB-chip CTC-iChip Graphene oxide-Chip	Microfluidics	Gentle CTC enrichment → high viability	Slow, only low volume of blood can be processed	Nagrath et al. [16], Stott et al. [17], Yoon et al. [18], Yu et al. [19]
CellCollector	In vivo EpCAM-based capture	In vivo detection → large volume of blood	Imaging of CTCs on the CellCollector is challenging	Saucedo-Zeni et al. [20]
EPISPOT	Assay to detect secreted proteins	Viable CTCs can be detected	Indirect detection, as only the secreted proteins remain	Alix-Panabieres [21]
Based on physical features (size, density)				
Ficoll, Oncoquick™	Density gradient centrifugation	Density based	Nonspecific cell loss	Gertler et al. [22], He et al. [23]
RosetteSep™	Negative enrichment	Simple and can capture viable CTCs	Nonspecific cell loss	He et al. [23]
ScreenCell	Filtration-based size exclusion	Simple, no additional equipment needed	Size bias, small CTCs may be lost	El-Heliebi et al. [24]
ISET	Filtration-based size exclusion	Simple and fast	Size bias, small CTCs may be lost. Additional equipment needed	Hou et al. [25], Vona et al. [26]

cancer [28–31]. Another promising technology branch is represented by microfluidic chips which allow isolation of CTCs with high viability. One of the first microfluidic chips for CTC isolation was the CTC-chip, based on EpCAM expression [16]. Several generations of new microfluidic chips followed [17, 32,

33]. Using the CTC-iChip, Yu et al. succeeded in culturing CTCs isolated from blood samples of patients with metastatic estrogen receptor (ER)-positive breast cancer [19]. Furthermore, making use of established CTC lines, tumorigenic tests were performed in mice, which provided encouraging strategies for functional characterization of CTCs in addition to a simple enumeration and one-time genetic analyses [19]. Another promising EpCAM-based enrichment system is the AdnaGen system. The AdnaGen system is an optimized combination of antibodies for cell selection and subsequent RT-qPCR for tumor-associated expression patterns. In the first step, CTCs are enriched by magnetic beads coupled to EpCAM antibodies. In the following steps, the cells are lysed, the RNA is reversely transcribed, and specific targets are analyzed using quantitative PCR. For example, in prostate cancer, it was shown that the AdnaGen test could reliably detect the androgen receptor splice variant 7 (AR-V7) in CTCs, which is associated with resistance to antihormonal therapy [15]. Although this technology offers specific molecular characterization of CTCs, it does not support the quantification of the original CTC numbers.

Due to the low abundance of CTCs in the circulation, one of the major limitations for their detection is the low volume that can be obtained from one blood draw. Therefore, the most effective way to increase the CTC detection rate would be to increase the sample volume within the clinically allowable range without burdening the patient. In a study of Lalmahomed et al., it was shown that the analysis of 30 mL instead of 7.5 mL of blood resulted in 20% more patients having detectable CTCs [34]. Another possibility of further increasing the analyzable blood volume is an in vivo enrichment of CTCs. The CellCollector is an in vivo device, based on a medical wire which is coated with anti-EpCAM antibodies enabling the harvest of CTCs expressing EpCAM on their cell surface. The CellCollector is applied by inserting the wire into a cubital vein for 30 min. Within 30 min, it is estimated that 1.5–3 L of blood pass the CellCollector, capturing CTCs as they pass by [20, 35]. Recent data show higher detection rates of CTCs compared to other technologies [36].

An indirect approach to isolate CTCs is based on a negative enrichment strategy. Negative enrichment involves a red blood cell lysis, followed by depletion of CD45+ leukocytes using a magnetic bead separation method [37]. This allows an efficient enrichment of the CTC fraction to a maximum of 1% purity (e.g., 1 CTC to 99 leukocytes) [37]. A similar approach is the RosetteSep system, which combines Ficoll density gradient to remove cells from whole blood with a subsequent depletion of unwanted blood cells [23].

Another method for the detection of viable CTCs is the EPISPOT technology that detects proteins secreted/released/shed from single epithelial cancer cells. After leukocyte depletion, the enriched samples are put into plates that are coated with specific antibodies, directed against specific proteins expressed on the CTC surface. EPISPOT is targeting secreted proteins of CTCs rather than the CTCs themselves [38]. By this technique, viable CTCs can be indirectly counted as they leave a “footprint” of their secreted proteins on a membrane, which can be visualized.

2. Exploiting the physical properties

   There exist several technologies which exploit the physical properties of CTCs for their isolation. Filtration-based size exclusion technologies have been developed, such as ISET (isolation by size of epithelial tumor cells) or ScreenCell, which allow for antigen-independent isolation of CTCs from blood based on their larger size in comparison to hematological cells [24, 25]. CTCs can be isolated from diluted blood using a polycarbonate membrane with 8 μm-sized pores. Blood cells can pass the membrane, while CTCs are captured on the filter and can then be analyzed by light microscopy and immunocytochemistry [24]. Although a promising technology, size filtration-based methods have their limitation in the lack of specificity for CTCs as many non-CTCs are isolated by the filtration devices and may lead to false positivity. Downstream analysis of DNA or RNA can be performed, but needs labor-intensive technologies, such as laser capture microdissection [24, 39–41]. A novel microfluidic platform, called Parsortix, utilizes the size and deformability of cells to enrich CTCs from blood [42, 43]. The technology is based on a chip with physical “steps” in which tumor cells move upward. The CTCs get arrested at the top steps as they get stuck between the most upper step and the top lid of the microfluidic chip [43]. Blood cells which are smaller and more deformable will pass through [43]. Cells can then be forwarded to mRNA and DNA downstream analysis [43], and thereby Parsortix represents an attractive EpCAM-independent solution.

12.2.3 Clinical Use of CTCs

Established routine procedures to investigate a tumor site include imaging technologies and biopsies. As biopsies are an invasive procedure, and usually not conducted in a metastatic setting, liquid biopsies, such as CTCs, can represent a promising alternative. Potential clinical applications of CTCs include the monitoring of cancer progression, the prediction of relapses or drug resistances, and the evaluation of treatment efficiency [44–47].

The most widely used CTC technology currently in clinical testing is still the CellSearch platform since it is the only technology to have received FDA approval for the enumeration of CTC in whole blood in specific cohorts of cancer patients. A landmark study for the clinical application of CTCs that actually led to FDA clearance was published by the group of Cristofanilli in 2004 [48]. The authors showed for the first time that CTC counts in metastatic breast cancer before treatment were an independent predictor of progression-free survival (PFS) and overall survival (OS) [48]. Investigation with several other tumor entities followed these tracks. In castration-resistant prostate cancer (CRPC), CTC enumeration is the most accurate and independent predictor of OS [29]. Furthermore, in metastatic colorectal cancer, the number of CTCs before and during treatment was reported as an independent predictor of PFS and OS in patients [28]. Similar results were obtained from metastatic non-small cell lung cancer (NSCLC), where the CTC numbers were shown to be the strongest predictor of OS [30].

Besides the prognostic utility of CTCs, an important question is whether CTCs can also be used as a predictive marker, i.e., to what extent CTCs can lead to a treatment decision which in the end improves health outcomes. In the SWOG S0500 clinical trial, a total of 595 patients with metastatic breast cancer were recruited and stratified based on repeated CTC counts during the treatment [49]. Patients with consistently high levels of CTCs before, during, and after the first cycle of chemotherapy were switched to a different treatment, while patients with decreasing CTC counts remained on the initial treatment [49]. However, the SWOG S0500 trial failed to show an improved outcome, based on CTC numbers and their resulting change of therapy [49]. Nevertheless, it is not clear yet whether the CTC counts failed as a predictive marker or if the available drugs for metastatic breast cancer failed to improve patient outcome. On the other hand, a recent study showed promising results in metastatic castration-resistant prostate cancer if CTCs were analyzed for androgen receptor splice variants [15]. Detection of the splice variant 7 (AR-V7) of the androgen receptor in CTCs was associated with a resistance to antihormonal therapy with enzalutamide or abiraterone [15]. Moreover, the authors stated that AR-V7 status may serve as a treatment selection marker in metastatic castration-resistant prostate cancer [50]. This study shows that for a clinical utility of CTCs, downstream analysis may pose an important factor.

12.2.4 Limitations and Challenges of CTCs

The greatest limitation to translate CTC research into a clinical application is the scarcity of CTCs in blood. Especially in patients with early cancer disease, the low number of patients with detectable CTCs remains an obstacle. Furthermore, most platforms for CTC isolation need qualified personal and usually come along with high costs. In addition, the molecular analysis of single cells is a challenging and expensive endeavor. These are major contributing factors for its infrequent use in the clinical routine workflow. Nevertheless, from a research point of view, technologies, such as next-generation sequencing, have evolved dramatically in the past years, which will potentially lead to a better and affordable molecular analysis of CTCs which finally could lead to improvements of therapeutic strategies.

12.2.5 Summary

Better insight into the biology of CTCs may help to understand the metastatic cascade. The aforementioned methods show a wide range of different properties of CTCs, such as physical or biological ones. Each technology has its advantages and disadvantages, and maybe each tumor entity will need a specific CTC technology. The clinical application of CTCs was first proven in 2004, and 10 years later, analysis of androgen receptor splice variants in prostate cancer CTCs was shown to be of relevance for a treatment selection. Improvements of CTC isolation and novel molecular analysis tools led to novel diagnostic

applications for patient stratification. Especially functional assays of CTCs, such as CTC culture or CTC-derived xenografts, will stimulate CTC research for the coming years.

12.3 Circulating Tumor DNA (ctDNA)

12.3.1 Biology of Cell-Free DNA

The presence of cell-free DNA (cfDNA) in blood of healthy individuals was already described by Mandel and Metais in 1948 [51]. Despite their pioneering work, it took several decades until its clinical utility as a potential biomarker was recognized. Only in the 1970s, the occurrence of higher concentrations of cfDNA in patients with benign conditions, such as systemic lupus erythematosus (SLE) or rheumatoid arthritis, compared to healthy individuals was observed [52, 53]. Leon et al. reported elevated levels of cfDNA in the circulation of cancer patients, and in some patients, even a decrease of cfDNA after successful anticancer therapy could be observed [52]. Another 10 years later, Stroun et al. demonstrated the presence of tumor-specific aberrations in the circulation and therefore provided evidence that certain circulating DNA fragments originate from tumor tissues [54]. These findings were then confirmed by several other groups [55–58]. In the following years, other tumor-specific aberrations, including mutations in tumor suppressors and oncogenes [59], LOH [56], MSI [60], and DNA methylation [61], were identified and provided concrete evidence that cfDNA is released into the circulation by tumors, which is referred to as circulating tumor DNA (ctDNA).

There are now numerous studies about ctDNA available, but little is known about the origin, mechanisms and kinetics of release or clearance of cfDNA and ctDNA. Although it is thought that necrosis and active secretion contribute to cfDNA, the driving force for the release may be apoptosis. Cell-free DNA is highly fragmented, and assessment of the size distribution of cfDNA reveals an enrichment of fragments in the size of nucleoprotein complexes or multiples of them [62, 63]. More specifically, peaks corresponding to nucleosomes (147 bp) and chromatosomes (nucleosome + linker histone 167 bp) have been noted. This was also confirmed in mice experiments where the predominant fragments in plasma from xenografted animals were mononucleosome derived, indicating that apoptosis is the major source of cfDNA [64]. The authors demonstrated that ctDNA features vary during colorectal cancer (CRC) tumor development in nude mice that were xenografted with the human colorectal carcinoma cell lines HT29 or SW620 [64]. In a study of Heitzer et al., total plasma DNA concentrations and tumor-specific KRAS mutations in CRC patients were analyzed, and these data showed that a higher amount of tumor-specific fragments and a higher number of CTCs were linked to biphasic size distributions of plasma DNA fragments. However, despite advanced tumor stage, not all patients had detectable levels of ctDNA in their circulation [62].

It is now clear that cfDNA constitutes of a mixture of DNA released from cells from different tissues of the body. Studies of pregnant women have shown that the placenta is the origin of the cell-free fetal DNA detectable in the maternal circulation [65, 66]. Moreover, the investigation of circulating DNA pools after organ and bone marrow transplantations shed light on the different origins of cfDNA [67, 68]. These studies suggested that in healthy individuals, cfDNA is primarily derived from apoptosis of normal cells of the hematopoietic lineage, and material from other solid tissues contributes only to a small part of cfDNA [67, 68]. These data were confirmed by the Lo group, which used organ-specific DNA methylation signatures established by whole-genome bisulfite sequencing in order to trace back the origin of cfDNA fragments in pregnant women, patients with hepatocellular carcinoma, and subjects following bone marrow and liver transplantation [69]. Consistent with previous reports, the most abundant signature could be attributed to hematopoietic cells. The placental contributions in plasma of pregnant women range from 12.1 to 41.0% [69]. The graft-derived contributions to the plasma in the transplant recipients correlated with those determined using donor-specific genetic markers [69]. In cancer patients, a large part of the circulating DNA fragments could be associated with the primary tumor tissue [69].

In a recent study conducted by Snyder et al., it was shown that cfDNA is the detritus of cell death and that nucleosome phasing is reflected in the fragmentation pattern of cfDNA [70]. Since the boundaries of cfDNA fragments are biased by their association with nucleosomes, the fragmentation patterns of cfDNA might contain evidence of the epigenetic landscape of their tissue(s) of origin [70]. This might be a useful approach for the identification of cfDNA-releasing cells independent of genotypic differences between contributing cell types.

The fact that cfDNA is also released from normal cells is one of the major limitations of cfDNA as a diagnostic marker in cancer patients. The amount of ctDNA is highly variable and can range from less than 1% to more than 90% of total cfDNA [62, 71–73]. Despite continuous improvements in precision and accuracy of sequencing technologies, the fraction of ctDNA can be below the detection limit of these methods, especially in early-stage cancer, where in most cases insufficient levels of ctDNA for comprehensive analyses are present.

Although more and more studies deal with cfDNA origins, the clearance mechanisms of cfDNA are still poorly understood. The short half-life of cfDNA in the circulation suggests a model of ongoing release from apoptotic cells and rapid degradation or filtration [74]. A more recent study of Dennis Lo's group revealed a biphasic clearance with half-lives of about 1 h for the rapid phase and a second phase of 13 h [75]. It is of note that these data come from studies in pregnant women, and it is not clear yet whether these findings can be transferred to cancer patients. In contrast to fetal DNA, the massive accumulation of ctDNA in some patients might be a consequence of massive cell death due to a fast turnover of cancer cells, an inefficient degradation, or a combination of both. Moreover, it is not known how other factors, such as circadian rhythms, inflammation, or particular therapies, influence release and clearance mechanisms.

12.3.2 Methodological Aspects for the Analysis of ctDNA

Due to its high fragmentation and the low abundance in the circulation, the analysis of plasma DNA is challenging. However, recent advances in molecular technologies now offer the necessary sensitivity and specificity to detect small amounts of ctDNA in circulation. Although significantly higher cfDNA concentrations are present in serum than in plasma, plasma turned out to be the better source for ctDNA analyses [76]. In serum, cfDNA is "contaminated" by high molecular weight genomic DNA due to the clotting of white blood cells in the collection tube, leading to their lysis [77]. To minimize cellular degradation, even plasma should be immediately processed after blood collection in standard EDTA tubes. By the addition of cell-preserving reagents, which prevent white blood cell degradation, and inhibit nuclease-mediated DNA degradation, tubes can be stored for up to 14 days at ambient temperature [78]. A variety of methods have been used for the quantification of cfDNA [79, 80]. Although most studies dealing with extraction and quantification methods come from fetal cfDNA, the same issues apply for cfDNA from tumor patients. These are several technical confounders, including storage conditions or processing delay before plasma separation [77, 81], DNA extraction method [82], amplicon size, and target gene choice [83] that can influence the quantitation of cfDNA and, therefore, complicate data analysis, comparability, and reproducibility of the tests. The lack of generally accepted units of measure for cfDNA quantification further aggravates the situation. However, in recent years, efforts were made in order to establish benchmarks for standardization of the extraction and quantification of cfDNA.

Turning to the analysis of ctDNA, there are two different approaches used for the analysis, i.e., targeted and untargeted methods. Targeted methods are limited to the analysis of single or few known mutation or hotspots with clinical implications for therapy decisions, e.g., mutations in KRAS or EGFR. Since the first targeted mutation analyses in plasma or serum in the 1990s, technological progress has brought a number of highly sensitive methods, such as ARMS [84], digital PCR [85, 86], or BEAMing [87], which allow for the identification of mutant alleles at very low frequencies. A particularly sensitive and specific approach is the so-called personalized analysis of rearranged ends (PARE) [72]. This method involves the identification of tumor-specific translocations from the primary tumor that are monitored in plasma by the use of dPCR. This method can be used to detect tumor-specific changes at very low levels, i.e., in early stages, or to identify minimal residual disease; however, the availability of tumor tissue is required [88]. In a study by Heitzer et al., they were able to identify structural rearrangements directly from plasma after targeted enrichment of chromosomal regions that are frequently involved in translocations [89].

Although these methods achieve a high resolution, most of them interrogate only few loci. Novel occurring mutations or mutations in genes that lack mutational hotspots, such as tumor suppressors, are missing. One possibility of including driver genes without hotspots is targeted resequencing of selected genes that are known to be associated with tumorigenesis and progression. To this end there is striving for

the establishment of targeted enrichment of larger number of genes or chromosomal regions. CAPP-Seq, an ultrasensitive method for quantifying ctDNA, was introduced by Newman et al. [90]. This method combines optimized library preparation methods for low-input DNA with a multiphase bioinformatics approach to design a "selector" consisting of biotinylated oligonucleotides that target recurrently mutated regions in the cancer of interest [90]. The researchers have shown to detect ctDNA in 100% of patients with stage II–IV nonsmall cell lung cancer and in 50% of patients with stage I and with 96% specificity for mutant allele fractions down to approximately 0.02%. Another approach was developed by Forshew et al., the so-called tagged-amplicon deep sequencing (TAm-Seq) [91], including 5995 genomic bases for low-frequency mutations. Using this approach, they identified cancer-specific mutations present at allele frequencies as low as 2%, with a sensitivity and specificity of >97%.

In contrast, genome-wide, untargeted approaches offer several advantages compared to targeted methods, including the fact that no a priori knowledge about the genetic makeup of the tumor is necessary and that they are not limited to recurrent changes [44, 89, 92]. Such comprehensively designed studies are of particular interest in late-stage cancer since tumors evolve rapidly due to progression and the selective pressure of therapies. The establishment of genome-wide copy number profiles from plasma DNA can now be performed very quickly and cost-effective [89]. Several studies have shown that the evolution and the plasticity of tumors can be effectively tracked using such genome-wide approaches [62, 89, 92–94]. However, these analyses are still lacking sensitivity and require a certain amount of tumor-specific DNA (about 5–10%), which does not apply to many samples from patients in earlier stages. Recent studies demonstrated that even in highly metastasized patients, there are clinical situations where ctDNA is present below optimal levels for the detection of mutations [62, 71, 95]. An untargeted pre-screening methods called mFAST-SeqS can identify samples with sufficient ctDNA levels that are suitable for subsequent analyses with genome-wide methods [96].

12.3.3 Clinical Use of ctDNA

The first effort to use cfDNA as a biomarker focused on the simple quantification of DNA. Several studies reported significant differences in the amount of cfDNA isolated from healthy individuals, patients with benign disease, and cancer patients [57, 97]. In lung cancer patients, higher levels of cfDNA have been observed compared to disease-free heavy smokers, suggesting a new, noninvasive approach for early detection of lung cancer [57]. Kim et al. recently reported that changes in the levels of cfDNA can act as reliable biomarkers to detect cancer early, predict tumor burden, and estimate curative resection and even prognosis in gastric cancer [98]. In contrast, other studies demonstrated no association between cfDNA concentrations and clinical, biological, or histological characteristics [56, 99, 100]. Soon it became clear that the amount of cfDNA alone is not a suitable marker for cancer patients due to the highly variable amounts of circulating DNA fragments that partially

overlap with those of healthy individuals [56, 71, 97, 100]. However, the use of tumor-specific alterations in the circulation has already been applied in different scenarios of the therapy management of cancer patients.

12.3.3.1 Monitoring Tumor Burden and Minimal Residual Disease

Studies of the University of Cambridge and the Johns Hopkins University showed that the analysis of ctDNA is a better marker for the detection of recurrence of breast and colorectal cancer than conventional protein tumor markers [71, 101]. Dawson et al. used a personalized assay for a minimally invasive monitoring of treatment response in breast cancer patients. Changes in ctDNA levels showed a greater dynamic range and greater correlation with changes in tumor burden than CA 15-3 or CTCs. Another group from Lund University made use of a combined approach, including whole-genome sequencing of the primary tumor for the identification of rearrangements and digital PCR for monitoring purposes [88]. They retrospectively analyzed a set of breast cancer patients with localized tumors. Patients with detectable amounts of ctDNA after curative surgery developed metastases within a median time frame of 11 months, whereas those patients with no detectable ctDNA showed long-term progression-free survival [88]. A similar study was already reported in 2008 by Bert Vogelstein’s group. The authors showed a significant association of decreasing levels of ctDNA with progression-free survival (PFS). On the other hand, in patients in whom tumor-specific mutations could be detected after surgery, the tumors recurred [87].

12.3.3.2 Detection of Resistance Mechanisms

Another paradigm for the clinical use of ctDNA is the early detection of resistance mechanisms, which can only be ensured by a tight monitoring. One of the first clinically used applications is the minimally invasive monitoring of patients with non-small cell lung cancers (NSCLC) that are treated with specific tyrosine kinase inhibitors (TKI). Approximately 10–15% of NSCLC harbor activating mutations in the EGFR gene, which codes for the epidermal growth factor receptor (EGFR), a protein involved in cell proliferation and division. Patients with an activated EGF pathway benefit from an intracellular blockade of the receptor. However, 50% of patients that initially respond well to the treatment develop resistance within several months. The underlying mechanism is in most cases a secondary mutation in EGFR that hinders the TKI from binding its target. A close monitoring of known resistance mechanisms can guide treatment decision and lead to an early adaption of further lines of therapies before the progression becomes clinically obvious. Sorensen et al. were able to monitor decreasing levels of the activating EGFR mutations and occurrence of the resistance-conferring mutation at the same time. The resistance mutation was detected up to 344 days before a clinically evident progression [102]. Other studies using different analysis methods achieved similar results [103–105].

Achievements in minimally invasive tumor monitoring could also be shown in patients with colorectal tumors. Similar to lung carcinoma, the EGF receptor is an important therapeutic target in the treatment of metastatic colorectal cancer. However, patients harboring activating mutations in the KRAS gene in their tumors

do not benefit from EGFR antibodies, such as cetuximab and panitumumab. Since KRAS is a downstream component of the EGFR signaling network, activating mutations lead to intrinsic activation of the signal transduction pathway. KRAS mutations are thus a negative predictor of response to EGFR-directed therapy. Although the majority of KRAS wild-type patients benefit from the EGFR blockade, resistance occurs within 3–6 months in almost all patients. Known resistance mechanisms include—in addition to KRAS mutations as the predominant mechanism of resistance—mutations of BRAF and activation of alternate signaling pathways or increased EGFR numbers [106]. All these mechanisms have already been identified in ctDNA. In 2012, Diaz and colleagues determined whether mutant KRAS DNA could be detected in the circulation of 28 CRC patients receiving the anti-EGFR antibody panitumumab [107]. They showed that the appearance of these mutations was very consistent, generally occurring between 5 and 6 months following treatment. In three out of nine cases, mutant KRAS could be identified before radiographic evidence of disease progression. The mean time interval from detectable ctDNA to radiographic evidence of progression was 21 weeks [107]. Other studies showed that a comprehensive genome-wide analysis based on copy number status of ctDNA can further contribute to early detection of resistance mechanisms. The development of resistance to anti-EGFR therapies was associated with acquired gains of KRAS which occurred either as novel focal amplifications or as high-level polysomy of chromosome 12p. Again, in some cases, the resistant clones were detectable in the circulation months before progression was clinically obvious [106, 108]. In addition, focal amplifications of other genes recently shown to be involved in acquired resistance to anti-EGFR therapies, such as MET and ERBB2 [106–109], were minimally and invasively identified in the plasma DNA [106].

Similar approaches are used for prostate cancer patients. A variety of novel agents targeting the androgen receptor (AR) have altered the treatment paradigm of metastatic prostate cancer. Nevertheless, all patients develop inevitable therapeutic resistance. Resistance-conferring aberrations, such as mutations or gene amplification of the AR gene, can be monitored in plasma and may help to quickly adapt treatment based on the molecular nature of the tumor [108, 109]. These data highlight the benefit of moving beyond specific mutations and toward the full spectrum of genomic alterations, i.e., aneuploidy, amplifications, deletions, and translocations, since these aberrations represent some of the most clinically useful genomic targets in cancer (e.g., ERBB2, AR, KRAS amplifications) [110].

The relevance of ctDNA was proven not only by the monitoring of targeted therapies but also in patients under chemotherapy. Sequencing of 15 clinically relevant genes demonstrated the benefits of ctDNA as a marker for treatment response to cytotoxic chemotherapy [111]. In more than 98% of patients, candidate mutations were detected in the tumor, which were then screened with the high resolution using the Safe-SeqS method in plasma [112]. Patients under chemotherapy, who had a significant reduction in the ctDNA levels, had a significantly better response and a better progression-free survival [111]. Using exome sequencing, the group led by Nitzan Rosenfeld was able to identify resistance mechanisms in more than 80% of patients [113]. By the application of genome-wide methods, i.e., low-coverage

genome sequencing for the detection of somatic copy number alterations (SCNA), even clonal shifts and the occurrence of focal alteration that contain driver genes can be detected at the chromosome level [89, 95].

12.3.4 Limitations and Challenges of ctDNA

Although the analysis of ctDNA has a great potential for improving therapy management in a cancer patient, there are several issues that have prevented the widespread implementation of ctDNA in clinical routine. The lack of pre-analytical and analytical standards is still a big issue, especially if it comes to comparability and reproducibility of results and the integration of ctDNA analysis in large clinical trials. In this respect, more and more efforts are being made to establish standard operation procedures by international consortiums, e.g., the IMI CANCER-ID project, which includes a number of experts in the field of ctDNA and CTCs (http://www.cancer-id.eu/). It is of utmost interest to find a consensus in which methods will find practical application and how to report results. Furthermore, it is still not clear yet whether the focus should be on specific targets that can be analyzed with high resolution or broader approaches should be used. In this respect, of course, also costs and time play a major role, as well as the question of who will bear the costs for such investigations.

Moreover, the actual clinical long-term benefit of ctDNA analyses for patients needs to be confirmed in large-scale studies with sufficient sample sizes. The discovery of resistance-conferring mutations neither saves a patient's life nor does it increase the quality of life, if there is no drug that bypasses the resistance or may be administered subsequently.

In addition, many of the questions regarding the biology and release of ctDNA are still unanswered: Does ctDNA represent a true portrait of the cancer? Do all tumor locations or all clones of a tumor release the same amounts of ctDNA, or is it just the most dominant and proliferative clone that can be found in the circulation? Is the prevalence of ctDNA in all tumor entities the same? Many of these issues could only be resolved by a comparison of ctDNA and all existing tumor sites by the use of "warm autopsies" which can be quite challenging from an ethical perspective.

12.3.5 Summary

All these studies and many more that could not be discussed in this chapter suggest that the analysis of ctDNA resents a very promising tool in the treatment management of cancer patients. The hitherto most comprehensive study of the group of Diaz showed that in at least 75% of cancer patients with advanced solid tumors, ctDNA can be detected [71]. In later stages, the analysis of ctDNA allows for a comprehensive therapy monitoring, which allows the physicians to respond as quickly as possible to changes in the tumor. Thus, treatments can be adjusted

rapidly, and patients can be spared from expensive treatments with very toxic drugs and side effects from the moment the drug is no longer effective. Moreover, novel therapeutic targets that occur in the course of the disease can be identified and offer new treatment options. ctDNA also provides a unique opportunity to learn more about metastasis processes and the related signaling pathways.

Furthermore, ctDNA levels can be used as prognostic markers in order to evaluate whether a patient needs adjuvant treatment after curative surgery in order to eliminate residual cancer cells. Especially in earlier stages, only a subset of patients relapse and therefore profit from adjuvant therapy after a curative resection of the tumor. ctDNA analyses could spare some patients a burdensome and costly therapy. In the near future, ctDNA might also be used as a diagnostic biomarker enabling early detection of cancer. Illumina, the current market leader in the field of NGS, has just founded a 100 million dollar start-up company called GRAIL with the aim to develop a test for early detection of cancer, which would be launched in 2019. Although the detection of cancer in its earliest stage is the "the Holy Grail" in oncology, one has to consider that the detection of specific mutations in the circulation in individuals who do not yet have visible tumor can be problematic.

12.4 Circulating RNA and Exosomes

12.4.1 Biology of Exosomes and Circulating RNA

Besides the two major topics in liquid biopsies, CTCs and ctDNA, circulating RNA is a small but promising field for clinical applications. The term circulating RNA refers primarily to microRNA (miRNA) which either travels as small fragments bound to proteins or encapsulated in exosomes [114, 115]. The number of publications increased tremendously in the past 10 years, of only 85 papers published in the year 2005 to 3680 in 2015, with a peak in 2014 of 4190 papers published with the topic "microRNA in cancer." Longer stretches of RNA transcripts outside of cells are considered to derive from dying cells and having no functional role. Furthermore, RNA molecules can originate from viruses and therefore can pose a serious threat to the whole organism. Therefore, it is not surprising that RNase activity is high in serum, as it was shown that >99% of mRNA added to serum is degraded within 15 s [116]. This is an explanation why long RNAs, like mRNAs, are usually not detectable in blood. However, smaller RNAs with a size below 25 nucleotides, like miRNAs which play an important role in gene silencing and post-transcriptional regulation of gene expression, are very stable in plasma and serum. This is due to the fact that these molecules are too small to be degraded by RNases. They are often bound to subcellular particles like Argonaute proteins or captured in microvesicles preventing them from degradation [117, 118]. Exosomes belong to these microvesicles that are thought to be actively released from cells. They are 30–100 nm in diameter and can be found in blood of cancer patients [119–124]. Exosomes contain proteins and nucleic acids, such as fragmented DNA, RNA, and miRNA [115, 125]. Being encapsulated by lipid bilayers, the molecules are stably preserved, making

exosome a good resource for the study of tumor-associated biomarkers. In the following paragraphs, we will focus on miRNA as blood-based biomarker. In this context, they are also discussed as a "liquid biopsy," although miRNAs are not necessarily derived from the tumor tissue, but can be a regulatory change of expression in response to cancer disease.

12.4.2 Methodological Aspects for the Isolation and Analysis of miRNA

miRNAs are exposed to lots of physical and chemical stresses prior to their isolation which affect their stability and quantity in plasma and serum. It is essential for a clinically reliable and robust biomarker to minimize the influence of pre-analytical parameters prior to their analysis. The following paragraphs will give more information about the most important steps in miRNA isolation and detection and some insights into current research.

Recent publications showed that in many pathologic conditions, miRNAs are expeditiously released from tissues into blood circulation. It was also demonstrated that circulating miRNA in peripheral blood was highly stable and protected from degradation conditions, such as extreme pH values or endogenous RNase activity. The protection is given as miRNAs are embedded in microvesicles or exosomes, thereby being inaccessible by degrading enzymes [114, 126]. This feature allows miRNAs to be used as noninvasive biomarkers [127]. MiRNA extraction can be applied to nearly every fluid or tissue of the body. Besides the commonly used sample types like serum and plasma, there are also kits available for miRNA extraction from cerebrospinal fluid, saliva, or urine [127]. As the composition of these body fluids is pretty dissimilar, the isolation methods of miRNA need to be adapted from one tissue/fluid to another [128]. Thus, depending on the specific miRNA and the corresponding sample material, the corresponding extraction kit should be used. Most studies use serum or plasma as sample starting material for miRNA extraction. Comparison of extracellular miRNA stability shows little to no difference in these biological fluids [127], though higher concentrations were steadily found in serum [129].

12.4.2.1 Storage of miRNA Samples

Sample storage conditions can seriously affect the accuracy and reliability of analytical results. Sourvinou et al. investigated the stability of circulating miRNAs in identical plasma samples under different temperature and time conditions [130]. The results showed that for the accurate quantification of cell-free miRNAs, the isolation process should be performed within 48 h after sample collection if plasma samples are kept at −20 °C or −70 °C [130]. Storage at 4 °C leads to a significant decrease in circulating miRNA levels within 24 h [130]. If long-term storage of plasma samples is needed, temperatures of −70 °C rather than −20 °C should be preferred to avoid extensive miRNA degradation [130]. Grasedieck et al. reported similar results with respect to the impact of serum storage conditions on miRNA stability [131].

Experiments indicate that extracted miRNAs are stable for more than 1 year at a temperature of −70 °C [130, 132]. However, results showed differences in the stability of stored miRNAs depending on the extraction buffer [130]. There are also studies that report significant degradation of miRNAs 3 days after isolation [133]. A study of Sourvinou et al. indicated that the use of different elution buffers for miRNA storage might be responsible for differential stability of miRNA [130]. Therefore, they suggested the use of mirVana PARIS kit for long-term storage [130].

12.4.2.2 Extraction of miRNA

Several extraction kits for miRNA are commercially available. As mentioned above for each source of material, the optimal miRNA extraction method needs to be experimentally determined. The following paragraph refers to protocol considerations for isolating miRNAs from blood plasma or serum.

12.4.2.3 Phenol/Chloroform Extraction for miRNA

This procedure relies on the different solubility of cellular components in organic solvents, such as phenol, chloroform, or ethanol. The main components of the phenol/chloroform protocol are phenol and guanidinium thiocyanate, usually marketed as TRIzol [134]. TRIzol denatures proteins, including RNases, which permits long-term storage of samples [135]. After phase separation, RNA gets precipitated with isopropyl alcohol. Due to the fact that miRNAs are small, ample time is needed for recovery. Recently, authors realized that there is selective loss of small RNA molecules with low GC content using TRIzol, especially by analyzing low numbers of cells [136]. Though this publication refers to miRNAs isolated from cells rather than body fluids, nevertheless, it is unknown if these factors also alter RNA extraction from blood plasma or serum [130, 134].

12.4.2.4 Silica-Based miRNA Recovery Methods

There are manifold kits available, of which the most frequently used are the miRVana PARIS and miRNeasy Mini kits. A direct comparison of these methods is difficult as the majority of publications do not report the actual yield and quality of miRNA. The miRVana PARIS kit is a commercially available method of separating nucleic acids and proteins. miRVana PARIS method is a two-part, sequential filtration with increasing ethanol concentrations, used for collection of a highly enriched fractions of RNA molecules shorter than 200 nucleotides. This method works very well for the isolation of miRNA from tissues and body fluids as well, as it requires fluid volumes from 100 μL up to 625 μL [134]. The miRNeasy Mini kit uses a silica-based column technique to recover miRNAs. Some groups report that this kit leads to a two- to threefold better yield than miRVana PARIS kit; however, the available literature of groups that use this kit for miRNA extraction from plasma or serum is very limited. The kit also uses a phenol/guanidine-based lysis to isolate the miRNA from other plasma components, by adsorption on a silica mini-column in the presence of ethanol. Remarkably, using the Qiagen QIAcube, the binding, washing, and elution step can be operated automatically. Consequently, this development can decrease working time and variability [134].

12.4.2.5 Analysis and Measurement of miRNA

Moldovan et al. give a good insight into the three most common miRNA profiling methods, (1) RT-qPCR, (2) microarrays which are a hybridization-based method to detect miRNA expression, and (3) next-generation sequencing and their advantages and disadvantages [134]. Most of the scientific interest is focused on the detection of circulating miRNA in plasma or serum, and the commonly used techniques, such as conventional RT-qPCR, offer both high sensitivity and specificity. The measured miRNA levels can vary depending on the extraction method and the body fluid used. This complicates the comparison of results from different methods as well as between fluids. Therefore, a normalization control needs to be implemented to minimize these variations [134]. There are two main normalization controls: One is the absolute quantification which is done by analyzing a series of probes with known, increasing concentrations [134]. Another approach involves relative quantification, where a small constantly expressed miRNA or a spiked-in miRNA is used for normalization of input amounts [134]. Microarrays offer high-throughput analysis of a large number of miRNAs and can be customized for high flexibility. Nevertheless, microarrays are less specific and sensitive than RT-qPCR, and results obtained by microarrays need to be confirmed by RT-qPCR. The third and probably most promising technology for miRNA analysis is next-generation sequencing. With its extreme sensitivity of one miRNA copy per cell, it has the ability to detect expressions over 6–7 log fold ranges. It is also the only one of the three technologies which is able to detect both known and novel miRNAs. Moreover, it can detect small RNAs like noncoding RNA (ncRNA), small interfering RNA (siRNA), etc. [134]. Even though these advantages are tremendous, the downside of RNA-Seq is its high cost as well as the tremendous amount of computational infrastructure and bioinformatics know-how needed [137].

12.4.3 Clinical Use of Circulating miRNA

At present, there are miRNA panels helping clinicians in determining the origins of cancer in disseminated tumors, as reviewed by Hydbring et al. [138]. These days, approximately 1600 human miRNAs have been placed into miRNA databases based on analyses of RNA deep sequencing data [139]. The majority of miRNA publications refer to the usage of solid tissues even though miRNAs can be readily detected in human serum, plasma, or total blood because of their small size and high stability as described above. The high potential of circulating miRNAs as biomarkers in serum was demonstrated by studies testing patients with diffuse large B-cell lymphoma, highlighting miR-21 as a potential biomarker [140, 141]. In another study, expression level of miR-143 allowed the discrimination between prostate cancer patients and healthy controls [127]. Subsequent studies reported on miRNA detection in patients suffering from breast cancer, colorectal cancer, or squamous cell lung cancer by using whole blood, plasma, or sputum samples [138]. It has been shown that circulating miRNAs may also be used for prognostic purposes. The group of Boeri et al. detected miRNAs with strong prognostic value in lung cancer

patients years before the onset of disease by analyzing expression in samples taken before diagnosis, at the time of disease detection, and in disease-free smokers [142].

By analyzing 863 miRNAs from 454 human blood samples, Keller et al. could show that in each disease an average of more than 100 miRNAs were deregulated [143]. The samples were taken from patients suffering from 14 different diseases, including lung cancer, prostate cancer, multiple sclerosis, pancreatic ductal adenocarcinoma, ovarian cancer, melanoma, gastric tumors, pancreatic tumors, chronic obstructive pulmonary disease, pancreatitis, sarcoidosis, periodontitis, and myocardial infarction [143]. By using this data and developing mathematical algorithms, the authors could precisely predict the disease in more than two-thirds of people involved in the study [143].

12.4.4 Limitations and Challenges of Circulating RNA

Even though the field of miRNA is very promising, there are also limitations and challenges. The main issue in miRNA quantification is the lack of validation and standardization. There are huge differences in detectable miRNA expression depending on pre-analytical conditions, such as temperature (e.g., freezing) and additives (e.g., anticoagulants), and the condition of the patient with respect to diet, lifestyle, or drug usage. Standard operating procedures (SOPs) are not in place yet, but need to be defined for sample preparation, extraction, and analysis. As already small differences in sample handling can result in big changes in outcome, published miRNA data which correlate with a specific disease should be critically questioned prior to using their miRNA signatures in clinically meaningful tests [144].

12.4.5 Summary

miRNA detection is an increasingly important field. Although still facing challenges mainly due to the lack of standardization, miRNA detection, not only from serum and plasma but also from other noninvasive collected fluids like saliva or urine, is a promising biomarker for a daily routine clinical approach. Importantly, miRNA diagnostic is not just a further approach to detect disease and its progression; it could also have the potential to initially identify the unknown origins of tumors and metastases.

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