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Cell free circulating tumor nucleic acids, a revolution in personalized cancer medicine

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ABSTRACT

Innovative diagnostics are becoming an essential component in personalized cancer medicine. These diagnostics are increasingly based on cell-free nucleic acids and membrane vesicles. Isolating and sequencing cell free circulating DNA (cfDNA) in plasma may progressively substitute tumor biopsies. A small albeit now detectable fraction of cfDNA correspond to circulating tumor DNA (ctDNA). In this review, we describe the pre-analytical procedures for collecting ctDNA from plasma, since these procedures should be optimized within laboratories depending on the available infrastructures. We also provide an overview of the technological breakthrough in ctDNA Isolation for instance digital PCR methods and next generation sequencing techniques and discuss their key challenges. The clinical implementations of liquid biopsy and more specifically ctDNA in cancer management are reviewed. We predict in the near future, ctDNA will be used more routinely to guide cancer treatment and provide a new approach to personalize treatment in precision medicine.

1. The history of liquid biopsy

In 1948, Mandel and Métais detected and quantified the presence of cell-free nucleic acid (cfNA) in human blood of both healthy and diseased patients for the first time (Schwarzenbach et al., 2011). In 1966, researchers discovered high levels of cfDNA in lupus patients (Thierry et al., 2016). These studies attracted some attention in the scientific community and the presence of tumor-specific mutations in cfNA was not reported by scientists until 1994. In the plasma of patients with pancreatic cancer and acute myelogenous leukemia, they used polymerase chain reactions (PCR) amplification to identify tumor specific mutations such as *NRAS* gene mutations. The mutations observed in circulating DNA originating from tumor cells, prompted later the designation as circulating tumor DNA (ctDNA). At that time, PCR was the only available technique that could detect the weak tumor signal. Since ctDNA is highly diluted by normal cfDNA, common sequencing methods of the time such as Sanger sequencing did not have the sensitivity to properly detect ctDNA. In 1997, Dennis Lo detected fetal cfDNA in the blood (Lo et al., 1997). The first liquid biopsy test, the cobas ® EGFR mutation test, was approved by the Food and Drug Administration (FDA) in June 2016 as a blood cell-free DNA (cfDNA) test for lung cancer patients (Kwapisz, 2017).

Today, the detection of ctDNA in bloodstream, provides tumor diagnostic possibilities, determining therapy reaction, tracking, and prognosis prediction. Different kinds of tumor-specific DNA aberrations may be recorded in cfDNA such as point mutations, modifications in the status of microsatellite biomarkers including loss of heterozygosity (LOH); gene amplifications; presence of viral oncogenic DNA hypermethylation of tumor suppressor gene promoter areas and hypomethylation of long interspersed nucleotide element-1 (Kerachian and Kerachian, 2019; Volik et al., 2016; Wang et al., 2017a).

In the majority of patients, a tumor biopsy is clinically challenging and relatively often difficult to achieve or leads to unusable material for DNA or RNA extraction (Karachaliou et al., 2015). Thus, ctDNA investigation in cancer patients as a type of liquid biopsy have generated lots of research interest. Currently, there is a growing number of companies involved in the development of the liquid biopsy assays for the diagnosis and stratification of cancer patients. Serial assessment of

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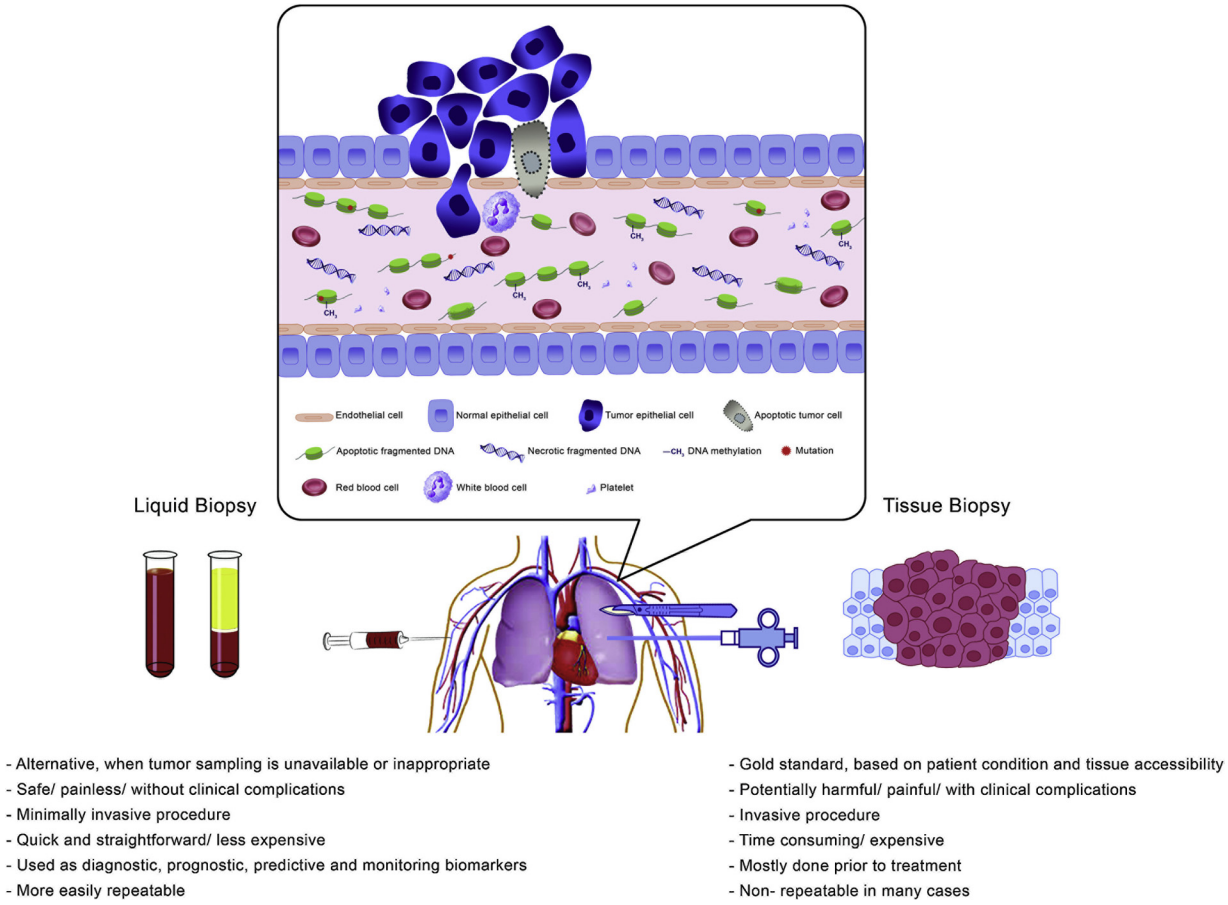

Fig. 1. Comparing the performance of liquid biopsy to tissue biopsy, and indicating the source of circulating tumor DNA (ctDNA) in blood.

ctDNA during therapy may show a vibrant image of genomic alteration, indicating that this non-invasive method may also be used to monitor the growth of therapeutic resistance and also recognize heterogeneous subclonal populations of tumor cells that develop during therapy. Fig. 1 compares the performance of liquid biopsy to tumor biopsy.

In addition to ctDNA, circulating tumor cells (CTCs) are another main source for nucleic acids. The presence of CTC in cancer patients was first reported by Thomas Ashworth in 1869 (Joosse and Pantel, 2013; Plaks et al., 2013). CTCs can be enriched from blood as a result of physicochemical properties or cell surface molecules that differentiate them from ordinary blood cells (Bettegowda et al., 2014). In 2000, Veridex introduced the first commercial liquid biopsy assay, the CELLSEARCH®CTC test (Neumann et al., 2018), a system based on the expression of the EpCAM antigen (Farace et al., 2011).

While the FDA has approved liquid biopsy of CTCs for prognosis of overall survival, the predictive value of ctDNA may become potentially more significant than CTCs (Table 1). The interaction of tumor cells with platelets has been shown to cause particular expression of mRNAs in platelets (Sol and Wurdinger, 2017). Tumor cells can release RNA into the circulation by different microvesicle-dependent or -independent mechanisms, which are directly absorbed by platelets (Zhang et al., 2017) known as tumor-educated platelets (TEPs) (Joosse and Pantel, 2015). Further evidence has revealed that TEPs identified in patients with glioma and prostate cancer contained *EGFRvIII* and prostate cancer antigen 3 (*PCA3*), defined as the cancer-associated RNA biomarkers (Karachaliou et al., 2015). Thus, the relative ease of platelet RNA isolation and analysis could provide an additional avenue for the non-invasive monitoring of cancer. Moreover, microvesicles, like exosomes, presented in plasma could provide a protective environment for tumor-derived RNA (Huang et al., 2013). On the other hand, genome-wide miRNA-expression-profiling studies using high-throughput technologies have shown that a particular profile of up- or down-regulated miRNAs is present in most (if not all) types of cancer. Both the characteristic expression of miRNA's profile within tumor cells and sparse post- transcriptional and translational modifications have generated interest in the implementation of miRNA as a potential tumor cell biomarker (Ha, 2011; Larrea et al., 2016; Li et al., 2009). The reasons mentioned above have incentivized the use of liquid biopsy to detect cancer biomarkers for diagnosis and therapy guidance.

In this review, we describe the source, characteristics, quantification, detection technology, implementation of cfDNA in diagnosis, monitoring, prognosis assessment and personalized cancer medicine for solid tumors.

2. The source of ctDNA

Circulating tumor DNA or ctDNA can be released from CTCs, primary and secondary tumors into the circulation of cancer patients. They could be presented in many forms; either cfDNA, bound to complex proteins, bound to cell surfaces, or vesicles (Thierry et al., 2016). Most ctDNA is made up of apoptotic and/or necrotic tumor cells that release their divided DNA into the blood (Wang et al., 2017b, 2003) (Fig. 1). Besides, viable tumor cells can release microvesicles (or exosomes), which are double-stranded DNA (Nurwidya et al., 2016) into the circulation, although this is still controversial.

Studies have shown that normal cfDNA is derived from all cell types in the body (Ha, 2011) and it originates from both nuclear and mitochondrial sources (Diehl et al., 2008). White blood cells compose 55%–93% cfDNA of healthy individuals (Fernando et al., 2017; Moss et al., 2018). The normal cfDNA dilutes the ctDNA in cancer patients, particularly after surgery, chemotherapy, or radiotherapy when normal tissue is damaged (Leung et al., 2016).

Table 1
The comparison of circulating tumor DNA (ctDNA) with circulating tumor cells (CTC).

	ctDNA	CTC
Composition	Double stranded, highly fragmented, with most molecules being ~ 150 bp in length	Released cells into the bloodstream as a result of the formation and growth of tumor cells
Source	Primary tumor, CTC, micrometastasis, or overt metastases into the blood	A primary tumor and/or a metastatic site
Half-life	From 15 minutes to several hours	1 – 2.4 h
Clearance mechanism	Mostly through the liver or kidneys & uptake by host cells	Rapid clearance mostly mediated by natural killer cells, monocytes/ macrophages and neutrophils
Properties used for detection	Presence of tumor-specific mutations aberrant methylation, copy number variants and chromosomal rearrangement	Physicochemical characteristics or cell surface molecules that distinguish them from normal blood cells;
Technologies for detection	PNA clamping PCR, BEAMing Safe-SeqS, TamSeq, and digital PCR; WGS	CellSearch platform; MACs
Diagnosis	Has been tested for diagnosis of CRC, gastroesophageal cancer, pancreatic cancer and lung cancer patients; with several ongoing clinical trials	Has been tested for diagnosis of lung, pancreatic and breast cancer; with several ongoing clinical trials
Diagnostic limitations	The presence of cancer-associated mutations in healthy people; limited number of ctDNA and their short size fragments; distinguishing a small change in DNA copy number in a high background of diploid genomes	Discriminating tumor cells circulating in the bloodstream from the vast majority of WBC & RBC; identifying the subpopulation of CTCs with the metastatic potential responsible for mortality

Tumor biomarkers can enter blood indirectly by inflammatory cells that phagocytose tumor cells or enter the circulation carrying tumor-derived nucleic acids or proteins. This alternative path could take place throughout all phases of tumorigenesis and could possibly allow pre-cancerous and cancerous lesions to be detected through a blood test (Alix-Panabieres and Pantel, 2016).

CfDNA is made up of single- or double-stranded and extremely fragmented DNA (Barbany et al., 2019), with most fragments being about 150 bp in length, which corresponds to the length of DNA occupied by a nucleosome (Volik et al., 2016; Plaks et al., 2013). Moreover, other molecules lengths DNA fragments corresponding to two or more nucleosomes (two and three units for 300-bp and 450-bp bands, respectively). Thus, cfDNA is usually about 170–500 bp fragments and thought to originate mainly from apoptotic cells (Jahr et al., 2001). It is still unclear if the higher or lower integrity of cfDNA is related to cancer (Volik et al., 2016). In 2003, Wang et al. studied the cfDNA integrity in breast and gynecologic patients with cancer compared to normal individuals and showed higher cfDNA integrity in cancer patients to controls (Wang et al., 2003), although there are some conflicting reports. Madhavan et al. demonstrated decreased cfDNA integrity in cancer patients, which reflects increased apoptotic rates in cancer (Madhavan et al., 2014). Similar data also suggested that the length of ctDNA is shorter than the cfDNA background, which occurs physiologically (Underhill et al., 2016) and about 90% of the total cfDNA in cancer patients is composed of low molecular weight bands, that are 150–180 bp (Volik et al., 2016).

3. The quantity of ctDNA in bloodstream

The number of cfDNA copies in cancer patients varies extensively (Lange and Laird, 2013), which depends on the tumor type, location and cancer stage (Han et al., 2017). While the concentration of cfDNA in cancer patients' blood is higher than in healthy individuals' blood and non-malignant patients, the concentration of cfDNA varies significantly with an average of 180 ng/mL (Barbany et al., 2019). However, in some studies less than 100 ng/mL has been reported for the most of cancer patients (Volik et al., 2016). The mean quantity of plasma circulating DNA in normal subjects is less than 10 ng/mL but sometimes to more than 1500 ng/mL (Pantel and Alix-Panabieres, 2017). The quantity of cfDNA in healthy controls has also been reported differently in several studies (Breitbach et al., 2014; Spisak et al., 2013).

Not surprisingly, in addition to the different absolute levels of cfDNA, the proportion of ctDNA to total cfDNA also varies. In 2001, Jahr et al. estimated the ratio of ctDNA to total cfDNA by measuring the amount of hypermethylated *CDKN2A* promoter expected to be tumor-specific. The percentage of tumor-specific hypermethylated sequences of *CDKN2A* varied from less than 10% to over 90% of the total cfDNA (Jahr et al., 2001). Diehl et al. published a study using digital PCR and BEAMing technology (acronym; beads, emulsion, amplification and magnetics) that regularly contained mutant adenomatous polyposis coli (*APC*) DNA molecules in patients' plasma with developed colorectal infections. They displayed the ctDNA/cfDNA ratio ranged from 0.01% to 1.7%. Surprisingly, the number of the total *APC* fragments (wild type and mutant) increased by 5- to 20-fold when the amplicon in PCR reduced from 1296 to 100 bp (Diehl et al., 2005). Several other studies showed comparable outcomes, e.g. Dawson et al. also reported that the ctDNA fraction in metastatic breast cancer is an average of 4% of the total cfDNA (Dawson et al., 2013).

The kinetics of cfDNA in blood, tumor cells and their constituents are sequestered variable throughout the reticuloendothelial system. Plasma includes DNases and proteases that may influence its stability and integrity (Ahlquist, 2010), although mechanisms of its clearance have not been fully explored. Importantly, the amount of plasma/serum DNA is strongly influenced by its rapid transition. The first report depicting cfDNA turnover come from 1963, when Tsumita and Iwanaga showed that almost all of the DNA injected into mice was eliminated from the bloodstream in 30 min. They also indicated that the kidney has the highest clearance of injected DNA followed by liver and spleen (Tsumita and Iwanaga, 1963). In contrary, Gauthier et al. showed liver was the major organ for removal of circulating nucleosomes in comparison to other organs (Gauthier et al., 1996). Once DNA reaches the circulation, it is speedily degraded by blood nucleases and removed by the liver, spleen, and kidneys, with a short half-life of about 10–15 minutes (Thierry et al., 2016; Alegre et al., 2015; Gould et al., 2015). In 1999, Lo et al. measured the half-life of the fetal cfDNA in mother's blood post-partum which was 16 min, and it was shown that by 2 h post-partum there was no fetal cfDNA detectable in the plasma in most women (Lo et al., 1999). However, a recent study revealed a biphasic clearance for the fetal cfDNA with half-lives of about 1 h for the rapid phase and a second phase of 13 h (Yu SC et al., 2013). Following intensive exercises, Fatouros et al. determined the kinetics of cfDNA concentrations in athletes. They reported that cfDNA increased (15-fold) 30-min after exercise, stabilized (13-fold) 30-min and normalized thereafter indicating the short half-life of cfDNA (Fatouros et al., 2010; Steen et al., 2010). Overall, cfDNA has a variable half-life between 15 min and several hours in circulation (Kidess and Jeffrey, 2013; Salvi et al., 2016; Yeh, 2015).

4. Detection methods

Circulating tumor DNA containing the same molecular aberrations as the solid tumor, found in the bloodstream, refers to DNA from cancer cells and tumors (Thierry et al., 2016). Several methods can detect the existence of tumor-derived DNA in cfDNA. Nonetheless, the low

amount, high degradation, and high mix of normal cfDNA with ctDNA cause serious challenges to choose the appropriate detection method. Since almost all tumors are marked by various subclonal populations, the problem would be further complicated by sharing only a subset of somatic genetic/epigenetic changes between the tumor cells (Andor et al., 2016). However, ctDNA can be detected using most of the methods used to detect genetic/epigenetic aberrations in tumor cells (Barbany et al., 2019). The detection of improved concentrations of *MYCN* sequences in blood of patients suffering from neuroblastoma by Combaret et al. in 2002, was one of the first applications for PCR-based assessment of ctDNA (Combaret et al., 2002). Methods such as single nucleotide polymorphism (SNP) arrays, chip-based microarray technology, genome-wide association studies (GWAS) and microarray-based comparative genome hybridization (CGH) have been adopted to address the sensitivity and accuracy issue of tumor genomes detection and have provided crucial insights into tumorgenesis (Han et al., 2017). However, over-all detection of copy number aberrations (CNA) in cfDNA using aCGH or methods targeting single and restricted loci such as digital PCR (dPCR) has several limitations, which make them less appropriate for quantification of ctDNA. Ultimately, in a large context of human diploid genome, the method should be able to differentiate a minute change in DNA copy number. The amount of copies of a locus of interest should be quantified, relative to a reference loci in order to solve this problem (Gevensleben et al. (2013)).

To date, PCR-based techniques have been widely used to detect particular mutations and can be widely classified as either qualitative or quantitative in nature, such as ARMS-PCR and PNA clamping PCR, respectively (Whitcombe et al., 1999; Wu et al., 2011). Meanwhile, real-time PCR have a limit of detection (LoD) of 0.5-0.1%. Some improved PCR-based methods such as the allele-specific quantitative PCR-based Intplex technology have a sensitivity ranging from 0.014 to 0.004% (Normanno et al., 2018; Thierry et al., 2014).

Various implementations of dPCR such as BEAMing digital PCR technology (Volik et al., 2016; Chen et al., 2013), microfluidic platforms (Dawson et al., 2013; Streets and Huang, 2013) and digital droplet-based systems (Dressman et al., 2003) have been recently developed. This group of approaches unites a number of methods used to enhance PCR's mutation detection precision by separating template molecules into individual response chips. These methods are mixed with either microfluidics, separating sample and PCR reagents into droplets in an oil emulsion with equal size. They have a high sensitivity to identify genomic changes with a LoD ranging from 0.01 to 0.001% (Normanno et al., 2018).

The restriction of the methods explained above is that, only a few loci can be questioned per assessment. Considering the longitudinal and spatial heterogeneity of cancer cells, gene alterations could vary among different tumor subclones; consequently, digital PCR technologies may miss some information during its procedure. To solve this problem, massively parallel or next generation sequencing (NGS) technologies are implemented to obtain a more inclusive view of the whole genome (Sol and Wurdinger, 2017), characterizing the whole genomic alterations in a single experiment. Several other cutting-edge technologies have emerged in recent years, resulting in the incorporation of methods for detecting ctDNA mutation with higher sensitivities such as Safe-Sequencing System (Safe-SeqS) (Kinde et al., 2011), targeted error correction sequencing (TEC-Seq) (Phallen et al., 2017) and tagged-amplicon deep sequencing (TAmSeq){Forshew, 2012 #109}. Forshew et al. in 2012 reported that *de novo* mutations can be detected through TAm-Seq assay, which allowed the re-sequencing of approximately 6000 nucleotides, while maintaining high depth analysis. They carried out an experiment by investigating ctDNA from an ovarian cancer patient. The patient ovarian tumor tissue was also sequenced and a *TP53* mutation was identified. TAm-Seq analysis manifested EGFR mutation in plasma samples as the cancer progressed, which was surprisingly not found in the original specimen (Forshew et al., 2012).

In the same year, Leary et al. offered “personalized rearranged end assessment” (PARE) to establish tumor-derived chromosomal changes by analyzing cancer patients’ circulating cfDNA (Leary et al., 2012). Another massive parallel sequencing method (MPS) named “Shotgun” was developed by Chan et al. in 2013 (Chan et al., 2013). In 2014, Maximilian Diehn's group created a novel strategy called "deep sequencing personalized cancer profiling" or "CAPP-Seq" initially used for non-small cell pulmonary cancer (NSCLC) with a model covering various classes of somatic changes (Neumann et al., 2018). Similarly, Pisapia and colleagues in 2017 used a next generation sequencing (NGS) assay, employing the SiRe® gene panel to detect on cfDNA mutations of *EGFR* and *KRAS*, *NRAS*, *BRAF*, *cKIT* and *PDGFR* genes in NSCLC patients (Pisapia et al., 2017). All the recent techniques combine DNA deep sequencing with molecular barcodes and appropriate bioinformatics filtering steps to create a very low sensitive approach. To analyze *de novo* mutations in serial plasma samples feasible, whole exome sequencing (WES) was conducted to track tumor alterations in reaction to treatment (Leary et al., 2010). Murtaza et al. used this approach for patients with metastatic tumors. Whole genome sequencing (WGS) could also screen a larger range of the genome but it is currently quite expensive for routine use especially to detect single nucleotide variants (SNVs). Besides, WES enables more in-depth interrogation of different regions, although the identification of differences in copy numbers is less susceptible (Murtaza et al., 2013).

In addition to high-throughput mutation detection techniques, other methods for methylation detection have also emerged now (Rokni et al., 2018). These techniques are divided into three categories: 1) methylation content assay: high-performance capillary electrophoresis (HPCE) or high-performance liquid chromatography (HPLC) ; 2) methylation pattern and profiling: restriction landmark genomic scanning (RLGS), methylated CpG-island amplification (MCA), amplification of inter-methylated sites (AIMS), reduced representation bisulfite sequencing (RRBS) (Smith et al., 2009), genome-wide methylated CpG tandem amplification and sequencing (MCTA-Seq) method (Schuster, 2008); 3) candidate gene approach: methylation-sensitive restriction endonuclease-PCR/Southern (MSRE-PCR/Southern), bisulfite sequencing, methylation-specific PCR (MS-PCR), quantitative multiplexed methylation-specific PCR (QM-PCR) (Han et al., 2017), MethyLight and HeavyLight technologies (Ma et al., 2015).

More recently, Sina et al. developed a novel electrochemical or colorimetric one-step assay method based on the DNA-gold affinity for detection of methylation patterns of ctDNA (Sina et al., 2018).

5. ctDNA as biomarkers

The detection of ctDNA in plasma could be useful for many diagnostic applications in addition to limiting the need for solid biopsies (Wang et al., 2017a). The discovery of a proportion of circulating DNA in cancer patients has created the potential for a so-called "liquid biopsy" to mark tumor genetic characteristics as an alternative to tissue biopsy (Esposito et al., 2016). CtDNAs are thought to be easily detected even in the early stages of cancer patients’ plasma (Alix-Panabieres and Pantel, 2016) although there are many challenges which will be discussed later in this article. Use of ctDNA as a biomarker leads to the possibility of taking repeated blood samples to trace the changes in cfDNA throughout the progress of the disease or cancer treatment (Schwarzenbach et al., 2011). In addition, it is also possible to consistently measure and reflect tumor-specific DNA methylation status within ctDNAs (Warton et al., 2016), which could be promising to improve diagnosis, planning, and monitoring of treatment. A liquid biopsy test is cost-effective with low side-effect (Guerra et al., 2016) and it could be helpful to predict the risk for early relapse, identify therapeutic targets, monitor and stratify patients to the most efficient therapy, and detect potential resistance mechanisms (Alix-Panabieres and Pantel, 2016).

Hence, to date the use of ctDNA to assess the tumor burden has focused on two areas: total concentration of ctDNA and the detection of

ctDNA as a diagnostic and prognostic marker tool (Aravanis et al., 2017). Detection of cancer by monitoring ctDNA is of high public interest. The Johns Hopkins team used digital PCR–based techniques to assess the ctDNA tumor detection capacity in 640 patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers and they found that ctDNA was detectable in more than 75% of them. This number was less than 50% for primary brain, renal, prostate, or thyroid cancers (Bettegowda et al., 2014). These rates of detection are noteworthy, but not sufficient for early detection of cancer.

In another study, Newman et al. used CAPP-Seq for NSCLC. They identified mutations in 100% of tumors in stage II-IV NSCLC and 50% of patients with stage I, with a specificity of 96% for mutant allele fractions down to proximately 0.02%. They realized that at early stage, the amount of ctDNA in the cfDNA fraction was approximately 10-fold lower than the advanced stages of the disease (Neumann et al., 2018). Pisapia et al. also represented, SiRe® NGS panel, a robust analytical tool for NSCLC patients enabling the possibility to test cfDNA mutational status in basal setting of when no tissue samples were available to assess EGFR mutational status for first line treatment decision making. They reported an overall mutation rate of 38% in their study (Pisapia et al., 2017).

In breast cancer using WGS, Bettegowda et al. detected ctDNA in more than 75% and 50% of patients with advanced and localized breast cancer, respectively (Bettegowda et al., 2014). In addition, in patients with metastatic breast cancer, Dawson et al. recognized somatic genomic changes in serially gathered plasma samples. Circulating ctDNA had been recognized in nearly all breast cancer patients whom have been detected somatic genomic modifications. The concentrations of ctDNA suggested a higher dynamic range, more correlated with tumor burden modifications than did CA 15-3 or tumor cells in circulation (Dawson et al., 2013).

In addition to genetic, epigenetic aberrations such as DNA methylation could be used as a type of biomarker for cancer detection, especially in early stage of cancer (Leygo et al., 2017). Compared to genetic alterations that are generally spread throughout the gene, changes in DNA methylation are more focused in the gene promoter making it easier to build biomarkers (Wang et al., 2017a; Houshmand et al., 2017). The methylated ctDNA has been considered as potential DNA methylation biomarkers to detect several cancers, including CRC (Farace et al., 2011; Leygo et al., 2017; Pantel, 2016), lung cancer (Lu et al., 2017), breast cancer (Tang et al., 2016; Van De Voorde et al., 2012), and pancreatic cancer (Angsuwatcharakon et al., 2017; Tan et al., 2009). Liquid biopsy is expected to provide the necessary acceleratory strength for the implementation of precision oncology and personalized medicine in clinical settings by contributing a comprehensive understanding of tumor heterogeneity and permitting the dynamic monitoring of treatment responses and genomic variations. However, widespread implementation of liquid biopsy based biomarker-driven therapy in the clinical practice is still in its preliminary stages. Technological achievements have resolved many of the obstacles faced in the liquid biopsy methodologies but sufficient clinical and technical validation for the performance of cfDNA detection assay has not yet been attained for routine clinical use (Mathai et al., 2019). To date in addition to cfDNA mutation biomarkers, a few DNA methylation-based biomarkers have been used clinically, for example the DNA methylation status of *MLH1* for the diagnosis of Lynch syndrome (Tost, 2015), Epi proColon test, which uses the methylated *SEPT9* gene to detect CRC (Church et al., 2014; Kormi et al., 2017; Potter et al., 2014) or the Epi proLung (methylated *SHOX2* gene) test for diagnosing lung cancer (Ilse et al., 2014). Panels with several genes can further improve the performance of DNA methylation signatures (Wang et al., 2017a). For instance, scientists have demonstrated a stool-based screening test for CRC, where a panel of two methylated genes were combined with mutation of *KRAS* gene along with an occult blood test. This assay obtained the first approval from the US-FDA for a diagnostic test based on DNA methylation in 2014 (Kormi et al., 2017; Imperiale et al., 2014).

On the other hand, some biomarkers have prognostic values in cancer. In CRC, acquired resistance to epithelial growth factor receptor (EGFR)-specific antibodies is related to mutations in the *RAS* pathway (Sforza et al., 2016), and patients with CRC who gained resistance to EGFR antibodies shows a heterogeneous pattern of mutation in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* (Hamzehzadeh et al., 2018; Hsu et al., 2016). In this regard, Mohan et al. performed WGS of ctDNA in CRC patients treated with anti-EGFR drug. Several modifications in copy numbers were discovered in all samples, including loss of chromosomal regions in 17p, 18q and 5q22 (retaining the *APC* gene) and amplifications in *ERBB2*, *MET*, and *KRAS* genes involved in resistance to *EGFR* blockade. Hence, they suggested that clonal genomic alterations during targeted therapies could be detected by analysis of ctDNA (Mohan et al., 2014). Hu et al. assessed the copy number of mutated *KRAS* and *EGFR* alleles in the pre/post surgery plasma of 168 patients with lung cancer and they found that ctDNA analysis in plasma is an alternative and complement to tissue analysis, which holds clinically promising application (Hu et al., 2017). Liang et al. in their meta-analysis showed that ctDNA is a potential predictive biomarker of recurrence in patients with early stage NSCLC (Liang et al., 2018). In reaction to treatment, Murtaza et al. also monitored genomic evolution of metastatic breast cancer patients on ctDNA and showed that ctDNA mutation could complement invasive biopsy associated with drug resistance. These authors evidenced an activating mutation in *PIK3CA* after paclitaxel therapy, a truncating mutation in *RB1* after cisplatin therapy; a truncating mutation in *MED1* after therapy with tamoxifen and trastuzumab, and following subsequent treatment with lapatinib, a splicing mutation in *GAS6* in the same patient; and a resistance-conferring mutation in EGFR after therapy with gefitinib (Murtaza et al., 2013).

There is a strong focus in prostate cancer on genomic aberrations linked to anti-androgen therapy resistance. In patients with metastatic prostate cancer, Heitzer et al. conducted a genome-wide study of ctDNA and discovered various copy number aberrations, including those recently reported in prostate tumors, such as 8p loss and 8q gain. In patients with castration-resistant prostate cancer (CRPC) but not with castration-resistant disease, high-level copy number gains in the androgen receptor (*AR*) locus were reported (Heitzer et al., 2013). More lately, Azad et al. discovered that in patients progressing with enzalutamide, *AR* amplification was considerably more prevalent than in abiraterone or other medicines (Azad et al., 2015). In addition to *AR* gene amplification, Joseph et al. reported that the *AR* F876 L mutant, a ligand-binding mutation in the *AR* domain, could be detectable in ARN-509-treated patients with progressive CRPC in ctDNA. (Joseph et al., 2013). Along these lines, Romanel et al. developed a targeted NGS approach for plasma cfDNA, covering all AR coding bases and genomic regions, which was extremely informative in prostate cancer (Romanel et al., 2015).

In addition to the biomarker serving role for ctDNA, it is also very much involved in cancer progression in a phenomenon termed “genometastasis”. Garcia-Olmo et al. indicated that CRC cell-derived cfDNA could not only induce oncogenic transformation of murine embryonic fibroblasts but also distant metastasis (Garcia-Olmo et al., 2010).

Concisely, the application of ctDNA for the early detection or screening of cancer or its prognostic and predictive implementations has received great attention as of now, but it encounters serious challenges especially regarding specificity and sensitivity of the current assays (Pantel and Alix-Panabieres, 2017).

6. Challenges for diagnostic and prognostic tests

As we move into the era of personalized cancer medicine, the need for more innovative cancer diagnostic and prognostic assays would seriously raise. Cellular nanoparticulates such as cfNAs (cfDNA, cfRNA) are now considered as important biomarkers with high performance in

medicine. There is huge hope that blood-borne cfNA could replace more invasive tumor solid biopsies in order to detect mutation/methylation in cancer and monitor treatment. Usually, conventional techniques for cfDNA biomarker blood isolation are time-consuming, complex and quite costly and inaccurate in many instances. They require quite large blood sample volumes at least mostly 10 ml, which must be processed to serum or preferably, plasma before isolation of biomarkers can be done (Medina Diaz et al., 2016). It is essential to know that while overall, serum may yield higher levels of cfDNA than plasma, the yield is more variable and the cfDNA quality may be highly influenced due to lysis of monocytes and other cells. Plasma is theoretically less likely to be contaminated with DNA from blood cells but, importantly, the time elapsed between blood collection and centrifugation can heavily impact it (El Messaoudi et al., 2013). In addition, the pre-analytic procedures have not been standardized yet. The sampling time, method of blood sampling, collection tubes, origin of blood sampling, blood and cfDNA storing conditions and so on are other cumbersomes, which restrict the widespread use of powerful downstream genomic analyses. New techniques that allow biomarkers to be quickly isolated straight from the blood could allow sophisticated molecular diagnostic testing (Lewis et al., 2015).

Although most cfDNA technologies have revealed high concordance with the molecular profile of the tumor tissue, there is still uncertainty whether molecular cfDNA testing can eventually replace tissue testing (Polivka et al., 2015).

The total amount of ctDNA could be as low as 0.01 percent of the total cfDNA. They occur in short fragments and are very limited in the number of genome equivalents presented in blood samples of early cancer patients. Hence, it could complicate molecular analysis and cause challenges for clinical detection (Genovese et al., 2014; Janku and Kurzrock, 2016; Elazezy and Joosse, 2018). Besides, there is a need for extensive study to identify possible combinations of cancer-specific genetic mutations and/or epigenetic changes to define precise quantitative thresholds in diagnosis. Based on the findings to date, DNA sequencing needs to be broad in order to embrace the high tumor heterogeneity and to display minute quantities of ctDNA fragments in the background of vast genetically normal cfDNA (Bettegowda et al., 2014).

The specificity of cfDNA measurements also faces many extra difficulties in relation to the need for greater sensitivity. With aging, cancer-associated mutations could happen even in people who have never developed cancer during their lifetime, restricting the chance of using somatic mutation assessment as a single strategy. For instance, clonal hematopoiesis with somatic mutations have been also shown in 10% of people over the age of 65 of age with an absolute risk of conversion from clonal hematopoiesis to hematologic cancer of 1.0% per year. Similarly, leukemia-associated mutation was reported to occur with increasing age. Although these mutations pose a statistically important danger of developing leukemia, over 90% of people with these mutations have never created leukemia during their lifetime (Genovese et al., 2014). Besides, in 11.4% of the 123 matched non-cancer controls, *TP53*-mutated cfDNA fragments were observed (Fernandez-Cuesta et al., 2016), as well as in the peritoneal fluid and peripheral blood of women with benign ovarian lesions (Krimmel et al., 2016). Gormally et al. showed *TP53* and *KRAS2* mutations in plasma DNA of healthy subjects (Gormally et al., 2006). Hence, non-cancerous cells, such as normal epidermal and blood cells, might possess genetic alterations in driver genes without evidence of neoplastic transformation (Genovese et al., 2014; Martincorena et al., 2015). In this regard, plenty of hallmark driver mutations for cancer including *BRAF*, *RAS*, *HER2*, *FGFR3*, *CDKN2A*, *PIK3CA*, *EGFR*, *TP53*, and *NF1/2* genes, can also be recognized in benign and premalignant conditions, even at frequencies higher than in their malignant forms (Kato et al., 2016). For instance, *BRAF* V600E mutations in benign nevi are unexpectedly more frequent than in dysplastic nevi or melanomas approximately 80%, 60% and 40–45%, respectively (Pollock et al., 2003). Another driver mutation i.e. *FGFR3* mutation which has been initially discovered in bladder cancer (Cappellen et al., 1999), also occur in seborrheic keratosis, a nonmalignant lesion (Hafner et al., 2006; Logie et al., 2005).

Consequently, the detection of a driving mutation on ctDNA may not therefore indicate that the individual tested already has cancer or will develop cancer in his or her lifetime. The mutation assay may only lead to so much anxiety for the patient and cause extensive diagnostic procedures like radiation with side effects. Accordingly, it is recommended to use NGS-based techniques, which interrogates multiple loci in a single reaction to identify several somatic mutations in oncogenes and/or tumor suppressor genes instead of a single driver mutation.

7. Future developments in personalized medicine

CTCs and ctDNA analyses have paved new diagnostic and prognostic avenues and are nominated to date the bases of liquid biopsy. To what extent in the future they might replace tumor solid biopsies is not completely known but their contribution in molecular personalized medicine is anticipated. However, they are not expected to entirely replace tumor biopsies since they cannot address many important factors such as changes in and interactions with the tumor microenvironment (Lewis et al., 2015). CTCs and ctDNA analyses can provide an alternative option in primary diagnosis of tumors that are troublesome to solid biopsy, such as lung or brain cancer, and for restaging metastatic lesions. Furthermore, liquid biopsy diagnostics could be used in cancer screening programs for the high-risk population, leading to reduced side effects (e.g., radiation in mammography) and health-care costs. Early detection of cancer by liquid biopsy, despite some promising preliminary outcomes and huge interest from diagnostic firms, faces important challenges in biomarker specificity and sensitivity. In order to recognize genomic aberrations, delicate and specific enough to detect early malignant lesions, extensive cohort studies with huge resources are required (Alix-Panabieres and Pantel, 2016). It is feasible to combine different types of biomarkers in order to improve sensitivity and specificity of the blood test. For instance, the CancerSEEK test is based on an algorithm that considers both mutations and protein markers detected in ctDNA and circulation in order to achieve high performance levels in early tumor stages (Logie et al., 2005).

CTCs and ctDNA monitoring during cancer treatment is an alternative potential application in clinical practice. Another significant clinical implementation in cancer patients undergoing surgery for their primary tumor is early detection of minimal residual disease (MRD). Postsurgical surveillance of MRD by cfDNA analysis has enabled investigators to seek for clonal evolution alterations in cancer patients such as CRC. Several clinical trials in cancer patients using liquid biopsy now focus on stratification and monitoring of therapies. For example, the DETECT-III study evaluates in breast cancer whether patients with HER2-negative primary tumors and HER2-positive CTCs will respond to lapatinib anti-HER2 therapy or not. In another example for breast cancer, the METABREAST trial is assessing the benefit of CTC counts in making a decision to prescribe chemotherapy or less aggressive endocrine therapy (Pantel, 2016). Therefore, these examples and similar ones indicate that liquid biopsies are becoming used to guide cancer treatment and provide the ideal approach to personalize treatment in precision medicine.

Developing low-cost NGS technology and complex data analysis of bioinformatics for the analysis of ctDNA provides a more comprehensive genetic profile and make this concept a potential future reality.

8. Conclusions

In the next few years, liquid biopsy especially cfDNA-based will be implemented in clinical studies and drug development and it will likely become an integral part of diagnostics in oncology but to reach that goal, a more laborious plan of devoted research with appropriate cohort studies and standardized analytical methods is necessary.

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Authors' contributions

MAK and AP designed the study. MAK and AP drafted the manuscript. MAK, and JPT coordinated, edited, and finalized the drafting of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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