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ctDNA as a cancer biomarker: a broad overview

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Abstract

Circulating tumor DNA (ctDNA) in fluids has gained attention because ctDNA seems to identify tumor-specific abnormalities, which could be used for diagnosis, follow-up of treatment, and prognosis: the so-called liquid biopsy. Liquid biopsy is a minimally invasive approach and presents the sum of ctDNA from primary and secondary tumor sites. It has been possible not only to quantify the amount of ctDNA but also to identify (epi)genetic changes. Specific mutations in genes have been identified in the plasma of patients with several types of cancer, which highlights ctDNA as a possible cancer biomarker. However, achieving detectable concentrations of ctDNA in body fluids is not an easy task. ctDNA fragments present a short half-life, and there are no cut-off values to discriminate high and low ctDNA concentrations. Here, we discuss the use of ctDNA as a cancer biomarker, the main methodologies, the inherent difficulties, and the clinical predictive value of ctDNA.

Keywords: biomarker, blood/plasma, cancer, ctDNA, liquid biopsy

1. Introduction

DNA circulates freely in the blood plasma of both healthy and sick individuals (Thierry et al., 2016). A crucial discovery was made when Mandel and Métais (1948) for the first time demonstrated the presence of extracellular nucleic acids in human plasma derived from patients with systemic lupus erythematosus (MANDEL and METAIS, 1948). Thirty years later, cfDNA (cell-

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free DNA) levels from patients with cancer were characterized by Leon and colleagues in 1977. It was shown that the total amount of circulating DNA was higher in patients than in healthy subjects (Leon et al., 1977). Although DNA is present in human body fluids, its molecular origin remains poorly understood, and several possible sources have been proposed. The term cirDNA (circulating DNA) or cfDNA describes any DNA existing in the extracellular environment, in spite of the structure (if associated with protein complexes or extracellular vesicles) (Aucamp et al., 2016; Bronkhorst et al., 2019).

Recently, the utility of molecular profiling of cfDNA in the diagnosis, prognosis and therapy of cancer and other diseases has been studied extensively, but the origin of cfDNA remains poorly understood (Aucamp et al., 2018). To date, many endogenous and exogenous sources of cfDNA have been identified, such as apoptosis, necrosis, and bacterial and viral DNA. Apoptosis and necrosis are considered to be the main processes for cfDNA origin. Necrotic and apoptotic cells are usually phagocytosed by macrophages, which can release digested DNA into the tissue environment (Schwarzenbach et al., 2011). On the other hand, numerous studies have demonstrated that cfDNA can be derived from an active cellular release mechanism (Anker et al., 1975; Gahan et al., 2008). Additionally, the terminal differentiation of erythrocytes and keratinocytes is a source of plasma and urine DNA. In this case, the differentiation of erythroid cells resembles apoptosis (Lichtenstein et al., 2001). This review will focus on the origin of cfDNA and ctDNA (circulating tumor DNA) in body fluids, with an emphasis on DNAs from blood and plasma and their clinical utility as a marker in liquid biopsy to diagnosis and follow-up the treatment and recurrence of tumors.

2. Cell-free DNA origin

Distinctive mechanisms that permit the translocation of DNA from the intracellular to extracellular compartment while maintaining biological stability of the molecule can be contemplated. Two opponents for the main origins of cfDNA are (I) cellular breakdown mechanisms and (II) active DNA release mechanisms. The structural characterization of cfDNA is reflected in one of these origins: apoptosis, necrosis, phagocytosis, oncosis and active secretion for particular structures (Aucamp et al., 2018; Thierry et al., 2016).

As mentioned earlier, it was demonstrated that patients with cancer show a higher total amount of circulating DNA than healthy individuals (Leon et al., 1977). In 1989, Stroun and colleagues suggested the presence of tumor-derived cfDNA in plasma/serum (Stroun et al., 1989). Shortly thereafter, the presence of point mutations of the N-Ras gene in plasma originating from tumor cells in the bone marrow and mutated K-Ras sequences from the plasma of patients with pancreatic cancer were detected, confirming that the mutant DNA

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fragments were of tumor origin (Sorenson et al., 1994; Vasioukhin et al., 1994). Nevertheless, an increase in cfDNA was not specific to malignancies. Elevated levels of cfDNA were also detected in the plasma of pregnant women (Dennis Lo et al., 1997) (Swaminathan and Butt, 2006). Other physiological and pathological processes also contribute to elevated cfDNA levels, such as exercise, inflammation, diabetes, sepsis and myocardial infarction (Kustanovich et al., 2019).

As cfDNA can be released by both dividing and nondividing cells and different cell populations, their fragment sizes range from 100 bp to up to 21 kbp. The short fragments can be related to the histone octamer structure due to apoptosis, and the longer fractions originate from necrotic cells. The majority of DNA produced by apoptosis has a size of approximately 166 bp, which corresponds to 147 bp of DNA wrapped around a nucleosome plus the stretch of DNA on histone H1, which links two nucleosome cores. Depending on the nuclease action, apoptosis can produce longer DNA fragments consistent with di-, tri-, or polynucleosomes (Bronkhorst et al., 2019; Jahr et al., 2001). However, a high proportion of cfDNA originating from tumors is more fragmented, with fragments smaller than 100 bp (Mouliere et al., 2011). Patients with cancer have elevated levels of cfDNA than healthy individuals, which can be explained by augmented levels of necrotic death in large tumors at advanced stages and by the association with tumor aggressiveness (Kustanovich et al., 2019).

Apart from the origin of apoptosis and necrosis, other DNA release mechanisms have been described. These include oncosis, pyroptosis, phagocytosis, active secretion, neutrophil extracellular trap release (NETosis) and excision repair (Aucamp et al., 2018). Active secretion occurs in live, functional cells that are facing the different forms of cell death, with the released DNA being either encapsulated in vesicles or in the form of complexes with glycolipoprotein and associated RNA, referred to as virtosomes (Gahan and Stroun, 2010). Recent studies have demonstrated the presence of cfDNA in culture medium at levels that do not correlate with the processes of apoptosis, necrosis or DNA replication (Bronkhorst et al., 2016).

The concentration of cfDNA in blood ranges from 0-5 to higher than 1000 ng/mL in patients with cancer and between 0 and 100 ng/mL in healthy subjects. In patients with metastatic cancer, DNA fragments are found at higher concentrations than those in patients with localized cancers (Bettegowda et al., 2014; Jahr et al., 2001).

It is necessary to consider the cellular origins of the DNA found in the blood of cancer patients. The demonstration that the tumor cell compartment is a source of cfDNA was possible due to the identification of genetic alterations specific to cancer cells. The tumor composition consists of a group of malignant tumor cells and cells that constitute the tumor microenvironment, such as

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stromal cells, endothelial cells, lymphocytes and other immune cells, all of which can constitute a potential source of circulating tumor DNA (ctDNA) (Thierry et al., 2016). Other sources of ctDNA include circulating tumor cells (CTCs) and exosomes. CTCs were discovered by Thomas Ashworth in the 1860s during an autopsy of a patient with metastatic cancer. These cells have the ability to detach from a primary or metastatic tumor site, penetrate the vessel wall and enter the circulatory system (Domínguez-Vigil et al., 2018; Zhang et al., 2017). CTCs can be isolated as single cells or clusters from the blood of patients with cancer. However, CTCs and exosomes are beyond our scope in this review.

Aside from the mechanisms of release, the characteristics of cfDNA are largely influenced by the rate of its clearance. The half-life estimated for cfDNA in circulation is between 16 minutes and 2.5 hours and depends on various factors, such as association with molecular complexes or the type and stage of the tumor, treatment and other factors (Yao et al., 2016; Yu et al., 2013). In blood, cfDNA degradation occurs essentially by circulating enzymes such as DNase I, renal excretion into urine, and uptake by the liver and spleen followed by macrophagic degradation, breakdown by nucleases and intake by cells (Bronkhorst et al., 2019; Gauthier et al., 1996; Kustanovich et al., 2019). The association of cfDNA with protein complexes, extracellular vesicles and serum proteins can influence the clearance mechanisms. Therefore, the rate of cfDNA uptake by different cells may affect its clearance rate (Bronkhorst et al., 2019).

3. Liquid biopsy

In this fashion, the cfDNA/ctDNA profile present in a single blood sample comprises a combination of both wild-type and genetically and epigenetically altered DNA fragments released by different tissues and organs by different mechanisms (Figure 1A). Analysis of ctDNA can identify tumor-specific abnormalities that can be used for highly specific testing strategies (Husain and Velculescu, 2017). Tissue-based assays have limitations in the clinical setting, which leads to overdiagnosis, false positives and low specificity. Additionally, biopsies can miss important drivers due to tumor heterogeneity or distant metastatic lesions (Morgeson et al., 2018). In this regard, liquid biopsy is a promising approach that could overcome these shortcomings as a minimally invasive approach that can provide tumor diagnostic possibilities, determining therapy response and prognostic prediction. Specific DNA aberrations related to tumors can be recorded, such as point mutations, loss of heterozygosity (LOH), gene amplifications and hypermethylation of the tumor suppressor gene promoter (Kerachian et al., 2019). The term liquid biopsy was first used to describe methods that can derive the same diagnostic information from a blood sample that is typically derived from a tissue biopsy sample, and in oncology, the term is used to refer to sampling and analysis from various biological fluids

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(Heitzer et al., 2019). Thus, liquid biopsy has a wide potential for clinical applications.

In addition to be a noninvasive alternative to surgical biopsies, advances in genomics and molecular technologies have created great interest in liquid biopsies. Liquid biopsy poses a relatively low risk to patients, and these measurements can be dynamic, permitting correlations between disease burden and progression to quantifiable biomarkers. Liquid biopsy based on ctDNA is superior to previous plasma biomarkers with respect to sensitivity and clinical correlations (Sun et al., 2018).

4. Main methodologies to detect ctDNA

The challenge in liquid biopsy is the identification of very low amounts of ctDNA in blood samples with variable amounts of cfDNA and the choice of the right panel for each neoplasia. Therefore, highly sensitive and specific methods have been developed to detect ctDNA (Figure 1B). Some of these methods include BEAMing Safe-Seq, TamSeq and digital droplet PCR to detect single nucleotide mutations or whole genome sequencing for untargeted approaches (Alix-Panabières and Pantel, 2016). Each technique is able to detect mutant allele frequencies with a sensitivity of at least 2%, but the cost and the standardization of each for use in the clinic is a significant consideration and can pose diverse technical difficulties (Crowley et al., 2013).

The majority of available commercial kits for ctDNA are real-time PCR-based, such as the Therascreen TM EGFR plasma RGQ PCR kit (Qiagen), cobas EGFR Mutation Test v2 (Roche Molecular Diagnostics) and Super-ARMS EGFR Detection Kit (Franczak et al., 2019). However, a variety of allele-specific assays have been introduced to improve the analytical sensitivity of conventional qPCR methods, and they can be characterized as targeted approaches. For example, digital PCR was developed by Vogelstein and Kinzler in order to transform the exponential nature of PCR to a linear and increasing the sensitivity (Vogelstein and Kinzler, 1999). Subsequently, different strategies have been developed using microchambers or microdroplets. In parallel to these systems, droplet-based digital PCR uses aqueous droplets dispersed in oil for compartmentalization of PCRs to realize high-throughput digital PCR by taking advantage of microfluidic circuits and surfactants. The sensitivity of this approach is approximately 0,1% (Franczak et al., 2019; Hindson et al., 2011; Postel et al., 2018). Today, numerous droplet-based digital PCR devices are being commercialized, including Raindrop ™ digital PCR (Raindance Technologies), Bio-Rad QX200™ Droplet Digital System (Bio-Rad Laboratories) and Naica™ System (Stilla Technologies) (Perkins et al., 2017). BEAMing (beads, emulsion, amplification and magnetics) was the first high-throughput droplet digital PCR system described for the detection and enumeration of

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genetic variations (Diehl et al., 2006). The sensitivity of this method is one mutant DNA molecule for 10,000 wild-type molecules, but the system requires a complicated procedure for routine clinical use (Postel et al., 2018). Other PCR-based methods are coamplification at lower denaturation temperature (COLD-PCR), real-time bidirectional pyrophosphorolysis-activated polymerization (Bi-PAP) and Intplex.

Characterized as an untargeted approach, next-generation sequencing (NGS) is theoretically applicable to all patients, as it allows de novo identification of molecular alterations, including single nucleotide substitutions, structural rearrangements and copy number variations (Volckmar et al., 2018). Notwithstanding, strategies such as whole exome or whole genome sequencing usually generate approximately 30 to 100 times average sequencing coverage, which leads to too low sensitivity to analyze rare mutations in ctDNA. Different improved NGS technologies, such as the Safe-Sequencing System (Safe-Seq) (Kinde et al., 2011) and Cancer Personalized Profiling by deep sequencing (CAPP-seq) (Newman et al., 2014), have allowed greatly improved detection sensitivity of ctDNA.

The first cfDNA-based test approved by the FDA was the Cobas EGFR Mutation Test v2 in 2015. This device is a real-time PCR test for the qualitative detection of exon 19 deletions and exon 21 substitution mutations of the EGFR gene from non-small cell lung cancer (NSCLC) patients (Kwapisz, 2017). Another FDA-approved test is Epi proColon (Epigenomics AG), which reports on the methylation status of the SEPT9 promoter in the plasma of colorectal cancer (CRC) patients (Heitzer et al., 2019). The clinical utility of these tests emphasizes the potential of ctDNA as a biomarker. In the next sections, we will further discuss the use of ctDNA as a biomarker in both diagnostic and treatment follow-up and assistance in choosing the suitable therapy.

5. Using plasma ctDNA in cancer diagnosis

The gold standard in the diagnosis of oncology is tissue biopsy, which has been used by clinicians for over 100 years. Biopsies allow histological definition, and with the advancement of molecular biology, it is also possible to describe the genetic profile of the tumor (Crowley et al., 2013). Although tissue biopsy is the gold standard for cancer diagnosis, there are some disadvantages. For example, they are invasive procedures, and they show heterogeneity with different genetic profiles depending on the fragment removed from the tumor, sometimes at high cost and with some risk to the patient (Diaz and Bardelli, 2014; Doval et al., 2017; Finotti et al., 2018; Mathai et al., 2019) To address the limitations of tissue biopsy, methodologies such as liquid biopsy have been extensively studied, as mentioned in sections 3 and 4. In terms of diagnosis,

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liquid biopsy can be a major contributor to early detection, metastasis, prognosis and case confirmation (Castro-Giner et al., 2018; Doval et al., 2017; Grölz et al., 2018; Mathai et al., 2019; Palmirotta et al., 2018).

Liquid biopsies, in addition to blood sampling, can be performed with other body fluids, including cerebrospinal fluid, urine, saliva, bronchial lavage, and pleural fluid. Many studies report that depending on the location of the tumor, non-blood samples are more sensitive in liquid biopsy, as many authors have shown that the combination of different fluids may increase the sensitivity of the tests (Lousada-Fernandez et al., 2018; Thierry and Tanos, 2018).

The detection of circulating DNA has been observed by qualitative and quantitative changes. Studies describe the relationship between cfDNA levels and prognosis and disease stage. Qualitative changes include genetic changes, such as point mutations, specific methylation and ctDNA copy number variations (Okajima et al., 2017). ctDNA analysis was officially employed as a diagnostic tool in leading reference center laboratories during the course of 2016 (Castro-Giner et al., 2018). Several genetic alterations have been studied in several existing tumor types. Studies show mutations in different genes as possible biomarkers for the diagnosis of cancer by liquid biopsy (Table 1) (Castro-Giner et al., 2018; Mathai et al., 2019)

Liquid biopsy is not yet a routine test in clinical practice, but its diagnostic potential for cancer is drawing the attention of oncologists (Palmirotta et al., 2018). Few of these biomarkers have been validated so far for clinical use (Thierry and Tanos, 2018).

Here, we highlight some studies that have demonstrated the importance and possibility of liquid biopsy for diagnosis in different types of neoplasms, classifying them in accordance with the relative abundancy of ctDNA found usually in the patient´s blood sample. The quantity of ctDNA in blood varies depending on the tumor localization (Méhes, 2019). Bettegowda et al. (2014) demonstrated that tumors such as lung cancer, CRC, breast cancer (BC), liver cancer and prostate cancer have higher quantities of ctDNA in blood than others. Instead, oral, pancreatic, gastric cancer and gliomas are described as yielding lower concentrations of ctDNA in the patient´s blood (Bettegowda et al., 2014).

5.1 Tumors with higher quantities of ctDNA in the patient´s blood

Prostate cancer is the second most common cancer in men worldwide, with mortality in the fifth position of cancer deaths (Neuhaus and Yang, 2018). Studies show that prostate cancer patients have higher plasma cfDNA levels than healthy individuals, and cfDNA levels may be related to disease stage (Moyer, 2012). The main genomic profile findings in prostate cancer describe changes in TP53, RB1, PTEN, AR, FOXA1, MYC, ERG, PI3K, and WNT (Hodara et al., 2019).

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In turn, CRC is described as the third most common cancer worldwide, representing one of the leading causes of cancer deaths (Klein-Scory et al., 2018; Mathai et al., 2019). Approximately half of patients with localized CRC have been reported to have metastases (Siegel et al., 2017). The gold standard for early CRC detection is colonoscopy, which can be supported by the detection of occult blood DNA mutations (Hench et al., 2018). Studies using CRC tissue biopsy often describe mutations in the BRAF, KRAS, APC, TP53, CEA, and SEPT9 genes. Therefore, these genes have been studied as possible biomarkers for liquid biopsy (Mathai et al., 2019). There are already studies demonstrating the relationship between ctDNA levels in CRC cases and the poor prognosis of these cases (Alix-Panabières and Pantel, 2016; Hardingham et al., 1995). Qualitative ctDNA detection studies have shown a high correlation between tissue biopsy and liquid biopsy (Hench et al., 2018).

Lung cancer represents one of the most common tumors in the world and the most common cause of cancer deaths (Ferlay et al., 2019; Mathai et al., 2019; Siegel et al., 2017). The high mortality rate of lung cancer patients can be attributed to the fact that these tumors are more frequently detected in advanced stages of the disease. Therefore, early detection in these cases demonstrates a life-enhancing possibility for patients (Santarpia et al., 2018). Studies report a broad spectrum of cfDNA concentrations in the blood of patients with lung cancer, and levels may be related to disease prognosis and tumor stage (Hench et al., 2018). Several studies describe mutations in different genes in lung cancer patients at different stages (Mathai et al., 2019).

In a study of blood samples from patients with small cell lung cancer (SCLC), mutations in the TP53 gene were detected in 49% of the cases tested (Thierry and Tanos, 2018). In another study, a mutation in TP53 was detected in patients with SCLC and in 11% of control patients (Aceto et al., 2014). A study in patients with NSCLS reported mutations in the KRAS gene (Ying et al., 2015). Other studies in patients with advanced lung cancer have found mutations in the EGFR, BRAF, ERBB2 and PIK3CA genes (Couraud et al., 2014; Del Re et al., 2017). In lung cancer patients, alterations in the growth factor receptor 1 (FGFR1), HER2, KRAS, ROS1 and RET genes are also commonly found (Doval et al., 2017).

Among liver tumors, the most common is hepatocarcinoma or hepatocellular carcinoma (HCC). HCC is the sixth most common cancer in the world and the second most common cause of cancer deaths (Howell and Sharma, 2016; Okajima et al., 2017; Su et al., 2018), featuring an overall survival rate of 0 to 10% over 5 years (Yin et al., 2016). The occurrence of genetic mutations in blood and urine samples from patients with HCC has been demonstrated in several studies (Lin et al., 2011). Mutations in the TP53, CTNNB1, PTEN, CDKN2A, ARID1A, MET, CDK6, EGFR, MYC, BRAF, RAF1, FGFR1, CCNE1, PIK3CA genes and ERBB2/HER2 have been described (Mathai et al., 2019). One study demonstrated the possible prognostic utility of ctDNA hTERT mutations, observing higher mutated ctDNA levels in patients

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with multinodular HCC than in patients with early-stage disease, and found that higher ctDNA levels could be associated with shorter survival (Howell and Sharma, 2016). Liquid biopsy proved useful in the early detection of HCC, and the detection of changes in DNA copy number in HBV patients with no previous history of HCC was observed in patients who developed HCC afterwards, demonstrating the possibility of liquid biopsy as a screening tool for the early detection of HCC (Jiang et al., 2015).

BC is considered the second most common cancer in the world and the fifth leading cause of cancer deaths (Mathai et al., 2019). This neoplasm presents with a heterogeneous set of diseases with distinct biological characteristics (Hench et al., 2018). Studies have shown the possibility of detecting ctDNA genetic alterations for diagnosis using liquid biopsy in BC patients (De Mattos-Arruda and Caldas, 2016). However, reports show that mutated genes are rare in BC cases, and few mutations in the TP53 and PIK3CA genes have been demonstrated (Nik-Zainal et al., 2012; Shah et al., 2009). As with other tumor types, detecting and evaluating plasma ctDNA mutations contributes to the early diagnosis of relapses in BC patients (Garcia-Murillas et al., 2015; Olsson et al., 2015). A correlation between cfDNA levels and disease malignancy was also demonstrated. There are studies describing the use of the HER2 receptor as a biomarker for liquid biopsy in BC patients, as well as mutations in the BRCA1 gene (Mathai et al., 2019).

Therefore, many studies have demonstrated the possibility of using ctDNA in blood for early detection and prognosis in lung cancers, CRC, BC, HCC and prostate cancers.

5.2 Tumors with a lower quantity of ctDNA in the patient´s blood

Head and neck cancers are the sixth most malignancies in the world (Jemal et al., 2011). These include several malignancies common to the mucosal and upper aerodigestive tract epithelia (Bellairs et al., 2017; Payne et al., 2019). A significant increase in the incidence and mortality of these cancers is observed in developing countries (Payne et al., 2019). Studies using plasma and saliva samples have demonstrated the importance of ctDNA as a biomarker for these tumors (Wang et al., 2004). A cohort study of 93 patients using plasma and saliva samples to detect mutations in the TP53, PIK3CA, NOTCH1, FBXW7, CDKN2A, NRAS and HRAS genes demonstrated greater sensitivity for detection of plasma ctDNA in the oropharynx, hypopharynx and larynx, while in oral cancer, salivary ctDNA showed a higher sensitivity (100% vs. 80%), which can be explained by its closer proximity to the tumor. In addition, plasma and saliva samples were tested in combination, which demonstrated a higher sensitivity rate for all cases (Wang et al., 2015). Studies conducted on 200 tumor patients showed a higher level of cfDNA, but there was no significant difference from the level in the control group (n = 15). However, it was noted that patients with oropharyngeal tumors had significantly higher levels of ctDNA

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(Mazurek et al., 2016). In a study using 47 patients' plasma and saliva, ctDNA was detected in 96% of the cases. When divided by disease stage, ctDNA was identified in 100% of the initial disease cases and 95% of the advanced disease cases. As for location, ctDNA was identified in all patients with oral lesions ranging from the larynx to hypopharynx and in 91% of patients with oropharyngeal tumors (Wang et al., 2015). In addition to the genes already described, one study detected mutations in ctDNA in the CASP8 and PTEN genes in plasma samples from 42 patients with early oral cancer (Lousada-Fernandez et al., 2018).

Pancreatic cancer (PC) is one of the most lethal cancers in the world, with a poor five-year survival, mainly due to the late diagnosis of the disease (Maire et al., 2002; Qi et al., 2018; Yadav et al., 2018). Shapiro et al. (1983) demonstrated elevated levels of ctDNA in PC patients compared to healthy controls, thus concluding that ctDNA may be a promising biomarker for pancreatic cancer diagnosis (Qi et al., 2018; Shapiro et al., 1983). The main mutation described for ctDNA in pancreatic cancer is the KRAS gene, which is often found in patients with this cancer (Lu et al., 2018; Qi et al., 2018). ctDNA PCR assays showed a sensitivity of 30-50% and a specificity of 90% in detecting blood KRAS (Maire et al., 2002). Thus, the KRAS mutation has been used for the diagnosis of the disease (Yadav et al., 2018). In addition to the KRAS mutation, genetic alterations in the BRAF gene have been identified and observed in 3% of PC patients without KRAS mutations (Witkiewicz et al., 2015).

Gastric cancer (GC) is the fourth most common cancer in the world and the second leading cause of cancer deaths (Matsuoka and Yashiro, 2019; Tsujiura et al., 2014). In recent years, certain studies have been performed yielding possible values for the diagnosis of gastric cancer by assessing cfDNA levels (Matsuoka and Yashiro, 2019). Most studies show a contribution to the detection of specific methylated promoter regions in the blood of patients with GC (Gao et al., 2017). An investigation involving liquid biopsy demonstrated the possibility of using HER2 gene ctDNA detection as a biomarker for the diagnosis of GC cases (Mathai et al., 2019). The use of new biomarkers, including proteins and genes such as FGFR, CDH1, PI3K, Met, VEGFR, TP53, and DP-1, enables new diagnostic and monitoring approaches for patients with GC (Matsuoka and Yashiro, 2019).

In turn, the detection rate and plasma ctDNA concentration of patients with central nervous system (CNS) neoplasms have been shown to be low compared to other tumor types (Bettegowda et al., 2014; Okajima et al., 2017). A cohort of 41 patients with primary brain tumors was evaluated by Bettegowda et al., seeking detection of ctDNA changes in plasma using digital PCR methodology. In this study, unlike other tumor types, a low detection was found (<50% for medulloblastoma and <10% for gliomas). The low sensitivity of liquid plasma biopsy for the diagnosis of CNS tumors has been suggested by the presence of the blood brain barrier (BBB), which hinders the circulation of tumor

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components in the blood. Therefore, the vast majority of liquid biopsy studies in these cases use cerebrospinal fluid as an alternative diagnostic sample, aiming at its direct contact with the CNS (Bertero et al., 2019). The feasibility of detecting ctDNA in patients with secondary CNS tumor involvement has been demonstrated in studies of breast carcinoma, melanoma and secondary hematologic malignancies (Li et al., 2016).

Therefore, in different types of cancer, liquid biopsy from the blood of patients has demonstrated its clinical usefulness as a minimally invasive methodology for diagnosis, prognosis and prediction. In addition, liquid biopsy can be used in the laboratory to further analyze mechanisms for the acquisition of drug resistance, recurrence and dissemination, which are further discussed in the next section. Thus, new possibilities can be opened with the study of circulating DNA to unravel the mechanisms involved in all these processes.

6. Using ctDNA to follow-up cancer treatment and recurrence

The profile of mutations and the behavior of cancer cells change in a dynamic way and may show additional features over time (Brown et al., 2017; Kim et al., 2015; Zheng et al., 2018). Recurrence or metastases are related to the selection of factors that favor the survival of subclones that potentially have adapted well to applied therapies (Goranova et al., 2011). In different periods of a malignant disease, repeated tests are required to learn about molecular factors of resistance and new therapeutic targets that are applicable (Nemecek et al., 2016). However, repeated tissue samples are a challenge for patients and physicians, especially for restricted anatomical locations and in the case of multiple affected organs, regarding the decision of which sites should be included in biopsy. Histology would be informative at any phase of the disease, but immunohistochemical subclassification, even if evaluated in a meticulous manner, has limited value to predict treatment response without molecular characterization (Louis et al., 2016; Méhes, 2019). Histological subtypes call for the profiling of specific gene panels focusing on therapeutic sensitivity. Although the paraffinized sample can be used in DNA or RNA-based studies, individual variables and technical impairments contribute to the low representation of tissue and biological heterogeneity in tumor composition, complicating the clinical analysis. Specific alterations can be present in different proportions or can be masked by the amount of normal or non-neoplastic tissue. Therefore, medicine is moving toward a noninvasive genetic test for cancers (Méhes, 2019). Tumor profiles in plasma, urine, liquor and other body fluids that are useful for the detection and follow-up of specific genes by the several methods already discussed here provide a good alternative (Savli et al., 2019).

DNA extracted from the serum is variably fragmented, but representative sequences with characteristic cancer-related aberrations could reproducibly demonstrate suitable amplification and accurate sequencing (Ma et al., 2017). Recently, there has been consensus in the literature that ctDNA obtained by liquid biopsy is a convenient carrier of the actual genetic composition and is

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potentially more informative than tissue-derived nucleic acids archived from the time of diagnosis (Li et al., 2019; Remon et al., 2019; Vitiello et al., 2019). Additionally, ctDNA is useful for finding therapeutic targets after several cycles of therapy (Bhangu et al., 2018; Garlan et al., 2017). Circulating free DNA, comparable with tissue-derived DNA, seems to work optimally for cancer-related single nucleotide variant (SNV) (Table 2) or in/del mutation detection (Méhes, 2019).

In an investigation of CRC patients, (Schøler et al., 2017) observed ctDNA post-surgery in all patients who relapsed but not in any of the nonrelapsing patients. The blood ctDNA detected cancer recidivism with an average lead-time of ~9 months compared to imaging. They also observed that changes in ctDNA levels induced by relapse showed agreement with changes in the tumor volume. Other studies achieved similar results (Lyskjær et al., 2019; Reinert et al., 2016). Furthermore, driver mutations of the epithelial growth factor receptor (EGFR) pathway, including the receptor kinase domain itself, and downstream signaling members, such as KRAS and BRAF, are good predictors for the efficacy of EGFR tyrosine kinase inhibitor (TKI) therapies in epithelial cancers (Afrăsânie et al., 2019; Lu et al., 2018; Roengvoraphoj et al., 2013). Currently, it is possible to identify 42 EGFR mutations, including exon 19 deletions, exon 20 insertions and S768I, L861Q, L858R and T790M mutations in both tissue and plasma that result in increased sensitivity to TKIs (St. Jude, 2016). These alterations are strong predictors for increased progression-free survival (PFS) in patients who receive EGFR-targeted therapies such as erlotinib (Tarceva). Additionally, the test can be used to detect the T790M EGFR mutation in patients after treatment with EGFR-targeted TKIs because this mutation leads to therapy resistance, and its detection is indicative for switching the treatment from erlotinib to the third-generation drug osimertinib (Heitzer et al., 2019).

CRC and NSCLC, for instance, share MAP kinase signaling as the most frequently targetable pathway (Liu et al., 2016; Méhes, 2019). RAS mutational status in plasma from 115 metastatic CRC patients showed 93% overall agreement with the analysis of RAS in tissue samples. Additionally, in patients with baseline mutant RAS tumors treated with antiangiogenic agents or chemotherapy, longitudinal analysis of RAS ctDNA reflected the response to these treatments (Vidal et al., 2017).

Other factors, such as microsatellite instability in CRC and translocations of the ALK, ROS1 and NTRK genes in NSCLC, differentiate their predictive molecular profiles for the clinic. As more drugs are released in multiple indications, mutational testing is becoming more generalized to cover large common panels representing several oncogenes, suppressors and other related genes (Méhes, 2019).

Undetectable baseline or early decline to undetectable ctDNA levels during the first 6–9 weeks was correlated with prolonged progression-free survival (PFS) and overall survival (OS) in melanoma patients treated with anti-PD1 therapy (Seremet et al., 2019). In addition, there was a case that reported a stage III hormone receptor-positive breast cancer (BC) patient who remained without symptoms after receiving surgery and adjuvant chemotherapy. Liquid biopsy analysis by NGS detected the presence of a ctDNA PIK3CA mutation

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five months before the detection of a tumor relapse with multiple liver metastases by regular clinical follow-up (Cheng et al., 2019).

In a study of epithelial ovarian cancer (OC) patients who were treated with bevacizumab, both PFS and OS were significantly shorter in patients with high levels of cfDNA in blood samples, suggesting that cfDNA could be an independent prognostic factor in patients treated with bevacizumab (Steffensen et al., 2014). Detection of mutant DNA in the plasma of patients with OC may also be useful in the follow-up of postsurgical assessments (Cheng et al., 2017). In another study analyzing plasma cfDNA from cisplatin-treated OC patients, an increase in truncating mutations of retinoblastoma 1 tumor suppressor (RB1) was observed (Murtaza et al., 2013). In addition, the loss of RB1 heterozygosity is associated with epithelial-mesenchymal transition (EMT) and linked with the chemotherapy response (Knudsen and Knudsen, 2008). Weigelt et al. found that BRCA1/2 reversion mutations were identified in cfDNA from 21% of platinum-resistant OC patients, indicating that cfDNA sequencing may aid the selection of patients for PARP inhibition therapy (Weigelt et al., 2017).

Follow-up of HCC patients who received surgical resection followed by adjuvant therapies showed consistency of the somatic profile between pre- and postsurgery plasma-derived ctDNA and primary tumor tissue (Mezzalira et al., 2019). A total of 44 samples collected from 35 HCC patients, with most having high-stage disease, analyzed 122 genetic alterations. Excluding variants with undetermined or synonymous alterations, the top genes altered in this cohort of patients were *TP53* (18%), *TERT* (14%), *CTNNB1* (13%), *ARID1A* (9%), *MYC* (5%), *BRAF* (4%), *CCND1* (4%), *CDK6* (4%), *MET* (4%), and *EGFR* (3%) (Mody et al., 2019). Another study showed that HCC tumors harboring oncogenic PI3K-mTOR mutations had worse outcomes on sorafenib treatment, while the presence of activating WNT/b-catenin alterations was associated with innate resistance to immune checkpoint inhibitors (Harding et al., 2019). Piciocchi and colleagues split the patient´s cohort into two groups with high or low levels of cfDNA (cut off: 2 ng/µL). The analysis revealed that patients with cfDNA levels below the cut off showed a median survival that was 13 months longer than patients with levels above the cut off (Piciocchi et al., 2013).

Several reports also investigated the correlation between mutations in ctDNA matching tumor biopsy in patients with PC, especially in the *KRAS* gene, with a concordance ranging from 25 to 70% (Del Re et al., 2017). Although few analyses have been carried out in PC, data reported showed that longitudinal monitoring of ctDNA can predict the response to chemotherapy and disease progression, simultaneously or even 5 months earlier than conventional monitoring approaches, such as radiological imaging and CA 19-9 protein detection in blood (Del Re et al., 2017; Tjensvoll et al., 2016). Liquid biopsy has been used in challenging cases to confirm the early recurrence of PC where acquisition of tissue is not feasible (Soyano et al., 2019).

Not only genetic alterations but also epigenetic changes have been studied in follow-up patients. Recently, for the first time, *ESR1* methylation in

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CTCs paired with ctDNA in BC patients was shown to be highly concordant. *ESR1* methylation was associated with a lack of response to everolimus/exemestane. *ESR1* methylation should be further evaluated as a potential liquid biopsy-based biomarker for the follow-up of BC patients treated with these therapy combinations (Mastoraki et al., 2018).

In general, postoperative detection of ctDNA in blood and other body fluids is significantly correlated with relapse-free survival. Therefore, ctDNA analysis appears to be strongly predictive of recurrence among patients. In this context, ctDNA could be exploited to closely monitor patients before and after surgery to identify high-risk patients for disease recurrence (Ponti et al., 2019). Moreover, the identification of punctual mutations and epigenetic changes has been useful to direct or redirect therapeutic arsenal (Heitzer et al., 2019; Mastoraki et al., 2018).

7. The “bottle neck” of ctDNA analysis

Although ctDNA has been appointed as a predictive marker in several cancers (Hironaka-Mitsuhashi et al., 2019), this analysis has several obstacles, including the comparison and standardization of different methods. Additionally, its clinical relevance studies have only recently started (Volckmar et al., 2018), as we will discuss in this section.

The amount and composition of free DNA is influenced by multiple factors, including the stage and anatomical localization of the cancer, the rate of tumor cell growth, death and necrosis, the relative mass of subclones, and the stromal and inflammatory components (Méhes, 2019). Aggressive cancers are generally poorly differentiated or undifferentiated and have considerable areas of necrosis, while well-differentiated tumors show a low cell-proliferation capacity with a limited area of necrosis. The total ctDNA represented in the body fluid is a sum of the tumor localization, phenotype and differentiation grade. Tumor samples with less than 10% neoplastic cell content may lose the low rate of genetic aberration sensitivity (Lorber et al., 2019; Méhes, 2019; Schwarzenbach et al., 2011). Therefore, the ctDNA concentration is not controllable, as tumor composition and localization are major preanalytical variables. The total amount of ctDNA is very diluted in the large body fluid volumes in a human body, and the concentration of ctDNA measurable in peripheral blood samples, for instance, is generally very low, highly variable and impairs the analysis (Fiala and Diamandis, 2018; Jahr et al., 2001). Other challenges associated with ctDNA analysis are the need for highly sensible technologies to distinguish mutant from wild-type alleles and the development of thresholds for mutations. Furthermore, ctDNA fragments present a short half-life of less than 2 h and must be processed quite shortly (Müller Bark et al., 2019).

There is no consensus about the proper cut-off value to apply for the discrimination of high and low cfDNA concentrations; this cut-off is strongly laboratory-dependent, resulting in poor reproducibility of the data. Moreover, the concentration has been evaluated in different sources (Mezzalira et al., 2019).

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The overall concordance between plasma and matched tumor tissues assessed by NGS is only approximately 25% (Pishvaian et al., 2017). Internal quality control guidelines and ctDNA reference materials are still not available to support strong and reliable liquid biopsy-based cancer diagnostics and follow-up (Li et al., 2019). To date, there are no cross-platform validations, and therefore, the clinical predictive value of variant detection must be considered with caution (Merker et al., 2018). In addition, there is no clear indication for serum DNA analysis in the context of therapeutic target identification, since most of the clinical validation studies so far were performed using solid biopsies. The genetic heterogeneity and subclonal variability of tumors, better reflected by ctDNA, represent a new level of complexity and require further experience for clinical interpretation (Méhes, 2019; Volckmar et al., 2018)

Furthermore, achieving detectable concentrations of ctDNA in a noninvasive way poses another difficulty for some types of tumors, including brain tumors such as gliomas (Bettegowda et al., 2014). These studies demonstrate a low yield of ctDNA in glioma patients' blood, mostly justified by the presence of the blood brain barrier (BBB). However, another study showed that tumor size and cell proliferation influenced the release of ctDNA in mice prior to treatment, while BBB integrity did not. This study used a patient-derived orthotopic xenograft model and found that cell death post therapy is an additional factor that can augment ctDNA release (Mair et al., 2019; Müller Bark et al., 2019). Piccioni et al. analyzed 419 patients with primary brain tumors, detecting ctDNA mutations in blood samples from 50% of all brain tumor patients. This work also included 222 patients with glioblastoma (GB), and the authors were able to detect ctDNA mutations in 55% of these GB patients (Piccioni et al., 2019). Therefore, further studies are necessary to evaluate whether the BBB impairs ctDNA release in the blood and which approach would be necessary to detect ctDNA from brain tumors, such as gliomas.

In addition to the cerebrospinal liquid already mentioned, analysis of circulating platelets seems to be another intriguing approach to achieve liquid biopsy of CNS tumors. Many biomolecules are transferred between tumors and platelets, and this process has been termed platelet "education" (Bertero et al., 2019; Best et al., 2018). Platelets can sequester tumor-shed extracellular vesicles, which allow the detection of tumor-derived biomarkers, including proteins and nucleic acids, with high sensitivity and specificity (Klement et al., 2009; Kuznetsov et al., 2012). In 114 samples (62 patients) with brain lesions, tumor-educated platelet analysis allowed assertive identification of cancer in 91% of patients and discernment between the mutational subtypes in 82% of cases (Bertero et al., 2019; Best et al., 2018).

We conclude that ctDNA seems to be a great predictive marker for certain types of cancer, such as lung, breast, ovarian, hepatic, pancreatic and colon cancer. However, especially for brain tumors and other poorly accessible tumors, it is necessary to pursue alternatives to detect ctDNA in an assertive manner to englobe the majority of positive patients. We are moving in the

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correct direction, but further studies are still necessary to achieve clinical validation of ctDNA analysis for diagnostic and treatment monitoring.

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Author contributions

Designed and wrote the manuscript: LP, MH and VF. Supervised the involved coworkers and edited the manuscript: VF.

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Legends

Figure 1. Liquid biopsy by using blood/plasma samples. (A) The bloodstream close to the tumor mass receives circulating tumor cells, extracellular vesicles and free nucleic acids from the tumor. By taking blood samples from cancer patients, it is possible to analyze point mutations, epigenetic modifications and amplifications, deletions and translocations in ctDNA. (B) The main methods that have been used to analyze ctDNA in blood samples are real-time PCR, COLD-PCR, digital PCR, BEAMing and next-generation sequencing.

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