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SPECIAL REPORT

Droplet-based digital PCR and next generation sequencing for monitoring circulating tumor DNA: a cancer diagnostic perspective

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ABSTRACT

Introduction: Early detection of cancers through the analysis of ctDNA could have a significant impact on morbidity and mortality of cancer patients. However, using ctDNA for early cancer diagnosis is challenging partly due to the low amount of tumor DNA released in the circulation and its dilution within DNA originating from non-tumor cells. Development of new technologies such as droplet-based digital PCR (ddPCR) or optimized next generation sequencing (NGS) has greatly improved the sensitivity, specificity and precision for the detection of rare sequences.

Areas covered: This paper will focus on the potential application of ddPCR and optimized NGS to detect ctDNA for detection of cancer recurrence and minimal residual disease as well as early diagnosis of cancer patients.

Expert commentary: Compared to tumor tissue biopsies, blood-based ctDNA analyses are minimally invasive and accessible for regular follow-up of cancer patients. They are also described as a better picture of patients' pathology allowing to highlight both tumor heterogeneity and multiple tumor sites. After a brief introduction on the application of the follow-up of ctDNA using genetic or epigenetic biomarkers for prognosis and surveillance of cancer patients, potential perspectives of using ctDNA for early diagnosis of cancers will be presented.

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1 Introduction

Cancers occur when the accumulation of deregulated genetic and epigenetic alterations leads to the abnormal survival, proliferation, and differentiation of cells, which finally escaped from immune surveillance [1–3]. Initially, the identification and quantification of these genetic and epigenetic alterations were mainly based on polymerase chain reaction (PCR). However, with the use of traditional bulk PCR-based technologies for biological sample analysis, rare genetic changes might be completely neglected as their amplification signal is largely diluted in those of wild-type DNA sequences, eventually preventing their identification. With the recent developments of droplet-based digital PCR (ddPCR) [4] and optimized next-generation sequencing (NGS) approaches [5–9], identification of rare genetic alterations is now permitted with a clearly improved detection sensitivity (i.e. % of mutant DNA detectable within corresponding wild-type DNA), reproducibility, and precision.

Nowadays, for certain type of cancers such as colorectal or lung cancer, molecular characterization of tumor is required in clinics to better tailor the treatment of cancer patients. Tissue biopsies remain the standard biological materials for assessing the tumor-specific alterations [10], but its analysis present several limitations. First, obtaining tissues biopsies requires surgical intervention, which limits largely the frequencies of biopsies sampling. Second, intra-tumor heterogeneity especially spatial heterogeneity could lead to unreliable results of biomarker detection notably when a single biopsy is tested [11–14]. Third, presence of multiple tumor sites also complicates the characterization of the patient pathology [14]. As an example, Sakamoto *et al*. described that multiple lesions, identified as precursor, preinvasive and invasive ones, found in lung resection of lung cancer patients can present different genetic profiles [15]. Finally, as it could be necessary to be able to perform serial monitoring of tumor progression and evolution in patients, the repetitive use of tissue biopsies is not feasible. Therefore, there is a strong need for the use of more accessible materials implying non- or minimally invasive procedures and that could allow systematic and real-time monitoring of the whole genomic alterations of cancer patient.

Late diagnosis is one of the reasons which reduce the chances of survival of cancer patients [16]. Early noninvasive diagnosis using easily accessible materials could dramatically improve the chances of survival of cancer patients as well as decrease associated costs. In recent years, there have been large developments on the use of circulating cell-free DNA (ccfDNA), and more precisely its tumor fraction (ctDNA), for diagnosis, prognosis, follow up, and treatment of cancer patients [17–19]. Therefore, combining noninvasive and easily

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accessible ccfDNA from blood-based samples (i.e. liquid biopsies) with highly sensitive procedures such as ddPCR or newly optimized NGS makes early diagnosis of cancers patients a promising avenue.

In this review, after a rapid introduction on ccfDNA, ctDNA, and the newly developed methods for its detection such as ddPCR and optimized NGS, we will focus on the clinical applications of this noninvasive method for early detection of recurrence or minimal residual disease as well as early diagnosis of cancer patients.

1.1. Origin and challenges associated with the detection of circulating tumor DNA

The presence of cell-free circulating nucleic acids in blood was shown for the first time in 1948 by Mandel and Metais, who detected ccfDNA in the blood stream of cancer patients [20]. In 1977, Leon *et al*. demonstrated the presence of larger amount of ccfDNA in serum of cancer patients compared to those of healthy subjects [21]. Following this, Stroun *et al*. described that a proportion of plasma ccfDNA was derived from tumor (ctDNA) and was carrying its molecular characteristics [22,23]. The concentration of ccfDNA in healthy subjects has a mean value of 13 ng per mL of plasma, whereas, in cancer patients, it shows an average of 180 ng per mL of plasma [24]. Recently, it has been reported that ccfDNA could also be detected in other bodily fluids, such as urine [25], cerebrospinal fluid [26], saliva [27], pleural fluid [28], or feces [29].

Although the mechanisms of ccfDNA release into circulation have not yet been fully understood, most reports considered it to be related with cell apoptosis [30] and necrosis [24]. Moreover, active secretion has also been suggested as a potential source of ccfDNA [31,32]. Based on the analysis performed in patients whose plasma was collected sequentially after complete resection of their tumor, Diehl *et al*. suggested that the half-life of ctDNA monitored by a specific tumor alteration was 114 min [33]. Therefore, to limit the potential nucleases cleavage, the conditions of storage of samples and delay of analysis need to be taken into consideration. Moreover, this will also avoid unwanted lysis of nucleated blood cells leading to normal cell DNA release and thus even higher dilution of tumor DNA [34–36]. It is now accepted that the average delay between sampling and blood processing should be around 2 h, whereas it seems to be less important for storage temperature of blood samples (4 to room temperature) [37–39]. Besides, it has been also suggested that the extraction of ccfDNA should ideally be carried out within few hours following collection in order to avoid any degradation [33]. The recent developments of ctDNA analysis have thus led to the commercialization of new tubes, involving nucleated blood cell stabilization, such as the cell-free DNA BCT blood collection tubes (Streck®, La Vista, NE). Although they present higher cost than standard EDTA tubes, these tubes permit to conserve the samples for up to 14 days between 6 and 37°C [40] facilitating the addressing of samples to centralized centers helping further ctDNA development. They also allow to stabilize better DNA and prevent its degradation [41]. Moreover, the methods used for ccfDNA extraction are also critical as they could cause discrepancies of analysis [37,42–44]. It has been shown that isolation efficiencies of ccfDNA as well as a representation of smaller DNA fragments (potentially enriched in ctDNA) [45] in the ccfDNA extract depend on the commercial extraction kits used [42,46]. Hence, it is necessary to apply standardized pre-analytical processes for ccfDNA studies [37,46].

Generally, for cancer biomarker study, plasma is preferred over serum. Indeed, serum has been described as presenting larger proportion of nontumor-derived DNA compared with plasma [15,35,47,48], which might be due to the clotting process of white blood cells in the collection tube leading to their lysis [34–36]. Therefore, plasma is described as more suitable source for the collection of ctDNA with a lower wide-type background ccfDNA.

As mentioned above, patients with cancers including lung, breast, ovary, pancreas, colon, melanoma, or prostate cancer generally present increased ccfDNA concentrations compared with healthy subjects [21,49,50]. However, increased level of ccfDNA has also been associated with pregnancy, noncancer pathological processes (diabetes, inflammation, infection, etc.), intensive exercise or soft-tissue injury [51–53]. The presence of ctDNA has been demonstrated by monitoring tumor-specific mutations and methylation alterations in different types of cancers including colorectal, lung, gastric, breast or pancreatic cancer, etc. [7,54–58]. Higher concentration of ctDNA is detected in advanced cancer compared with localized ones [57–59] and a correlation between the quantity of ctDNA and tumor burden has also been observed [6,60,61]. Furthermore, higher ctDNA concentration was associated with the presence of liver metastases in advanced lung cancer patients [7].

The main challenge of detecting cancer-specific genetic or epigenetic alterations in ctDNA is to achieve acceptable levels of sensitivity and specificity. Dilution of ctDNA molecules within the pool of ccfDNA hampers its quantitative evaluation. With technologies such as qPCR or conventional NGS, the sensitivity of mutation detection are generally higher than 2–5% which prevents the identification of rare mutations especially in early-stage cancer patients [62]. Recent developments in NGS [5–8] and ddPCR have allowed the quantitative detection of rare mutation with a sensitivity below 0.001% [4].

1.2. Methods with improved sensitivity for detecting ctDNA

1.2.1. Droplet-based digital PCR

In 1999, with a newly developed microtiter plate-based technology, Vogelstein and Kinzler detected very rare sequences using limiting dilutions as previously described [63,64]. This technology was named as digital PCR or dPCR [65]. With the use of dPCR, individual target sequences from a complex mixture could be partitioned and individually tested within separate compartments, allowing rare event to be detected and quantified at the level of single molecule. The sensitivity of dPCR depends mainly on the number of individual compartments and individual sequences that could be created and analyzed, respectively and the false-positive rate of each assay. However, the technical constraints of this microtiter plate-based technology including the limited number of

compartments and large reaction volume have greatly limited its possible applications in clinics [4]. Different strategies have thus been developed [66–71] based on the dramatic decrease of reaction volumes using either microchambers or microdroplets [72–74]. The advantage of the platforms using microchambers is their ease of use and the possibility to automate the different steps from the sample injection to the reaction analysis. However, in order to minimize the dimensions of the chip, the number of compartments for the different systems is often limited to a few thousands [67], which can limit the detection sensitivity. Actually, several systems using microchambers have been commercialized including Constellation Digital PCR, Quantstudio™ or Clarity™ digital PCR system (JN Medsys) [4]. In parallel to these systems, emulsion PCR (ePCR) or ddPCR uses aqueous droplets with volumes ranging from few femtoliters to nanoliters dispersed in oil for compartmentalization of PCR reactions, opening up the possibility of having a theoretically unlimited number of compartments [75] thus largely increasing the detection sensitivity.

BEAMing (beads, emulsion, amplification, and magnetics, today commercialized by Sysmex Inostics) was the first high-throughput ddPCR systems described in 2003 for the detection and enumeration of genetic variants [76]. The detection limit of this procedure has been described as one mutant DNA molecule in a background of 10,000 wild-type molecules [77]. However, it requires a relatively cumbersome and complicated procedure for routine clinical use [47,77].

Coupling microfluidic systems with ePCR has finally allowed precise control of droplet volumes in an emulsion [73]. Today, several ddPCR microfluidic platforms are being commercialized, including Raindrop™ digital PCR (Raindance Technologies, recently acquired by Bio-Rad), Bio-Rad QX200™ Droplet Digital™ system (Bio-Rad Laboratories) or Naica™ System (Stilla Technologies) [4]. In such systems, ctDNA samples is partitioned in aqueous droplets (ranging from nanoliter to few picoliter size) acting as independent micro-compartments. Each droplet ideally contains no more than one haploid genome and all reagents allowing to perform PCR assay including specific TaqMan® probes, generally one targeting mutant sequence and the other one wild-type allele, being labeled with different fluorophores (Figure 1). By counting the droplets with different fluorescent signals, the identification of mutant sequences within wild-type ones is then carried out, which has been demonstrated with a detection sensitivity below 0.001% [4]. Multiplex analyses have been performed in these systems based on the variation of the concentrations of probes and/or amplicon size (Raindance Technologies [78], Biorad Laboratories [79,80]) or using a third fluorescence signal (Stilla Technologies) [81]. However, the capabilities of actual strategies are limited to 5–10 multiplex for precise identification of each single tested sequences [79]. Other multiplex strategies have been described allowing to screen for a pool of mutations such as *RAS/RAF* mutations [82] or EGFR exon 19 deletions [83]. Such strategies have then to be followed by conventional duplex ddPCR if the identification of the particular mutations is needed.

1.2.2. Next-generation sequencing

NGS technology allows to analyze millions of ctDNA molecules at the same time and the sequence data are then aligned against a reference genome in order to identify genetic or epigenetic changes (Figure 2a). However, strategies such as whole-exome sequencing or whole-genome sequencing usually generates around 30–100× average sequencing coverage leading to too low detection sensitivity to analyze rare mutations in ctDNA within ccfDNA. Conventional NGS

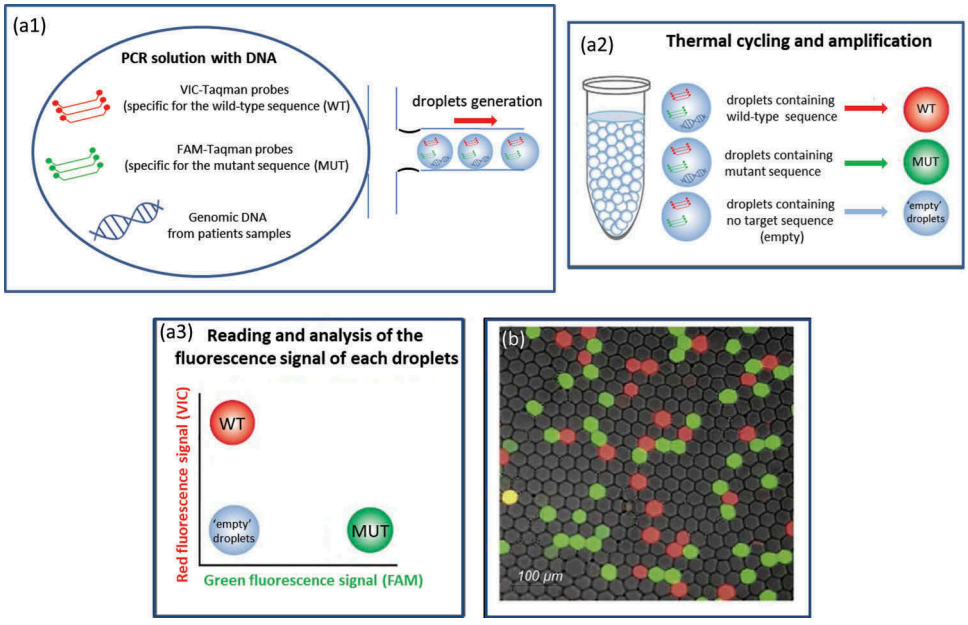
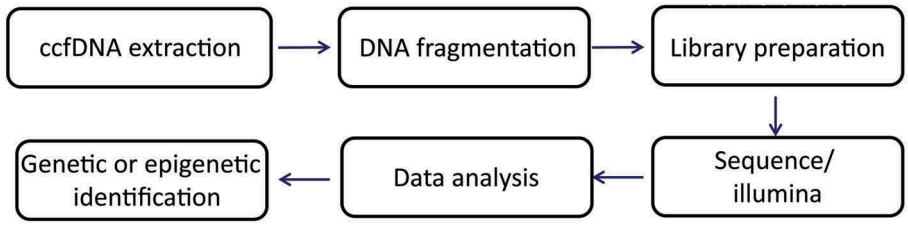


Figure 1. Workflow for picoliter droplet-based digital PCR. A mixture of PCR reagents including fluorescent probes, primers and genomic DNA is partitioned into picoliter to nanoliter droplets where theoretically less than one DNA molecule is distributed in one droplet (Figure 1a1). After thermal-cycling and amplification (Figure 1a2), the fluorescence intensity of each individual droplet is analyzed by instrument which detect the fluorescence signals (Figure 1a3 and 1b). Modified from V. Taly, P. Nizard, P. Laurent-Puig, Circulating DNA, digital PCR and colorectal cancers, Correspondances en Onco-Theranostic 2 (2013) 188–193 with their permission and reproduced from Ref [131]. with permission from (Lab on a Chip) The Royal Society of Chemistry.

a.



b.

	Method	Detection sensitivity	Advantages	Disadvantages
Digital PCR	BEAMing dPCR	0.01%	1. ddPCR: ease to use 2. ddPCR: fast 3. No need of informatics expert support 4. Less expensive	1. Need to know the target in advance
	ddPCR	0.001%		
NGS	Safe-seq	0.1%	1. Capable of the detection of genetic or epigenetic changes without knowing the target in advance; ability of detecting new mutation	1. Take more time, slow 2. Expensive 3. Necessary of informatics expert support
	CAPP-seq	0.01%		
	iDES-CAPP-seq	0.001%		
	Bper	0.1%		

Figure 2. Schematic illustration of optimized highly sensitive next generation sequencing (NGS) procedure (a) and comparison of several technologies of digital PCR and NGS (b). BEAMing, beads, emulsion, amplification, magnetics; ddPCR, droplet digital PCR; SAFE-SeqS, safe-sequencing system; CAPP-Seq, cancer personalized profiling by deep sequencing; iDES, integrated digital error suppression; BPER, Base-Position Error Rate Analysis.

technologies such as Ion AmpliSeq targeted sequencing present detection sensitivity higher than 2% [84]. This makes conventional NGS less adaptable for the detection of rare mutations in ctDNA.

Detection sensitivity and detection specificity are two critical points for permitting the utilization of ctDNA analysis for clinic use as too low detection sensitivity would prevent the detection of ctDNA within ccfDNA and false-positive detection could bring serious psychological consequences on patients. Recently, different improved NGS technologies such as Safe-Sequencing System (Safe-seq) [5], CAncer Personalized Profiling by deep Sequencing (CAPP-seq) [6], integrated digital error suppression-enhanced CAPP-seq (iDES-enhanced CAPP-seq) [9], or newly developed analysis method like Base-Position Error Rate (BPER) [8] have allowed to greatly improve detection sensitivity and specificity of ctDNA within ccfDNA. In CAPP-seq [6], ctDNAs are hybridized and captured by a predesigned selector probe set detecting the regions for their high driver mutation frequencies in the cancer type of interest followed then by sequencing, which makes the interest sequence to be sequenced with a much higher sequencing coverage (around 10,000× coverage). Meanwhile, by incorporating optimized bioinformatics analysis methods, CAPP-Seq achieves lower background error rate, which greatly improves the detection sensitivity of ctDNA within ccfDNA to 0.01% but sequencing artifacts remain a problem for this sequencing strategy [6]. iDES-enhanced CAPP-seq [9], developed from CAPP-seq, is using a unique molecule identifier (UID) for each template molecule as used by Safe-seq. These templates are then amplified resulting in families of the same UID molecules. True rare mutations should be present in most molecules of the same family making it possible to distinguish sequencing errors from true rare mutations. Using a computational pipeline that performs barcode-mediated error suppression to maximize molecule retention and suppress background error, the detection sensitivity (0.001%) and specificity (96%) of iDES-enhanced CAPP-seq are further improved for rare mutation detection in ctDNA. The sensitivity of rare mutation detection in plasma ctDNA can also be improved, using well-designed algorithms such as BPER, to 0.1% [8]. This method calculates the base position error rates for all sequenced bases using control plasma samples and for all potential SNV/indel in a given patient plasma sample. A binomial test is then run comparing the current mutation frequency in ctDNA against the associated base position error rate previously calculated to identify true mutations in ctDNA. Finally, because the BPER method takes into account the error-rates found at all tested positions for each sample using outlier detection, its specificity for the detection of rare mutations in ctDNA is also improved to 95% compared to conventional targeted sequencing.

1.2.3. Comparison of two highly sensitive technologies: ddPCR and optimized NGS for detecting ctDNA

In addition to the increased detection sensitivity and specificity, ddPCR and optimized NGS strategies possess their own advantages and disadvantages for the analysis of ccfDNA (Figure 2b). Compared to NGS, ddPCR experiments are easier to set up, faster, present higher sensitivity, and do not require complex informatics support for analysis. However, it also necessitates the knowledge of genetic or epigenetic changes to be detected and also present limited multiplex abilities [78,79] . A 5-plex assay has been developed to detect ctDNA *KRAS* mutations in plasma from CRC patients and showed concordance with duplex ddPCR assay [78]. In contrast, NGS could identify novel genetic or epigenetic modifications, present high multiplexing capabilities but is time consuming and requires a powerful informatic support. Many strategies have thus combined the use of NGS and ddPCR for liquid biopsy analysis [7,19,54,57]. For example, Pécuchet *et al*. used BPER NGS and

ddPCR for the detection of ctDNA from pancreatic or lung cancer patients and observed high detection sensitivity and specificity for both methods. Moreover, significant consistency between these two methods for *EGFR* and *KRAS* mutations detection was observed [kappa 0.90 (0.73 ± 1.06)] [8].

With these recent developments of ddPCR and optimized NGS technologies, the limit of detection of ctDNA could currently be as low as 0.001% with high accuracy allowing opening a large area for the clinical use of ctDNA. However, it is also important to mention that in most cases, and especially in case of early cancers, very limited quantity of ctDNA could be released in the circulation. Limitations of the use of ctDNA could often come from the low amount of DNA to be tested. More researches are thus needed not only to understand better mechanisms of ctDNA release but also potentially to find ways to optimize sample collection and processing including handling of large volume of plasma.

2. Different ctDNA mutation and methylation biomarkers described for prognosis and surveillance of cancer patients

Researchers and clinicians have largely investigated the potentialities of different plasma ctDNA biomarkers including genetic alterations [85,86], aberrant methylation [58,87–89] for prognosis, and surveillance of cancer patients. Bettegowda *et al*. have reported that cancer-related hotspot gene mutations in ctDNA could be detected in more than 80% of advanced colorectal, melanoma, and pancreatic cancer patients (NGS and ddPCR technologies) [57]. Similar to that, Taly *et al*. have also detected plasma ctDNA mutations in melanoma (ddPCR) [90], CRC (ddPCR) [54,78], lung cancer (ddPCR and NGS) [7], and pancreatic cancer (ddPCR and NGS) [19] (Figure 3a and b).

By performing a systematic review of data from published studies, Fan *et al*. confirmed that ctDNA mutations could be used as prognostic biomarkers in CRC, negatively correlated with the survival of patients [92]. Pietraz *et al*. showed that the presence of ctDNA was strongly correlated with poor overall survival [OS; 6.5 vs. 19.0 months; $P < 0.001$] in advanced pancreatic adenocarcinomas patients and it could be used as an indicator of shorter disease-free survival in resected pancreatic cancer patients when detected after surgery [19]. Tie *et al*. demonstrated that stage II CRC patients with ctDNA-positive status postoperatively had a markedly reduced recurrence-free survival (RFS) compared to those with a ctDNA-negative status [hazard ratio (HR), 18; 95% confidence interval (CI), 7.9 to 40; $P = 2.6 \times 10^{-12}$], which reinforced further the message of ctDNA being a prognostic biomarker [93].

Recently, detection of DNA methylation changes in ctDNA for diagnosis, prognosis, and surveillance of cancer patients has been central to several studies [58,87–89,94]. DNA methylation is one of the epigenetic modifications that regulate gene expression by altering transcriptional accessibility of gene regulatory regions, which occurs at early stage in cancer and are stable [95]. Schröck *et al*. have demonstrated that 59% of head and neck squamous cell carcinomas patients were *SEPT9* and *SHOX2* methylation positive (96% specificity). Methylation levels correlated with tumor and nodal category ($P < 0.001$) [94]. Compared to stage I cancer and healthy subjects, higher *BRCA1* promoter methylation frequencies have also been described in stage II and III ovarian cancers [96].

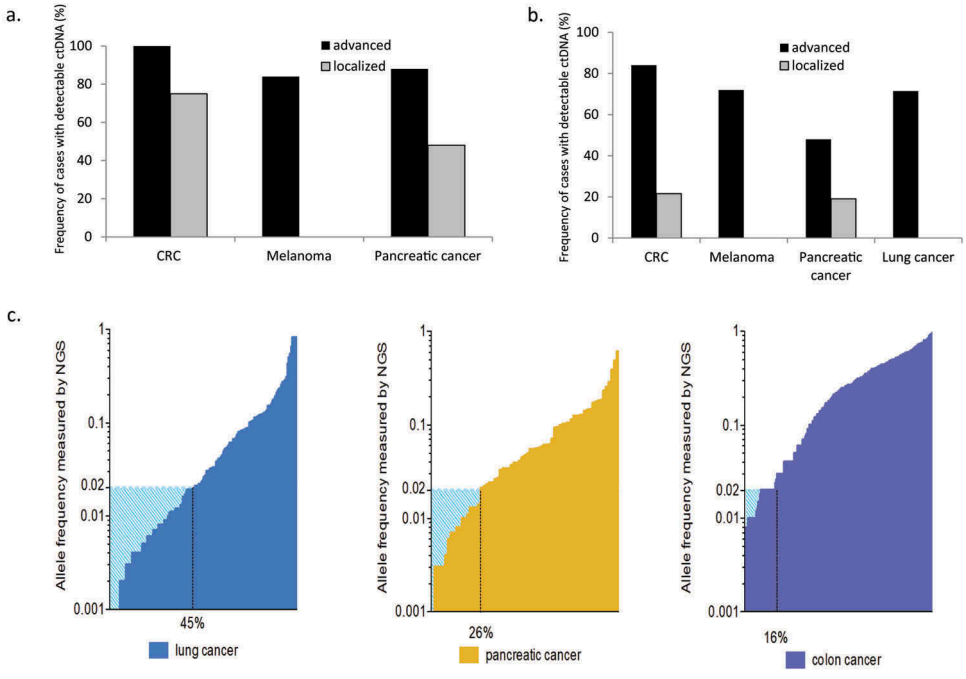


Figure 3. Frequency of cases with detectable ctDNA in different cancers from different laboratories (a) and (b) and distribution of mutated allele frequencies found in pancreatic, lung (republished with permission of American Association for Clinical Chemistry Inc. from ref [Analysis of Base-Position Error Rate of Next-Generation Sequencing to Detect Tumor Mutations in Circulating DNA, Pecuchet, N., et al., 62, 11, 2016; permission conveyed through Copyright Clearance Centre Inc.) and colorectal cancer (CRC) [91] (Laurent-puig, unpublished data) plasma samples (c). The percentage of patients with detectable ctDNA for patients with either localized or advanced cancers (a), modified and permitted from The American Association for the Advancement of Science from ref [Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med, 2014. 6(224): p. 224ra24] [57]; permission conveyed through Copyright Clearance Centre Inc.). n = 24 for advanced CRC patients; n = 18 for advanced melanoma patients; n = 34 for advanced pancretic cancer patients (a). The percentage of patients with detectable ctDNA (b) summarized from previous studies [7,19,78,90]. n = 50 for advanced and n = 250 for localized CRC patients; n = 11 for advanced melanoma patients; n = 105 for advanced lung cancer patients; n = 104 for advanced and n = 31 for localized pancretic cancer patients (c).

However, Ruscito *et al*. have found that there were no significant differences within BRCA1 promoter methylation status between primary and metastatic tissue in ovarian cancers [97]. Garrigou *et al*. have detected methylated ctDNA (MetctDNA) (*WIF1* and *NPY*) in 80% of metastatic CRC and 45% of localized CRC with the use of ddPCR. Meanwhile, *WIF1* and *NPY* hypermethylation has been detected in 100% of stage I to IV CRC tumor tissues [58]. Garlan *et al*. further used this test in combination with mutation analysis to demonstrate the potentiality of using ctDNA as a pertinent biomarker for follow up of the treatment efficacy [54].

3. Potential application of ctDNA biomarkers for early cancer diagnosis

Even if less ctDNA is released at early stage of cancer, detection of hotspot mutations in ctDNA showed potentiality for early cancer diagnosis, including mutations in *KRAS* (colorectal cancer, pancreatic cancer) [98,99], *APC* (colorectal cancer) [47], *BRAF* (melanoma), *EGFR* (non-small cell lung cancer), *HER2* (gastric cancer), *PIK3CA* (breast cancer), *TP53* (pancreatic cancer), or *CDKN2A* (pancreatic cancer) [100–104]. By investigating a panel of hotspot gene mutations, Bettegowda *et al*. have detected ctDNA in 48–73% of patients with localized (stage I–III) CRC, gastroesophageal cancer, breast, and pancreatic cancer [57]. In early stage of non-small cell lung cancer patients (stages IA, IB, and IIA), Chen *et al*. described that ctDNA could be detected in more than 32.8% of patients using Ion Ampliseq cancer hotspot panel V2 (Life technologies, USA) [105]. The fraction of patients with measurable ctDNA might be further improved if more sensitive methods such as ddPCR or optimized NGS have been used. Similarly, Diehl *et al*. have demonstrated the detection of mutant *APC* molecules in more than 60% of patients with early and probably curable CRC using BEAMing [47]. Beaver *et al*. have detected PIK3CA mutation in ctDNA in 48.3% of early-stage breast cancer (stage I, II, III) patients using ddPCR [106]. Garcia-Murillas *et al*. also demonstrated that ctDNA mutations tracking could monitor minimal residual disease (MRD) and early relapse in early breast cancer patients at high accuracy [HR, 25.1; 95% CI, 4.08 to 130.5] [107].

To permit efficient patient follow up, a large panel of different genes and hotspot codons should be tested since gene mutations and hotspot codons vary largely in individual cancer patients. This obviously complicates the detection procedure. Analysis of 12 most frequently reported CRC mutations [58] in primary tumors of CRC patients using cBioPortal database reveals that 46.2% of the patients were positive for one of these mutations in tumor tissue. However, increasing the number of screened mutations to 30 would only improve the detection to 54.2% of the patients [108,109]. In contrast, several works have recently shown that analysis of few methylation markers could lead to screening of large number of, if not all, cancer patients [58,110]. This could clearly decrease the tests to perform, simplify the detection procedure and allow the monitoring of tumor DNA dynamics without the need of developing individualized assays for each cancer patient.

Uehiro *et al*. have demonstrated a sensitivity and specificity of 86% (stages 0–I 84.6%, IIA 86.2%, IIB-III 81.8%) and 83%, respectively, for detecting early breast cancer with the use of a panel of ctDNA methylation biomarkers including *RASGRF1, CPXM1, HOXA10*, and *DACH1* [111]. *PENK* and *CDKN2A* ctDNA methylation were detected in the plasma of 21.4 and 45.4% of patients with localized pancreatic cancer [112]. Garrigou *et al*. have found that methylated ctDNA (MetctDNA) (*WIF1* or *NPY*) was detected in 45% of localized CRC (stage II and III) with the use of ddPCR [58]. More interestingly, a strong correlation between mutated ctDNA (MutctDNA) and MetctDNA was observed in this study [58]. Therefore, the use of both ctDNA mutation and methylation biomarkers might make a breakthrough for early stage cancer diagnosis.

World health organization (WHO) defined early diagnosis as the early identification of cancer in patients who have symptoms of the disease [113]. This contrasts with cancer screening that seeks to identify unrecognized (preclinical) cancer or precancerous lesions in an apparently healthy target population. Several studies have targeted the development of new markers for cancer screening applications. A blood Epi proColon test for CRC screening based on the methylation of the *SEPT9* promoter region has been approved by the Federal Drug Administration (FDA) for clinical use [114]. This test is indicated to screen adults of either sex, 50 years or older, defined as average risk for CRC, who have been offered and have a history of not completing CRC screening [115]. Kisiel *et al*. described a panel of methylated biomarkers *CD1D, KCNK12, CLEC11A, NDRG4, IKZF1, PKRCB*, and *KRAS* resulting in 75% sensitivity and 95% specificity comparing pancreatic cancer to normal pancreas and pancreatitis [116]. Presently, a larger clinical study is being performed to assess its accuracy [117].

4. Detection of cancer-associated mutations in noncancer individuals

Recent reports have shown that cancer-associated mutations could also be detected in noncancer patients. *TP53*-mutation in plasma ccfDNA was observed in 11.4% of 123 matched healthy controls in a study of early detection of small cell lung cancer [118]. Calvez-Kelm *et al*. detected plasma *KRAS* mutations in ccfDNA in 3.7% (N = 14 of 394 individuals) of healthy controls and in 4.3% (N = 6 of 141 individuals) of subjects with chronic pancreatitis but the fraction of mutations and the incidence was much lower than those from pancreatic cancer patients (21.1%, N = 92 of 437 patients) [119]. In another study, no *KRAS* mutations were found in ccfDNA from patients with chronic pancreatitis (N = 10) [53]. *KRAS* mutations were also detected in benign colon tumor tissues: 9 of 50 (18%) hyperplastic polyps, 6 of 10 (60%) mixed hyperplastic polyp/adenoma (HP/AD) [120]. However, it has been suggested that benign tumors do not generally give rise to circulating DNA [47]. Moreover, *TP53* mutations are detected in rheumatoid arthritis synovial tissue sections [121] but no such mutations in ccfDNA were described in those patients. *BRAF* V600E mutation has been described in 50% of benign nevi [122] but is undetectable in ccfDNA of healthy subjects with nevi [123].

For ctDNA methylation biomarkers, certain aberrant ctDNA methylation in cancer patients could be detected in noncancer individuals [53]. For examples, Ibanez De Caceres *et al*. found methylation of *RASSF1A* and *BRCA1* promoters in plasma in 25/50 (50%) and 9/50 (18%) of ovarian cancer samples, respectively, with neither promoter methylated in any of 20 controls [124]. In other studies [125–128], *RASSF1A* and *BRCA1* have also been

found methylated in a small number of healthy controls, which might be also induced by the low specificity of these markers.

The findings of cancer-associated mutations and aberrant DNA methylation in noncancer individuals could complicate their applications on ctDNA analysis. However, based on previous studies [47,53,119,123], the fact that no or very low fractions of cancer-associated alterations in ccfDNA in benign or non-neoplastic individuals renders ctDNA detection for early cancer diagnosis less problematic. Besides, the advantage of using ctDNA biomarkers is being able to combine different mutation and methylation markers for ctDNA analysis, which could be performed to decrease false-positive detection. Therefore, application of highly specific ctDNA mutation and methylation biomarkers is promising for early cancer diagnosis.

5. Expert commentary

Early detection of cancers through the analysis of ctDNA could have a significant impact on morbidity and mortality of patients. Different biomarkers have shown clinical significance for the prognosis and follow up of cancer patients [57,58,92,129]. However, very few data exist for early cancer diagnosis using ctDNA as several challenges exist. First, compared to metastatic stages, there are very limited amount of ctDNA released from tumors at early stage of cancer, which is then largely diluted in DNA coming from normal cells [130]. ctDNA concentration in early-stage lung cancer patients has been described as low as one genome equivalent in 5 mL blood [9]. Even in metastatic stages, around 45% of lung cancer patients release less than 2% of ctDNA, 26% for pancreas patients [8] and 16% for CRC patients [91] (Laurent-Puig *et al*. unpublished data) (Figure 3c). Second, false-positive rate of detection methods could hamper the application of ctDNA biomarkers for early detection of cancers. Third, as mentioned above, the existence of cancer-related mutations and methylation modification in cancer-free individuals have to be investigated since it could complicate their use as biomarkers for detection of cancers. Therefore, different strategies are needed to overcome these obstacles. First, robust standardized pre-analytical procedures might improve the quantity and quality of ccfDNA available for ctDNA detection. Second, very sensitive and specific cancer biomarkers (not detected in healthy individuals) combined with highly sensitive and accurate detection methods such as ddPCR are certainly needed for early cancer diagnosis, which might track the least trace of ctDNA released in the circulation. Finally, larger volume of blood for plasma ccfDNA extraction might be another solution for the detection of rare sequences because of increased starting materials. However, one should always keep in mind that some tumors might not be able to release ctDNA in the circulation, either controlled by tumor microenvironment or by the patients' own immune system.

6. Five-year view

The prevention of cancer should aim at early detection, which might be achieved by regular screening. This screening should aim first at population with average risk of certain cancer, such as blood Epi proColon test for CRC screening. A main advantage of using ctDNA biomarkers relies on the possibility to detect and monitor cancer more easily and regularly, which might lead to early detection of cancer and increase the overall survival of the patients.

Technology has been evolving rapidly and lots of efforts have been made to develop highly sensitive approaches for ctDNA biomarker discovery and validation. Among the existing methods, ddPCR is the most sensitive approach available for the detection of ctDNA in cancer patients but presents lower multiplexing capabilities. With the use of ctDNA mutation or methylation biomarkers, different studies have shown its potentiality for early cancer diagnosis (Table 1). However, high detection specificity of such ctDNA biomarkers for early cancer diagnosis is necessary. Therefore, excluding the potential ctDNA biomarkers, which could be detected, even to a lesser extent, in plasma from healthy individuals is mandatory for a future application of ctDNA biomarkers in clinics.

The approval of ctDNA *SEPT9* promoter methylation assay for CRC screening by FDA supports the pertinence of using ctDNA biomarkers for cancer diagnosis. However, a meta-analysis of recently published articles reveals that the sensitivity of this assay for early stage cancer detection still remains unsatisfactory (17.1–84.0%) [110]. Putting aside biological limitations linked to the low amount of ctDNA released in the circulation at early cancer stages, more efforts should be made on identifying new highly sensitive and specific biomarkers and combining the application of different ctDNA mutation and methylation biomarkers to finally broaden its use for early cancer diagnosis.

Key issues

- Several limitations exist for molecular characterization of tumors in clinics to better tailor the treatment of cancer patients.
- Compared to tumor tissues, blood-based ctDNA is minimally invasive. It could be used for regular follow-up and real-time monitoring of the whole genomic alterations of the patient cancer.
- Very limited amount of ctDNA is released from tumors at early stage of cancer, which is then largely diluted in DNA coming from normal cells. With technologies such as qPCR or conventional NGS, the sensitivity of mutation detection are generally higher than 2–5% which prevents the identification of rare mutations especially in early-stage cancer patients.
- Newly developed technologies such as ddPCR and optimized NGS increased largely the detection sensitivity, specificity and precision of the analysis of rare sequences, allowing their detection in ccfDNA.
- Different plasma ctDNA biomarkers including genetic alterations and aberrant methylation have been reported for prognosis and surveillance of cancer patients.
- Such ctDNA biomarkers present pertinent candidates for early diagnosis of cancer.
- Compared to the use of a panel of different genes and hotspot codons, fewer ctDNA methylation biomarkers could be used for the same purpose of diagnosis, follow-up of all cancer patients. This could drastically decrease the number of tests to perform and simplify the detection procedure.
- Putting aside biological limitations linked to the low amount of ctDNA released in the circulation at early cancer stages, other challenges such as false positive detection or

Table 1. Applications of ctDNA mutation or methylation biomarkers for early-stage cancer diagnosis.

Cancer	Genetic or epigenetic changes detected	Technology	Year	Reference
Localized CRC(Stage II, III)	ctDNA methylation: *WIF1* and *NPY*	ddPCR	2016	Garrigou S. et al. [51]
Breast cancerStage 0–I, IIA, IIB	ctDNA methylation: *RASGRF1, CPXM1, HOXA10*, and *DACH1* etc. 12 markers	ddPCR	2016	Uehiro N. et al. [98]
Localized pancreatic cancer	ctDNA methylation: *ppENK* and *p16*	MSP	2007	Jiao L. et al. [99]
Localized CRC, gastroesophageal,pancreatic cancer,breast cancer (stage I–III)	ctDNA mutation: *KRAS, NRAS, PIK3CA*, and *BRAF* etc.	BEAMing dPCR/Safe-seq	2014	Bettegowda C. et al. [50]
Localized pancreatic cancer (stage IIIB)	ctDNA mutation: *EGFR, KRAS*, and *BRAF, etc.)*	BPER NGS/ddPCR	2017	Pietrasz D. et al. [16]
Localized CRC (Stage II, III)	ctDNA mutation: *KRAS, BRAF, PIK3CA* and *NRAS* etc.	ddPCR	2016	Garrigou S. et al. [51]
Localized CRC	ctDNA mutation: *APC, KRAS, TP53, PIK3CA* etc.	BEAMing dPCR	2005	Diehl F. et al. [35]
Early stage breast cancer(stage I, II, III)	ctDNA mutation: *PIK3CA*	ddPCR	2015	Beaver J.A. et al. [95]
Early-stage non-small cell lung cancer(stages IA, IB, and IIA)	ctDNA mutation: *EGFR, KRAS, PIK3CA*, and *TP53* etc.	AmpliSeq	2016	Chen KZ. et al. [94]

CRC: colorectal cancer; BEAMing: beads, emulsion, amplification, magnetics; ddPCR: droplet digital PCR; SAFE-SeqS: safe-sequencing system; MSP: methylation specific PCR; BPER: Base-Position Error Rate Analysis.

presence of genetic alterations, described in cancer patients, in healthy individuals hampers the use of such markers for early diagnosis of cancer.

- Identifying new highly sensitive and specific ctDNA biomarkers and the use of both ctDNA mutation and methylation biomarkers might make a breakthrough for early stage cancer diagnosis in the future.

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Declaration of Interest

V Taly discloses honoraries from RainDance Technologies and Boehringer Ingelheim. P Laurent-Puig discloses honoraries from Astra-Zeneca, Boehringer Ingelheim, Amgen, Integragen Roche, Sanofi. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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