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Review Article

Progress in quantitative technique of circulating cell free DNA and its role in cancer diagnosis and prognosis

Jia-Lei Weng[a,b], Manar Atyah[a,b], Chen-Hao Zhou[a,b], Ning Ren[a,c,*]

[a] Department of Liver Surgery and Transplantation, Liver Cancer Institute, Zhongshan Hospital, Fudan University; Shanghai 200032, China
[b] Key Laboratory of Carcinogenesis and Cancer Invasion of Ministry of Education, Shanghai, China
[c] Department of Surgery, Minhang Hospital, Fudan University, Shanghai 201199, China

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ABSTRACT

The interest in the potential application value of circulating cell free DNA (ccfDNA) has increased rapidly in recent years, as numerous researchers have demonstrated that the change of its level in the blood is associated with many diseases. Its potential role in cancer management is of particular concern. In comparison with traditional invasive tissue biopsy, quantitative analysis of ccfDNA level for the detection of cancer is advantageous due to the non-invasiveness of blood collection. Moreover, its clinical significance in prognosis prediction and dynamic monitoring of disease progression in cancer patients is equally worthy of attention. At the same time, quantitative detection of ccfDNA is being improved to pursue higher sensitivity due to its low concentration in the blood sample. In this review, we will summarize the progress in quantitative technology of ccfDNA and describe the possible relationship between ccfDNA level and cancer diagnosis and prognosis prediction.

Contents

Introduction

The free DNA fragments in plasma or serum, namely circulating cell free DNA (ccfDNA), originates from necrotic and apoptotic cells, or activated lymphocytes and hematopoietic cells. Although ccfDNA was detected by Mandel et al as early as 1948 [1], it was not until 1977 when Leon et al [2] reported the significantly elevated serum DNA level in cancer patients that this discovery was gradually taken seriously. With the rapid development of molecular biology techniques such as DNA quantification and sequencing, great progress has been achieved in the study of ccfDNA. The correlation between ccfDNA level and several diseases such as trauma, autoimmune diseases, and cancer makes it a promising biomarker in disease screening, early diagnosis, prognosis assessment, disease recurrence and so on.

* Corresponding author.
E-mail addresses: 16211210127@fudan.edu.cn (M. Atyah), 17111210068@fudan.edu.cn (C.-H. Zhou), renning@fudan.edu.cn (N. Ren).

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Table 1
Optimization and standardization of various pre-analytic factors before ccfDNA quantification.

Pre-analytic factors	Optimization and standardization
Choice of matrix	Plasma can avoid release of DNA from blood cells during clotting and is preferred over serum.
Collection tube	K3EDTA collection tubes is preferred as PCR can be inhibited by heparin.
Drawing and processing of blood sample	Blood samples should be drawn carefully and avoid being agitated; It is recommended to process whole-blood samples as soon as possible; Storage conditions of blood should be room temperature or +4 °C up to 4–6 h; A double-centrifugation protocol is recommended for retrieval of plasma: first centrifugation at 1600 g for 10 min followed by a centrifugation at 16,000 g for 10 min.
Processing of plasma sample	Plasma samples should be kept at +4 °C up to 3 h before DNA extraction and could be stored at −80 °C if extraction is delayed; Plasma samples should be aliquoted and can submit at most two freeze-thaw cycles.
Choice of DNA extraction method	Automated cfDNA extraction methods are recommended.
Processing of ccfDNA extracts	ccfDNA extracts could be stored at −20 °C and may submit at most three freeze-thaw cycles.

Despite its enormous potential for application and easy accessibility in clinical collection, further research on ccfDNA is limited considerably due to its low concentration in blood and lack of sensitive quantitative methods. Although multiple quantitative techniques have been used in ccfDNA, there is no standard technology and the main methods are generally based on the optimization or improvement of PCR technology.

At present, the tissue biopsy is still the gold standard of cancer diagnosis, which requires an invasive operation and has significant potential risk. Not only that, just a few tumors have specific biomarkers for assistant diagnosis and disease monitoring, such as alpha-fetoprotein (AFP) and prostate-specific antigen (PSA). In this context, ccfDNA has attracted considerable attention in view of its easy accessibility and potential tumor specificity through quantitative detection or specific sequencing. Hence, great efforts have been made to find its potential clinical value in cancer management. Furthermore, given that ccfDNA in cancer patients originate from either non-cancer cells or cancer cells, identification of circulating tumour DNA (ctDNA) and selective detection and quantification of this subpopulation of ccfDNA can contribute to better exploration and understanding of biology of ctDNA and improved accuracy of researches in this area.

The present review will describe the current advancement in quantitative detection of ccfDNA and provide an overview of the latest results on the potential role of ccfDNA level in cancer diagnosis and prognosis from available studies.

Advances in quantitative technique of circulating cell free DNA

Before ccfDNA quantitative analysis, there are various pre-analytic factors from blood drawing to storage of ccfDNA extracts that can influence the concentration and fragmentation of ccfDNA [3]. Optimization and standardization of these pre-analytic factors is essential to maintain the quality of samples for accurate quantification and achieve reproducibility and robustness of results (Table1) [3–5].

Real-time quantitative PCR (qPCR) Developed in the 1990s, this method achieves the purpose of monitoring the process of PCR reaction in real time by adding fluorophore (TaqMan probe or fluorescent dyes) into its system and subsequently collecting the accumulated fluorescent signals. Using a standard curve, an unknown sample can be quantified by the C_T value, which correlates linearly with the logarithm of initial DNA copy number (Fig. 1a). The C_T value should range from 15 to 25 and this requires appropriate initial template concentration, reasonable primer design, reduction of PCR inhibitors or contamination in reagents, and 95–105% amplification efficiency. Compare to other PCR methods, qPCR is more accurate and labor-saving. Therefore, the advent of qPCR obviously restores the interest in ccfDNA and promotes its research in many diseases like cancer, trauma and preeclampsia.

In order to quantify low-abundance ctDNA and achieve increased sensitivity and specificity of detection, several qPCR-based methods have been developed, such as allele-specific PCR (AS-PCR) [6], allele-specific, non-extendable primer blocker PCR (AS-NEPB-PCR) [7], coamplification at lower denaturation temperature PCR (COLD-PCR) [8], and peptide nucleic acid-locked nucleic acid PCR (PNA-LNA PCR) [9]. Take PNA-LNA PCR as an example, the utilization of PNA could hinder amplification of wild-type alleles and mutation sequences could be effectively detected by LNA probe during the process of qPCR [9]. A further increase of detection sensitivity and specificity of these methods is possible, like strategies of wild-type blocking [7,8] and use of smaller PCR products and increased PCR cycles [9].

However, it should be noted that qPCR actually determines the C_T value of the target gene like β-globin gene [10] or β-actin gene [11] to calculate the total amount of ccfDNA. Whether the concentration of various target genes can completely represent that of total ccfDNA level is unknown, resulting in the data obtained from studies using different target genes uncomparable. For this reason, the accuracy and standardization of absolute quantification of ccfDNA still need further discussion. Technologies targeting total ccfDNA fragments as the means of detection, such as fluorescent dye, can relatively avoid this problem. Another issue is that the step of ccfDNA extraction could cause loss of approximately 5–10% of ccfDNA fragments, especially small ones (<100–150 bp) [12]. This bias may affect the selection of amplified target sequence length and primer design in qPCR.

Direct qPCR The essence of “direct” means no conventional DNA extraction from plasma or serum before qPCR measurement. The process of DNA isolation not only increases the possibility of contamination like PCR inhibitors but also cause the loss of ccfDNA of a specific length to a certain extent [12]. Therefore, Umetani et al [13] developed a robust, highly sensitive and high-throughput method called direct ALU-qPCR. After treated with a buffer and proteinase K, the sample is directly quantified by qPCR through amplification of two ALU sequences (ALU115 and ALU 247) without the need for DNA purification (Fig. 1b). Using ALU repeat sequences in qPCR can significantly improve the sensitivity of ccfDNA quantification as it is the most abundant repetitive sequence in the human genome. Another direct qPCR approach has been reported in 2014, simply requiring dilution of plasma and water in a 1:40 ratio before qPCR and an addition of a special polymerase to amplify difficult templates [12] (Fig. 1b). The authors emphasized that this procedure can avoid the loss or rupture of ccfDNA fragments during the extraction and thus increase its concentration and integrity.

Similarly, varied methods of DNA extraction impede the comparability of ccfDNA values from different studies. The development of direct qPCR may standardize the processing of samples, though its reliability still demands more similar or comparative research to verify.

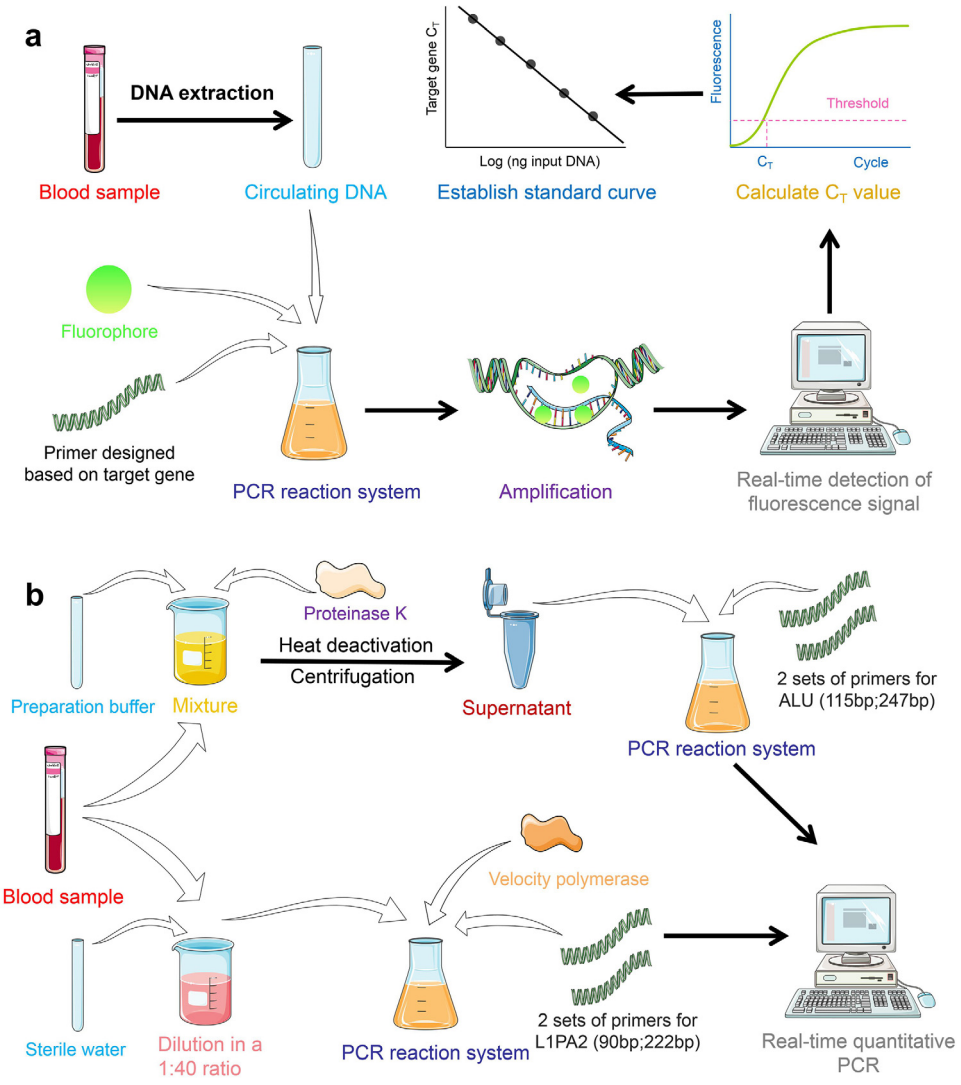

Fig. 1. Schematic diagrams summarizing the most commonly used methods for quantifying circulating cell free DNA. a Real time quantitative PCR (qPCR): Addition of fluorophore into PCR reaction system to count the cycles of DNA amplification in real time; **b** Direct qPCR: Pre-treatment with a buffer and proteinase K or dilution of plasma and water in a 1:40 ratio with an addition of velocity polymerase before qPCR quantification; **c** Duplex qPCR: Simultaneous amplification of internal standard and target gene in qPCR reaction to minimize the bias; **d** Digital PCR (dPCR): Distribution of PCR reaction system into numerous units to achieve single molecule PCR amplification and absolute quantification of ccfDNA; **e** Branched DNA (bDNA): Quantification of ccfDNA based on the bind of a series of probes and target gene and the reaction of enzyme and substrate; **f** Fluorescent dye: Detection of enhanced luminous intensity released by fluorochrome bound to dsDNA. LE: Label extender probe; CE: Capture extender probe.

Duplex qPCR In 2009, Horlitz et al [14] presented a duplex qPCR targeting DYS14 gene (synonyme for testis-specific protein 1) and 18S-1 gene that allows for unbiased quantification of male DNA in the male/female mixture of ccfDNA. Hence, this assay could be utilized to accurately measure the concentration of male fetal DNA in maternal plasma. It may provide a novel idea for designing more comprehensive quantitative methods in the future that could be able to compute the percentage of DNA fragments of a specific length in total ccfDNA. For example, this assay might be applicable to measure the fraction of ctDNA in ccfDNA owing to distinct differences in fragment length between normal ccfDNA and ctDNA. However, previous reports presented contradictory results on the ctDNA fragment length size compared to normal ccfDNA [15–17], and this issue will be discussed in detail below.

In order to eliminate the variation during DNA extraction, another duplex real-time PCR is recommended for quantification of plasma DNA in 2016 [18]. This technology includes four steps (Fig. 1c): (i) construction of a recombinant plasmid DNA as an internal standard using an artificial 41-bp dsDNA corresponding to the human β-actin gene. (ii) extraction of DNA from plasma sample containing the recombinant plasmid DNA with known concentration. (iii) simultaneous amplification of the internal standard and β-actin gene in the same tube with a common reverse primer located in 41-bp corresponding sequence by duplex real-time PCR. (iv) separate detection of fluorescent signals from the internal standard and target gene through two Taq-man probes. The internal standard plays a role as a calibration tool to minimize bias in different extraction methods and the process of DNA amplification in qPCR. Considering the inclusion of amplification efficiency in the final concentration calculation, its quality assurance is as important as C_T value. Optimization of qPCR steps and conduction of parallel repeated experiments are required.

Digital PCR (dPCR) The conception of digital PCR was proposed formally in 1999. A standard PCR reaction system is evenly distributed into numerous independent units with Poisson's distribution of the target genes. Most units contain zero or only one molecule template theoretically to achieve single molecule template PCR amplification. Finally, the absolute copy number

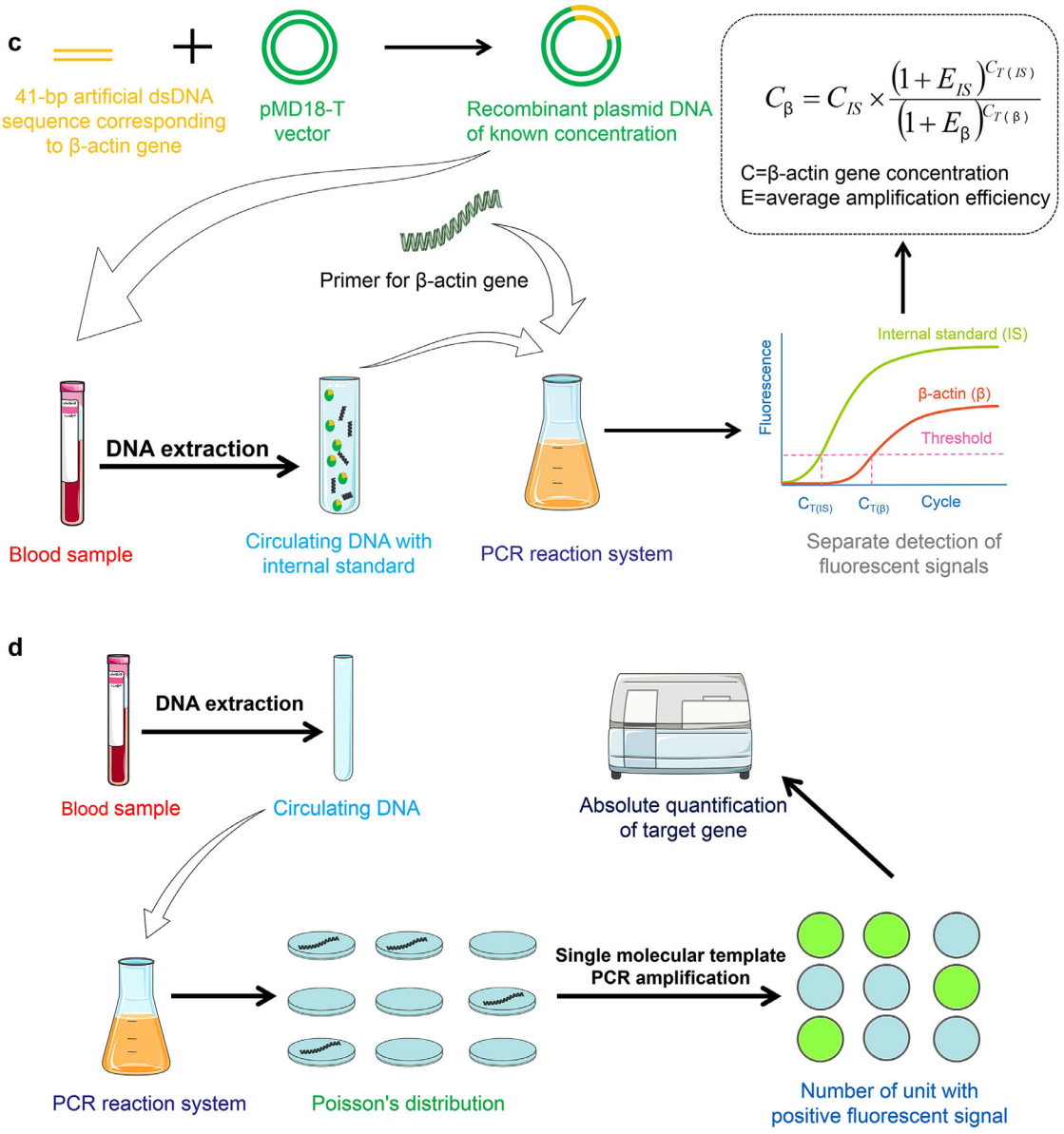

Fig. 1. Continued

of target genes can be quantified accurately according to the proportion of units of positive fluorescence signal in all units (Fig. 1d). The sensitivity here is just limited by the number of units you could analyze [19]. Furthermore, three straightforward parameters need to be considered and optimized [20]: (i) Single amplification product: increasing the specificity of the primer binding by raising the annealing temperature; (ii) Peak resolution: optimising the final concentrations of primers and probe in a reaction based on targets; (iii) The amount of stragglers or 'rain': avoiding and removing 'rain' by re-designing primers, running more cycles, using sonication, and adding PCR enhancers. The advantage of high sensitivity and accuracy allows this technology quite suitable in detection of rare gene mutations and copy number variations, and other fields. In 2003, Bert Vogelstein et al proposed an approach called BEAMing (beads, emulsion, amplification and magnetics) with highly selective and sensitive on the basis of combination of digital PCR technology and flow cytometry [21]. An extension of BEAMing was developed later to examine more template molecules in a convenient fashion and was applied to quantify ctDNA in patients with colorectal cancer [22]. Furthermore, multiplex picodroplet digital PCR, based on the droplet digital PCR (ddPCR) technology, could screen for multiple mutations in ctDNA simultaneously with a sufficient sensitivity [19].

Since this technology can sensitively detect low-abundance target sequence from a large background of ccfDNA, the most widely applied field of this technology is the detection and quantification of ctDNA [19,22]. Therefore, its application in the future may tend to a comprehensive analysis of overall ccfDNA level and ctDNA to improve its clinical value.

Branched DNA (bDNA) Branched DNA is a quantitative technology based on DNA hybridization. The ends of capture extender probe (CE) are combined with target gene and capture probe fixed on 96-well plate, respectively. Subsequently, the target gene is sequentially connected with label extender probe (LE), signal amplification probe and 3'-alkaline phosphatase label probe. Just like ELISA, the concentration of Alu sequence can be determined through the reaction of enzyme and substrate and generation of the chemiluminescent signal (Fig. 1e). The number of photons emitted in the luminometer is a critical parameter and better assay performance can be achieved through following

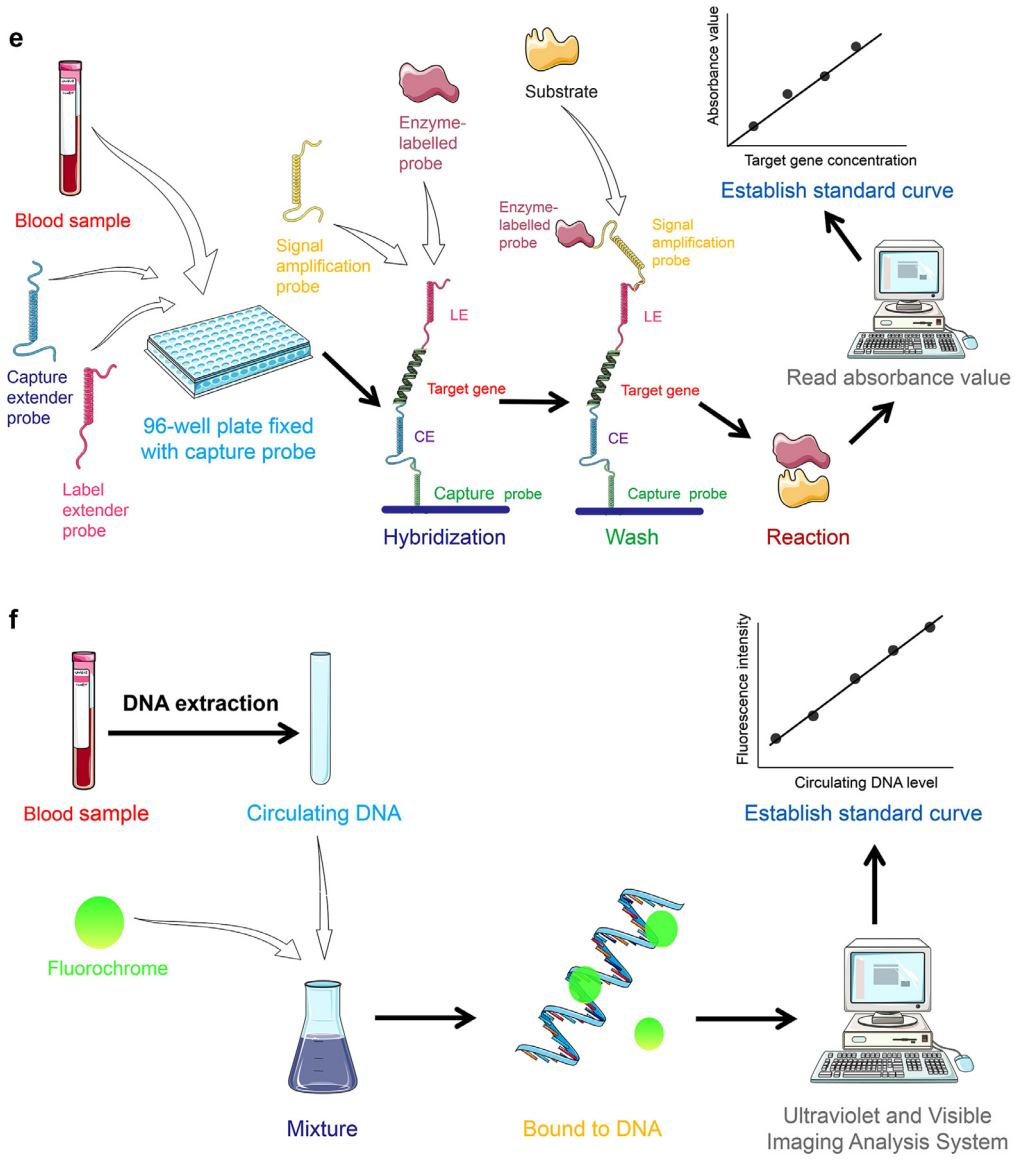

Fig. 1. Continued

Table 2
Comparison of the characteristics of quantitative technique of ccfDNA.

Technique	DNA extraction	Sensitivity	Specificity	Accuracy	Cost	Standardization	Other advantages
qPCR	Need	High	High	High	High	No	/
Direct qPCR	No need	High	High	Higher	High	No	Reduce loss or rupture of ccfDNA
Duplex qPCR	Need	High	High	Higher	High	No	Minimize bias in DNA extraction and amplification
Digital PCR	Need	Higher	High	Higher	High	No	Suitable in detection of rare gene mutations
Branched DNA	No need	High	High	High	Moderate	No	Less sample
Fluorescent dye	Need	Higher	Moderate	Moderate	Low	Yes	Simple procedure

improvements [23]: (i) Increasing the hybridization stringency of target and capture probes; (ii)Enhancing signal amplification by using preamplifier molecules; (iii) Incorporation of isoC and isoG into oligonucleotide probe sequences to reduce non-specific hybridization of bDNA assay components.

Although the total ccfDNA level is still represented by the level of the target gene, it needs less sample and avoids DNA extraction compared with qPCR. (Table 2)

Fluorescent dye Briefly, diluted sample is mixed with fluorochrome like SYBR Green I or PicoGreen and then a fluorometer at corresponding excitation and emission wavelength is used to obtain the luminous intensity of the solution (Fig. 1f). The fluorochrome should be protected from light and introduction of bubbles into mixture should be avoided. Take PicoGreen assay as an example, its fluorescence can be extremely enhanced when selectively bound to dsDNA. Compared to qPCR, it can serve as an advantageous alternative for ccfDNA quantification with higher sensitivity, simpler procedure and less cost. Another commonly used fluorescence-based method, the Qubit dsDNA HS assay, is also an accurate and highly sensitive quantitation system, espe-

Table 3
Studies on ccfDNA level between various cancer patients and healthy volunteers.

Cancer type	Source	Method	No.of patients/controls	DNA(ng/ml) in patients/controls	AUC	Reference
Lung cancer						
2015	plasma	real-time PCR	50/40	8.02/2.27	0.76	[11]
2009	plasma	real-time PCR	151/79	12.8/2.9	0.79	[25]
2008	plasma	real-time PCR	76/66	60/5	0.82	[26]
Gastric cancer						
2014	plasma	real-time PCR	30/34	113.11/79.78	0.991	[27]
2012	plasma	real-time PCR	54/59	71.4/29.8	0.784	[28]
Colorectal cancer						
2017	plasma	real-time PCR	74/36	54.12/11.98	0.875	[29]
2014	serum	real-time PCR	104/110	1046/385.4	0.85	[30]
2006	serum	real-time PCR	75/75	35.8/7.7	0.86	[31]
Liver cancer						
2012	serum	real-time PCR	80/50	477.5/103.1	0.82	[32]
2012	plasma	real-time PCR	72/41	173/9	0.949	[33]
Breast cancer						
2018	serum	real-time PCR	40/40	1083.66/228.19	0.7	[17]
2015	serum	fluorescent dye	38/16	1010/395	0.83	[34]
2006	plasma	real-time PCR	61/27	65/13	0.946	[35]
2004	serum	real-time PCR	96/24	221/63	0.92	[10]

cially in the quantification of small amounts of pure dsDNA [24]. Furthermore, the uniform protocol makes it convenient to compare data from different laboratories.

It is anticipated that there will be more application of this method in the future. However, its sensitivity might be affected by the high background fluorescence, requiring further optimization in materials or procedures.

Circulating cell free DNA level and cancer diagnosis

Numerous studies have demonstrated significantly elevated levels of ccfDNA in various cancer patients (Table 3), suggesting that ccfDNA might be a potential diagnostic marker for cancer. The value of AUC (area under the ROC curve), ranging from 0.7 to 0.991, shows the great discriminatory power of ccfDNA level. A cutoff point was also set up in some studies to evaluate its sensitivity and specificity. For example, Szpechcinski et al [11] established a cutoff point of >2.8 ng/ml, which provided 90% sensitivity and 80.5% specificity in discriminating non-small-cell lung cancer (NSCLC) from normal people. In addition, the diagnostic sensitivity of serum ccfDNA is generally lower than plasma ccfDNA [17,29,30,32,35], indicating that plasma is more suitable as the sample source for ccfDNA. One possible reason is that ccfDNA level in serum may be affected by the clotting, during which intracellular DNA tends to be released from circulating cells. Furthermore, the diagnostic sensitivity and clinical utility of ccfDNA level among these common cancers varies. The diagnostic sensitivity of ccfDNA level in lung cancer, ranging from 79% to 85% [25,26], was consistent with a recent meta-analysis (sensitivity of 81%) [36] and thus confirmed that it was a promising and effective biomarker in diagnosis of lung cancer. However, the sensitivity of ccfDNA in breast cancer fluctuated between 65% and 72% [10,17,34] and was lower than that in a meta-analysis (sensitivity of 78%) published in 2017 [37]. Similarly, a meta-analysis including 19 articles showed a sensitivity of 73% for ccfDNA quantitative analysis in prostate cancer and suggested that it could serve as an adjuvant tool for screening [38]. Intriguingly, the significant inconsistence of diagnostic sensitivity of ccfDNA level (ranging from 56.4% to 90.2%) in liver cancer makes its value ambiguous [32,33]. A meta-analysis involving 1280 HCC patients in 22 studies concluded a diagnostic sensitivity of 74% for ccfDNA and recommended a combination of ccfDNA and AFP to improve diagnostic performance rather than independent use [39].

Moreover, the diagnostic accuracy of ccfDNA quantification is compared with the routine tumor biomarkers. It was confirmed that the capacity of ccfDNA in discriminating between hepatitis C virus (HCV) - associated HCC and HCV carriers was superior to that of AFP, which has been predominant marker for HCC screening for decades [40]. What's more, a markedly higher sensitivity of ccfDNA than carbohydrate antigen 15-3 (CA15-3) was described in breast cancer [34]. Hence, ccfDNA level may be a noninvasive and valid auxiliary diagnostic tool for screening cancer in healthy population. The healthy population here refers to the normal people without any disease, including individuals with minor comorbidities, not persons in pathological conditions that may raise the ccfDNA level like chronic diseases, acute infection and autoimmune diseases, as Agassi et al [34] emphasized.

However, there are some issues limiting its diagnostic value at present. Firstly, it is obvious that the absolute ccfDNA concentrations obtained from various groups are quite different. This might be explained by discrepancies in patient selection, sample processing, method choice etc, deeply revealing the present dilemma of research and an urgent need for standardization. Besides, ccfDNA levels can vary considerably even between patients with the same cancer type and stage due in part to differences in histology, tumour volume, tumour vascularization, extent of metastatic spread or disease burden, perfusion, turnover activity, and proliferation rate [4,5]. Thus, only after the standardization of quantitative procedures can it be possible to establish a recognized threshold and further validate its clinical practicability through larger case-control studies. Secondly, a remarkable overlap in ccfDNA concentration among various cancer types indicates that it can only predict the presence of cancer, but unable to determine the definite type. This may be the inherent limitation of quantitative analysis. Last and most important, since most cancers occur on the basis of benign diseases like cirrhosis, it is more critical to ascertain the ability of ccfDNA level to discriminate between corresponding benign diseases and cancer.

Unfortunately, the results from different studies of this aspect are quite contradictory. In a few studies, researchers found a significant difference in ccfDNA level between cancer patients and controls with benign disease (Table 4), though the sensitivity shown in some papers was not high as expected [11,33,41,42]. Notably, a few of researches measuring plasma ccfDNA concentration by qPCR showed satisfactory diagnostic sensitivity of this assay in breast cancer and liver cancer (93.4% [35] and 91% [40] respectively). Although its diagnostic sensitivity in lung cancer is

Table 4
Studies on ccfDNA level between cancer patients and patients with benign diseases.

Cancer type	Control group	No. of patients/ controls	DNA(ng/ml) in patients/controls	Cutoff (ng/ml)	Sensitivity(%)	Specificity(%)	AUC	Reference
Lung cancer								
2015	CRI	50/101	8.02/3.36	>5.25	56	91	0.76	[11]
2010	benign lung diseases	100/100	122.7/74	104.5	52	95	/	[41]
Colorectal cancer								
2014	IP	104/63	1046/423.3	/	/	/	/	[30]
Gastric cancer								
2014	GP	81/13	1034/394.2	/	/	/	/	[30]
Liver cancer								
2013	CH	66/41	9.5/1.6(ng/μl)	1(ng/μl)	91	54	0.78	[40]
2012	CIRR /CH	72/37	173/46	143	59.7	78.4	0.705	[33]
Breast cancer								
2018	benign mammary hyperplasia	40/40	1083.66/145.87	/	/	/	/	[17]
2015	noncancerous breast lesion	38/2	1010/386	/	/	/	/	[34]
2006	benign breast diseases	61/33	65/22	22	93.4	66.7	0.845	[35]
Ovarian cancer								
2010	benign ovarian neoplasm	164/49	10113/2365 (GE/ml)	≥4500 (GE/ml)	55	87.1	/	[42]

CRI chronic respiratory inflammation; IP intestinal polyp; GP gastric polyp; CH chronic hepatitis; CIRR liver cirrhosis; GE genome equivalent.

only about 55% in two previous studies [11,41], a recent study demonstrated that plasma ccfDNA level provided 86.4% sensitivity in discriminating NSCLC from benign lung nodules [43]. Obviously, further conclusion requires more supportable evidence to reference and comparison. In other studies, however, no significant difference of ccfDNA or ctDNA levels between various cancers and corresponding benign diseases like hepatitis B virus (HBV) infection [32,44]. The critical issue for these conflicting results may be the inclusion criteria of the control group as inclusion of benign disease patients in the inflammatory stage might lead to the reduction of difference with cancer patients. Moreover, the sample size of most studies is too small to avoid accidental errors. Hence, a definite conclusion requires multicenter, large-scale and prospective studies with the premise of standardized methodology.

On the other hand, the combination of ccfDNA level and other diagnostic indicators was proposed to improve the sensitivity and diagnostic accuracy [17,30,31,33]. In HCC, the combined detection of ccfDNA with AFP or AFU or both could improve the diagnostic sensitivity for the disease [33]. Similar results were obtained in the studies on diagnosis of early-stage colorectal cancer (CRC), combining ccfDNA level with carcinoembryonic antigen (CEA) [31]. Notably, the decreased specificity of these combined detection may lead to an increase in misdiagnosis rate [33]. Thus, the balance between sensitivity and specificity needs further consideration. In this regard, studies on combined assessment of the levels of circulating proteins and genetic mutations in ccfDNA for cancer detection have also made great progress. A study suggested that the combination of *KRAS* gene mutations in plasma ctDNA with four protein biomarkers could increase the sensitivity for detection of resectable pancreatic cancer while retaining high specificity [45]. In 2018, a multi-analyte blood test called CancerSEEK was designed to evaluate levels of eight tumor-specific proteins and the presence of mutations in 1933 distinct genomic positions in ctDNA [46]. This test among eight common cancer types showed a median sensitivity of 70% with the specificity greater than 99%.

Based on the hypothesis that normal tissues may release short-segment DNA into the circulatory system via apoptosis while tumor tissues mainly generate long DNA fragments [17], the concept of DNA integrity was proposed. Its essence is an extension of ccfDNA quantification, using two sets of primers with different length to amplify the target gene fragments in PCR and DNA integrity is calculated as the ratio of long fragment to short fragment like ALU 247/115. The diagnostic value of ALU247/115, when combined with other tumor markers like CEA and CA15-3, was proved to be superior to ALU115 alone [17,30]. Intriguingly, this hypothesis is being challenged as more and more recent studies have demonstrated shorter fragment size of ctDNA compared to normal ccfDNA [4,5,15,16] and enhanced detection of ctDNA by selecting short ccfDNA fragments [15]. Here are some potential explanations for this problem: There are indeed aberrantly short and long DNA molecules existing in plasma of cancer patients [16]**.** Short ctDNA fragments may be released by tumour cells via apoptosis while the long ones may indicate an origin from tumour necrosis[4,5,8]. Furthermore, the short ctDNA fragments may preferentially carry tumor-associated aberrations [16]. Consequently, investigators directly measuring the length of ccfDNA fragments predispose to notice aberrantly long ctDNA fragments and conclude an increased integrity of ccfDNA in cancer patients. Conversely, studies targeting cancer-related mutations through sequencing technology tend to find a substantially high mutant frequency in short ctDNA fragments and thus get the opposite conclusion.

Circulating cell free DNA level and cancer prognosis

An accurate and readily available prognostic indicator contributes to early clinical decision-making, dynamic monitoring of cancer progression and timely treatment intervention. A lot of studies have been devoted to this aspect to reveal the potential association between ccfDNA level and clinicopathological parameters or various prognostic indicators, as well as its potential application in monitoring therapeutic efficacy, cancer progression or recurrence.

Clinicopathological parameters

Numerous studies have demonstrated a direct correlation between ccfDNA levels and tumor size [17,27,32–35,40,41], stage or grade [27,30,32,34,42], lymphovascular invasion or distant metastasis [17,29,33,34,47] among cancer patients. Therefore, it may have a potential reference value for evaluating tumor burden, invasiveness of tumor, degree of differentiation or disease status. Notably, the strikingly higher levels of ccfDNA were observed in breast cancer patients with axillary node involvement [34], suggesting that ccfDNA level is more closely related to the characteristics and biological behavior of this tumor. There are some analyses and hypotheses to elucidate these phenomena. One is that large tumors or aggressive growth may release more ctDNA into the bloodstream [29], and the other is that release of ctDNA may be aug-

Table 5
Multivariate analysis of baseline ccfDNA level for prognosis of various cancer patients.

Date	Cancer type	Source	No.of patients	cut-off	Indicator	HR(95%CI)	P value	Reference
2011	NSCLC	plasma	446	49.8 ng/ml	OS	1.33(1.08–1.63)	0.007	[48]
					TTP	1.44(1.18–1.75)	<0.001	
2011	Lung adenocarcinoma	plasma	134	5.26 ng/ml	OS	2.7(1.16–4.91)	0.041	[47]
2019	MBC	plasma	117	/	OS	1.75(1.08–2.84)	0.023	[51]
					PFS	1.68(1.28–2.21)	<0.001	
2018	MBC	plasma	268	/	OS	2.16(1.49–3.14)	<0.001	[49]
					PFS	1.53(1.15–2.03)	0.003	
2017	MBC	plasma	112	/	OS	2.2(1.3–3.6)	0.03	[52]
2019	mCRC	plasma	397	/	OS	1.54(1.21–1.96)	<0.001	[53]
2019	mCRC	plasma	132	50 ng/mL	OS	2.46(1.51–4.03)	0.0003	[54]
					PFS	2.51(1.69–3.73)	<0.001	
2018	mPC	plasma	571	/	OS	1.53(1.18–1.97)	0.001	[55]
					PFS	1.54(1.15–2.08)	0.004	
2010	Ovarian cancer	plasma	164	22,000GE/ml	DSS	2.52(1.16–5.48)	0.02	[42]

HR hazard ratio; CI confidence interval; NSCLC non-small-cell lung cancer; MBC metastatic breast cancer; mCRC metastatic colorectal cancer; mPC metastatic prostate cancer;
GE genome equivalent; OS overall survival; TTP time to progression; PFS progress-free survival;
DFS disease-free survival; DSS disease specific survival.

mented in the course of their invasion of vessels or metastasis. In addition, the relationship between ccfDNA level and some known prognostic factors was also delineated in some papers. For example, a strong and positive correlation of plasma ccfDNA with lactate dehydrogenase (LDH) level was observed in advanced NSCLC [41]. However, there was also a considerable amount of literature reporting no correlation between ccfDNA level and these clinicopathological parameters of tumor [10,11,17,25,26,29,33,35,40,47,48], possibly due to the small sample size of studies [34].

Treatment effect

A stepwise drop in ccfDNA levels over time was quite visible during the follow-up time in disease-free patients and even reached the levels observed in normal subjects, whereas patients with recurrence or metastasis showed a reappearance of high levels [26,27,30,35]. There was no remarkable difference in presurgery values of ccfDNA between individuals with or without disease progression later. Similar results have been obtained in studies on radiotherapy or chemotherapy of cancer [41,49,50]. For example, in 66 patients with acute myeloid leukemia (AML) receiving chemotherapy, the plasma ccfDNA levels in complete and partial remission groups decreased significantly, while levels of non-remission and relapsed patients remained high [50]. This indicates that dynamic detection of ccfDNA levels during cancer treatment may help clinicians monitor the therapy effectiveness or clinical status of patients and identify recurrence at a preclinical stage. A sudden spike in ccfDNA level observed during patient follow-up may be indicative of disease progression and poor prognosis. However, a study on advanced NSCLC (III_B and IV) showed a poor correlation between the kinetics of total plasma ccfDNA and the response to treatment, likely due to its poor specificity for total tumor burden [47].

Another important issue is whether baseline ccfDNA levels before treatment can also predict treatment effect or possibility of recurrence. Some researchers found that pre-treatment levels of ccfDNA were positively correlated with postoperative recurrence and reduced chemotherapy efficacy in NSCLC patients [26,48]. The authors deduced that micro-metastasis might have occurred at the time of diagnosis in patients with high baseline ccfDNA levels [26]. Interestingly, a study demonstrated that lower baseline levels of ccfDNA were observed in NSCLC patients with lower chemotherapy response rate [47]. The possible reasons for the contrary results will be discussed in detail below.

Prognostic indicators

A series of studies have confirmed by performing Kaplan–Meier curve or univariate analysis that elevated ccfDNA levels of various cancer patients are associated with decreased overall survival (OS) [33,40,44,49] or disease-specific survival (DSS) [42], as well as shorter disease-free survival (DFS) [44] or progress-free survival (PFS) [49]. Moreover, a COX multivariate survival analysis was applied to further identify the role of ccfDNA level as an independent predictor in cancer prognosis (Table 5). Reportedly, the prognostic value of ccfDNA level was even superior to that of some routine prognostic markers like AFP [40] and carbohydrate antigen 125 (CA125) [42].

On the contrary, the baseline levels of ccfDNA in some articles did not seem to correlate with several prognostic indicators in both early-stage and advanced NSCLC [26,41]. Most surprisingly, some researchers found that the survival percentage was better in breast cancer patients with higher pre-therapeutic serum ccfDNA concentrations [10]. These findings were in line with the study by Huang et al [35], which showed a little longer survival in breast cancer patients with plasma ccfDNA level more than 65 ng/ml, though without statistical difference ($P = 0.12$). Some authors attributed the discrepancy in survival results to the different quantitative methods across various studies, as well as the heterogeneity in patient inclusion criteria and sample selection [41], as discussed repeatedly earlier. Others, innovatively, assumed that high levels of ccfDNA may mean a stronger host response to tumor and therefore better survival rate [10]. For this cause, a deeper disclosure of mechanism of DNA release from tumor tissue into the blood is needed to explain whether elevated ccfDNA level represents an aggressive tumor behavior or better host anti-tumor response.

Several studies have tried to evaluate the increased prognostic value of ccfDNA level when combined with other biomarkers. For example, a study by Cheng et al [49] showed that ccfDNA concentration in metastatic breast cancer patients, together with ccfDNA integrity and circulating tumor cell (CTC) status, had the lowest integrated prediction error (IPE) scores, which meant a better prediction accuracy. These results further illuminate the value of ccfDNA level in multi-parameter combination for prognosis prediction of cancer.

Conclusion

At present, real-time qPCR and new qPCR-based technologies are widely used in the field of ccfDNA, characterized by higher accuracy and sensitivity. However, the choice of varied target gene

primers hampers the comparison of data from different studies, and the high expense also limits their clinical applicability. On the other hand, the application of fluorescent dye is gradually recognized with the advantages of cheap, rapid and identical procedure. Anyway, these technologies will be developed in a trend of optimization and standardization to make the result of ccfDNA quantity reproducible, comparable and more reliable.

The clinical significance of ccfDNA in diagnosis of cancer is as follows: (i) Significantly elevated ccfDNA levels in cancer patients compared with normal subjects suggests that it might be able to preliminarily screen cancer in healthy population or high-risk group and help to judge whether further examination is necessary; (ii) Although ccfDNA as a sole tumor marker does not seem to be an ideal diagnostic tool for differentiation between benign and malignant diseases, its combination with other markers could constitute potent diagnostic indicators with higher sensitivity.

As for the role of ccfDNA level in cancer prognosis, given that its elevation is likely to indicate more aggressive cancer behavior and worse therapy efficacy, its baseline level and dynamics during follow-up can contribute to the judgement of survival time. The conclusion remains to be further confirmed.

Overall, ccfDNA, the main part of liquid biopsy, has been paid more and more attention. Especially, quality analysis of ccfDNA like gene mutation and DNA methylation, is promising in the field of precision medicine for cancer. But the unique value of ccfDNA quantity analysis should not be ignored as well. Besides, given the importance of differentiating between ctDNA and normal ccfDNA, quantification of ctDNA rather than total ccfDNA by targeting cancer-associated mutant genes should be considered in future studies. Nowadays, there is a trend of multi-parameter combination in cancer diagnosis and prognosis prediction. Based on the numerous studies above, it can be speculated that in the near future, with the standardization of technology and the conduction of multicenter large-scale studies, ccfDNA level will be a powerful choice of biomarkers in cancer management.

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Declaration of competing interest

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Jia-Lei Weng: Investigation, Writing - original draft, Writing - review & editing. **Manar Atyah:** Writing - review & editing. **Chen-Hao Zhou:** Writing - review & editing. **Ning Ren:** Writing - review & editing.

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