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Research Article

Quantification of Circulating Cell-Free DNA and its Clinical Applications in Cancer Management

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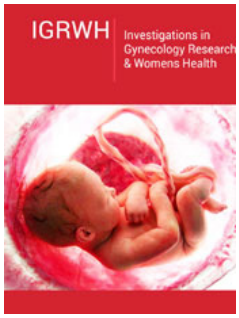

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Abstract

As liquid biopsy gains more traction, quantification of the level of circulating cell-free DNA (cfDNA) in plasma has become an important biomarker to study in cancer diagnosis, tumor progression and treatment response monitoring in the whole process of cancer management. Here we summarized the commonly used plasma cfDNA quantification methods and reviewed cfDNA quantification in clinical applications in cancer management based on publications. The aim of the review is to provide scientific information and practical guidance for clinicians to choose the appropriate method for cfDNA quantitation despite the lack of standardization. We also hope to see the cfDNA quantification biomarker can be validated in larger clinical studies or clinical trials with different clinical indications in cancer management in the near future.

Introduction

Cell-free DNA (cfDNA) is released from dying or damaged cells into the body's circulatory system. First discovered in plasma in 1948 by Mandel and Metais [1]), cfDNA were also discovered in other body fluids such as Cerebral Spinal Fluid (CSF) [2], urine [3,4], pleural fluid [5], saliva [6,7]. It is hypothesized that cfDNA is generated through both active (secretion) and passive (apoptosis in normal cells and necrosis in cancer cells) mechanisms [8]. In healthy individuals, most of the plasma cfDNA originates from the hematopoietic cells [9], while in cancer patients, tumor cells could release DNA, called circulating tumor DNA (ctDNA), into the blood stream as well as from the normal cells [10]. It is estimated that ctDNA accounts for a small fraction (0.01% to 1%) in early-stage cancer patients [11]. In advanced and metastatic cancer, the ctDNA fraction can go up to even 90% of the total cfDNA [12]. However, the increase of cfDNA in cancer patients is not totally from cancer cells. New studies indicate that the majority of the ctDNA is not from tumor cells, but from leukocytes in the blood [13]. More studies are needed to study the cfDNA origin in tumor cells to resolve the contradictions from the above observations.

Cell-free DNA are small pieces of DNA fragments in range of 120-220 bp with a maximum peak at 167 bp [14]. Unlike uniformly fragmented DNA released from apoptotic healthy cells, ctDNA released from tumor cells through necrosis varies in size [15]. Some researchers state that the ctDNA is around 50 to 100 bp [16]. This is supported by the evidence that enrichment of shorter fragments of DNA increases the ctDNA assay sensitivity [17]. In the human circulatory system, the cfDNA can exist in unbound/naked form or bound form internalized in extracellular vesicle (EVs)/exosomes [18].

cfDNA concentrations are found to be different between healthy/benign tumor patients and cancer patients [19]. The normal cfDNA concentration is normally in the range of 0 to 100ng/ml with an average of 30ng/ml whereas the cfDNA concentration ranges from 0 to 1000ng/ml with an average of 180ng/ml in cancer patients [20].

Accurate quantification of cfDNA is challenging at several levels. First of all, quantitation of cfDNA is sensitive to genomic DNA (gDNA) contamination derived from lysed blood cells in poorly manipulated samples [14], and it is critical to prevent hemolysis during processing and storage of cfDNA. Secondly, different DNA extraction methods showed varied DNA yield

and fragment size distribution [18], causing underestimate of the real cfDNA concentration. Finally, varied DNA quantitation methods also give different cfDNA concentration [21], adding further variation. Hence, there is a need to standardize collection, handling, and preservation methods of blood, cfDNA isolation and cfDNA quantitation. In this review, we focused on the cfDNA quantitation area and summarized the commonly used cfDNA measurement methods from the literatures. With the comparison of these methods, we hope to provide comprehensive information and guidance for clinicians to choose the appropriate cfDNA quantification tools for their future clinical practice. We finally briefly reviewed the clinical utility and applications of cfDNA quantification as a biomarker.

Cell-free DNA Quantitation Methods

Direct and indirect quantification

The cfDNA quantitation methods include both direct quantitation and indirect quantitation methods. The direct method directly measures cfDNA concentration without DNA purification, such as direct PCR [22,23], direct SYBR Gold assay [23,24], and QuantiDNA™ direct cfDNA test [25-27]. In indirect cfDNA quantitation method, the cfDNA needs to be purified from plasma or other body fluids and then quantitated with quantitative PCR (qPCR) [21,28-30], droplet digital PCR (ddPCR) [31], fluorescent dye (e.g. Qubit), or UV-spectrophotometry (e.g. NanoDrop). The advantages of the direct quantification methods are that they are quick and simple, but the drawback is the lack of accuracy due to matrix, reaction, or other interferences. The advantages of the indirect quantification methods lie in the elimination of all sources of interferences, but the major drawback is underestimation of cfDNA concentration due to sample loss during cfDNA extraction. The sample loss varies depending on the extraction methods used (column purification or magnetic beads purification) or the commercial vendors of the extraction kits. The cfDNA loss after extraction using QIAamp DNA Blood Mini Kit can be as high as 63.3% [22]. Unless a complex extraction control is included in extraction procedure to calibrate the loss, the cfDNA quantification after cfDNA extraction is unreliable. Although this cfDNA mis-quantification may not always affect the conclusion of clinical monitoring in a clinical study, it may have contributed to the contradictory results in some studies [32,33]. Inaccuracy and non- standardization of DNA quantification methods has also become the hurdle for the use of the cfDNA quantification in clinical practice [34]. These methods are summarized in (Table 1).

Table 1: Commonly used cfDNA quantitation methods.

Methods	Target for Quantification	cfDNA Extraction needs	Technology	Sensitivity	Sequence Specific	Fragment Size Limit
Nanodrop	DNA, RNA	YES	UV-spectrophotometry	nanogram	No	No
qPCR	Alu, GAPDH, TERT, ACTB	Yes	PCR	Varied on targets	Yes	amplicon size
Qubit dsDNA HS	dsDNA	YES	dsDNA specific fluorescent dye	5 ng/ml	No	No
direct PCR	Alu	No	PCR	0.1ng/ml	Yes	amplicon size
direct SYBR Gold assay	naked dsDNA	No	dsDNA specific fluorescent dye	170 ng/ml	No	No
QuantiDNA	Alu for naked DNA and exosomal DNA	No	bDNA	0.09 ng/ml	Yes	>50bases

Nanodrop method

The common bench UV-spectrophotometric method such as NanoDrop cannot detect DNA below nanogram levels [3]. It can't be used for direct measurement due to blood matrix interference. In addition, the UV-spectrophotometry cannot differentiate DNA from RNA and oligonucleotides, even single nucleotides. To measure the cfDNA quantitatively for healthy individuals, one to a few milliliters of plasma or other body fluids is needed to purify the cfDNA before quantification. Even after purification, the nanodrop method shows very poor correlation with other quantification methods such as ddPCR methods [32].

Qubit method

Fluorescent dye-based methods such as Qubit uses the double stranded specific dye such as SYBR gold, PicoGreen to measure the cfDNA concentration directly from plasma or purified DNA. The direct SYBR Gold assay quantitates cfDNA in microtiter plate directly from plasma or serum with Qubit Fluorometer. This method is simple and quick, but low sensitivity with a detection limit at only 170 ng/ml [25], which is far above the 0-100ng/ml of healthy individual [10]. In addition, in direct quantitation method, only freely unbound cfDNA is measured, as SYBR Green dye can only bind to free/naked DNA, not the DNA internalized in EV or exosomes [24]. However, Qubit quantification has been used for cfDNA quantification after DNA purification [32]. When compared to qPCR or ddPCR quantification methods, Qubit quantification method shows good correlation with these methods [32]. When a specific source of DNA is quantified from another, such as human cfDNA from mouse cfDNA, unlike the methods described below (e.g. qPCR, ddPCR or QuantiDNA™ methods), both Nanodrop and Qubit methods have limitations to quantitate species-specific cfDNA as they both detect DNA without sequence specificity.

qPCR and ddPCR methods

The qPCR method can also be used to quantitate cfDNA directly and indirectly. Breitbach et al developed a direct qPCR method with

primers designed in a multi-locus L1PA2 sequence with loading 2ul of 1:40 diluted plasma [22]. Its LOQs were determined at 100 copies per reaction. It was reported that the majority of cfDNA in the blood of cancer patients was contained within exosomes, rather than floating freely DNA [18,35] Umetani et al. [15] developed another qPCR method with primers designed in ALU regions [23]. In order to measure the bound form of cfDNA, a proteinase K pretreatment was added. This ALU-qPCR method had a detection limit of 0.01pg of DNA which is equivalent to the plasma cfDNA concentration of 0.1ng/ml. Most of the qPCR quantification methods use purified cfDNA for quantification. These methods use either 1 million copies of conserved repeats such as ALU [23] or single copy housekeeping genes such as GAPDH [29], TERT [30], ACTB [21,31] as the reference gene to quantify.

A few parameters may affect the qPCR method accuracy. Single copy gene amplicon quantification may not always be the representative of the total amount of cfDNA [36], which may underestimate the total cfDNA in the clinical samples. qPCR-based cfDNA quantification is also highly variable according to the amplicon length [37]. Comparing with the total DNA amount gauged by PicoGreen, the DNA content determined by qPCR was several-fold lower [21]. This difference could be explained by the fact that PicoGreen or other dyes can detect nearly all DNA fragments while qPCR only quantitates amplifiable DNA.

ddPCR method is an absolute quantification tool for cfDNA copies, but the same principle of primer and amplicon design makes ddPCR carry the same drawbacks as qPCR does despite a more sensitive quantification method.

QuantiDNA™ Direct cfDNA test and QuantiDNA™ DNA Measurement Assay

QuantiDNA™ tests for cfDNA is highly sensitive and specific signal amplification nucleic acid probe assay based on the branched DNA (bDNA) technology [27] This assay is a direct hybridization assay without DNA/RNA extraction or PCR amplification. It uses microliters of plasma and detect 0.39ng/ml of cfDNA using the luminometer (QuantiDNA™ DNA Measurement Assay) and 0.09ng/ml cfDNA using Luminex MagPix (QuantiDNA™ Direct cfDNA Test) after plasma dilution. This assay contains the proteinase K and detergents in its assay protocol therefore it can measure both the unbound freely cfDNA and protein/exosome bound cfDNA. The assay quantitates the cfDNA by hybridization with a seven oligos probeset covering a 200base ALU region [27]. The probeset has both CE (capturing the DNA targets to beads or plate well surface) and LE (hybridizing with bDNA molecules for signal amplification) types of oligos where any two neighboring CE, LE oligo can quantitate DNA fragments over 50 bases.

QuantiDNA™ DNA Measurement Assay, have shown the accuracy of quantification with 5 to 10% variation from the standards (DiaCarta, unpublished), exceeding the accuracy of the methods that requires cfDNA extraction, that may underestimate the actual cfDNA DNA by 40 to 60%. This quantification method only requires 10 to 20 microliters of plasma and is currently used in many clinical applications [26,28,38] [Table 1].

In summary, there is a need for method standardization of cfDNA quantification before the clinical studies can be carefully compared and solid conclusion is generated. Although Qubit and qPCR are often used by researchers, many of them have not included an extraction control to calibrate the accuracy of the quantification methods. It is necessary to evaluate QuantiDNA™ cfDNA quantification methods side by side with the traditional methods before picking a reliable cfDNA quantification method for clinical applications.

cfDNA quantification in clinical applications of cancer management

Many studies have indicated that the cfDNA levels are much higher in cancer patients than in healthy controls, and patients in late-stage cancer than in early-stage cancer [39], indicating the role of cfDNA levels as a biomarker for cancer prognosis. Fan et al. reported that plasma cfDNA concentration was markedly higher among preoperative lung cancer patients when compared to healthy subjects [40] Austin et al. measured the cfDNA concentrations in 178 patients with colon, pancreatic, lung or ovarian cancer and 64 healthy individuals and found the levels of cfDNA among the cancer patients are substantially higher than healthy subjects [13]. In breast cancer, the level of cfDNA is significantly higher in cancer patients than in healthy people and people with benign tumors [41,42], higher in metastatic patients than in progression-free patients [42,43]. Although the cfDNA quantification marker has better sensitivity and specificity than traditional protein biomarkers such as CEA and CA (cancer antigen), the cfDNA is less than ideal to be used as a diagnostic marker.

In management of different types of cancers, cfDNA quantitation can also be used for response monitoring of treatments of various types, including radiotherapy, chemotherapy, target therapy, and immunotherapy [44-47]. cfDNA level changes in breast cancer patients is correlated with chemotherapy response [48,49]. When compared with the traditional biomarkers such as CA 15-3 and alkaline phosphatase, total cfDNA is a better predictor for both treatment response and overall survival [50]. The cfDNA level is also higher in recurrent breast cancer patients than in non-recurrent patients [51]. These studies have shown that cfDNA concentrations may have a great prognostic value [14,52]. In Ovarian Cancer (OC), cfDNA quantification has been demonstrated to be an effective biomarker for treatment (e.g. chemotherapy) response in OC [53,54] and predicts disease-specific survival in OC [55,56].

There are recent reviews on cfDNA quantification and ctDNA analysis [35,56] and their applications in clinical review. Here, we will not discuss the ctDNA analyses (e.g. mutation analysis and methylation analysis) that are used for cancer screening, Molecular Residual Disease (MRD) detection for recurrence prediction, or treatment responses. Although ctDNA is tumor-specific, they are more time consuming and costly, and hard to be applied for frequent monitoring compared to cfDNA quantification monitoring. The two types of analysis can complement each other and provide more comprehensive information in the whole cancer management process.

Conclusion

Current clinical studies use qPCR and Quibit methods for cfDNA quantification by ignoring the fact that these methods underestimate cfDNA level. This may cause wrong conclusions when studying the correlation of cfDNA with cancer progression and treatment responses. QuantiDNA™ direct cfDNA quantitation and other methods should also be evaluated in clinical studies side by side with the traditional methods. After all, it is critical to have a simple and reliable cfDNA quantification method if the biomarker is to be used in clinical settings.

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