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Review

Digital PCR analysis of circulating nucleic acids

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ABSTRACT

Detection of plasma circulating nucleic acids (CNAs) requires the use of extremely sensitive and precise methods. The commonly used quantitative real-time polymerase chain reaction (PCR) poses certain technical limitations in relation to the precise measurement of CNAs whereas the costs of massively parallel sequencing are still relatively high. Digital PCR (dPCR) now represents an affordable and powerful single molecule counting strategy to detect minute amounts of genetic material with performance surpassing many quantitative methods. Microfluidic (chip) and emulsion (droplet)-based technologies have already been integrated into platforms offering hundreds to millions of nanoliter- or even picoliter-scale reaction partitions. The compelling observations reported in the field of cancer research, prenatal testing, transplantation medicine and virology support translation of this technology into routine use. Extremely sensitive plasma detection of rare mutations originating from tumor or placental cells among a large background of homologous sequences facilitates unraveling of the early stages of cancer or the detection of fetal mutations. Digital measurement of quantitative changes in plasma CNAs associated with cancer or graft rejection provides valuable information on the monitoring of disease burden or the recipient's immune response and subsequent therapy treatment. Furthermore, careful quantitative assessment of the viral load offers great value for effective monitoring of antiviral therapy for immunosuppressed or transplant patients. The present review describes the inherent features of dPCR that make it exceptionally robust in precise and sensitive quantification of CNAs. Moreover, I provide an insight into the types of potential clinical applications that have been developed by researchers to date.

Contents

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1. Introduction

Blood plasma is a dynamic medium that contains much molecular information that is reflective of an individual's health condition. The fact that nucleic acids freely circulate in human bloodstream has been known for more than 60 years [1]. More recent studies have shown unequivocal evidence that the majority of circulating nucleic acids (CNAs) in plasma of healthy individual is derived from hematopoietic cells undergoing apoptosis [2,3]. Tumor tissue, transplanted organs, and placenta also contribute to the pool of plasma CNAs [4–8]. Circulating nucleic acids therefore give the unique opportunity to noninvasively diagnose or monitor pathological states in cancer, organ transplant patients, as well as women over the course of pregnancy.

However, there are many technical challenges associated with the biological nature of plasma CNAs, i.e. degraded nucleic acids originating from tumor, organ graft, or placental cells represent a minority of the total amount of CNAs floating in plasma [9,10]. Therefore, only highly precise and robust methods would be able to detect and quantify the minor species of CNAs contributed by the additional source. The unceasing technological advancement moves the frontiers in research of CNAs constantly forward. By counting individual molecules in the sample as offered by dPCR technique, one can achieve this goal with enhanced sensitivity and precision.

2. Digital PCR technology

2.1. Principles of digital PCR

The concept of absolute measurement of nucleic acids by single molecule counting was reported in 1992 by Sykes et al. recognizing the merit of limiting dilution and Poisson statistical analysis [11]. Diluted template is distributed into individual PCR reactions and the original template amount is gauged by the number of partitions with a positive amplification with respect to the total number of analyzed partitions. The distribution of the molecules throughout partitions is a random and independent process [12] and optimal template dilution usually assures that only one target molecule per reaction partition is examined. When using a higher template concentration, the actual number of molecules would be underestimated because some partitions contain more than one template molecule. The Poisson statistics can correct for this distortion to some extent. Fluorescence chemistry is used to interrogate the presence or absence of specific PCR product [13]. Ultimately, data interpretation does not depend heavily on sophisticated bioinformatics analysis. For some applications, the sequential probability ratio test may be used to measure the strength of evidence for the allele distribution being different from normal [14].

2.2. Digital PCR platforms

To transform the powerful potential of dPCR into efficiently working platforms, the process of single molecule amplification must take place in a highly stable environment. The choice of platform depends mainly on the trade-off between the degree of precision, throughput and the costs of the system and the assay.

The first generation of dPCR platforms were based on chips containing microfluidic channels such as those made by Fluidigm [15] and plates with hydrophilic and hydrophobic surfaces such as those by Life Technologies [16]. The target molecule is monitored in real-time conditions, thus false positive reactions could be inferred from the amplification curve of each reaction. With the demand for higher sensitivity and precision, platforms providing much higher number of digital partitions were needed. Hence, companies have launched systems where each dPCR reaction takes place in aqueous droplets in oil coupled with endpoint reading of the PCRs. Despite the fact that these platforms cannot perform real-time PCR measurement, the immense number of reaction partitions with uniform size led to a dramatic extension of the dynamic range. Twenty thousand reaction droplets could be generated per reaction by the platform developed in the BioRad Laboratories, which provides quantitative measurement across 4 orders of magnitude [17,18]. On the other hand, millions of reaction partitions could be handled by the system by RainDance Technologies, further expanding the dynamic range [19]. The BEAMing digital PCR technology (beads, emulsion, amplification and magnetics) provided by Sysmex Inostics [20,21] clonally amplifies nucleic acids in the presence of magnetic particles and assesses its quantity by using a flow cytometry. This dPCR strategy has found a wide application especially in cancer research [22–27].

2.3. Advantages of digital PCR

Currently, quantitative real-time PCR (qPCR) is still a more popular choice for nucleic acid measurement, mainly because of the lower costs. The use of external calibrators is the core for analytical performance in qPCR and may vary among laboratories. In contrast, quantification of nucleic acids in dPCR does not rely on external references and shows an increased tolerance to enzyme-inhibiting substances [28,29]. Even though both technologies share essentially the same fluorescence chemistry for nucleic acid detection, the distinct power of dPCR for sensitive and precise measurement lies in the number of partitions allowing for simultaneous template amplification, concurrently dictating the dynamic range. The latest development of dPCR technology employing thousands to millions of reaction partitions thus provides a scalable environment [17,19]. Consequently, dPCR offers measurement of nucleic acids with superior precision, sensitivity and reproducibility over qPCR [30–33]. The coefficient of variation, corresponding to a measure of analytical precision, has been shown to be significantly lower for dPCR compared to qPCR [10,28,34–37]. Moreover, a template compartmentalization reduces the background DNA and contaminant levels which consequently increases the signal-to-noise ratio in positive reaction partitions and thus improves detection sensitivity [38].

The technique is extremely powerful in detection of minute traces of nucleic acids without the need for a pre-amplification step, thus preventing introduction of an assay-specific bias [30,32]. Duplex dPCR has been shown to provide even more precise measurement than uniplex dPCR for the detection of limited concentrations of plasma CNAs [32]. By adjusting the amplicon length, primer concentration, and annealing temperature, one can achieve equally precise discrimination of multiple targets in one reaction using DNA-binding dye over the same dynamic range as with TaqMan chemistry [39].

3. General applications of digital PCR

The aforesaid inherent features of dPCR, which are far beyond the scope of detection abilities of other methods, make this approach unique for several research applications. The dPCR technology allows

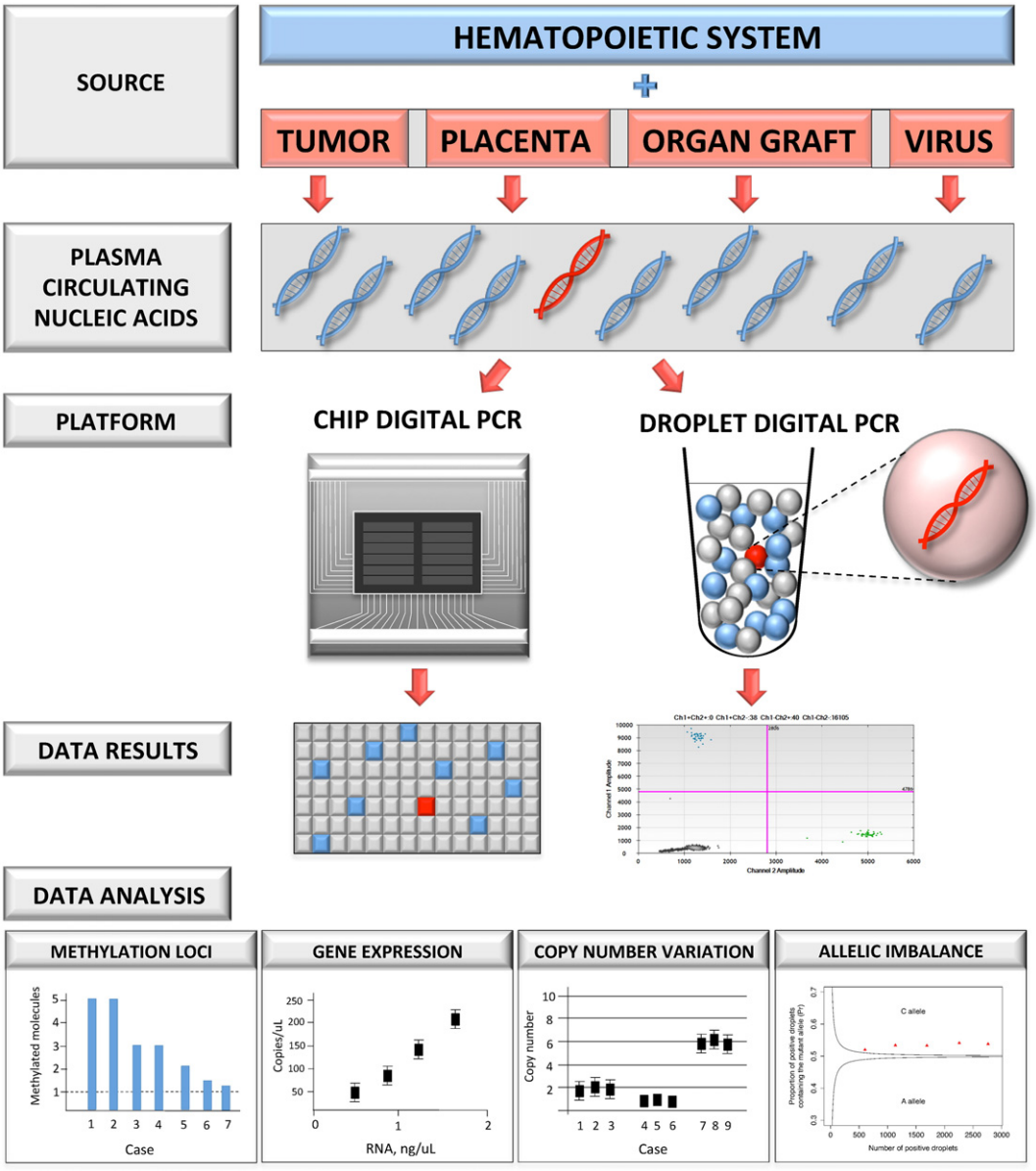

Fig. 1. Schematic representation of the general application of chip or droplet digital PCR technology for the research of circulating nucleic acids.

for sensitive detection of rare mutations, accurate quantification of slight alterations in copy number variations, differentiation between changes in gene expression, or assessment of the methylation status (Fig. 1).

3.1. Rare variant detection

The early stages of tumor formation are associated with minute amounts of tumor-derived CNAs released into the bloodstream requiring employment of extremely sensitive detection methods. Cancer research therefore benefits considerably from the ability of dPCR to trace such a scarce genetic material. By effective partitioning of nucleic acids and counting thousands to millions of reaction partitions, one can detect tumor-derived mutations or rare variants present in extremely low abundance. The analytical power of dPCR allows single-nucleotide-variant detection below one copy per 100,000 wild-type sequences [40]. For example, it is possible to trace one *KRAS* mutation in the background of 200,000 wild-type molecules using the picolitre scale droplets [19]. Similarly, a single-color assay based on non-specific double-stranded binding dye could detect arbitrarily prepared mutation in concentration of less than 1% among the wild-type *BRAF* variant, suggesting that the approach is applicable for research of CNAs [41].

3.2. Gene copy number variation analysis

Copy number variations (CNVs) are among the most prevalent causes of structural variability of DNA responsible for the human genomic diversity [42]. At the molecular level, CNVs are composed of gene amplifications or deletions, a genetic variability often observed in a process of tumorigenesis [43].

To date, the potential of dPCR technology has not been extensively explored in detection of CNVs by testing plasma circulating nucleic acids. Nonetheless, previous studies indicate that dPCR would be an ideal molecular tool in this research field [33,44,45]. Investigations on the breast cancer cell lines showed superior precision and sensitivity of dPCR compared to qPCR in detection of subtle fold-differences of *HER2* gene copy number variation [33]. Quantitative real-time PCR revealed presence of *HER2* gene amplification in plasma of patients with breast cancer [44], thus giving the opportunity to refine its detection by the digital single molecule counting strategy [46]. Furthermore,

detection of multiple regions by using a strategy called Multiplex Template Sampling described by Petriv et al. can further increase the template concentrations for CNVs assessment [45]. The use of non-specific double-stranded binding dye in dPCR similarly provides a highly sensitive and specific quantification of CNVs in a multiplex format simply by adjusting the size of the targeted region [41].

3.3. Gene expression analysis

Regulatory RNA molecules modulating the gene expression, namely microRNA (miRNA) and long non-coding RNA (lncRNA), affect basic cellular processes like cell cycle progression, cell differentiation and apoptosis [47]. Several studies report that miRNAs have a high potential to be a blood-based biomarker in cancer detection [48] as well as in prenatal testing research [49–51]. Ma et al. reported the use of dPCR as a potential tool for quantitative assessment of miRNA in plasma of lung cancer patients by observing significantly higher copy number of miRNA compared to controls [52]. Similarly, encouraging results were observed in a study focused on the measurement of circulating lncRNA expression in human serum [53]. Nevertheless, as stated in the dMIQE (Minimum Information for Publication of Quantitative Digital PCR Experiments) guidelines, the quantitative analysis of RNA using RT-dPCR still requires careful consideration of the experimental design and final data reporting [54].

3.4. Analysis of methylation loci

DNA methylation plays a central role in epigenetic inheritance and its alteration is often involved in cancer or pathological processes resulting in genome instability and deregulation of cell growth [55]. Similarly, changes in methylation pattern have been observed in pregnancy-associated pathological states [56,57]. Bisulfide treatment modification of plasma DNA may reveal the DNA methylation alterations of placenta DNA associated with pathological conditions and serve as effective noninvasive biomarkers.

Digital PCR has been used to quantitatively assess methylation status at specific loci in plasma of patients with colorectal cancer [25] and breast cancer [38]. Li et al. developed a methyl-BEAMing technology whereby increasing the ability to detect curable early-stage colorectal cancers with the same precision as currently used next-generation sequencing platforms [25]. Additionally, a method called microfluidic digital MethyLight has been successfully tested for cancer-specific DNA hypermethylation events present in plasma of breast cancer patients [38].

4. Bringing digital PCR into clinical use for nucleic acid detection

In 1999, Vogelstein and Kinzler's pioneering study on quantification of *ras* oncogene mutations associated with a colorectal cancer highlighted the potential clinical utility of dPCR [13]. However, laborious and low-throughput setup hindered the possibility for routine application in a clinical setting, thus urging a focus on its practical improvement. Development of microfluidic devices and emulsion PCR with reaction volume minimized to nanoliter up to picoliter extent represented a substantial advancement [17,19].

Subsequently, diverse applications of dPCR for research of CNAs, facilitated by the practicality of currently available platforms, have paved the path into clinical use in several areas. Cancer research as well as the field of noninvasive prenatal testing (NIPT) highly benefit from the analytical sensitivity of dPCR to detect low concentration of target CNAs. Additionally, precise quantitative assessment of viral load and organ graft-derived CNAs hold the promise for effective monitoring of treatment therapy for immunosuppressed or transplant patients. Likewise, the possibility to precisely measure levels of circulating mitochondrial DNA would help to provide further insight into the pathology of several diseases.

4.1. Noninvasive prenatal testing (NIPT)

The discovery of fetal nucleic acids circulating in maternal blood [6,7] began the journey of developing noninvasive prenatal tests in pregnancy management. The risk for fetal chromosomal aneuploidies or single-gene disorders is an indication for performing prenatal diagnostic tests, which conventionally required the invasive sampling of fetal genetic material by amniocentesis or chorionic villus sampling. CNAs have the potential to enhance the efficacy of prenatal care without the risk associated with the invasive procedures and could be performed regardless of the gestational window.

The fact that fetal CNAs constitute only a minor fraction of the total pool hindered the initial attempts to develop such a noninvasive molecular protocol [10]. Moreover, a discrimination of the maternal causative mutations inherited by the fetus appeared to be another challenge. Methods for single molecule counting, including digital PCR, provided an elegant solution for a direct assessment of fetal mutation status.

4.1.1. NIPT of fetal aneuploidies

To noninvasively detect fetal aneuploidy, a method must be able to distinguish subtle alterations in plasma DNA concentration with a degree of deviation associated with the aneuploid chromosome linearly proportional to the fetal DNA fraction. Lower fetal DNA fractions ultimately result in a demand for higher number of molecules to be examined. In the first trimester pregnancies, the proportion of fetal DNA in maternal plasma accounts for about 10% of total circulating DNA [10]. Digital PCR-based data simulation of Evans et al. shows that if only 2% of fetal DNA enhancement can be achieved, an extremely high number of counts would be needed to achieve a clinical significance for aneuploidy detection [58]. Consequently, taking into consideration approximately 1000 DNA copies per milliliter [10], the approach using even the latest dPCR technology accommodating such high number of molecules would require impractical volume of maternal plasma (100 mL) for pregnancies with extremely low fetal DNA fractions. On the other hand, with respect to already established methods for fetal aneuploidy detection, digital PCR would represent a time- and cost-effective alternative for pregnancies with sufficient levels of fetal DNA.

Two groups simultaneously outlined a principle of digital PCR-based trisomy 21 detection of the fetus [59,60]. Using artificial DNA mixtures, the authors successfully assessed gene dosage between chromosome 21 and a reference chromosome. Unlike the RNA-SNP method for trisomy 21 detection developed previously [59], this approach was polymorphism-independent, although still limited by a high initial plasma volume to assess fetal trisomy 21 status.

Later, by targeting fetal hypermethylated epigenetic marker on chromosome 21 and *ZFY* gene on Y chromosome, a method called digital epigenetic–genetic chromosome dosage strategy allowed an accurate discrimination between normal and affected pregnancies [61]. Alternatively, to demonstrate application of the same approach in a gender-independent manner, paternally inherited fetal SNPs on a reference autosomal chromosome were used instead of the Y chromosome marker, although still restricting the analysis to cases with informative SNPs [62]. Therefore, in order to increase the population coverage, Tsui et al. examined mRNA encoded by *PLAC4* gene for prenatal screening of trisomy 21 using two approaches, namely *PLAC4* RNA–SNP approach for heterozygous cases and *PLAC4* mRNA-quantification approach for homozygous cases [63]. As measured by microfluidic digital PCR, a significant increase in plasma *PLAC4* cDNA concentration in the trisomy 21 pregnancies has been observed, thus supporting its potential application as a screening tool for fetuses homozygous for the targeted *PLAC4* SNP.

4.1.2. NIPT of single-gene disorders

Driven by the curiosity to explore the use of dPCR for single-gene disorders, Lun et al. developed a strategy for the detection of fetal alleles inherited from the mother heterozygous for beta-thalasemia mutation

[64]. The relative mutation dosage (RMD) method together with a size enrichment strategy, namely digital nucleic acid size selection (NASS), has proven the applicability for noninvasive prenatal diagnosis of single-gene disorders. Furthermore, the RMD approach has been found to be extremely robust for the detection of fetal hemophilia mutations, representing the X-linked disease model, clinically manifested in male fetuses [65]. In addition, Barrett et al. proved the feasibility of the RMD approach using dPCR in pregnancies at risk of sickle cell anemia and recommended further optimization of fetal DNA fractions by designing a series of biallelic indel markers with shorter amplicons in order to improve its performance [66]. As the study of Barrett et al. was informative only in 65% of the female-bearing pregnancies, Gu et al. proposed an alternative strategy exemplified by an autosomal recessive disorder, methylmalonic acidemia [67]. A set of multiple SNPs was used to determine fetal DNA fractions regardless of fetal gender. To assess the mutational state of the fetus two methods were developed: a direct strategy, targeting the mutation, and an indirect strategy, using a set of multiple SNP markers linked to the mutation to be evaluated by z score statistics. Since all of the described methods are based on detection of allelic imbalance, the accurate measurement of fetal DNA fractions is a critical factor in the successful determination of fetal single-gene disorders.

4.1.3. NIPT of other pregnancy-related complications

Even though prenatal *RHD* genotyping is already established as a clinical service for pregnant women at risk for complications related to Rh immunization, traditional qPCR approach seems to have limitations in particular instances. Presence of an intact but dysfunctional variant *RHD* gene caused by point mutations would result in RhD negative phenotype, prone to maternal sensitization to fetal D antigens [68]. Using dPCR technology, Tsui et al. were able to correctly identify fetal wild-type *RHD* genotype against the background of maternal *RHD*(IVS3 + 1G > A) mutation in maternal plasma [69].

Whitehead and Tong have outlined another exciting application of dPCR to monitor the degree of fetal hypoxia to prevent stillbirth or childhood disability associated with hypoxia [70]. The authors previously showed that circulating hypoxia-induced RNA transcripts were significantly increased and appear to be correlated with a degree of fetal hypoxia [71] and dPCR would find its place in the validation of candidate biomarkers.

4.2. Cancer research

Identification and characterization of CNAs in plasma of cancer individuals [4,5] have sparked an interest in developing noninvasive approaches facilitating early detection, treatment monitoring, and screening for disease recurrence. As the tumors are highly heterogeneous, plasma CNAs derived directly from the tumor tissue are expected to represent a full repertoire of mutations [72]. New somatic mutations can develop during the tumor treatment, thus contributing to nonresponsiveness of the originally effective drug and an urgent need for therapy replacement [73–75].

With the technological advances to sensitively detect CNAs originating from the tumor, the term “liquid biopsy” has started to be pronounced even more frequently [76]. To track tumor development at an early stage, an extremely sensitive method must be able to pick up only few copies of mutation-associated allele released into the bloodstream by a tumor and recognize them from the normal variant background. A recent study of Bettegowda et al. examines different tumor types in a large cohort of patients and confirms the wide use of dPCR in research of circulating tumor DNA (ctDNA) to effectively screen for somatic mutations in *KRAS* gene and to concurrently monitor the effect of therapy by detecting of newly developed *EGFR* mutations [77]. Altered quantities of circulating tumor nucleic acids have been described in several cancer types [9,78–83] and may play a role as a molecular biomarker of tumor progression and therapy efficacy.

4.2.1. Breast cancer

Abnormality in regulation of the phosphoinositide 3-kinase (PI3K) pathway in patients with breast tumors is caused by somatic mutations of the gene encoding PI3K catalytic subunit p110alpha (*PIK3CA*) [84]. The BEAMing technology allowed detection of ctDNA harboring *PIK3CA* mutations in plasma of breast cancer patients [27]. As observed in this study, fast tumor evolution of *PIK3CA* mutational status upon disease recurrence reinforces the need for contemporary assessment to administer efficient therapy. In a study by Jelovac et al., the same technology revealed the presence of *PIK3CA* mutation harbored by the breast tumor, at concentration 0.0759% of the total circulating DNA in pre-operative plasma, and was found undetectable after surgery [85]. Recently, droplet dPCR technology accurately detected *PIK3CA* mutations in plasma of patients with early-stage breast cancer in both pre- and post-surgery samples, thus allowing stratification of patients by measuring residual disease as being at higher or lower risk for recurrence [86]. Moreover, by quantification of ctDNA carrying specific *PIK3CA* and *TP53* somatic mutations in plasma of breast cancer patients, the microfluidic dPCR provided the earliest measure of treatment response in half of patients receiving systematic therapy [87].

In addition to common mutations in *PIK3CA* gene, the human epidermal growth factor receptor 2 (*HER2*) gene has been found amplified in 25 to 30% of human breast cancer [88]. The existence of amplified *HER2* gene in circulating DNA has been reported previously [44] implying its use as a potential marker for breast cancer patients. The BEAMing strategy has shown a high accuracy in detection of oncogenic amplification of the *HER2* copy number, reporting a 90% concordance with tumor-derived status, which suggests the use of this approach for any amplified locus in cancer [46].

4.2.2. Lung cancer

Activating mutations in the epidermal growth factor receptor (*EGFR*) tyrosine kinase have been shown to be responsible for the responsiveness to tyrosine kinase inhibitors (TKI) [89–91]. A microfluidic dPCR system accurately detected and quantified two common *EGFR* mutations in plasma of non-small cell lung cancer (NSCLC) patients [92]. A strong association between plasma *EGFR* mutation levels and the clinical response seems encouraging to use this strategy to monitor the effect of treatment therapy. Furthermore, a recent study quantitatively assessed *EGFR* and *KRAS* mutant alleles using droplet dPCR in serial plasma samples of NSCLC patients and shows that this approach allows for the prediction of development of clinical resistance several weeks ahead, and thus guides the treatment [93]. A major problem in TKI therapy represents an acquired resistance attributed to T790M secondary mutation in *EGFR* gene [73]. By using the BEAMing technology, a ratio of the resistance forms of mutation and the number of activating mutations may be useful in monitoring patients with advanced stages of lung cancer [26].

4.2.3. Colorectal cancer

Inactivation of the tumor suppressor genes, *APC* and *p53*, and activation of the oncogene Kirsten-ras (*KRAS*) play an important role in the colorectal tumor development and its progression [94]. It has been described earlier, that CNAs harboring mutations associated with these genes can be found in plasma of individuals with colorectal cancer [95–98]. A BEAMing technology allowed for characterization of the nature, quantity, dynamics and mechanism of the CNAs release by investigating tumor-associated genes in plasma of colorectal carcinoma patients [22,23]. Importantly, the authors report that more than 60% of patients who were not yet metastasized exhibited detectable mutant fragments in plasma [22]. The same research group characterized parameters critical for detecting colorectal tumor-associated mutations using the BEAMing technology on plasma and stool samples [24]. They confirmed the mutational status only in 50% of plasma cases attributing this observation to a lower plasma volume and suggested using stool samples of patients with colorectal cancer rather than plasma. On the

other hand, a picodroplet dPCR detected tumor-associated mutations in plasma of colorectal cancer patients in a multiplex manner and demonstrated that this strategy is suitable for a sensitive screening of rare mutations in a clinical practice [99].

4.3. Mitochondrial DNA research

Mitochondrial DNA, contrary to the nuclear genome, is only a 16.5 kb long, circular double-stranded nucleic acid present in many copies in a mammalian cell, inherited by maternal lineage and found to be associated with the pathogenesis of diseases [100]. In 2000, circulating mitochondrial DNA (cmtDNA) has been observed in plasma of patients with type 2 diabetes mellitus [101]. In plasma of healthy individuals, mtDNA is present as both a particle-associated as well as a free form of nucleic acid, with concentration highly affected by the blood-processing protocol [102]. The distinct nature of these plasma nucleic acids has been further corroborated in studies observing no correlation between the levels of cmtDNA and gDNA [103,104].

Altered quantities in cmtDNA of cancer patients, as measured by qPCR, support the notion that mitochondrial nucleic acids may be potentially useful biomarker in early detection of neoplastic transformation process, tumor progress monitoring and post-treatment follow-up procedure. Several studies indicate that levels of cmtDNA appear to be tumor specific; circulating mtDNA levels have been found elevated in patients with epithelial ovarian cancers [104] and urological malignancies [103,105,106]. In contrast, significantly decreased cmtDNA levels have been observed in breast cancer patients, presumably due to altered replication rate and a subsequent decrease of mtDNA copy number [107]. Moreover, in advanced prostate cancer patients, cmtRNA seems to be the strongest predictor of overall survival and an independent prognostic factor for cancer-related death [103]. To date, only one study reports on dPCR used for cmtDNA levels assessment [108]. In cerebrospinal fluid samples of Alzheimer's disease patients, the cmtDNA shows a significant reduction observed even before the appearance of clinical symptoms for age-related disease.

To verify whether using dPCR on plasma cmtDNA can bring an insight into management of cancer patients, scrutiny in the whole spectrum of tumor types would be of clinical importance. Further studies in different research fields would reveal a possible significance of cmtDNA for other clinical scenarios.

4.4. Transplantation medicine

Organ transplantation between individuals with different genetic makeup results in activation of recipient's immunological response against the transplant and subsequent liberation of an increased amount of donor's genetic material into bloodstream [109]. Circulating DNA released from the graft has been observed in plasma of transplant recipients [2,8,110] and its quantitative changes have been found to be associated with acute graft rejection [111,112]. Therefore, Snyder et al. explored the use of microfluidic dPCR in sex-mismatched transplants in female patients with heart transplantation [110]. They tracked the patient's response by quantification of chromosome Y marker at multiple time points to show that donor DNA levels higher than 0.5% appeared to be indicative of organ damage. Similarly, droplet dPCR has been used on a set of SNPs to differentiate between donor and recipient DNA to measure proportion of fragments released by the transplanted organ in plasma of recipients after liver, kidney and heart transplantation [113]. Recently, Oellerich et al. further confirmed the use of graft-derived cell-free DNA as an organ integrity biomarker to establish minimally effective immunosuppressant concentrations for patients after liver transplantation [114]. Digital PCR technology therefore seems to significantly improve post-transplant monitoring, prevent patients from invasive biopsies and eventually set a personalized immunosuppression therapy by measuring donor's DNA reflecting the health of the graft organ.

4.5. Virology

Presence of cell-free viral nucleic acids has been reported in 1999 in plasma of patients with nasopharyngeal carcinoma (NPC) by detecting EBV-associated DNA and has been shown to be associated with the disease stage [115]. Plasma EBV DNA levels were found approximately eight times higher in advanced NPC than in early-stage disease and were not detectable in patients with complete tumor regression, while the patients with disease persistence still showed higher levels.

Quantitative analysis of viral-derived nucleic acids is already used as a tool for screening and monitoring of immunosuppressed, transplant and cancer patients using standard qPCR. However, several recent studies show superior performance of dPCR technology by measurement of DNA levels of HIV virus in infected patients [36] or an occult RNA virus in cell culture supernatants [116]. Brunetto et al. further confirm the use of droplet dPCR by quantification of human T-lymphotropic virus 1 proviral loads in peripheral blood and cerebrospinal fluid of HAM/TSP patients to be of clinical relevance [35]. Recently, Sedlak et al. and Hayden et al. were the first to assess viral load in post-transplant monitoring for cytomegalovirus [37,117]. Their results show that clinical application of droplet dPCR would be beneficial, although still premature at the current stage, thus urging for further investigations in this field.

5. Limitations, suggested improvements and future perspectives

Even though the first commercial platform for digital PCR has already been on the market since 2006, compared to already well-established qPCR, revelation of its full potential in clinical setting is only at the beginning. The first step towards implementing dPCR into clinical application has already been taken by publishing the dMIQE guidelines providing a framework to report experimental details for improving reproducibility among different laboratories [54].

Huggett et al. comprehensively describe factors, such as the susceptibility to systematic bias leading to underestimation, that have to be taken into consideration to reliably interpret dPCR data before its implementation as a diagnostic tool [118]. Whale et al. recently reported one of the reasons for analytical imprecision in dPCR [32]. A phenomenon called “molecular dropout” is a source of technical variation caused by a failure to detect presence of a target molecule attributed to failed template amplification due to its secondary structure. Since the occurrence of molecular dropout in dPCR is observed for more complex templates like genomic DNA, the influence on the analysis of CNAs seems to be negligible. Presence of concatemer molecules for the *HER2* copy number detection may represent another potential limitation [33] and structural chromosomal abnormalities, such as balanced translocations or inversions are challenging to be directly detected [119]. Moreover, the detection specificity may be decreased in the presence of pseudogene or multiple sequences on the same DNA strand.

Despite the fact that measurement of low copy RNA targets has been proven accurate [31], variability and inefficiency related to the reverse transcription step adds to the complexity of RNA quantification and further urges for requirement of calibration controls [54]. A report on quantification of cell-associated *HIV-1* RNA underlines the awareness of the false positive signals in dPCR [120]. As reported by others, there are still unexplained false positive events occurring when detecting HIV viral load [36]. Therefore, an optimal assay design is critical to prevent cross-reactivity and occurrence of false positives [118].

Undoubtedly, dPCR is a cost-effective alternative to the currently used next-generation sequencing platforms, however suitable only for analyses when the prior knowledge about the mutation is available, thus relying on a personalized assay design. Additionally, this technology may be used to work in concert with next-generation sequencing platforms to determine the input material by providing accurate quantification of DNA libraries [28]. Sequencing technology can also benefit from obtaining separate heterogeneous templates generated in dPCR

for the detection of heterogeneous methylation patterns in cancer research [38].

At present, dPCR is one of the most powerful methods available for an accurate quantification of a scarce amount of CNAs in plasma. It is therefore expected that more research in this area will establish this technology in a broad spectrum of clinical scenarios where the reliance on precision and sensitivity offered by dPCR is of the highest priority.

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