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Review

Applicability of digital PCR to the investigation of pediatric-onset genetic disorders

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ABSTRACT

Early-onset rare diseases have a strong impact on child healthcare even though the incidence of each of these diseases is relatively low. In order to better manage the care of these children, it is imperative to quickly diagnose the molecular bases for these disorders as well as to develop technologies with prognostic potential. Digital PCR (dPCR) is well suited for this role by providing an absolute quantification of the target DNA within a sample. This review illustrates how dPCR can be used to identify genes associated with pediatric-onset disorders, to identify copy number status of important disease-causing genes and variants and to quantify modifier genes. It is also a powerful technology to track changes in genomic biomarkers with disease progression. Based on its capability to accurately and reliably detect genomic alterations with high sensitivity and a large dynamic detection range, dPCR has the potential to become the tool of choice for the verification of pediatric disease-associated mutations identified by next generation sequencing, copy number determination and noninvasive prenatal screening.

Contents

1. Introduction

Genetic disorders account for about one fifth of pediatric hospitalizations and create an even greater burden on inpatient care [1,2]. Even though genetic disorders are uncommon when considered individually, they affect *in toto* more than 20 million people in the United States of America [3]. Additionally, it is becoming increasingly important to accurately measure the amount of mod-

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ifier genes whose copy number – like *SMN2* in spinal muscular atrophy (SMA) [4] – is related to disease severity. Thus there is a real need for a reliable, accurate and sensitive means to measure genomic variants. Most of the currently available technologies for the identification of disease-associated single nucleotide and copy number variants are not sufficiently sensitive, not quantitative without the use of external calibrator or too expensive for routine implementation.

Digital PCR (dPCR) provides a way for the absolute quantification of a target locus in a DNA sample. In dPCR, the target locus is distributed across a large number of partitions by limiting dilution so that a single DNA molecule is present in some, but not all, of the partitions [5,6]. As a result, some of the partitions may be devoid of target molecule to amplify during PCR. The abundance of the target locus can be measured by counting the number of partitions with a positive end-point reaction as well as the negative partitions. The Poisson equation ($\lambda = -\ln(1 - p)$ where λ is the average number of molecules in the sample and p is the fraction of positive reactions) corrects for the underestimation of the true number of molecules by counting negative end-point reactions [7]. Because of this end-point measurement of abundance, dPCR does not require the use of external calibration curve for calculating abundance of the target locus making this assay an absolute quantification.

Early incarnations of dPCR used limiting dilution so that each PCR reaction contained a single DNA molecule. Unfortunately, this approach was very labor intensive and not very precise. Partitioning the DNA so that a single molecule would be present in a PCR would be a more efficient approach for dPCR. dPCR currently uses one of two platforms to partition target DNA into very small volumes: nanofluidic chambers, or arrays, and nanodroplet emulsion [7]. Nanofluidic arrays divide the target DNA into numerous reaction chambers of nanoliter volumes wherein PCRs are run and the number of positive reactions is counted by fluorescence imaging [8–11]. Another means of partitioning involves the emulsification of target DNA and PCR master mixes into thousands of nanodroplet; the nanodroplets are counted after the PCR run for the number of positive reactions [12–14]. Both approaches allow for the accurate absolute quantification of the target DNA in a sample.

2. Applications of digital PCR to pediatric genetics

2.1. Identification of genetic alterations associated with disease

Chromosomal abnormalities involving multiple genes or whole chromosomes have been detected historically with fluorescence *in situ* hybridization (FISH) or array comparative genomic hybridization (aCGH) panels. These approaches are labor intensive and costly. Chromosomal polyploidies like trisomy 21, trisomy 18 and trisomy 13 can also be readily and easily measured with dPCR [15,16]. Droplet dPCR can also readily detect the presence of a mosaic supernumerary marker isochromosome 12p (iso12p) in DNA samples from patients with Pallister-Killian syndrome [17]. Chromosome 22q11 microdeletion syndrome, which is associated with neurological, cognitive and behavioral deficits, can be rapidly detected in patient DNA samples using dPCR to quantify copy number changes within the deleted region [18,19]. Furthermore, dPCR determines the endpoints of this deletion within chromosome 22q11 [19]. Deletions within the *DFNB1* locus – which include the connexin genes *GJB2* and *GJB6* – can be quantitatively measured in DNA from patients with autosomal recessive nonsyndromic sensorineural hearing loss [20]. In addition to identifying larger-scale changes within the genome, dPCR can detect deletions of single genes associated with disorders like SMA (*SMN1* (*survival motor neuron 1*)) [21,22] and South East Asian-type α(0)-thalassemia (*HBA1*/*HBA2* (*α-globin*)) [23].

dPCR can be used to screen patient samples for intragenic mutations linked to numerous monogenic disorders. Examples include verification of point mutations in *GCM2* (*glial cells missing homolog 2*; *GCM2(T370M)* and *GCM2(R367Tfs*)*) associated with hypoparathyroidism [24], in *MAP3K3* (*mitogen-activated protein kinase kinase kinase 3*; *MAP3K3(I441M)*) with verrucous venus malformation [25], in *PIK3CA* (α *catalytic subunit of phosphatidylinositol-4,5-bisphosphate 3-kinase*; *PIK3CA(C420R)*, *PIK3CA(E542K)*, *PIK3CA(E545K)*, *PIK3CA(H1047R)* and *PIK3CA(H1047L)*) with lympathic malformation and Klippel-Trenaunay syndrome [26], in *SMN1* (*SMN1(Y272C)*) with SMA [21] and in *GNAS* (*stimulatory* α *subunit of G protein*, $G_s\alpha$; *GNAS(R201C)*) with McCune-Albright syndrome [27]. In many cases, the disease-associated intragenic mutations were initially identified using next generation sequencing [24–26]. The disease-associated *GNAS* mutation associated with McCune-Albright syndrome could not be detected in the patient until after death and only in certain tissues, suggesting somatic mosaicism [27]. With the assistance of a peptide nucleic acid (PNA) oligomer to lower the detection limit, Uchiyama et al. [28] can detect low frequency somatic mutations of *GNAQ* (*G protein* α *subunit q*, $G_q\alpha$; *GNAQ(R183Q)*) in patients with Sturge-Weber syndrome, a rare congenital neurocutaneous multisystem disorder, using droplet dPCR.

In addition to identifying early-onset genetic diseases, dPCR can detect subclonal mutations in children with various cancers. Using dPCR, subclonal *SETBP1* (*SET binding protein 1*) point mutations are detectable in a cohort of patients with juvenile myelomonocytic leukemia (JMML) [29]. Standard deep sequencing could not detect these subclonal point mutations. These somatic *SETBP1* mutations were associated with poor prognoses in these patients. dPCR shows somatic loss of one wild-type *NF1* (*neurofibromin-1*) allele within a malignant melanoma of a patient with neurofibromatosis type I [30]. Congenital hemangiomas are rare vascular tumors that develop prenatally as a result of somatic mutations; ddPCR can detect subclonal mutations in *GNAQ* (*GNAQ(E209L)* and *GNAQ(E209P)*) and *GNA11* (*G protein subunit* α *11*, $G_{11}\alpha$; *GNA11(E209L)*) in these tumors [31]. Within tumors, dPCR is a powerful tool to detect subclonal mutations affecting prognosis as well as loss of heterozygosity.

2.2. Noninvasive detection of genetic alterations in plasma

Cell-free fetal DNA (cffDNA) containing small fragments of fetal genomic DNA represent a small proportion (about 10%) of the DNA present in maternal plasma [32]. dPCR can reliably detected very small quantities of cffDNA in maternal plasma making noninvasive prenatal diagnosis feasible [12]. Noninvasive prenatal diagnosis of genetic disorders can be accomplished by comparing either the copy number of a target chromosome against that of a reference chromosome (relative chromosome dosage; RCD) or the amount of the mutant allele relative to the wild-type allele (relative mutation dosage; RMD). RCD can detect fetal trisomy 21 in maternal plasma [33]. RCD can also determine the sex of the fetus from cffDNA [34]. As a proof of concept, RMD detects single nucleotide changes or small deletions in *HBB* (*β-globin*; HbE, (*HBB(E26K)*) and CD41/42 allele (a 4 nucleotide deletion at codons 41 and 42)) associated with β-thalassemia [35]. RMD can be used to prenatally diagnose hemophilia (*F8*, *coagulation factor VIII*; *F9*, *coagulation factor IX*), sickle cell anemia (HbS, *HBB(E6V)*) and cystic fibrosis (*CFTRΔF508*; loss of phenylalanine 508 in *cystic fibrosis transmembrane conductance regulator* (*CFTR*)) [36–38]. Using massively parallel deep sequencing, cffDNA can be scanned globally for paternally inherited single nucleotide polymorphisms (SNPs) by relative haplotype dosage (RHDO) [39]. Lam and colleagues identify mutations in *HBB* that are associated with β-thalassemia via a targeted RHDO approach [40].