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Application of digital PCR with chip-in-a-tube format to analyze *Adenomatous polyposis coli* (*APC*) somatic mosaicism

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ABSTRACT

Background: Over the past decade, digital PCR (dPCR) technology has significantly improved, and its application in clinical diagnostics is rapidly advancing. The Clarity™ dPCR platform, which employs the chip-in-a-tube format to broaden its range of applications, has been used to determine gene copy number. However, detection of mutations in human samples, the most demanding task in clinical practice, has not yet been reported using this platform.

Methods: The Clarity™ dPCR platform was used to detect somatic *Adenomatous polyposis coli* mosaicism c.834 + 2 T > C, which had been identified using next-generation sequencing (NGS) technology in a patient with sporadic familial adenomatous polyposis. In addition, we were able to determine the size of the dPCR product.

Results: The mutation rate in the peripheral blood of the patient calculated using the dPCR platform was 13.2%. This was similar to that determined using NGS (12.7%). In contrast, in healthy donors, the mutation rate was < 0.1%. Furthermore, it was confirmed that the dPCR product size was consistent with its theoretical value.

Conclusion: Our results show that the dPCR platform with the chip-in-a-tube format is suitable for the analysis of mosaicism and enables the validation of the dPCR product size.

1. Introduction

Digital PCR (dPCR) enables the absolute quantitation of DNA in a reaction mixture and is considered to be one of the most effective molecular diagnostic tools [1]. The basic concept of dPCR was first described in 1992 [2], and the term "digital PCR" was first used in 1999 [3]. A situation wherein DNA templates are absent or present as a single molecule per partition can be created by diluting DNA templates and dividing them into partitions. After PCR, the amplified fluorescent signal in each partition is determined as negative or positive, and their proportions are determined. The target copy number is calculated using positive proportion based on Poisson statistics.

Partitioning techniques of commercially available dPCR platforms are roughly categorized into two types: droplet-based and chip-based partitioning. In the former, DNA templates are divided into water-in-oil droplets using microfluidics and proprietary surfactants [4,5]. In the latter, DNA templates are divided into tens of thousands of partitions on a chip using microfluidics [6]. Key features of dPCR systems are their sensitivity, accuracy, productivity, broad utility, usability, and cost. Through optimization of these aspects, dPCR technologies have impacted medical studies, such as detection of copy number alteration, base substitution using liquid biopsy in oncology [7–9], and highly precise virus detection in infectious diseases [10,11]. Therefore, dPCR technologies are being recognized as next-generation molecular diagnostic tools.

In our previous report, we identified a novel somatic *Adenomatous polyposis coli* (*APC*) mosaicism corresponding to a splice donor site in a patient with sporadic familial adenomatous polyposis using next-generation sequencing (NGS) technology [12]. The aim of this study was to investigate whether the latest dPCR platform in which DNA templates are distributed by capillary action into partitions on chips built into PCR tubes [13] is useful for the detection of somatic *APC* mosaicism.

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2. Materials and methods

2.1. Patients

A 40-year-old male (AGFAP001-1) visited the Hamamatsu University Hospital owing to the result of a fecal occult blood examination for colorectal cancer. Upon endoscopic examination, several colorectal adenomatous polyps and fundic gland polyposis were identified [12]. Peripheral blood samples were collected from the proband and his parents (father, AGFAP001-2; mother, AGFAP001-3). Peripheral blood samples from a donor population aged ≥ 60 years were collected in the Iwata City Hospital [14]. The design of this study was approved by the Institutional Review Boards of Hamamatsu University School of Medicine (G-260-4), and written informed consent was obtained from the patient and his parents.

2.2. DNA extraction

Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA, USA). Subsequently, it was subjected to genomic DNA screen tape assay using 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA) to determine the amount of genomic DNA (> 200 bp) and the DNA integrity number (DIN).

2.3. Digital PCR

Reaction mixtures (total volume = 15 μL) were prepared with 1 × PCR Master mix (JN Medsys, Singapore), 1 × JN solution (JN Medsys, Singapore), 10–20 ng of genomic DNA (3 μL), 1 pmol each of Locked Nucleic Acid (LNA) probes modified using HEX™ and FAM™ fluorescent dyes (LNA PrimeTime®, Integrated DNA Technologies, Coralville, IA, USA), and 0.5 pmol each of forward and reverse primers (5′-GGTCAAGGAGTGGGAGAAATC-3′ and 5′-TCTTAGAACCATCTTGCT TCATACT-3′, respectively). Detailed information on LNA probes is shown in Fig. 2A. Melting temperature values of LNA probes were calculated using the supplier tool (http://biophysics.idtdna.com/) under the following conditions: 50 mM Na^+/K^+, 0.80 mM dNTPs, 66.6 nM oligonucleotides, and 3 mM Mg^{2+}. Sample partitioning and fluorescence detection were performed using the Clarity™ dPCR system (JN Medsys) [13]. The reaction mixture was loaded onto a chip with an auto loader, and partitions were sealed using a sealing enhancer and 230 μL of a proprietary sealing fluid. Thermal cycling was performed under the following conditions: 95 °C for 5 min, 42 cycles of 95 °C for 50 s and 58 °C for 90 s, and 70 °C for 5 min. Ramp rate was set to 1 °C/s (Life Eco, Bioer Technology, Hangzhou, China). Fluorescent signals of HEX™ and FAM™ were detected using the Clarity™ reader, and the obtained data were analyzed using the Clarity™ software (ver. 2.0), which determines the DNA copy number according to Poisson statistics. The mean of triplicate assays was calculated in each experiment; the final mean value and relative standard deviation (RSD) was obtained from three independent experiments (Table 1). RSD was expressed using the following formula: standard deviation/mean. Mutation rate (%) was calculated using the following formula: $C_{mut} / (C_{ref} + C_{mut}) \times 100$, where C_{ref} and C_{mut} are copy numbers of the reference and mutant alleles, respectively. For the calculation of a detection limit, C_{ref} and C_{mut} were measured using a dilution series of AGFAP001-1 genomic DNA (0.11–0.45 ng) mixed with AGFAP001-2 genomic DNA (10.3 ng) (Fig. 3). The assays were repeated 20 times. The maximally diluted point at which the mean − 3 × standard deviation (SD) of C_{mut} was greater than the mean + 3 × SD of C_{mut} for AGFAP001-2 was determined as the detection limit of mutation rate (%).

2.4. Collection of PCR products

To collect digital PCR products from the chips built in PCR tubes, sealing fluid was removed from the tube, and 100 μL of TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) was added. After vigorous vortexing for 30 s, the solution was transferred to another tube and concentrated by the ethanol precipitation method. To assess PCR product size, one chip equivalent of concentrated solution was subjected to the High Sensitivity D1000 screen tape assay (Agilent Technologies).

3. Results

3.1. Digital PCR with the chip-in-a-tube format

To generate robust data, it is essential to fully understand the nature of the technical platform used. In the Clarity™ dPCR platform, high density partitions (> 10,000) are crafted on a chip built into a 0.2-mL 8-strip PCR tube (Fig. 1A, I) [13]. After preparing the reaction mixture, it was loaded onto the chip using an autoloader device (Fig. 1A, II). To avoid cross-contamination between partitions, the chips were sealed using a sealing enhancer and proprietary sealing fluid (Fig. 1A, III, and IV). Eight chip dPCR reactions (8-tube PCR strip) were simultaneously conducted following the above described steps (II–IV). The PCR was performed using a conventional thermal cycler (Fig. 1A, V), and fluorescent signals were detected using the Clarity™ reader (Fig. 1A, VI). The procedure took < 4 h in total. Fluorescent signals on the chip were imaged as shown in Fig. 1B, wherein positive and negative partitions are displayed as yellow and blue dots, respectively. Positive and negative partitions indicate the presence and absence of a target region, respectively. Black background corresponds to the partitions that did not receive any reaction mixture. Therefore, in most cases, the whole image of the fluorescent signals is shaped like the chip. The Clarity™ dPCR platform is adjusted such that at least 10,000 partitions are filled with the reaction mixture. In the present study, 95.5% of the experiments had a frequency of > 10,000 total signal counts per assay (Fig. 1C).

3.2. Dual LNA probe assay by the Clarity™ dPCR platform

The Clarity™ dPCR platform for life science research has been used in four previous studies [13,15–17] that investigated gene copy number. However, mutation detection in human samples has not yet been reported. We have previously identified a novel somatic *APC* mosaicism corresponding to the splice donor site (c.834 + 2 T > C) from a patient with sporadic familial adenomatous polyposis using NGS technology [12]. To investigate whether the Clarity™ dPCR platform can be used to detect a gene mutation in human samples, we used the platform to validate *APC* mosaic mutations. Fluorescent probes containing LNAs, known to bind to complementary target molecules with very high affinity, were designed for the reference (T) and mutation (C) alleles (Fig. 2A) [18]. FAM™ emission is detected when the DNA template with the mutant allele is present in a partition, whereas HEX™ emission is detected in the case of the reference allele. As a result, HEX™ and FAM™ emissions were detected in a large number of partitions in the peripheral blood genomic DNA of the patient when compared with the no-template control (NTC) (Fig. 2B). However, only HEX™ emission was detected from a large number of partitions in the control paternal sample. Positive partitions were distributed throughout the chip indicating that loading and sealing procedures were effective (Fig. 2C). Copy numbers of the reference and mutation sites were determined on the basis of Poisson statistics, and the mutation rate was calculated (Table 1). The mutation rate calculated from the patient's genomic DNA was 13.2%, which was similar to that calculated by NGS (12.7%).The mutation rates calculated from the patient's parents and healthy donors genomic DNA were < 0.1%. Detection limit for the *APC* c.834 + 2 T > C mutation site was calculated as 0.298% using diluted