Droplet-based digital PCR for non-invasive prenatal genetic diagnosis of α and β-thalassemia

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Abstract. Non-invasive prenatal diagnosis (NIPD) of isolated cell-free DNA from maternal plasma has been applied to detect monogenic diseases in the fetus. Droplet digital PCR (ddPCR) is a sensitive and quantitative technique for NIPD. In the present study, the development and evaluation of ddPCR-based assays for common α and β-thalassemia variants amongst the Asian population was described; specifically, Southeast Asian (SEA) deletion, HbE, and 41/42 (-CTTT). SEA is caused by deletion of a 20 kb region surrounding the α-globin gene, whilst HbE and 41/42 (-CTTT) are caused by point mutations on the β-globin gene. Cell-free DNA samples from 46 singleton pregnant women who were carriers of these mutations were isolated and quantified using ddPCR with specially designed probes for each target allele. Allelic copy number calculation and likelihood ratio tests were used to classify fetal genotypes. Classification performances were evaluated against ground truth fetal genotypes obtained from conventional amniocentesis. Copy number variation analysis of SEA deletion accurately classified fetal genotypes in 20 out of 22 cases with an area under the receiver operating characteristic curve of 0.98 for detecting Hb Bart's hydrops fetalis. For HbE cases, 10 out of 16 samples were correctly classified, and three were inconclusive. For 41/42 (-CTTT) cases, 2 out of 8 were correctly classified, and four were inconclusive. The correct genotype was not rejected in any inconclusive case and may be resolved with additional ddPCR experiments. These results indicate that ddPCR-based analysis of maternal plasma can become an accurate and effective NIPD for SEA deletion α-(0) thalassemia. Although the performance of ddPCR on HbE and 41/42 (-CTTT) mutations were not sufficient for clinical application, these results may serve as a foundation for future works in this field.

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Introduction

Thalassemia is an autosomal recessive disease that is caused by the reduction in hemoglobin chain synthesis. It is estimated that thalassemia affects 17% of over 330,000 newborns worldwide each year (1). The two major types of thalassemia are α- and β-thalassemia. α-thalassemia is caused by the loss of one or both of the α-globin genes (2). The Southeast Asian deletion (SEA) variant is a large deletion of the α-globin gene region, and is the most common cause of α-thalassemia in the Asian population. Couples carrying the SEA deletion do not show symptoms, but will have a 25% chance of conceiving a baby with Hb Bart's hydrops fetalis and experiencing toxemia during pregnancy (3). β-thalassemia is caused by mutations in β-globin genes, which lead to reduced β-globin chain synthesis (4). Children born with β-thalassemia may show symptoms of anemia and other complications throughout their lives (5). Thailand has a high (30-50%) prevalence of α- and β-thalassemia (6), particularly in the northern and north-eastern regions of the country (7). As thalassemia treatments are costly and time-consuming, the most effective means of limiting thalassemia incidence is to screen for the mutant genes before birth. Towards this goal, prenatal screening for thalassemia is becoming a part of primary health services in the majority of developed countries, particularly in Asia (8). Nonetheless, traditional prenatal diagnoses, such as chorionic villi sampling and amniocentesis, are invasive and may cause infection and miscarriage (9,10).

The discovery of cell-free fetal DNA (11), which is released from placental tissue into the maternal bloodstream, has enabled the development of non-invasive prenatal diagnosis (NIPD) for detecting disease-causing mutations in the fetus (12,13). In recent years, there has been substantial interest in developing NIPD for thalassemia. Reverse transcription-quantitative

PCR (RT-qPCR) using the gap-PCR technique with primers designed to bind at the deletion breakpoints have been used to diagnose common deletions such as $-a^{3.7}$, $-a^{4.2}$, $-^{THAI}$, $-^{MED}$ and $-^{SEA}$ (14,15). Similarly, the PCR-based techniques were also applied to diagnose Hb Bart's hydrops fetalis (16) and other forms of α-thalassemia (17-19). Next-generation sequencing (NGS) is a powerful technique for NIPD, as it has high sensitivity and enables the detection of low abundance cell free-fetal DNA (20-23). Several studies have shown high accuracy of NGS-based genome-wide single-nucleotide polymorphism genotyping and targeted sequencing for diagnosing β-thalassemia (24-26). However, the high costs of NGS-based techniques make them inappropriate in routine clinical practice.

Droplet digital PCR (ddPCR) is a sensitive and quantitative PCR-based technique that can amplify a low initial amount of target DNA molecules and enumerate different PCR products using probe-specific fluorescent signals (27,28). ddPCR has been used to detect circulating tumor DNA and cell-free fetal DNA (29-31). A study on SEA deletion α-(0) thalassemia showed a promising prospect of ddPCR (32). However, it is unclear whether the fetal DNA analyzed came directly from fetal tissues or cell-free fetal DNA in the maternal bloodstream (32). Furthermore, digital PCR-based NIPD could be improved by utilizing a nucleic acid size selection technique to enrich fetal DNA molecules (33).

The present study developed ddPCR-based assays for detecting fetal α- and β-thalassemia mutations in cell-free DNA from maternal plasma. The assays were evaluated on 22 pregnant women carrying SEA deletions, 16 carrying HbE mutations, and 8 carrying 41/42 (-CTTT) mutations.

Materials and methods

Patient recruitment. In the present study 46 singleton pregnant women who were thalassemia carriers [22 cases with SEA deletion, 16 cases with HbE (G>A), and 8 cases with 41/42 (-CTTT)] were recruited at Radjavithi Hospital, Bangkok, Thailand between March and July 2018 with approval from the Institutional Review Board of the hospital. Peripheral blood samples (10 ml) were collected from each participant for subsequent cell-free DNA extraction and ddPCR analysis. The α- and β-thalassemia status of each parent and fetus was determined by Hb typing, complete blood count tests and genotyping by PCR from amniotic fluids. Samples were collected with the approval from the Institutional Review Board of Radjavithi Hospital, Bangkok (approval no. 59194; date of approval 17th November 2016). Written informed consent was obtained from all subjects prior to inclusion in the study. Written informed consent from the parent or legal guardian of all subjects under the age of 18 was also obtained.

Cell-free DNA extraction from blood samples. Maternal blood samples (10 ml) were placed in BCT tubes (Streck™) and centrifuged at 1,600 x g for 10 min at 4˚C. The supernatant was centrifuged again at 16,000 x g for 10 min at 4˚C. The pellet was discarded and the supernatant was stored kept at -20˚C until required for extraction. Cell-free DNA was extracted from plasma (3 ml) using a QIAamp Circulating Nucleic acid kit according to the manufacturer's protocol (Qiagen GmbH).

Amniotic fluid DNA extraction. Amniotic fluids (5 ml) were collected during weeks 15-18 of gestation and centrifuged at 16,000 x g for 10 min at 4˚C. The supernatant was discarded, the pellet was washed twice with 1X PBS (1 ml) and stored at -80˚C in a sterile tube. The extraction step was performed according to the instruction manual of the QIAamp Blood Mini kit (Qiagen GmbH).

ddPCR. A ddPCR assay was developed for determining the copy number of the SEA allele. The HEX probe was designed to bind the genomic region inside the SEA deletion (NG_000006.1:g.26264_45564del19301) (34) whereas the FAM probe was designed to bind the genomic region just outside of the SEA locus (NG_000006.1:g.26140 to 26262), which is expected to be present in both a wild type fetus and a fetus with an SEA deletion (Table SI). For detecting HbE (G>A) and 41/42 (-CTTT), a rare mutation assay with specific probes designed to target the mutant and wild type amplicons was developed (Table SI).

ddPCR experiments were performed according to the manufacturer's protocol (Bio-Rad Laboratories, Inc.) with some modifications as described here. The master mix solution was prepared from 10 µl 2X ddPCR SuperMix for probes (Bio-Rad Laboratories, Inc.), 1 µl 20X prime PCR assay (Bio-Rad Laboratories, Inc.), and either 8 µl DNA (12 µg/ml) from plasma or 1 µl DNA (20 µg/ml) from amniotic fluid. Nuclease free water was added to adjust the final volume to 20 µl. A total of 13,500-15,000 droplets per reaction were created in a DG8 cartridge with 70 µl oil using a QX200 Droplet Generator (Bio-Rad Laboratories, Inc.). Droplets (40 µl) were transferred to a 96-well PCR plate and sealed with aluminium foil using PX1 (Bio-Rad Laboratories, Inc.) for 5 sec at 180˚C. Next the plate was placed in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc.). PCR reactions began with a cycle of 10 min at 95˚C, followed by 40 cycles of 30 sec at 94˚C and 1 min at 54˚C, with an enzyme deactivation step for 10 min at 98˚C. Fluorescent signals from PCR products were measured using a QX200 Droplet Reader (Bio-Rad Laboratories, Inc.) and analyzed using QuantaSoft version 1.7.4 (Bio-Rad Laboratories, Inc.). ddPCR reactions for SEA cases were performed in duplicates. ddPCR reactions for HbE and 41/42 (-CTTT) cases were performed in triplicates.

Estimation of fetal fraction. Fetal fractions were estimated as previously described (27,35). Briefly, *RASSF1A* and *ACTB* alleles in plasma samples were digested with *Bst*U I (Vivantis Technologies) at a ratio of 1 µg DNA to 1 U of enzyme for 1 h at 60˚C. The abundance of *RASSF1A* and *ACTB* alleles before and after digestion were measured using ddPCR analyses. *ACTB* locus, which is always unmethylated, acts as a control of *Bst*U I activity (36). Conversely, as only the fetal *RASSF1A* locus is hypermethylated, the ratio of *RASSF1A* positive droplets before and after *Bst*U I digestion approximate the fractional abundance of fetal DNA content of the sample.

Copy number variation (CNV) analysis to detect SEA deletion. QuantaSoft was used to determine the number of positive and negative droplets and analyze the relative CNV of SEA deletions. The average CNV value from triplicate wells was used to classify the fetal genotype using the following equation:

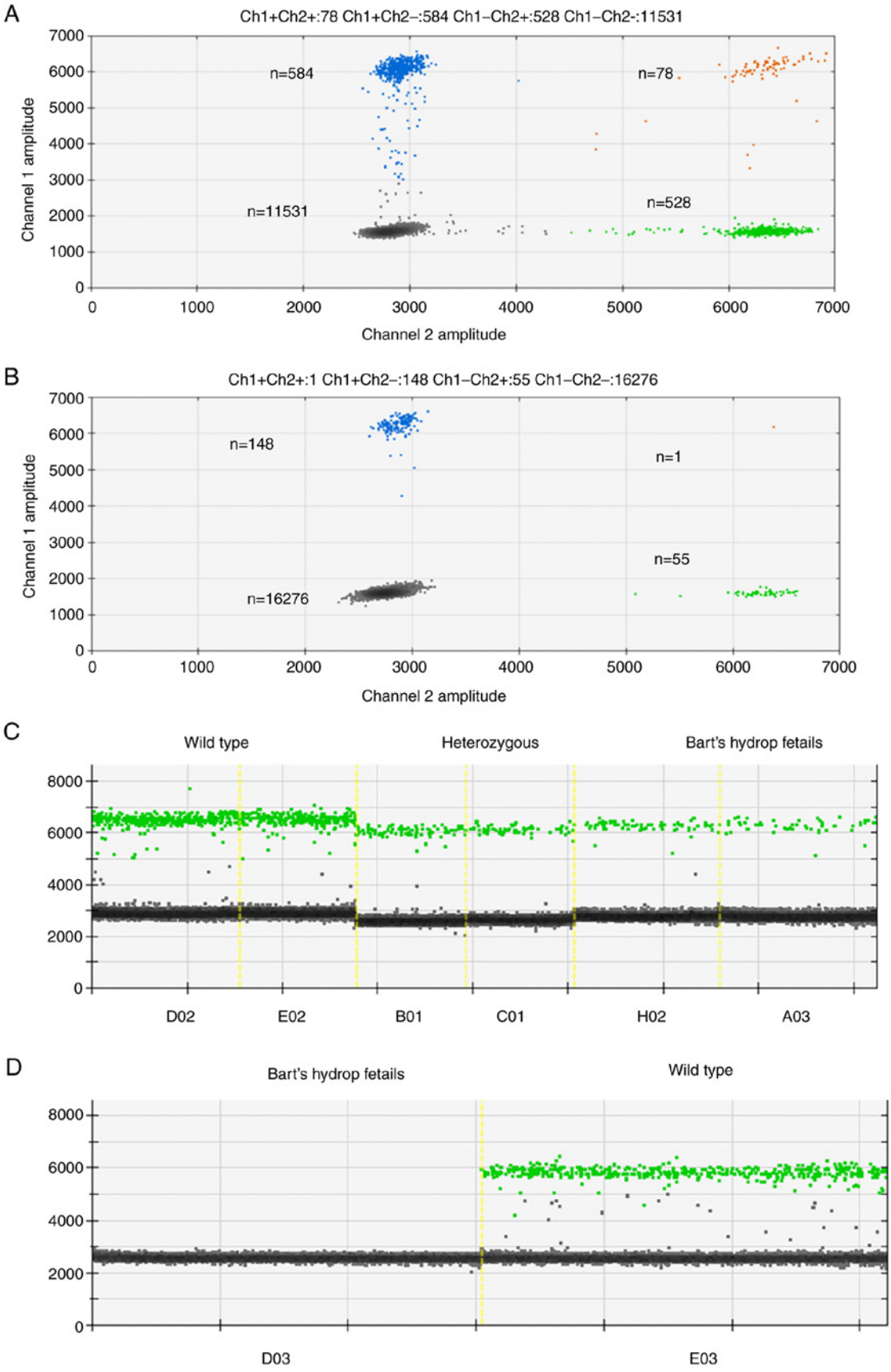

Figure 1. Representative droplet digital PCR results for detecting the SEA deletion. (A and B) show the scatter plots of two fluorescent channels: FAM (blue) which detects the wild type allele and HEX (green) which detects the SEA locus. Negative droplets are shown in black and double-positive droplets are shown in orange. The number of droplets in each group is indicated. (A) Shows a case of a heterozygous SEA deletion and (B) shows a Bart's hydrops fetalis case. The ratio of SEA droplets (HEX) to wild type droplets (FAM) decreased from 1:1 to 1:3 in the Bart's hydrops fetalis case. (C and D) One-dimensional visualizations of HEX (green) which detects the SEA locus. (C) Results from maternal cell-free DNA samples and (D) from amniocentesis samples. No SEA droplets (HEX) were observed in the amniocentesis sample from a Bart's hydrops fetalis case as would be expected. SEA, Southeast Asian; Ch, fluorescent channels; A03, B01, C01, D02, D03, E02, E03 and H02 refer to the position in the 96-well plate.

CNV=(concentration of SEA allele/concentration of wild-type allele) x copy number of wild type allele.

Rare mutation assay to detect HbE and 41/42 (-CTTT) mutations. QuantaSoft was used to determine the number of positive droplets, negative droplets and double-positive droplets. A previously described sequential probability ratio test (SPRT) (33) was adopted to classify fetal HbE and 41/42 (-CTTT) genotypes. Briefly, the total number of DNA molecules in each sample was calculated under the assumption that the number of positive droplets (droplets with DNA materials) followed the Poisson distribution: The probability of observing k positive droplets is $(\lambda^k e^{-\lambda})/k!$, where λ indicates the average number of DNA molecules per a droplet. Hence, by setting k=0, the number of DNA molecules can be calculated as: -ln(fraction of negative droplets) x number of droplets.

Next, the likelihood that the fetus has a specific genotype was calculated under the assumption that the numbers of negative and positive droplets for each allele followed the Binomial distribution. For example, if the fetal genotype is a homozygous mutant, then the fraction of mutant allele would be (1+fetal fraction)/2, whereas the fraction of wild type allele would be (1-fetal fraction)/2. Then, letting N be the total number of droplets, M be the total number of DNA molecules, mt be the number of positive droplets for the mutant allele, wt be the number of positive droplets for the wild type allele, and ff be the estimated fetal fraction, the likelihood of this observation can be expressed as:

$$\binom{N}{wt}\left(1-e^{-\frac{M(1-ff)}{2N}}\right)^{wt}e^{-\frac{M(1-ff)(N-wt)}{2N}}\binom{N}{mt}\left(1-e^{-\frac{M(1+ff)}{2N}}\right)^{mt}e^{-\frac{M(1+ff)(N-mt)}{2N}}$$

Two likelihood ratio tests were performed for each sample: One between the hypotheses that the fetal genotype is homozygous mutant and heterozygous, and another between the hypotheses that the fetal genotype is wild type and heterozygous. The threshold for accepting each test was set at type I and type II errors of 5% according to the SPRT criteria: ±log [(1-0.05)/0.05], which is ~±1.27 (33,37).

Real time PCR for melting curve analysis of SEA deletion from amniocentesis. The fetal genotypes were confirmed by high-resolution melting curve analysis in 10 samples were randomly selected from singleton parents for confirmation. Total reaction volume of 20 μl contained 10 μl Precision melt supermix (Bio-Rad Laboratories, Inc.), 1 μl of each primer and 5 μl of DNA sample. The sequences of the primers were NaI forward, 5'AGAAGCTGAGTGATGGGTCCG-3' and reverse, 5'ACAAACGCCCGTCCGACTCAA-3'; and NaIII reverse 5'-TGGACTTAAGTGATCCTCCTGCCC-3',. The amplification step began at 95˚C for 3 min followed by 40 PCR cycles at 95˚C for 20 sec, 60˚C for 20 sec, and 72˚C for 20 sec. Finally, a high-resolution melting cycle from 85-95˚C at a rate of 0.1˚C per 2 sec was performed (38). Dissociation curve analysis was performed using CFX96 Manager version 1.3 (Bio-Rad Laboratories, Inc.).

Statistical analyses. The sequential probability ratio test (SPRT) technique was used to analyze ddPCR results to identify the most likely fetal genotypes (39). For analysis, 5% type I and type II error thresholds were set. Numerical calculations were performed in Microsoft Excel according to the Poisson and Binomial distribution models described above.

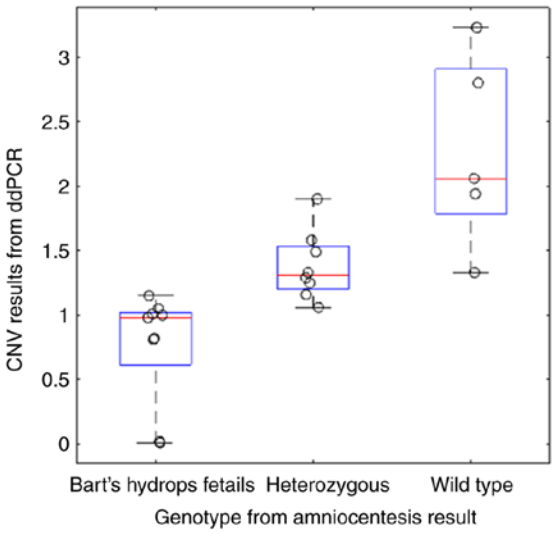

Figure 2. Plasma-derived CNVs distinguish fetal SEA deletion genotype groups. Boxplots show the distribution of CNV results from ddPCR for each fetal genotype group. The genotypes from amniocentesis results were determined from analyses of amniotic fluids at the same gestation age blood sampling. Red bars indicate the medians. Blue boxes indicate the 25-75th percentile. Black whiskers indicate the minimum and maximum values. Black circles indicate CNVs for individual cases. CNV, copy number variations; ddPCR, droplet digital PCR.

Results

Patient characteristics. A total of 46 couples who were at high risk of having children with thalassemia were recruited in the present study. The median age (age range) of pregnant mothers was 27 (15-39) years old. The median gestational age (range) was 18 (17-27) weeks. The median BMI (range) of participants was 21.8 (15.7-32.8). The cohort consisted of 22 (48%) couples with SEA deletion, 16 (35%) couples with an HbE (G>A) mutation, and 8 (17%) couples with an 41/42 (-CTTT) mutation.

Classification performance of fetal SEA genotype. Analysis of ddPCR fluorescent signals showed clear separations between negative and positive droplets as well as lower abundances of the SEA locus in Bart's hydrops fetalis case as would be expected (Fig. 1A and B). The complete absence of positive droplets for the SEA locus in the amniocentesis sample of the Bart's hydrops fetalis case confirmed that the designed probe was highly specific to the SEA locus (Fig. 1C and D). SEA locus copy numbers estimated from cell-free DNA samples significantly differ across wild type, heterozygous and Bart's hydrops fetalis samples (Mann Whitney U test P=0.0124 for the comparison between the wild type and heterozygous cases; P=0.000311 for the comparison between heterozygous and Bart's hydrops fetalis cases). The average copy numbers for the wild type, heterozygous and Bart's hydrops fetalis groups were 2.27, 1.38 and 0.76, respectively (Fig. 2). To ensure the high quality of ground truth fetal genotypes, high resolution melting curve analyses on selected samples was also performed and 100% concordance was obtained (Fig. S1). Most samples exhibited the expected copy numbers of the SEA locus, except

Table I. Detailed parental genotypes and ddPCR test results for α (0) thalassemia (SEA).

Sample ID	Paternal genotype	Maternal genotype	Fetal fraction,%	SEA deletion conc.	Wild type conc.	CNV by ddPCR	Interpretation	Fetal Genotype from conventional amniocentesis
1	(αα/αα)	($—^{SEA}$/αα)	10	141.5	87.5	3.23	Wild type	Wild type
2	($—^{SEA}$/αα)	($—^{SEA}$/αα)	15	15.7	11.2	2.8	Wild type	Wild type
3	($—^{SEA}$/αα)	($—^{SEA}$/αα)	16	11.2	10.9	2.06	Wild type	Wild type
4	($—^{SEA}$/αα)	($—^{SEA}$/αα)	7	23.55	24.3	1.94	Wild type	Wild type
5	($—^{SEA}$/αα)	($—^{SEA}$/αα)	17	57.95	60.95	1.9	Heterozygous	Heterozygous
6	($—^{SEA}$/αα)	($—^{SEA}$/αα)	8	8.45	10.7	1.58	Heterozygous	Heterozygous
7	($—^{SEA}$/αα)	($—^{SEA}$/αα)	9	44.45	59.8	1.49	Heterozygous	Heterozygous
8	(αα/αα)	($—^{SEA}$/αα)	10	53.3	80.3	1.33	Heterozygous	Heterozygous
9	($—^{SEA}$/αα)	($—^{SEA}$/αα)	3	3.15	4.8	1.33	Heterozygous[a]	Wild type
10	($—^{SEA}$/αα)	($—^{SEA}$/αα)	11	7.05	10.9	1.29	Heterozygous	Heterozygous
11	($—^{SEA}$/αα)	($—^{SEA}$/αα)	9	11.5	18.45	1.25	Heterozygous	Heterozygous
12	($—^{SEA}$/αα)	($—^{SEA}$/αα)	8	11.8	20.35	1.16	Heterozygous	Heterozygous
13	($—^{SEA}$/αα)	($—^{SEA}$/αα)	10	8.05	14.05	1.15	Heterozygous[a]	Bart
14	($—^{SEA}$/αα)	($—^{SEA}$/αα)	15	10.75	20.35	1.06	Heterozygous	Heterozygous
15	($—^{SEA}$/αα)	($—^{SEA}$/αα)	11	15.3	29.15	1.05	Bart	Bart
16	($—^{SEA}$/αα)	($—^{SEA}$/αα)	11	173.5	344	1.01	Bart	Bart
17	($—^{SEA}$/αα)	($—^{SEA}$/αα)	10	5.05	10.15	1	Bart	Bart
18	($—^{SEA}$/αα)	($—^{SEA}$/αα)	15	4.85	9.85	0.98	Bart	Bart
19	($—^{SEA}$/αα)	($—^{SEA}$/αα)	15	4.4	10.7	0.82	Bart	Bart
20	($—^{SEA}$/αα)	($—^{SEA}$/αα)	10	8.1	20.1	0.81	Bart	Bart
21	($—^{SEA}$/αα)	($—^{SEA}$/αα)	15	0.22	21.3	0.02	Bart	Bart
22	($—^{SEA}$/αα)	($—^{SEA}$/αα)	10	0.14	27.15	0.01	Bart	Bart

[a]Misclassified cases. SEA, Southeast Asian; CNV, copy number variation; ddPCR, droplet digital PCR; αα, α-globin genes.

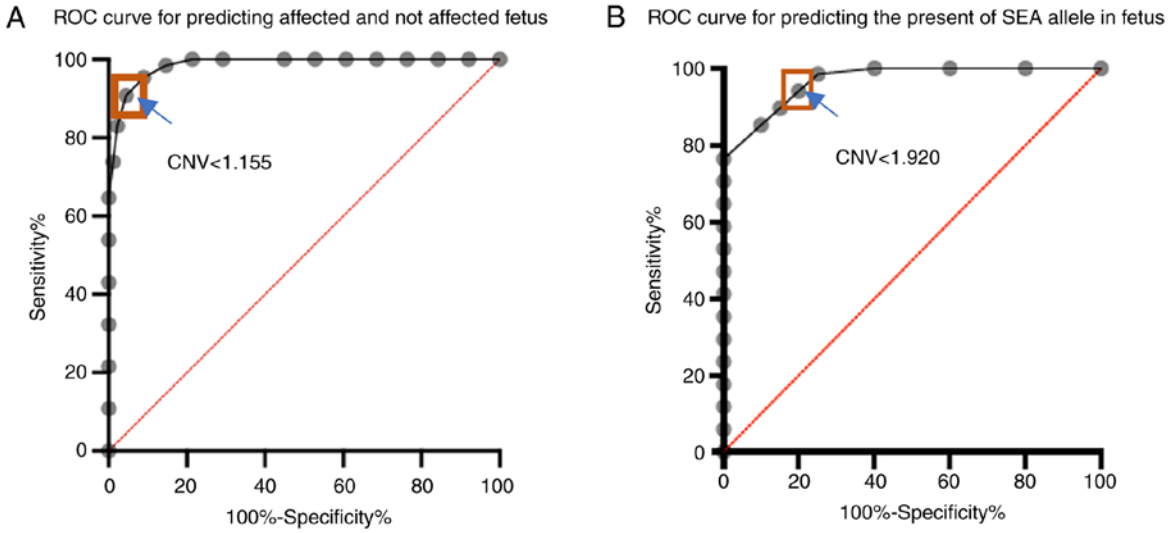

Figure 3. ROC curves of the performance of the droplet digital PCR assays on SEA detection. Orange boxes indicate the best CNV cutoff. (A) ROC curve for distinguishing between affected (Bart's hydrops fetalis) vs. unaffected (wild type and heterozygous). The AUROC curve was 0.98. The optimal CNV cutoff was 1.155. (B) ROC curve for detecting the presence of the SEA allele in the fetus (Bart's hydrops fetalis and heterozygous). The AUROC curve was 0.96. The optimal CNV cutoff was 1.920. ROC, receiver operating characteristic; AUROC, area under the ROC; CNV, copy number variations; SEA, Southeast Asian.

for one wild type case that may have been affected by a very low (3%) fetal fraction (Table I). The optimal copy number cut-off for distinguishing affected (Bart's hydrops fetalis) from unaffected (wild type and heterozygous) fetuses was ~1.155, which yielded 95.38% sensitivity [95% confidence interval (CI): 87.29-98.74] and 91.01% specificity (95% CI: 83.25-95.37) (Fig. 3A). The area under the receiver operating characteristic (AUROC) curve was 0.98. The optimal copy number cut-off for distinguishing the presence of SEA deletion in the fetus (Bart's hydrops fetalis and heterozygous) was 1.920, which yielded 98.53% sensitivity (95% CI: 92.13-99.92) and 75% specificity (95% CI: 53.13-88.81) (Fig. 3B). The AUROC curve in this scenario was 0.96.

Amniocentesis results from high resolution melting curve analysis. Amniocentesis DNA samples were collected from 10 randomly selected singleton parents to confirm the SEA genotypes. Amongst the 10 samples, 4 were diagnosed as Bart's hydrops fetalis, 5 were from normal fetuses and 1 was from a heterozygous fetus. The median maternal age was 27 (15-36) years old. The median gestational age was 18 (17-19) weeks. Dissociation curve analysis showed that the melting temperature for the wild-type fetuses was 91.5±0.15˚C, whereas the melting temperature for Bart's hydrops fetalis was 87.8±0.02˚C. Heterozygous fetuses showed peaks at the melting temperatures of both the wild-type and Bart's hydrops fetalis as expected.

Classification performance of fetal HbE (G>A) and 41/42 (-CTTT) genotype. Cell-free DNA from maternal plasma of 16 HbE carriers and 8 41/42(-CTTT) carriers was next investigated. ddPCR was performed using probes targeting either the wild type or mutant allele. The ground truth genotypes for several samples were confirmed with high-resolution melting curve analyses (Figs. S1 and 2). ddPCR results were then analyzed based on a published sequential probability ratio test method (33). At type I and type II errors of 5%, out of 16 HbE

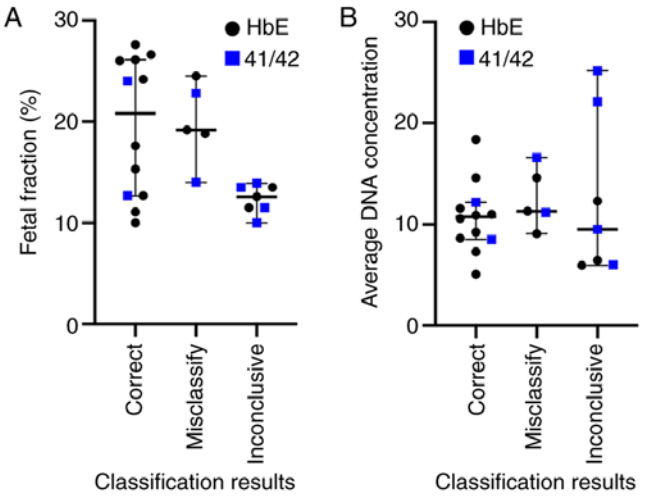

Figure 4. Low fetal fractions leads to inconclusive HbE and 41/42 (-CTTT) detections. Scatter plots showing the distribution of (A) fetal fractions and (B) average DNA concentration across 24 triplicate droplet digital PCR experiments for HbE and 41/42 (-CTTT) cases. The distributions for correctly classified, misclassified and inconclusive cases are shown. Black dots and blue boxes indicate data from HbE and 41/42 (-CTTT), respectively.

cases, the results for 3 patients were inconclusive, and 10 out of the remaining 13 cases were correctly classified. For 41/42 (-CTTT), the results for 4 out of 8 cases were inconclusive, and 2 out of the remaining 4 cases were correctly classified (Tables II and III). It should be noted that all inconclusive results still retain the correct genotypes and may be correctly classified if more ddPCR reactions are performed.

Typical causes of poor ddPCR assay performances include a low fetal fraction, low amount of amplifiable DNA, and poor binding between ddPCR probes and target alleles, all of which would hinder confident determination of the fetal genotype. Although a low fetal fraction is linked to inconclusive results (Fig. 4A, lower fetal fractions among inconclusive cases; Mann-Whitney U test P=0.0173), it cannot

Table II. Detailed parental genotypes and droplet digital PCR test results for HbE β-thalassemia.

Sample ID	Total droplet	Wild type droplet	Mutant droplet	Double-positive droplet	Average concentration	Fetal fraction, %	Mutant/mutant to mutant/wild type LLR	P<0.05	Wild type/wild type to mutant/wild type LLR	P<0.05	Interpretation	Fetal genotype from conventional amniocentesis
1	38,941	396	234	205	10.9	10	-8.5	Yes	5.7	Yes	Wild type	Wild type
2	41,227	315	321	117	9.1	24.5	-7.9	Yes	-9.2	Yes	Heterozygous[a]	Wild type
3	44,109	243	141	56	14.6	26.1	-17.8	Yes	6	Yes	Wild type	Wild type
4	38,124	130	89	143	7.3	12.7	-3	Yes	1.5	Yes	Wild type	Wild type
5	37,237	510	389	134	10.6	11.1	-8.3	Yes	3.5	Yes	Wild type	Wild type
6	42,706	548	102	84	9.23	15.3	-33.4	Yes	26.7	Yes	Wild type	Wild type
7	43,713	75	66	61	12.3	11.5	-0.9	No	0	No	Inconclusive	Heterozygous
8	42,939	407	405	401	11.6	26.6	-13.2	Yes	-12.7	Yes	Heterozygous	Heterozygous
9	37,535	332	348	338	11	26	-8.5	Yes	-12.2	Yes	Heterozygous	Heterozygous
10	37,001	175	181	178	6.46	12.6	-0.9	No	-1.6	Yes	Mutant or Heterozygous	Heterozygous
11	37,322	234	243	225	8.64	27.6	-7.1	Yes	-9.3	Yes	Heterozygous	Heterozygous
12	42,845	863	191	186	14.62	19.2	-65.8	Yes	48.6	Yes	Wild type[a]	Heterozygous
13	37,355	145	150	144	5.1	24.2	-3.3	Yes	-4.4	Yes	Heterozygous	Heterozygous
14	39,795	713	761	713	18.4	17.6	-6.3	Yes	-13.8	Yes	Heterozygous	Heterozygous
15	37,309	94	15	91	11.32	18.8	-7.4	Yes	5.7	Yes	Wild type[a]	Heterozygous
16	38,696	113	91	76	5.97	13.5	-2.1	Yes	0.5	No	Wild type or heterozygous	Heterozygous

[a]Misclassified cases. LLR, Likelihood ratio.

Table III. Detailed parental genotypes and droplet digital PCR test results for 41/42 (-CTTT) β thalassemia.

Sample ID	Total droplet	Wild type droplet	Mutant droplet	Double-positive droplet	Average concentration	Fetal fraction, %	Mutant/mutant to mutant/wild type LLR	P<0.05	Wild type/wild type to mutant/wild type LLR	P<0.05	Interpretation	Fetal genotype from conventional amniocentesis
1	38,196	139	205	137	16.6	24	2.6	Yes	-11.5	Yes	Mutant[a]	Wild type
2	33,835	357	17	15	8.5	14	-22.5	Yes	19.3	Yes	Wild type	Wild type
3	29,144	505	440	108	22.1	13.5	-7.7	Yes	0.1	No	Wild type or heterozygous	Heterozygous
4	31,830	197	159	7	9.5	13.9	-3.8	Yes	0.8	No	Wild type or heterozygous	Heterozygous
5	33,596	549	631	37	25.2	12.7	0.4	No	-8.8	Yes	Mutant or heterozygous	Heterozygous
6	32,369	254	277	0	12.2	22.8	-3.8	Yes	-8.5	Yes	Heterozygous	Heterozygous
7	33,437	157	123	12	6.02	10	-2.1	Yes	0.9	No	Wild type or heterozygous	Heterozygous
8	37,846	571	35	23	12.2	11.5	-28.8	Yes	25.3	Yes	Wild type[a]	Heterozygous

[a]Misclassified cases. LLR, Likelihood ratio.

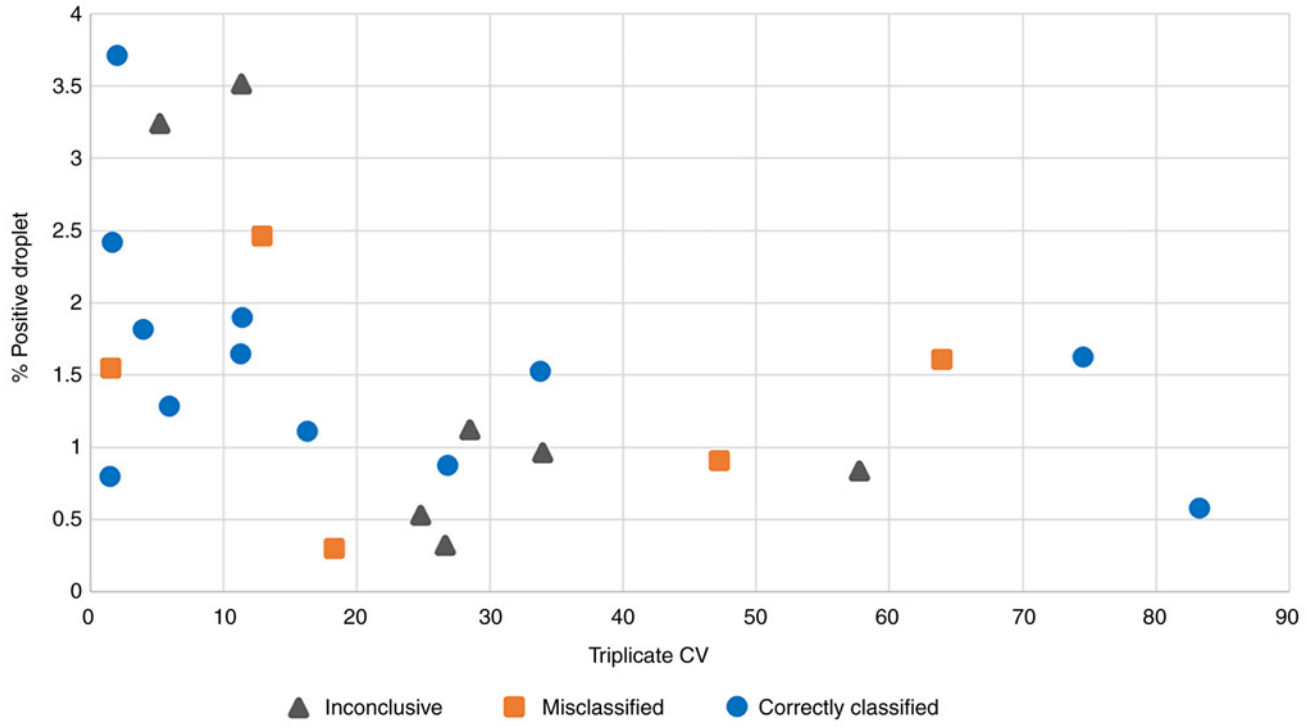

Figure 5. Coefficients of variation and percentage of positive droplets are good indicators of genotyping classification quality. Scatter plot of the coefficient of variations and the average percentages of positive droplets calculated from triplicate droplet digital PCR experiments of 24 HbE and 41/42 (-CTTT) cases.

explain misclassifications. The 12-25% fetal fractions amongst misclassified samples are high and should be adequate for NIPD applications. There is also no apparent difference in the amount of amplified DNA between correctly classified and misclassified cases (Fig. 4B). Finally, to test whether poor probe binding could explain the observed results, the coefficient of variation (CV) of the mutant-to-wild type droplet ratios from the triplicate ddPCR experiments and the percentage of positive droplets of each patient sample to the classification result were compared. The rationale here was that poor probe binding should result in a low percentage of positive droplets and a high variability across replicate experiments. This revealed a clear association between the variability of triplicate ddPCR experiments and the genotype classification accuracy (Fig. 5). Amongst the 11 samples with <15% CV, seven were correctly classified, two were misclassified, and two were inconclusive. Conversely, amongst the remaining 13 samples with 16-83% CV, only five were correctly classified, three were misclassified and five were inconclusive. The analysis also showed a borderline significant correlation between the CV of triplicate ddPCR results and the fraction of total positive droplets (Spearman Rank correlation=-0.4417 with permutation test P=0.0306).

Discussion

The growing interest in non-invasive prenatal testing has driven the development of various assays for diagnosing monogenic diseases from cell-free fetal DNA (24-26,30,31,33). One of the main challenges is the interference from high maternal DNA background that can overwhelm the signal from circulating fetal DNA fragments (40). Prior studies have focused on autosomal dominant monogenic diseases, such as achondroplasia, myotonic dystrophy and some forms of β-thalassemia, where the mutated fetal allele could be clearly distinguished from wild type maternal DNA (41-44). However, this consideration does not apply to autosomal recessive diseases and several other situations that involve maternal inherited alleles (44). Notably, the digital relative mutation dosage and SPRT methods have improved the confidence of assessment of the fetal genotype in the presence of the background maternal DNA (33,45). These techniques enabled interpretation of fetal status and detection of small allelic imbalances when the mother carries the pathologic allele.

ddPCR is a high-sensitivity and high-specificity technique that improves upon conventional PCR and has been utilized in prenatal diagnoses of thalassemia (29,46). Early studies of SEA deletion α-(0) thalassemia using RT-qPCR demonstrated high sensitivities for Bart's hydrops fetalis detection. However, these methods could not clearly distinguish between heterozygous and wild type fetuses (37,46,47). A study using the ddPCR technique on fetal genomic DNA samples provided the first accurate classification of fetal SEA genotype, suggesting that ddPCR is suitable for non-invasive prenatal diagnosis of SEA deletion (32). Nonetheless, no prior study had applied ddPCR to cell-free DNA without sample preprocessing, such as enrichment of fetal DNA by size selection, to the best of our knowledge.

The present study is the first to showcase the application of ddPCR to identify the copy number of SEA deletion in unprocessed cell-free DNA obtained from maternal plasma, thereby eliminating the need for invasive fetal DNA collection and sample preprocessing. The SEA CNV assay accurately classified 20 out of 22 samples, and detected Hb Bart's hydrops

fetalis with 95% sensitivity and 91% specificity. A possible explanation for the misclassified cases may be a low fetal fraction (3% in one case) and instability of the cell-free fetal DNA in the SEA region (16). These findings demonstrate the readiness of ddPCR-based assay for NIPD of SEA deletion α-(0) thalassemia.

Although the current performances of ddPCR for HbE and 41/42 (-CTTT) point mutations still do not meet the requirements for clinical use, the results for HbE are promising. Out of 16 samples, 10 were correctly classified, and three may be correctly classified with additional ddPCR experiments, whilst three misclassifications were made. The association between inaccurate classifications and high variability amongst triplicate ddPCR experiments and a low percentage of positive droplets suggests that these errors were likely caused by poor binding between ddPCR probes and target alleles. Some remedies for this issue include designing additional probes for targeting mutated DNA molecules, pre-amplifying the sample, and performing size selection to enrich fetal DNA fragments (48,49).

The present study developed ddPCR-based assays for identifying the fetal thalassemia status from unprocessed cell-free DNA extracted from maternal plasma. Although the 20 kb deletions of the SEA region can be reliably detected, the detection accuracies for HbE and 41/42 (-CTTT) point mutations were too low for recommendation of this technique for clinical use. The critical limitation of this work is the small number of HbE and 41/42 (-CTTT) samples, especially the complete lack of cases with a homozygous mutant allele. Further studies with a larger sample size and additional ddPCR probe designs are required to improve and validate the utility of these assays.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SPO, SJ managed the project. SPO, WQ, SC and KS conceived and designed the study. SJ recruited patients and collected data. WN, SPR and KT collected samples and extracted cell-free DNA. KS performed the experiments. SS and KS analyzed the data and interpreted the results. KS, SPO and SS wrote and revised the manuscript. SS, SJ and SPO supervised the project. All authors provided critical comments and contributed to the revision of the manuscript. KS and SPO confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Samples were collected with the approval from the Institutional Review Board of Radjavithi Hospital, Bangkok (approval no. 59194 date of approval, 17th November 2016). Written informed consent was obtained from all subjects prior to inclusion in the study. Written informed consent from the parents or legal guardians of all subjects under the age of 18 was also obtained.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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