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Diagnosis of α-thalassemia Using Droplet Digital PCR

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Abstract Most α-thalassemia occurs due to a large deletion in the *α-globin* gene. Common α-thalassemia with known mutations, such as Southeast Asian (SEA) type is readily diagnosed by Gap-PCR. However, there are many unknown mutations in α-thalassemia that need dosage or copy number assessment of the *α-globin* gene for diagnosis. Unlike β-thalassemia, a quantitative or real-time PCR approach for gene dosage study is not available for α-thalassemia. In real-time PCR, the gene needs to be amplified by two-fold at each cycle for dosage studies, which does not seem to be the case for the *α-globin* gene. The droplet digital PCR is not affected by amplification efficiency, and accurate quantification or copy number determination (copy/μg DNA) can be obtained. Here, we evaluated droplet digital PCR for detecting *α-globin* gene deletions. We analyzed DNA from 292 blood samples, including 62 normal samples, 35 heterozygous -$\alpha^{3.7}$, 19 homozygous -$\alpha^{3.7}$, 83 SEA type, 17 Filipino (FIL) type, 23 hemoglobin H disease (-$\alpha^{3.7}$/SEA or FIL), 4 α^{CS}/SEA and 49 non-SEA/FIL type α-thalassemia. Results of all α-thalassemia cases conformed to predicted values of copy numbers, except for six non-SEA/FIL α-thalassemias that had no deletion of the *α-globin* gene, but had deletions in a region containing multispecies conserved sequence R2 (DNase I hypersensitive sites 40) that is located 40 kb upstream of the *ζ-globin* gene and associates with the regulation of the *α-globin* gene. The α^{CS} allele or α^{T} has no deletion, and is diagnosed by sequencing. Thus, we show that digital droplet PCR gives accurate copy number of the *α-globin* gene, and is a reliable tool for determination of α-thalassemia.

Key words: Droplet Digital PCR (ddPCR), α-thalassemia, quntitative PCR, copy number variation

Introduction

Thalassemia is a congenital hemolytic anemia that occurs due to the defective production of either α- or β-globin. Alpha-thalassemia results from the decreased production of α-globin.[1] Two *α-globin* genes are present adjacent to each other in *cis* on chromosome 16p13.3 (5'-α_2-α_1-). Thus, four *α-globin* genes are present (αα/αα) in a diploid. Far upstream of these *α-globin* genes on chromosome 16 is located a regulator called multispecies conserved sequence R2 (MCSR2) or hypersensitive sites 40 (HS40) (Fig. 1).[1-4]

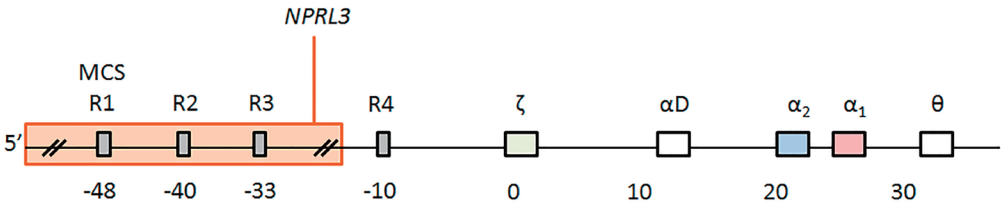

Fig. 1 Organization of *α-globin* gene cluster. The order of genes *ζ*, *αD*, α_2, α_1 and *θ* from 5' is depicted. The *ζ*, α_2 and α_1 are functional genes (solid box). Four upstream highly conserved elements known as multispecies conserved sequences (MCS) R1, R2, R3 and R4 or *NPRL3* gene composed of R1, R2, and R3 are associated with expression of *α-globin* gene cluster. Among them, especially within the fifth intron of *NPRL3* or MCS-R2, there is a 1.4 kb DNA segment (previously called HS-40) that is known to exert a strong enhancer effect for expression of *α-globin* gene.[4]

Deletion of all four *α-globin* genes (--/--) causes hemoglobin Bart hydrops fetalis (Hb Bart) syndrome, which is fatal.[2,5] Hemoglobin H (HbH) disease is caused by the deletion of three genes (--/-α) and has hemolytic involvement, demonstrating mild to severe hemolytic anemia. These two clinically significant forms are categorized as symptomatic thalassemia with hemolytic anemia. Thalassemia minor, or two gene deletion (--/αα, -α/-α) shows no hemolysis but inherent microcytosis with or without slight anemia. Thalassemia minor may exceptionally cause mild hemolysis on pregnancy and infection.[6,7] Thalassemia minor as well as silent carriers (-α/αα) are considered reservoirs for developing symptomatic α-thalassemia. Thus, correct diagnosis is necessary not only for symptomatic thalassemia, but also for thalassemia minor.[2,8] Thalassemia minor has been relatively rare in Japan, but is increasing in number with increased immigration from Southeast Asia where thalassemia is endemic. Most α-thalassemia is caused by large gene deletions involving the *α-globin* gene. Southeast Asian (SEA) type (--/) and $\alpha^{-3.7}$ type (-α/) thalassemia chromosomes are most prevalent in Japan, and Filipino (FIL) type (--/) follows SEA in frequency. Hemoglobin Constant Spring (α^{CS}) or α142 Term>Gln [TAA>CAA] in α_2 gives rise to α-thalassemia allele (α^{T}) without deletion and is sometimes found among immigrants.[9]

Thalassemia minor is readily detected by clinical data, and is diagnosed using Gap-PCR for SEA and FIL type α-thalassemia, which constitutes 49 and 4% of the chromosomes (--/), respectively, is found in Japan.[10] Nearly half of α-thalassemia minor (--/αα) have unknown deletions. Gap-PCR is not available for the non-SEA/FIL type thalassemia, and requires determination of copy number of the *α-globin* gene.[1,10] Unlike β-thalassemia for which real-time PCR is available for gene dosage analysis, this method is not applicable to α-thalassemia. The real-time PCR theoretically needs strict two-fold amplification at each cycle for correct estimation of gene dosage and often may be incorrect, especially for *α-globin* genes. PCR is inherently affected by contaminating inhibitors and base sequence structures.[11-13] Therefore, we abandoned our efforts with real-time PCR for α-thalassemia detection, and focused on droplet digital PCR (ddPCR), which is not affected by amplification efficiency. Here, we applied ddPCR

to 292 samples, including various types of α-thalassemia (n = 230). In ddPCR, samples containing the PCR reagents are dispersed to numerous droplets, and PCR is conducted in each droplet to amplify a specific target gene. During the PCR, hydrolyzed fluorescent substrate emits fluorescence, which is detected like in flow cytometry for the presence (positive) or absence (negative) of amplification. If this partition follows Poisson distribution, absolute copy number can be determined. Thus, ddPCR is likely to be less affected by PCR inefficiency.

Materials and methods

This work was approved by Yamaguchi University Ethics Review Committee (management number: H25-39) and Gene analysis research (174).

DNA was obtained from EDTA-anticoagulated blood with phenol-chloroform extraction. Blood samples from α-thalassemia patients that were referred to our laboratory from entire Japan for thalassemia testing, had been diagnosed as α-thalassemia by gene diagnosis. They contained the heterozygote for $-\alpha^{3.7}$ type (n = 35), homozygote for $-\alpha^{3.7}$ (n = 19), SEA (n = 83), FIL (n = 17), HbH disease ($\alpha^{-3.7}$/SEA or $\alpha^{-3.7}$/-FIL: n = 23) and another type of HbH (α^{CS}/SEA: n = 4). In addition, α-thalassemia minor (--/αα) that was diagnosed by routine laboratory analysis but not confirmed by Gap-PCR for SEA or FIL was designated as non-SEA/FIL α-thalassemia (n = 49) and included in this study. Thus, in total, 292 samples, including normal control (n = 62) were analyzed. All samples were verified not to carry $\alpha\alpha\alpha^{-3.7}$ and $\alpha^{-4.2}$ through Gap-PCR.

Sample DNA (50 ng/μL) was fragmented using *Mse* I (NEB) at 37 ℃ for 1 h and 20-fold diluted hydrolysate was used as template. To each well of twin. tec semi-skirted 96-well plate (Eppendorf), 8 μL of template, 10 μL of 2 × ddPCR Master Mix (Bio-Rad), 0.4 μL of each primer (50 μM), and 0.1 μL of probe (50 μM) for detection of α_1 or α_2 *globin* genes was added, and the total volume was made up to 22 μL with distilled water. Droplets were prepared using Automated Droplet Generator (Bio-Rad). Amplification primers common to both α_1 and α_2 *globin* genes were, forward 5'-GGTTGCGGGAGGTGTAG-3' and reverse 5'-CAGTGGCTTAGGAGCTGTG-3'. Hydrolysis probes specific to α_1 and α_2 *globin* genes are 5'-CTGGGCCCTCGGCC-3' and 5'-CTGGGCCGCACTGA-3', respectively, where underlined is a specific sequence of each gene. They had a fluorescent dye FAM™ (Applied Biosystems) and a quencher IBFQ (Iowa Black® FQ) labelled at 5'- and 3'-termini. *RNase P* was used as a control gene, and its amplification primers were, forward 5'-GATTTGGACCTGCGAGCG-3' and reverse 5'-GCGGCTGTCTCCACAAGT-3' (Fig. 2). The probe for *RNase P* was labeled with VIC fluorescence and MGBNFQ quencher.

After initial activation of DNA polymerase at 95 ℃ for 10 min, PCR was conducted with 40 cycles of 94 ℃ for 30 sec and 60 ℃ for 1 min, followed by stabilization of the droplets at 98 ℃ for 10 min and final hold at 4 ℃. QX200 Droplet Reader (Bio-Rad) was used for measurement and analysis of about 20,000 droplets. Results were evaluated by copy number variation (CNV), or ratio of the copy number

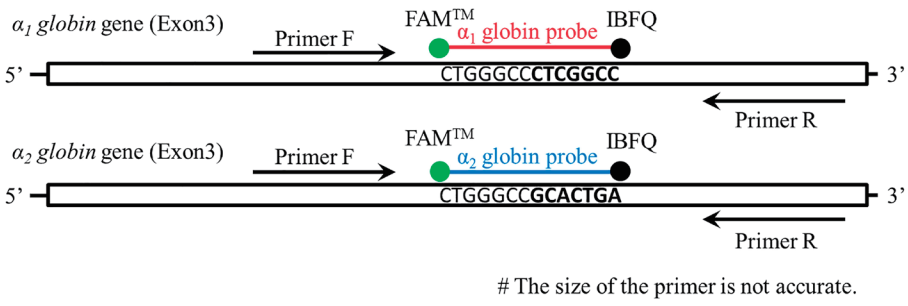

Fig. 2 Position of primers and probes. Primers and probe were made in third exon of each α globin gene. A portion of bold letters is an unique sequence for each α globin gene. The primers are positioned so as to sandwich the probe.

of the target gene and control gene, where 2 is normal (without any deletion), while deletion of either or both *α-globin* genes result in 1 or 0, respectively.

Results

Determination of protocol using normal samples

The α_2 *globin* gene was amplified with the PCR conditions described above with 40 cycles and satisfactorily used to discriminate positive and negative results. Blue and green dots stand for positive and gray dots for negative results (Fig. 3(A)). However, this method did not work well for α_1 *globin* gene (Fig. 3(B)). Thus, the PCR amplification cycles were increased from 40 to 45, which resulted in sufficient amplification of the α_1 *globin* gene, enabling satisfactory discrimination of positive and negative results (Fig. 3(C)). Thus, the PCR conditions for amplification of the α_1 *globin* gene were modified to 95 ℃ for 10 min, 45 cycles of 94 ℃ for 30 sec, and 60 ℃ for 1 min, followed by stabilization of the droplets at 98 ℃ for 10 min and final hold at 4 ℃.

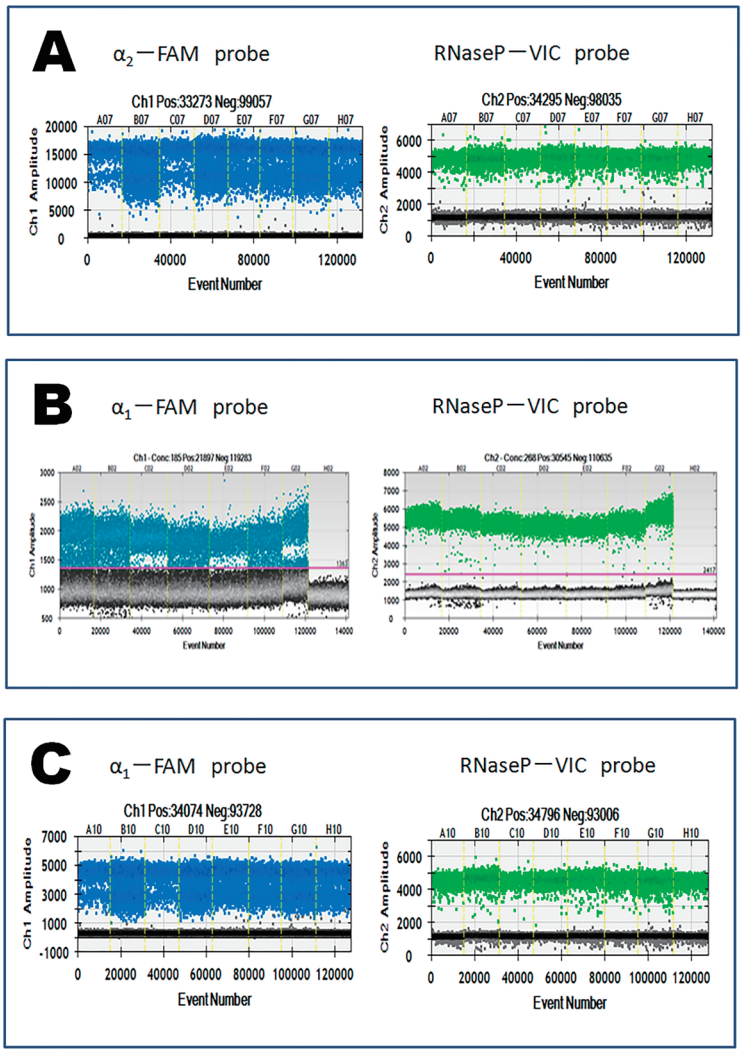

Fig. 3 ddPCR in normal subjects. Ordinate: fluorescent intensity, abscissa: number of events (or droplets). (A) α_2 *globin* gene, 40 PCR cycles. (B) α_1 *globin* gene, 40 PCR cycles. (C) α1 globin gene, 45 PCR cycles. The positive and negative results of ddPCR were discriminated in 40 and 45 cycles for α_2 *globin* and α_1 *globin* gene, respectively.
Positivity and negativity are clearly divided in the figures A and C, while they are obscure in the figure B where cut-off line is drawn by hand.

We evaluated the CNV and found that ddPCR accurately determines absolute copy number of the target gene, but the results for *α-globin* genes varied considerably (Fig. 4(A)), which could be ascribed to inaccurate concentration of DNA (50 ng/μL) adjusted spectrophotometrically. Thus, evaluation of the CNV was modified as relative copy number of α_1 *globin* gene adjusted by that of a reference *RNase P* gene (Fig. 4(B)).

Measurement of CNV of normal individuals and patients with α-thalassemia

The genotype of normal control is αα/αα, and expected CNV for α_2 and α_1 is 2 and 2, respectively, or (α_2, α_1) = (2, 2). Average CNV values of the normal control samples were (α_2, α_1) = (1.94, 1.95), with α_2 CNV showing a minimum of 1.79, maximum of 2.22 for a reference range of 1.76-2.12 and α_1 showing a minimum of 1.77, maximum of 2.16 for a reference range of 1.80-2.11. Thus, the actual values were close to the predicted values. The heterozygous alleles for $-\alpha^{3.7}$ and $-\alpha^{4.2}$ or (-α/αα) are detected as deletion of α2 depending on the position of our probe, and predicted CNV is (α_2, α_1) = (1, 2). Actual average CNV was (α_2, α_1) = (0.97, 1.94) with CNV of α_2 at a minimum of 0.86, maximum of 1.07 for reference range of 0.87-1.07 and α_1 at a minimum of 1.75, maximum of 2.07 for the reference range 1.79-2.06.

Likewise, the expected CNVs for homozygous alleles of $-\alpha^{3.7}$ and $-\alpha^{4.2}$ (-α/-α) are (α_2, α_1) = (0, 2), and those for heterozygous SEA and FIL (--/αα) are (α_2, α_1) = (1, 1). HbH disease that is a combination of these (--/-α) gives the CNV of (α_2, α_1) = (0, 1). However, HbH (α^{CS}/SEA) or (α^{T}α/--) is (α_2, α_1) = (1, 1), because α^{CS} has no deletion but a point mutation at the termination codon. Thus, actual CNV of both types conformed to the expected CNV (Table 1).

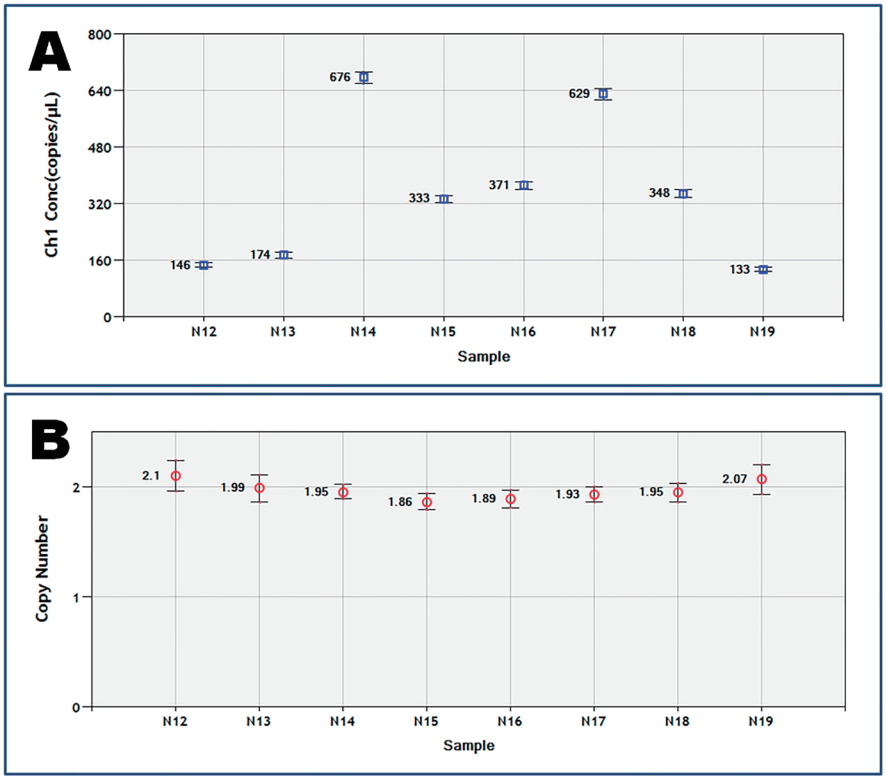

Fig. 4 Evaluation of CNV. (A) copy number of *α-globin* gene per μL (ordinate) for each normal sample (N12-N19) (abscissa). Evaluation was infeasible, because results varied by sample. DNA concentration was measured spectrophotometrically and adjusted to 50 ng/μL. (B) CNV value where the copy number of a target gene is divided by that of control gene (*RNase P* gene) (ordinate) for each sample. This allowed evaluation using CNV of 2 for no deletion.

Table 1 (A) The predicted CNV value and the measurement result. (B) Reference Range.

A

Type	n	Predicted CNV value		CNV value mean	
		α_2	α_1	α_2	α_1
Normal(αα/αα)	62	2	2	1.94	1.95
$-\alpha^{3.7or4.2}$Hetero(-α/αα)	35	1	2	0.97	1.94
$-\alpha^{3.7\text{ or }4.2}$Homo(-α/-α)	19	0	2	0.01	1.96
SEA(--/αα)	83	1	1	0.98	0.98
FIL(--/αα)	17	1	1	0.98	0.99
HbH(-3.7/SEA・FIL) (-α/--)	23	0	1	0.00	0.96
HbH(α^{CS}/SEA) (α^{T}α/--)	4	1	1	1.04	1.03
Non SEA・FIL(--/αα)#1	43	1	1	0.97	0.97
Non SEA・FIL(αα/αα)#2	6	2	2	1.92	1.95

B

Type	n	α_2			α_1		
		Min	Max	Reference Range	Min	Max	Reference Range
Normal(αα/αα)	62	1.79	2.22	1.76~2.12	1.77	2.16	1.80~2.11
$-\alpha^{3.7or4.2}$Hetero(-α/αα)	35	0.86	1.07	0.87~1.07	1.75	2.07	1.79~2.06
$-\alpha^{3.7\text{ or }4.2}$Homo(-α/-α)	19	0.00	0.04	0.00~0.03	1.85	2.22	1.74~2.18
SEA(--/αα)	83	0.75	1.23	0.84~1.11	0.82	1.25	0.83~1.12
FIL(--/αα)	17	0.9	1.29	0.82~1.19	0.89	1.19	0.85~1.15
HbH(-3.7/SEA・FIL) (-α/--)	23	0.01	0.08	0.00~0.04	0.93	1.00	0.84~1.09
HbH(α^{CS}/SEA) (α^{T}α/--)	4	1.01	1.11	0.94~1.14	0.96	1.12	0.89~1.16
Non SEA・FIL(--/αα)#1	43	0.88	1.12	0.87~1.07	0.81	1.05	0.86~1.05
Non SEA・FIL(αα/αα)#2	6	1.85	1.97	1.82~2.01	1.78	2.04	1.78~2.12

#1 Samples that have deletion at α1 and α2 globin genes
#2 Samples that don't have deletion at α1 and α2 globin genes, but have at MCRS2.

Non-SEA/FIL type α-thalassemia patients were suspected by hematological and biochemical data as (--/αα), but they had neither SEA nor FIL allele by Gap-PCR. Their expected CNV was (α_2, α_1) = (1, 1), whereas the actual value was (0.97, 0.97) with α_2 having minimum of 0.88, maximum of 2.04 for reference range of 0.86-1.07 and α1 having minimum of 0.73, maximum of 2.04 and reference range of 0.86-1.05. Among these, six samples gave (α_2, α_1) = (2, 2), indicating that they had no deletion at *α-globin* genes. Further analysis by real-time PCR (not presented) showed that all six of them had deleted MCSR2, a regulator of the *α-globin* gene, which validated their α-thalassemia phenotype. The reason why the maximum CNV for (--/αα) was 2.04 is that these six samples with deletion at MCSR2 but not at *α-globin* genes, were included together in the analysis for the group of non-SEA/FIL type α-thalassemia.

These results indicate that ddPCR gives accurate copy number of the α_2 and α_1 globin genes in α-thalassemia patients.

Discussion

α-Thalassemia minor is similar to iron deficiency anemia, because both involve microcytic anemia, and sometimes, unnecessary iron supplementation may be prescribed for α-thalassemia. In addition, Hb Bart (--/--) that emerges when both parents are α-thalassemia minor was seen in Japan.[14]

Thus, accurate and rapid gene diagnosis of α-thalassemia is mandatory.

Dosage measurement is commonly performed using real-time PCR, which was successful for *β-globin*, but not *α-globin* gene. In real-time PCR analysis, the amplification products need to be exactly doubled in a single PCR cycle to measure dosage against the standard curve. However, the amplification of *α-globin* gene is not doubled, probably because neighboring α_2 and α_1 *globin* genes have high homology and high GC content. In fact, fluorescence intensity of each droplet in ddPCR is widely distributed. This could be because a few genes instead of a single one is present in a single droplet. In contrast, ddPCR doesn't need the exact two-fold amplification and if the fluorescence is a little higher than the negative control, it is interpreted as positive and the bias inherent to PCR is overcome.

Another method for the quantification of specific DNA is multiplex ligation-dependent probe amplification (MLPA), which can detect subtle alteration in copy number as a multiplex analysis.[15] Since its description in 2002 by Schouten et al., there have been more than 500 reports citing its use. However, it needs a capillary electrophoretic system, involves a number of steps, and takes about 24 h without accounting for DNA extraction. In contrast, the ddPCR provides results within 4 h. MLPA needs high quality DNA, while the ddPCR does not. In addition, ddPCR gives more accurate results with lesser amount of DNA allowing the analysis of samples with low DNA content. Thus, ddPCR appears to be more suited for the clinical setting.

A few articles have reported the diagnosis of α-thalassemia using ddPCR.[16,17] However, the number of reports is small and the types of α-thalassemia examined are limited. Our study not only used normal control, but as many as 230 α-thalassemia samples covering almost all α-thalassemia mutations found in Japan.

This study gave satisfactory results for all normal and α-thalassemia samples even if the fluorescent intensity was at minimum or maximum level, and the results enabled the diagnosis of α-thalassemia cases that have been difficult before. Thus, non-SEA/FIL type α-thalassemia that was diagnosed by routine examination but not genetically evidenced were diagnosed by ddPCR.

Six cases from four families that were clinically suspected of α-thalassemia but not evidenced by ddPCR for *α-globin* gene had deletions at MCSR2, which is a regulatory gene for the *α-globin* gene cluster and locates far upstream of it on chromosome 16.[8,18-22] The remaining 43 of 49 non-SEA/FIL type thalassemia had deletion of the *α-globin* gene. Deletion at MCSR2 in the six α-thalassemia samples are being further analyzed. Precise determination of the range of each deletion will be disclosed in the future.

We propose a two-step strategy for genetic diagnosis of deletion-type α-thalassemia. First, α-thalassemia with high frequency mutation should be analyzed by Gap-PCR, followed by α-thalassemia with unknown mutation being analyzed using ddPCR. We designed primers and probes that are specific to α_1 and α_2 globin genes, to confirm the presence of deletion in each *α-globin* gene. Finally, real-time PCR for MCSR2 may be necessary for α-thalassemia that are undetermined using the above two steps. Real-time PCR is cheaper than ddPCR. Thus, all deletion-type α-thalassemia can be diagnosed with the former method. The non-deletion type α-thalassemia (α^T) is analyzed by DNA sequencing, dot blot or allele specific amplification. The ddPCR allows for accurate diagnosis of α-thalassemia.

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Conflict of Interest

The authors declare no conflict of interest.

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