Diagnosis of the accurate genotype of HK$\alpha\alpha$ carriers in patients with thalassemia using multiplex ligation-dependent probe amplification combined with nested polymerase chain reaction

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Abstract

Background: Patients carrying the HongKong$\alpha\alpha$ (HK$\alpha\alpha$) allele and -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$ could be misdiagnosed as -$\alpha^{3.7}$/$\alpha\alpha$ by the current conventional thalassemia detection methods, leading to inaccurate genetic counseling and an incorrect prenatal diagnosis. This study was aimed to accurately analyze the genotypes of HK$\alpha\alpha$ carriers and -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$.

Methods: Samples were collected in our hospital from July 2017 to October 2019. Twenty-four common types of Chinese thalassemia were screened by gap-polymerase chain reaction (Gap-PCR) and reverse dot blot (RDB). Anti-4.2 multiplex-PCR was used to confirm carriers of the $\alpha\alpha\alpha^{anti\text{-}4.2}$ duplication with -$\alpha^{3.7}$ deletion. Two-round nested PCR and multiplex ligation-dependent probe amplification (MLPA) were applied to accurately identify and confirm their genotypes. For data analysis, we used descriptive statistics and Fisher's exact tests.

Results: Two thousand five hundred and forty-four cases were identified as thalassemia in 5488 peripheral blood samples. The results showed that α, β, and $\alpha\beta$ compound thalassemia were identified in 1190 (46.78%), 1286 (50.55%), and 68 (2.67%) cases, respectively. A total of 227 samples from thalassemia patients were identified as -$\alpha^{3.7}$/$\alpha\alpha$ by Gap-PCR, and the genotypes of two samples were uncertain. There was a difference between Gap-PCR and combined groups (Gap-PCR combined with nested PCR and MLPA) in detecting HK$\alpha\alpha$ ($P < 0.05$). Among the 229 patients, 20 patients were identified as HK$\alpha\alpha$ carriers and one was identified as -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$ by two-round nested PCR and MLPA, including 15 patients with HK$\alpha\alpha$/$\alpha\alpha$, three with HK$\alpha\alpha$/$\alpha\alpha$ and β-thalassemia coinheritance, one with HK$\alpha\alpha$/–^{SEA}, one with HK$\alpha\alpha$/-$\alpha^{4.2}$ and β-thalassemia coinheritance, and one with -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$ and β-thalassemia coinheritance.

Conclusions: $\alpha\alpha\alpha^{anti\text{-}4.2}$ and HK$\alpha\alpha$ genotypes of patients carrying -$\alpha^{3.7}$ need to be detected to reduce the misdiagnosis rate of patients carrying HK$\alpha\alpha$ and -α3.7/$\alpha\alpha\alpha^{anti\text{-}4.2}$ alleles. More accurate genetic counseling can be provided in the clinic using nested PCR combined with MLPA.

Keywords: Thalassemia; HongKong$\alpha\alpha$; Nested polymerase chain reaction; Multiplex ligation-dependent probe amplification; Gene dosage

Introduction

Thalassemia is one of the most common single gene inheritance diseases in the world.[1] It is caused by the interruption of α- or β-globin chain synthesis, leading to an imbalance in the ratio of α/non-α chains. -$\alpha^{3.7}$, -$\alpha^{4.2}$, and –^{SEA} are the most common α-thalassemia (α-thal) deletions found in China.[2] The overall prevalence of –^{SEA}, -$\alpha^{3.7}$, and -$\alpha^{4.2}$ was 68.06%, 25.0%, and 2.78%, respectively, in Chengdu, Sichuan, China.[3] In 2005, Wang *et al*[4] found a rare rearrangement of the α-globin gene cluster, HongKong$\alpha\alpha$ (HK$\alpha\alpha$) allele, which contains both -$\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti\text{-}4.2}$ crossover junctions. The -$\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti\text{-}4.2}$ fragments are located on the same chromosome as HK$\alpha\alpha$, whereas on two chromosomes, it is -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$. However, both HK$\alpha\alpha$ and -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$ are misdiagnosed as -$\alpha^{3.7}$/$\alpha\alpha$ by the current conventional thalassemia detection methods.

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The HK$\alpha\alpha$ allele contains neither deletion nor triplication, and carriers of this allele are unlikely to suffer any deleterious effects. Moreover, there were no differences between HK$\alpha\alpha$/$--^{SEA}$ and $-\alpha^{3.7}/\alpha\alpha$ in erythrocyte parameters or hemoglobin electrophoresis results.[4,5] Once HK$\alpha\alpha$ is misdiagnosed as the $-\alpha^{3.7}$ deletion, and the patient's spouse is $--^{SEA}/\alpha\alpha$, while unnecessary amniocentesis is required for a clear diagnosis, increasing the risk of abortion and economic burden, causing psychological stress.

The clinical manifestations of $-\alpha^{3.7}/\alpha\alpha\alpha^{anti\text{-}4.2}$ are similar to HK$\alpha\alpha$ in theory. However, as the genotype is rare, the number of reports is small.[6] When the patient's spouse is a carrier of β-thalassemia (β-thal), their offspring have a 1/4 probability of $\alpha\alpha\alpha^{anti\text{-}4.2}$ and β-thal coinheritance. $\alpha\alpha\alpha$ triploid aggravates the imbalance of α/β due to the increase in the α-globin chain, which aggravates the clinical manifestation of β-thalassemia.[7-11] Therefore, when $-\alpha^{3.7}/\alpha\alpha\alpha^{anti\text{-}4.2}$ is misdiagnosed as $-\alpha^{3.7}/\alpha\alpha$, the genetic probability of moderate to severe thalassemia may be misjudged by clinicians, and may exacerbate the birth of anemic children.

At present, routine testing for thalassemia cannot detect HK$\alpha\alpha$ and $-\alpha^{3.7}/\alpha\alpha\alpha^{anti\text{-}4.2}$. Gap-polymerase chain reaction (Gap-PCR) and reverse dot blot (RDB) assays are widely used to detect the common α-thal and β-thal mutations.[12,13] The HK$\alpha\alpha$ allele and some additional genotypes (HK$\alpha\alpha$/$--^{SEA}$, HK$\alpha\alpha/-\alpha^{4.2}$, and HK$\alpha\alpha/\alpha^{CS}\alpha$) formed by HK$\alpha\alpha$ can be detected by multicolor melting curve analysis with real-time PCR.[14] However, none of the three methods can detect triplication of the α-globin gene. Nested PCR is a cost-effective way to detect the HK$\alpha\alpha$ allele, but it cannot identify additional genotypes. Sequencing is well suited to the detection of small sequence changes and is less well adapted for detecting structural variants.[15] Multiplex ligation-dependent probe amplification (MLPA) is a useful technique for detecting and identifying copy number variation (deletions/duplications) in any region of the genome.[16,17] This technique has been applied to detect the *HBA* and *HBB* gene.[18-21] Xie *et al*[22] detected HK$\alpha\alpha/\alpha\alpha$ with MLPA. However, when the balanced translocation of chromosomes coexists in samples, the results of MLPA are incorrect. Some genotypes, such as $-\alpha^{4.2}/\alpha\alpha\alpha^{anti\text{-}4.2}$ and $-\alpha^{3.7}/\alpha\alpha\alpha^{anti\text{-}3.7}$, may be diagnosed in normal subjects by this technology.

Therefore, nested PCR combined with MLPA was used to analyze the genotypes of $\alpha\alpha\alpha^{anti\text{-}4.2}$ and HK$\alpha\alpha$ in patients with $-\alpha^{3.7}$ deletion thalassemia, and to ascertain whether it coexists with other genotypes of thalassemia.

Methods

Ethical approval

The study was followed the principles of *Declaration of Helsinki* and approved by the Institutional Review Committee of Hospital of University of Electronic Science and Technology of China and Sichuan Provincial People's Hospital (No. 2019-1). Informed consent was obtained from each patient and their family members before genetic testing was performed.

Samples and hematologic analysis

A total of 5488 peripheral blood samples for thalassemia testing were collected from July 2017 to October 2019 in Sichuan Provincial People's Hospital, China. Of these, 1461 (26.62%) were male and 4027 (74.38%) female. The patients were in the age range of 2 month-60 years (27.72 ±10.53 years). The hematological parameters of the samples were determined by an automatic analyzer XN-10 (Sysmex Corporation, Kobe, Japan). A capillary electrophoresis device (Capillarys; Sebia, Montpellier, France) was used for hemoglobin electrophoresis analysis.

Analysis of common thalassemia mutations by Gap-PCR and RDB

Genomic DNA was extracted from peripheral blood. Single-tube multiplex Gap-PCR was used to test the four α-globin gene deletion ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{SEA}$, and $--^{THAI}$) (Shenzhen Yishengtang Biological Enterprise Co., Ltd, Shenzhen, China). All DNA samples with the $-\alpha^{3.7}$ deletion were collected.

The RDB assay was used for three common non-deletional α-thal mutations (Hb Constant Spring [Hb CS] *HBA2*: c.427T>C, Hb Quong Sze [Hb QS] *HBA2*: c.377T>C, and Hb Westmead [Hb WS] *HBA2*: c.369122C>G] and 17 known Chinese β-thal mutations[23] (Shenzhen Yishengtang Biological Enterprise Co., Ltd).

Analysis of the genotypes of $\alpha\alpha\alpha^{anti\text{-}4.2}$ and HK$\alpha\alpha$ allele

The $\alpha\alpha\alpha^{anti\text{-}4.2}$ junction was assessed by the anti-4.2 multiplex-PCR assay for samples diagnosed as $-\alpha^{3.7}$. All primers were used to detect the HK$\alpha\alpha$ allele [Table 1].[6,13] Each 20 μL reaction contained the following: 10 ng/μL DNA, 1× GC Buffer I, 0.4 mmol/L dNTP, 0.3 μmol/L anti-4.2-F, 0.3 μmol/L anti-4.2-R, 0.15 μmol/L LIS1-2.5-F, 0.15 μmol/L LIS1-2.5-R, 0.05 IU/μL Takara LA-Taq (Takara Bio, Shiga, Japan). Double-distilled water was added to a volume of 20 μL. The PCR procedure was as follows: 95° C for 1 min initially, followed by 35 cycles of 94° C for 30 s and 68° C for 3 min, then a final 3 min at 68° C.

Two-round nested PCR was carried out in samples with both $-\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti\text{-}4.2}$ [Figure 1]. The first-round PCR amplified a DNA segment from X1 to Z1 boxes and a DNA segment for internal quality control. Each 20-μL reaction contained the following: 10 ng/μL DNA, 1× GC Buffer I, 0.4 mmol/L dNTP, 0.4 μmol/L L-anti-4.2-F, 0.4 μmol/L L-α3.7-R, 0.1 μmol/L LIS1-2.5-F, 0.1 μmol/L LIS1-2.5-R, 0.05 IU/μL Takara LA-Taq, and double-distilled water was added to a volume of 20 μL. The PCR procedure was as follows: 95° C for 1 min initially, followed by 35 cycles of 94° C for 30 s and 68° C for 5 min, then a final 3 min at 68° C.

The second-round PCR was used to amplify the $\alpha\alpha\alpha^{anti\text{-}4.2}$ duplication. For general amplification success, the LIS1-2.0 primers were also used as the internal quality control. The primers used in the nested PCR except for LIS1-2.5 and LIS1-2.0 primers are labeled in Figure 1. Each 20-μL reaction contained the following: 1 μL of the first-round

Table 1: Primers for $\alpha\alpha\alpha^{anti\text{-}4.2}$ triplication and HK$\alpha\alpha$ allele.

Application	Name	5′→3′ Sequence	Amplicon (size)
First-round PCR/anti-4.2 multiplex-PCR	LIS1-2.5-F	GTCGTCACTGGCAGCGTAGATC	2503 bp
	LIS1-2.5-R	GATTCCAGGTTGTAGACGGACTG	
First-round PCR	L-anti-4.2-F	CCTTGCACCGGCCCTTCCTGGTC	4000–4500 bp
	L-α3.7-R	CCTCAAAGCACTCTAGGGTCCAGCG	
Anti-4.2 multiplex-PCR	anti-4.2-F	CCTTGCACCGGCCCTTCCTG	1711 bp
	anti-4.2-R	GAACTGGCTGAAAGGGATGCAG	
Second-round PCR	AT4.2-F	AATGTGCCAACAATGGAGGT	1566 bp
	AT4.2-R	TCCAAAAAGAGAGCCTGTGG	
	LIS1-2.0-F	TGCTATCTGTTGGTGCCTGA	2003 bp
	LIS1-2.0-R	TGTTCGCTGCTTCTCACATG	

F: Forward; R: Reverse; HK$\alpha\alpha$: HongKong$\alpha\alpha$; PCR: Polymerase chain reaction.

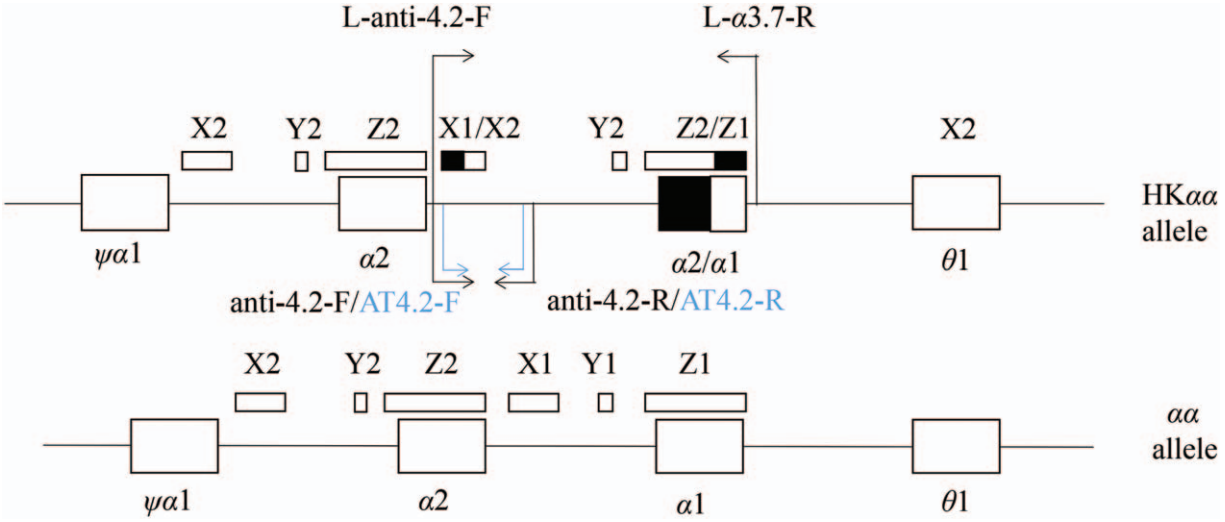

Figure 1: The strategy to confirm HongKong$\alpha\alpha$ (HK$\alpha\alpha$) allele. For screening the HK$\alpha\alpha$ allele, anti-4.2 multiplex polymerase chain reaction (PCR) amplifies an ~1.7 kb fragment flanked by X1/X2 hybrid box using primers anti-4.2-F and anti-4.2-R. A two-round nested PCR strategy to confirm the presence of the HK$\alpha\alpha$ allele. The first-round PCR amplifies ~4.0 and ~4.5 kb segments flanked by the X1 and Z1 boxes using primers L-anti-4.2-F and L-α3.7-R when HK$\alpha\alpha/\alpha\alpha$ is present; the second-round PCR detects an ~1.5 kb amplification fragment using primers AT4.2-F and AT4.2-R in the presence of the X1/X2 hybrid box (ie, the $\alpha\alpha\alpha^{anti\text{-}4.2}$ junction fragment). F: Forward; R: Reverse.

PCR products diluted 1600 times as template, 0.4 mmol/L $MgCL_2$, 1× EX Buffer (Mg^{2+} free), 0.4 mmol/L dNTP, 0.3 μmol/L AT4.2-F, 0.3 μmol/L AT4.2-R, 0.05 μmol/L LIS1-2.0-F, 0.05 μmol/L LIS1-2.0-R, and 0.05 IU/μL Takara EX Taq, and double-distilled water was added to a volume of 20 μL. The PCR procedure was as follows:95°C for 1 min initially, followed by 35 cycles of 95°C for 30 s, 56°C for 45 s, and 72°C for 60 s, then a final 3 min at 72°C.

Assessment of the copy number of the HBA gene by MLPA

For samples carrying both $-\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti\text{-}4.2}$, MLPA analysis was carried out using the SALSA MLPA KIT HBA140-C1 kit (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. Three wild-type subjects were collected as references, and three common α-thal deletions ($-\alpha^{3.7}/\alpha\alpha$, $-\alpha^{4.2}/\alpha\alpha$, and $-^{SEA}/\alpha\alpha$) were used as positive controls of the α-globin gene cluster for MLPA. In short, the genomic DNA was denatured and hybridized with SALS-MLPA probes specific to the α-globin gene cluster. After ligation, PCR was performed using primers specific to the probes. The amplification results were analyzed on an ABI PRISM 3730 (Applied Biosystems, Foster City, CA, USA) Genetic Analyzer. The data were analyzed by coffalyser.net (MRC-Holland).

Statistical analysis

The statistical software SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis. Fisher's exact test was used to detect a statistically significant difference between the Gap-PCR and Gap-PCR combined with nested PCR and MLPA in detecting HK$\alpha\alpha$ allele. Statistical significance was set as $P < 0.05$.

Results

Identification of thalassemia mutations by Gap-PCR and RDB assays

Total 2544 cases were identified as thalassemia in 5488 peripheral blood samples. The results showed that α, β,

and $\alpha\beta$ compound thalassemia were identified in 1190 (46.78%), 1286 (50.55%), and 68 (2.67%) cases, respectively. A total of 227 samples from thalassemia patients were identified as -$\alpha^{3.7}$/$\alpha\alpha$ by Gap-PCR. The other two patients were suspected to be HK$\alpha\alpha$ carriers due to the presence of three bands (2.0, 1.7, and 1.2/1.4 kb, respectively) in the electrophoresis study.

Analysis of the genotypes of $\alpha\alpha\alpha^{anti\text{-}4.2}$ and the HK$\alpha\alpha$ allele

Of the 229 patients who were identified as -$\alpha^{3.7}$/$\alpha\alpha$ or suspected to be HK$\alpha\alpha$ carriers, 20 patients were identified as HK$\alpha\alpha$ carriers, and one patient was identified as a -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$ carrier by two-round nested PCR and MLPA, including 15 patients of HK$\alpha\alpha$/$\alpha\alpha$, three patients of HK$\alpha\alpha$/$\alpha\alpha$ and β-thalassemia coinheritance, one patient with

Table 2: The phenotype, genotype of cases carrying the -$\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti\text{-}4.2}$.

Patient no.	Sex-age (years)	Hb (g/L)	MCV (fL)	MCH (pg)	HbA_2 (%)	Gap-PCR α-genetype	Nested PCR and MLPA α-genetype	β-Genetype
1	M-27	122	78.4	25.8	ND	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
2	F-7	115	57	17.4	4.9	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	$\beta^{IVS\text{-}2\text{-}654}/\beta^N$
3	M-28	101	113.4	38.5	1.7	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
4	M-30	149	83.1	27.6	2.5	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
5	M-54	147	93.0	31.3	2.2	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
6	M-27	161	87.7	29.5	2.8	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
7	M-29	70	64.8	18.7	2.3	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
8	M-52	129	95.1	31.4	2.4	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
9	M-29	94	111.3	36.7	1.8	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
10	F-26	106	67.1	20.8	5.2	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	$\beta^{codon41\text{-}42}/\beta^N$
11	F-19	101	68.2	21.5	ND	HK$\alpha\alpha$/-$\alpha^{4.2}$*	HK$\alpha\alpha$/-$\alpha^{4.2}$	$\beta^{IVS\text{-}2\text{-}654}/\beta^N$
12	F-28	136	69.5	21.4	ND	HK$\alpha\alpha$/–^{SEA}*	HK$\alpha\alpha$/–^{SEA}	β^N/β^N
13	M-61	144	96.1	31.1	2.5	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
14	M-33	185	91.4	31.8	2.7	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
15	F-7	131	82.9	28.4	2.8	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
16	M-29	133	61.5	18.2	4.9	-$\alpha^{3.7}$/$\alpha\alpha$	-$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$	$\beta^{IVS\text{-}2\text{-}654}/\beta^N$
17	M-29	157	86.2	29.6	ND	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
18	F-48	117	98.2	30.9	2.4	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
19	F-26	96	61.8	19.2	6.5	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	$\beta^{codon41\text{-}42}/\beta^N$
20	M-28	156	95.8	32.6	2.6	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N
21	M-27	126	77.5	24.5	2.6	-$\alpha^{3.7}$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha$	β^N/β^N

Patient no.: Patient number; Hb: Hemoglobin; MCV: Mean corpuscular volume; MCH: Mean cell Hb; PCR: Polymerase chain reaction; MLPA: Multiplex ligation-dependent probe amplification; HK$\alpha\alpha$: HongKong$\alpha\alpha$; β^N: Normal β-genotype; codons 41/42: HBB:c.124_127delTTCT; IVS-2-654: HBB: c.316-197C>T; ND: No detection; M: male; F: female. *The result was suspected to be HK$\alpha\alpha$ heterozygote and need further verification.

Table 3: Interpretation of PCR and MLPA results.

Genotype		HK$\alpha\alpha$/$\alpha\alpha$	HK$\alpha\alpha$/$\alpha\alpha\alpha^{anti\text{-}4.2}$	HK$\alpha\alpha$/$\alpha\alpha\alpha^{anti\text{-}3.7}$	HK$\alpha\alpha$/HK$\alpha\alpha$	HK$\alpha\alpha$/-$\alpha^{3.7}$	HK$\alpha\alpha$/-$\alpha^{4.2}$	HK$\alpha\alpha$/–^{SEA}	-$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$
Gap-PCR	1.2 kb	–	–	–	–	–	–	+	–
	1.4 kb	–	–	–	–	–	+	–	–
	1.7 kb	+	+	+	+	+	+	+	+
	2.0 kb	+	+	+	+	+	+	+	+
Anti-4.2	1.7 kb	+	+	+	+	+	+	+	+
	2.5 kb	+	+	+	+	+	+	+	+
First round PCR	2.5 kb	+	+	+	+	+	+	+	+
	4.0–4.5 kb	+	+	+	+	+	+	+	+
Second round PCR	1.5 kb	+	+	+	+	+	+	+	–
	2.0 kb	+	+	+	+	+	+	+	+
	Gene-exon								
MLPA	*HBA2*-up	1.5C	2.0C	1.5C	3C	1.5C	1.0C	1C	1.0C
	HBA1-up	0.5C	0.5C	1.0C	0C	0C	0.5C	0C	0.5C
	Other exons							0.5C	

PCR: Polymerase chain reaction; MLPA: Multiplex ligation-dependent probe amplification; HK$\alpha\alpha$: HongKong$\alpha\alpha$; +: The corresponding size of the product can be obtained; –: There is no product can be obtained; *HBA2*-up: The unequal exchange between homologous sequences of *HBA2*-up can result in the deletion of single α-globin gene -$\alpha^{4.2}$ and the triplications ($\alpha\alpha\alpha^{anti\text{-}4.2}$); *HBA1*-up: The unequal exchange between homologous sequences of *HBA1*-up can result in the deletion of single α-globin gene -$\alpha^{3.7}$ and the triplication ($\alpha\alpha\alpha^{anti\text{-}3.7}$); Other exons: It contains *HBM* region-up, *HBA1&2*-1, *HBA2*-intr.2, *HBA1*-intr.2, *HBA1*-3, *HBA1*-down, *HBQ1*-3, deletions of those exons and *HBA1*-up and *HBA2*-up are represented to –^{SEA} deletion.

HK$\alpha\alpha$/–^{SEA}, one patient with HK$\alpha\alpha$/-$\alpha^{4.2}$ and β-thalassemia coinheritance, and one patient with -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$ and β-thalassemia coinheritance. The phenotypes and genotypes of the cases carrying the -$\alpha^{3.7}$ deletion and $\alpha\alpha\alpha^{anti\text{-}4.2}$ duplication are summarized in Table 2. The interpretation of the PCR and MLPA results is shown in Table 3.

Comparison between Gap-PCR and the combination of Gap-PCR, nested PCR and MLPA in detecting HK$\alpha\alpha$ allele

There was a significantly difference between Gap-PCR and Gap-PCR combined with nested PCR and MLPA in detecting HK$\alpha\alpha$ ($P < 0.05$) [Table 4]. Of the 229 patients, two patients were suspected of carrying HK$\alpha\alpha$ by Gap-PCR. Gap-PCR combined with nested PCR and MLPA found that 21 patients were HK$\alpha\alpha$ carriers or -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$. The error rate of diagnosis was 9.17% (21/229) by Gap-PCR in -$\alpha^{3.7}$ deletion.

Table 4: Comparison of Gap-PCR and Gap-PCR combined with nested PCR and MLPA in detecting HK$\alpha\alpha$.

	Combined group			
Gap-PCR group	-$\alpha^{3.7}$/$\alpha\alpha$	Other	Total, *n*	*P*
-$\alpha^{3.7}$/$\alpha\alpha$	208	19	227	
Other	0	2	2	0.008
Total, *n*	208	21	229	

Error rate = 21/(208+21) × 100% = 9.17%. Gap-PCR: Gap-polymerase chain reaction; MLPA: Multiplex ligation-dependent probe amplification; HK$\alpha\alpha$: HongKong$\alpha\alpha$; Other: HK$\alpha\alpha$ carriers and -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$.

Discussion

In a clinical test, -$\alpha^{3.7}$/$\alpha\alpha$, HK$\alpha\alpha$/$\alpha\alpha$, HK$\alpha\alpha$/-$\alpha^{3.7}$, HK$\alpha\alpha$/$\alpha\alpha\alpha^{anti\text{-}4.2}$, HK$\alpha\alpha$/$\alpha\alpha\alpha^{anti\text{-}3.7}$, and -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$ may be misdiagnosed as -$\alpha^{3.7}$/$\alpha\alpha$. Different genotypes may lead to different clinical phenotypes. According to the literature, there is a considerable difference in the carrier frequency of HK$\alpha\alpha$ in different geographical populations, and the carrying rate is about 0.07% to 2.27%.[5,24] In this study, of the 229 patients, 20 patients were identified as HK$\alpha\alpha$ carriers, and one patient was identified as -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$ by two-round nested PCR and MLPA. The frequency of HK$\alpha\alpha$ allele was as high as 8.81% among the -$\alpha^{3.7}$ carriers. The error rate of diagnosis is 9.17% (21/229) by Gap-PCR in -$\alpha^{3.7}$ deletion. Thus, to obtain a more accurate diagnosis and treatment, it was necessary to distinguish -$\alpha^{3.7}$ from HK$\alpha\alpha$ and -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$.

In this study, when people were diagnosed as carriers of -$\alpha^{3.7}$, anti-4.2 multiplex-PCR was adopted to determine whether to continue supplementary experiments. However, HK$\alpha\alpha$/$\alpha\alpha$, HK$\alpha\alpha$/$\alpha\alpha\alpha^{anti\text{-}4.2}$, HK$\alpha\alpha$/$\alpha\alpha\alpha^{anti\text{-}3.7}$, HK$\alpha\alpha$/HK$\alpha\alpha$, and HK$\alpha\alpha$/-$\alpha^{3.7}$ could not be discerned from each

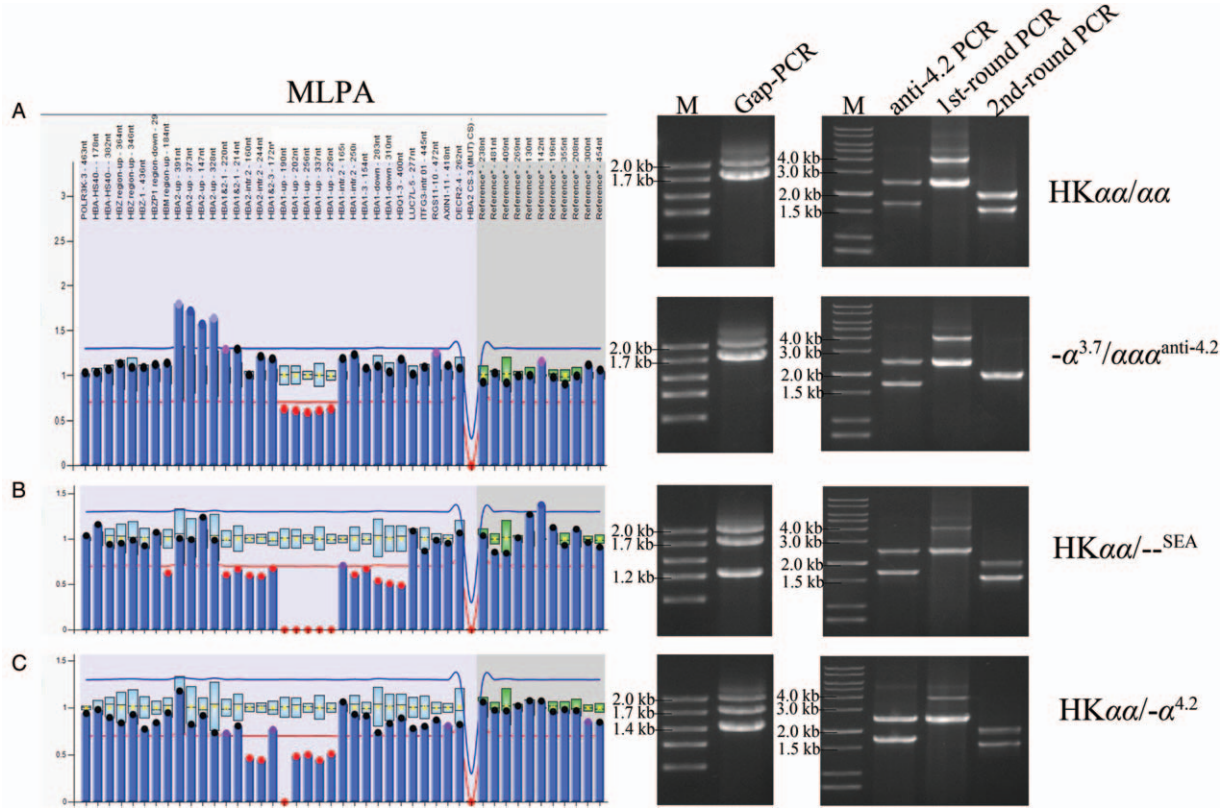

Figure 2: The analysis by multiplex ligation-dependent probe amplification (MLPA) integrating with nested polymerase chain reaction (PCR) (MRC-Holland, Amsterdam, The Netherlands). The left images were the results by MLPA, and the right images were the results by gap-PCR (Gap-PCR), anti-4.2 multiplex-PCR, and nested PCR. The *y*-axis represented the ratio signal as compared to the normal control (ratio 1); on the *x*-axis, the MLPA-probe numbers were shown on the figure. (A) HK$\alpha\alpha$/$\alpha\alpha$ or -$\alpha^{3.7}$/$\alpha\alpha\alpha^{anti\text{-}4.2}$; (B) HK$\alpha\alpha$/–^{SEA}; (C) HK$\alpha\alpha$/-$\alpha^{4.2}$. HK$\alpha\alpha$: HongKong$\alpha\alpha$.

other by Gap-PCR, anti-4.2 multiplex-PCR or nested PCR [Table 3]. MLPA analysis was used to determine the number of copies of the *HBA* gene. However, when there are balanced translocations of chromosomes in specimens, they could not be correctly assessed by MLPA [Figure 2C]. In addition, MLPA is unable to determine whether deletions and duplications are located on the same chromosome. Thus, HK$\alpha\alpha/\alpha\alpha$ could not be distinguished from $-\alpha^{3.7}/\alpha\alpha\alpha^{\text{anti-4.2}}$ [Figure 2A]. Therefore, in this study, nested PCR was performed to detect the HK$\alpha\alpha$ allele, and MLPA analysis was not only used to ensure the results of the nested PCR but also to find extra deletions or duplications. Thus, these two techniques could aid and verify each other [Figure 2].

All parameters were normal except for patients 2, 7, 10, 11, 12, 16, and 19. They might have been caused by the coinheritance of β-thal, $-\alpha^{4.2}$, or $-^{\text{SEA}}$ deletions. The other patients were HK$\alpha\alpha$ heterozygotes, of which patient 7 showed obvious small-cell hypochromic anemia, which was confirmed by clinical analysis and combined with iron deficiency anemia. The hematological parameters of the other patients were almost normal, which was consistent with the results of Wang *et al*[4] and Wu *et al*.[24] Patient 16 is $-\alpha^{3.7}/\alpha\alpha\alpha^{\text{anti-4.2}}$ combined with $\beta^{\text{IVS-2-654}}/\beta^{\text{N}}$, which is rarer than HK$\alpha\alpha$. After testing, the patient's spouse was found to have a normal genotype, and their offspring have a 1/4 probability of $-\alpha^{3.7}/\alpha\alpha$ complex $\beta^{\text{N}}/\beta^{\text{N}}$, 1/4 probability of $-\alpha^{3.7}/\alpha\alpha$ complex $\beta^{\text{N}}/\beta^{\text{IVS-2-654}}$, 1/4 probability of $\alpha\alpha/\alpha\alpha\alpha^{\text{anti4.2}}$ complex $\beta^{\text{N}}/\beta^{\text{N}}$, and 1/4 probability of $\alpha\alpha/\alpha\alpha\alpha^{\text{anti-4.2}}$ complex $\beta^{\text{N}}/\beta^{\text{IVS-2-654}}$. The $\alpha\alpha/\alpha\alpha\alpha^{\text{anti4.2}}$ complex $\beta^{\text{N}}/\beta^{\text{IVS-2-654}}$ genotype can aggravate the clinical manifestations of β-thal. Therefore, genetic counseling should be carried out during pregnancy.

At present, there is no gold standard for testing the accurate genotypes of HK$\alpha\alpha$ carriers as a reference. Thus we did not determine precise rates of this combination. In addition, samples of HK$\alpha\alpha/\alpha\alpha\alpha^{\text{anti-4.2}}$, HK$\alpha\alpha/\alpha\alpha\alpha^{\text{anti-3.7}}$, and HK$\alpha\alpha$/HK$\alpha\alpha$ have not yet been collected. So, more samples should be collected and tested to confirm the findings of our study.

Generally, patients carrying the $-\alpha^{3.7}$ deletion of thalassemia must undergo screening for the $\alpha\alpha\alpha^{\text{anti-4.2}}$ and HK$\alpha\alpha$ genotypes because of the high carrier frequency. Using nested PCR combined with MLPA can reduce the misdiagnosis rate of the HK$\alpha\alpha$ allele and $-\alpha^{3.7}/\alpha\alpha\alpha^{\text{anti-4.2}}$ and enable more accurate genetic counseling.

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Conflicts of interest

None.

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