Diagnosis of the accurate genotype of HKαα 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 HongKongα (HKαα) allele and -α3.7/ααanti-4.2 could be misdiagnosed as -α3.7/αα 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 HKαα carriers and -α3.7/ααanti-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 -α3.7/ααanti-4.2 duplication with -α3.7 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 four hundred and forty-four cases were identified as thalassemia in 5488 peripheral blood samples. The results showed that α, β, and αβ 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 -α3.7/αα 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 HKαα (P < 0.05). Among the 229 patients, 20 patients were identified as HKαα carriers and one was identified as -α3.7/ααanti-4.2 by two-round nested PCR and MLPA, including 15 patients with HKαα, three with HKαα and -β-thalassemia coinheritance, one with HKαα, one with HKαα and -α3.7 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.

Conclusions: ααanti-4.2 and HKαα genotypes of patients carrying -α3.7 need to be detected to reduce the misdiagnosis rate of patients carrying HKαα and -α3.7/ααanti-4.2 alleles. More accurate genetic counseling can be provided in the clinic using nested PCR combined with MLPA.

Keywords: Thalassemia; HongKongα; 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. -α3.7, -α4.5, and -SEA are the most common α-thalassemia (α-thal) deletions found in China.[2] The overall prevalence of -SEA, -α3.7, and -α4.2 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, HongKongα (HKαα) allele, which contains both -α3.7 and ααanti-4.2 crossover junctions. The -α3.7 and ααanti-4.2 fragments are located on the same chromosome as HKαα, whereas on two chromosomes, it is -α3.7/ααanti-4.2. However, both HKαα and -α3.7/ααanti-4.2 are misdiagnosed as -α3.7/αα by the current conventional thalassemia detection methods.

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The HKαα allele contains neither deletion nor triplication, and carriers of this allele are unlikely to suffer any deleterious effects. Moreover, there were no differences from each patient and their family members before genetic testing was performed.

The clinical manifestations of \(3.7^{-\alpha}\) allele are similar to HKαα allele in theory. However, as the genotype is rare, the number of reports is small. When the patient’s spouse is a carrier of \(\beta\)-thalassemia (\(\beta\)-thal), their offspring have a 1/4 probability of \(\alpha\alpha\) and \(\beta\)-thal coinheritance. \(\alpha\alpha\) triploid aggravates the imbalance of \(\alpha\beta\) due to the increase in the \(\alpha\)-globin chain, which aggravates the clinical manifestation of \(\beta\)-thalassemia. Therefore, when \(\alpha\alpha\) is misdiagnosed as \(\alpha\beta\), 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 HKαα allele and \(3.7^{-\alpha}\) allele. Gap-polymerase chain reaction (Gap-PCR) and reverse dot blot (RDB) assays are widely used to detect the common \(\alpha\)-globin and \(\beta\)-thal mutations. The HKαα allele and some additional genotypes (HKαα, \(3.7^{-\alpha}\), HKαa/\(\alpha\alpha\), and HKαa/\(\alpha\beta\)) formed by HKαα allele can be detected by multicolor melting curve analysis with real-time PCR. None of the three methods can detect triplication of the \(\alpha\)-globin gene. Nested PCR is a cost-effective way to detect the \(\alpha\)-globin gene. Sequencing is well suited to the detection of small sequence changes and is less well adapted for detecting structural variants. 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. This technique has been applied to detect the HBA and HBB gene. Xie et al. detected HKαα allele with MLPA. However, when the balanced translocation of chromosomes coexists in samples, the results of MLPA are incorrect. Some genotypes, such as \(3.7^{-\alpha}\) and \(3.7^{-\alpha}\) allele, may be diagnosed in normal subjects by this technology.

Therefore, nested PCR combined with MLPA was used to analyze the genotypes of \(3.7^{-\alpha}\) and HKαα allele in patients with \(3.7^{-\alpha}\) 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 samples were in the age range of 2 month-60 years (27.72 ±10.53 years). The hematologic parameters of the samples were determined by an automatic analyzer XN-10 (Sysmex Corporation, Kobe, Japan). A capillary electrophoresis device (Capillary; 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 \(\alpha\)-globin gene deletion \(3.7^{-\alpha}\). The HKαα allele and some additional genotypes (HKαα, \(3.7^{-\alpha}\), HKαa/\(\alpha\alpha\), and HKαa/\(\alpha\beta\)) formed by HKαα allele can be detected by multicolor melting curve analysis with real-time PCR. None of the three methods can detect triplication of the \(\alpha\)-globin gene. Nested PCR is a cost-effective way to detect the \(\alpha\)-globin gene. Sequencing is well suited to the detection of small sequence changes and is less well adapted for detecting structural variants. 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. This technique has been applied to detect the HBA and HBB gene. Xie et al. detected HKαα allele with MLPA. However, when the balanced translocation of chromosomes coexists in samples, the results of MLPA are incorrect. Some genotypes, such as \(3.7^{-\alpha}\) and \(3.7^{-\alpha}\) allele, may be diagnosed in normal subjects by this technology.
PCR products diluted 1600 times as template, 0.4 mmol/L MgCl₂, 1× EX Buffer (Mg²⁺ 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 -α3.7 and ααα anti-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 (-α3.7/αα, -α4.2/αα, and -SEAP/αα) 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 HKα 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 αβ compound thalassemia were identified in 1190 (46.78%), 1286 (50.53%), and 68 (2.67%) cases, respectively. A total of 227 samples from thalassemia patients were identified as α3.7/-/αα by Gap-PCR. The other two patients were suspected to be HKαα carriers due to the presence of three bands (2.0, 1.7, and 1.2/1.4 kb, respectively) in the electrophoresis study.

### Table 2: The phenotype, genotype of cases carrying the α3.7 and αα anti-4.2.

| Patient no. | Sex-age (years) | Hb (g/L) | MCV (fl) | MCH (pg) | HbA2 (%) | Gap-PCR | Nested PCR and MLPA genotype | α-genotype | β-Genotype |
|-------------|-----------------|----------|----------|----------|-----------|---------|--------------------------------|------------|------------|
| 1           | M-27            | 122      | 78.4     | 25.8     | ND        | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 2           | F-7             | 115      | 57       | 17.4     | 4.9       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 3           | M-28            | 101      | 113.4    | 38.5     | 1.7       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 4           | M-30            | 149      | 83.1     | 27.6     | 2.5       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 5           | M-54            | 147      | 93.0     | 31.3     | 2.2       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 6           | M-27            | 161      | 87.7     | 29.5     | 2.8       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 7           | M-29            | 70       | 64.8     | 18.7     | 2.3       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 8           | M-52            | 129      | 95.1     | 31.4     | 3.2       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 9           | M-29            | 94       | 111.3    | 36.7     | 1.8       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 10          | F-26            | 106      | 67.1     | 20.8     | 5.2       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 11          | F-19            | 101      | 68.2     | 21.5     | ND        | HKαα/-/αα  | HKαα/-/αα                         | αα         | β4.2^{-}N  |
| 12          | F-28            | 136      | 69.5     | 21.4     | ND        | HKαα/-/αα  | HKαα/-/αα                         | αα         | β4.2^{-}N  |
| 13          | M-61            | 144      | 96.1     | 31.1     | 2.5       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 14          | M-33            | 185      | 91.4     | 31.8     | 2.7       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 15          | M-7             | 131      | 82.9     | 28.4     | 2.8       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 16          | M-29            | 133      | 61.5     | 18.2     | 4.9       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 17          | M-29            | 157      | 86.2     | 29.6     | ND        | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 18          | F-48            | 117      | 98.2     | 30.9     | 2.4       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 19          | F-26            | 96       | 61.8     | 19.2     | 6.5       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 20          | M-28            | 156      | 95.8     | 32.6     | 2.6       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}N  |
| 21          | M-27            | 126      | 77.5     | 24.5     | 2.6       | α3.7/-/αα | HKαα/αα                         | αα         | β4.2^{-}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; HKαα: Hong Kong; αα: Normal α genotype; codons 41/42: HBBc.124_127delTTCT; IVS-2-654: HBB: c.316-197C>T; ND: No detection; M: male; F: female. The result was suspected to be HKαα heterozygote and need further verification.

### Table 3: Interpretation of PCR and MLPA results.

| Genotype | HKαα/αα | HKαα/αα anti-4.2 | HKαα/αα-αα-3.7 | HKαα/αα | HKαα/αα-αα-3.7 | HKαα/αα | HKαα/αα-αα-3.7 | HKαα/αα | HKαα/αα-αα-3.7 | HKαα/αα | HKαα/αα-αα-3.7 | HKαα/αα | HKαα/αα-αα-3.7 | HKαα/αα | HKαα/αα-αα-3.7 |
|----------|---------|-----------------|-----------------|----------|-----------------|----------|-----------------|----------|-----------------|----------|-----------------|----------|-----------------|----------|-----------------|
| Gap-PCR  | 1.2 kb  | 1.4 kb          | 1.7 kb          | 2.0 kb   | 2.5 kb          | 4.0–4.5 kb| 1.5 kb          | 2.0 kb   | 1.5 kb          | 3.7 kb  | 0.5C            | 0.5C    | 0.5C            | 0.5C    | 0.5C            |
| Anti-4.2 | 1.7 kb  | 1.7 kb          | 2.5 kb          | 4.0–4.5 kb| 1.5 kb          | 2.0 kb   | 1.5 kb          | 3.7 kb  | 0.5C            | 0.5C    | 0.5C            | 0.5C    | 0.5C            | 0.5C    | 0.5C            |

PCR: Polymerase chain reaction; MLPA: Multiplex ligation-dependent probe amplification; HKαα: Hong Kong; αα: Normal α genotype; codons 41/42: HBBc.124_127delTTCT; IVS-2-654: HBB: c.316-197C>T; ND: No detection; M: male; F: female. 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 αα-4.2 and the triplications (ααanti-4.2); HBA1-up: The unequal exchange between homologous sequences of HBA1-up can result in the deletion of single α-globin gene αα-3.7 and the triplications (ααanti-3.7); Other exons: It contains HBM region-up, HBA1-αα-2, HBA2-intr-2, HBA1-intr-2, HBA1-β, HBA1-down, HBQ3-3, deletions of those exons and HBA1-up and HBA2-up are represented to ααanti-3.7 deletion.

Analysis of the genotypes of ααanti-4.2 and the HKαα allele

Of the 229 patients who were identified as αα-7/αα or suspected to be HKαα carriers, 20 patients were identified as HKαα carriers, and one patient was identified as a ααanti-4.2 carrier by two-round nested PCR and MLPA, including 15 patients of HKαα, three patients of HKαα and two-thalassemia cotainers, one patient was
HKαα/SEA, one patient with HKαα/αα⁻²⁻ and β-thalassemia coinheritance, and one patient with α⁺⁻³⁻/αααα⁻⁻⁻⁻ and β-thalassemia coinheritance. The phenotypes and genotypes of the cases carrying the α⁻⁻³⁻ deletion and αααα⁻⁻⁻⁻ 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 HKαα allele**

There was a significantly difference between Gap-PCR and Gap-PCR combined with nested PCR and MLPA in detecting HKαα (P < 0.05) [Table 4]. Of the 229 patients, two patients were suspected of carrying HKαα by Gap-PCR. Gap-PCR combined with nested PCR and MLPA found that 21 patients were HKαα carriers or α⁺⁻³⁻/αααα⁻⁻⁻⁻. The error rate of diagnosis was 9.17% (21/229) by Gap-PCR in α⁻⁻³⁻ deletion.

**Discussion**

In a clinical test, α⁺⁻³⁻/αα, HKαα/αα, HKαα/αα⁻⁻⁻⁻, HKαα/αααα⁻⁻⁻⁻, and α⁺⁻³⁻/αααα⁻⁻⁻⁻ may be misdiagnosed as α⁻⁻³⁻/αα. Different genotypes may lead to different clinical phenotypes. According to the literature, there is a considerable difference in the carrier frequency of HKαα 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 HKαα carriers, and one patient was identified as α⁺⁻³⁻/αααα⁻⁻⁻⁻ by two-round nested PCR and MLPA. The frequency of HKαα allele was as high as 8.81% among the α⁻⁻³⁻ carriers. The error rate of diagnosis is 9.17% (21/229) by Gap-PCR in α⁻⁻³⁻ deletion. Thus, to obtain a more accurate diagnosis and treatment, it was necessary to distinguish α⁻⁻³⁻ from HKαα and α⁺⁻³⁻/αααα⁻⁻⁻⁻.

In this study, when people were diagnosed as carriers of α⁺⁻³⁻, anti-4.2 multiplex-PCR was adopted to determine whether to continue supplementary experiments. However, HKαα/αα, HKαα/αααα⁻⁻⁻⁻, HKαα/αααα⁻⁻⁻⁻, and HKαα could not be discerned from each other.

**Table 4: Comparison of Gap-PCR and Gap-PCR combined with nested PCR and MLPA in detecting HKαα.**

| Combined group | -α⁺⁻³⁻/αα | Other | Total, n | P |
|----------------|-----------|-------|---------|---|
| -α⁺⁻³⁻/αα      | 208       | 19    | 227     |   |
| Other          | 0         | 2     | 2       |   |
| 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; HKαα: HongKongαα; Other: HKαα carriers and -α⁺⁻³⁻/αααα⁻⁻⁻⁻.

**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) HKαα/αα or -α⁺⁻³⁻/αααα⁻⁻⁻⁻, (B) HKαα/αα⁻⁻⁻⁻, (C) HKαα/αα⁻⁻⁻⁻, (D) HKαα.
thus, HKα/αα could not be distinguished from -α/ααα- [Figure 2A]. Therefore, in this study, nested PCR was performed to detect the HKα 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, -α or -αβ deletions. The other patients were HKα 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 [24] and Wu et al [24]. Patient 16 is -α/ααα- combined with ββN-2.654/βNβ, which is rarer than HKα. After testing, the patient’s spouse was found to have a normal genotype, and their offspring have a 1/4 probability of -α/αα complex ββNββ, a 1/4 probability of -α/αα complex ββNββ, a 1/4 probability of ααα/ααα complex ββNβ, and a 1/4 probability of ααaa/ααα complex ββNββ. The ααα/ααα complex ββNββ 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 HKα carriers as a reference. Thus we did not determine precise rates of this combination. In addition, samples of HKααα/αααα- [24], HKααα/αααα- [24] and HKααα/HKααα have not yet been collected. So, more samples should be collected and tested to confirm the findings of our study.

Generally, patients carrying the -α/ααα- deletion of thalassemia must undergo screening for the ααaa/αααα- and HKα genotypes because of the high carrier frequency. Using nested PCR combined with MLPA can reduce the misdiagnosis rate of the HKα allele and -α/ααα- and enable more accurate genetic counseling.

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**Conflicts of interest**

None.

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