The pedigree analysis and prenatal diagnosis of Hong Kongαα Thalassemia and the sequence analysis of Hong Kongαα Allele

Wenjuan Wang1,2 | Haiqing Zheng1 | Dan Zeng1 | Linbin Jiang3 | Donglan Yu1 | Yuzhong Yang4 | Qiao Feng1 | Yang Xia5 | Chunjiang Zhu1

1Department of Genetics, Affiliated Hospital of Guilin Medical University, Guilin, People’s Republic of China
2Northwest Women’s and Children’s Hospital, Xi’an, People’s Republic of China
3College of Biotechnology, Guilin Medical University, Guilin, People’s Republic of China
4Department of Pathology, Affiliated Hospital of Guilin Medical University, Guilin, People’s Republic of China
5Department of Biochemistry and Molecular Biology, University of Texas Health Science Center at Houston, Houston, TX, USA

Correspondence
Chunjiang Zhu, Department of Genetics, Affiliated Hospital of Guilin Medical University, Guilin, PRC.
Email: zcjiang2003@qq.com

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Abstract
Background: Thalassemia is one of the most common monogenic hemolytic disorders in the world. Hong Kongαα (HKαα) thalassemia was initially found among the people of southern China. Because of the complexity of genetic changes in HKαα thalassemia, we lack a precise sequence analysis of the HKαα allele. Here we aim to detect the specific genotype and trace the law of inheritance of this rare genotype.

Methods: We recruited an unprecedented huge pedigree containing 11 individuals carrying the HKαα thalassemia gene and 4 nongenetic-related patients suffering from HKαα from south China. Regular hematological analysis and routine genetic screening were performed on the pedigree and two-round nested PCR (polymerase chain reaction) for HKαα thalassemia were performed on each individual. The first-generation gene sequencing was performed on six individuals, including four nongenetic-related patients.

Result: We found that five family members were positive for the HKαα allele. Patients II-2, III-1, and II-3 with only HKαα/-SEA or HKαα/-α4.2 presented with α-thalassemia minor trait. I-1, the carrier of both HKαα/-α3.7 and β41-42/αN, showed a typical β-thalassemia trait. Fetus with genotype HKαα/-α4.2 alone was not likely to suffer from any deleterious effects after birth. The whole sequence of HKαα allele revealed that HKαα alleles in the six patients shared a high similarity, implying that all HKαα alleles are likely from the same ancestor. Moreover, pedigree and sequencing analyses demonstrated that the HKαα allele contained αααanti4.2 mutation, -α3.7 mutation, and a fragment from α-hemoglobin gene; thus, the composition and formation of HKαα allele was revealed. Finally, the high similarity and composition of HKαα alleles implies that once HKαα formed, αααanti4.2 and -α3.7 mutations tended to be a fusion gene and quite impossible to be inherited separately.

Conclusion: The two-round nested PCR is an effective method to detect HKαα allele. Besides, our study for the first time revealed the sequence of the HKαα allele, the evidence of the same ancestor with HKαα thalassemia and enriched the composition as well as the formation mechanism of HKαα allele, and immediately opened up novel potential diagnosis and prenatal counseling for HKαα thalassemia.
1 | INTRODUCTION

Thalassemia, the most common monogenic disorder in the world (Weatherall & Clegg, 1996), is caused by genetic defects affecting hemoglobin gene expression. The reported carrier rate of α-thalassemia in tropical and subtropical populations is about 1%, and has plateaued in some areas (Harteved & Higgs, 2010). In Guangxi, China, the total heterozygous frequency of thalassemias and other hemoglobinopathies is 24.51%, in which α-thalassemia accounts for 17.55% (Xiong et al., 2010). Mutations affecting the α-globin gene lead to the pathogenic deficit of the alpha globin electrophoresis tests were conducted on a capillary electrophoresis (CE) device (Capillarys, Sebia, Montpellier, France).

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

Informed consent forms were acquired from all of the participants. This study was approved by the ethics committee of Guilin Medical University, Guilin, Guangxi, PRC.

2.2 | Patients

A pedigree containing 11 individuals (10 adults and 1 unborn fetus) carrying the HKαα thalassemia gene and 4 nongenetic-related patients (S1, S2, S3, and S4) suffering from HKαα was recruited from different areas of Guangxi province, south China. The pedigree with 10 individuals shows no anemic phenotypes, except a patient with -α3.7/-.SEA. The five males and five females in the pedigree developed normally and showed neither skeletal deformity nor hepatosplenomegaly, and none of them ever took any treatment for thalassemia. Besides, the suspicious α2 junction fragment was detected in four nongenetic-related individuals (S1, S2, S3, and S4).

Thus, the samples of S1, S2, S3, and S4 were preserved for the subsequent gene sequence analysis.

2.3 | Collection of samples

Peripheral blood of all of the participants (except the unborn fetus) was collected in EDTA-containing tubes for hematological phenotype analysis. Genomic DNA was extracted to determine their thalassemia genotypes. A quantity of 8 ml of amniotic fluid was collected from the proband's spouse (II-1) for the extraction of the fetal genomic DNA to determine the genotype of the unborn fetus. We failed to obtain the sample from the spouse of II-3 because of some insurmountable difficulties. A magnetic bead adsorption/automatic nucleic acid extraction method (Zhishan Biotechnology) was utilized for DNA extraction.

2.4 | Hematological analysis

Whole blood cell counts were performed on all the participants, except III-2, with an automated cell counter (Model Sysmex F-820; Sysmex Co Ltd, Kobe, Japan), and hemoglobin electrophoresis tests were conducted on a capillary electrophoresis (CE) device (Capillarys, Sebia, Montpellier, France).

KEYWORDS

gene sequence, Hong Kong α thalassemia, pedigree, prenatal diagnosis
2.5 Gene analysis

2.5.1 Detection of deletional genotype of α Thalassemia

The single-tube multiplex polymerase chain reaction (PCR) (Tan, Quah, Low, & Chong, 2001) for four Chinese common deletional α-thalassemia types (-α3.7, -α4.2, -SEA, and -THAI) was performed on the pedigree to determine the deletional genotype of α-thalassemia and the suspicious HKαα allele (Figure 1).

2.5.2 ααanti4.2 allele detection

Polymerase chain reaction for the detection of the ααanti4.2 allele was performed to determine the probability of the presence of HKαα allele and to identify the genotype of HKαα/aa, HKαα/α3.7, and -α3.7/αα as all of them show similar results in conventional detection methods of deletional genotypes of α-thalassemia. The primer sequences and the PCR reaction were performed as described by Wen et al. (2003) (Figure 2).

2.5.3 Two-round nested PCR on the pedigree

The two-round nested PCR on pedigree members was employed to determine the presence of the HKαα allele. The primer sequences and the reaction conditions were same as that described in previous articles (Wang et al., 2005; Tan et al., 2001; Wen et al., 2003; Figure 3).

2.5.4 Detection of point mutation of α- and β-thalassemia

A reverse dot blot (RDB) analysis was performed on the pedigree to detect nondeletional mutations of α- and β-thalassemias (Figure 4).

2.5.5 Two-round nested PCR on unrelated individuals

We redesigned the primer for the first round of the two-round nestedPCR as follows: ZW-F: 5′-CTCGGTAGCCGTTCCTCCTGC-3′, ZW-R 5′-AAGTCTGGGAATAAAACTCGGGA-3′.
FIGURE 4  Results of the reverse dot blot for β- and α-gene mutation detection. The reverse dot blot for β-gene mutation detection showed that I-1 and II-5 were the carriers of β41–42 mutation, while I-2, II-1, II-2, II-3, II-4, II-6, III-1, III-2, and III-3 are negative for β-thalassemia. All the participants are negative in nondeletional α-gene mutation.
The reaction procedure was optimized as follows: initial denaturation at 95°C for 5 min was followed by five cycles: 94°C for 30 s, 64°C for 30 s, decreased 1°C for every cycle, 72°C for 4 min, and then 35 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 4 min, and a final 72°C for 5 min. Primers for round 2 were also optimized as follows: initial denaturation at 95°C for 5 min was followed by five cycles: 94°C for 30 s, 64°C for 30 s, decreased 1°C for every cycle, 72°C for 4 min, and then, 35 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 4 min, and a final 72°C for 5 min (Figure 5).

2.6 Sequencing the genomic DNA

DNA samples of S1, S2, S3, S4, II-3, and III-1 were sequenced using first-generation gene sequencing on ABI (3730XL) to explain the sequence, the structure, the origin, and to explore the probability of simplifying the diagnosis of HKαα thalassemia allele.

2.7 Explanation of HKαα thalassemia allele

The HKαα thalassemia alleles of all six sequenced samples were compared with each other. The HKαα thalassemia allele was separated into three sections: the -α^{3.7} allele, the αααanti4.2 allele, and a special allele.

3 RESULTS

3.1 Recruitment of an unprecedented huge pedigree containing 11 individuals carrying the HKαα thalassemia allele

The proband’s wife (II-1) was diagnosed as the carrier of thalassemia gene -α^{1.2}/αα when pregnant. Next, routine thalassemia screening tests and genetic tests were given to the proband (II-2). Surprisingly, a suspicious α2 junction fragment was discovered besides the -α^{3.7} and -SEA junction fragments. This unexpected result led us to suspect the probable presence of the HKαα allele as reported. To investigate this possibility, to explore the law of the inheritance of this genotype, as well as to provide accurate and effective prenatal counseling regarding the proband’s unborn fetus (III-2), she and 10 other family members were recruited in this research.

3.2 Genotyping the pedigree containing 11 individuals carrying HKαα thalassemia gene

The five males (I-1, II-2, II-5, III-1, and III-3), five females (I-2, II-1, II-3, II-4, and II-6), and the proband’s unborn fetus (III-2) involved in this research were genetically related. First, we conducted the RDB analysis and found that I-1 and II-5 were carriers of the CD 41–42(-CTTT) mutation (Figure 4). Moreover, none of the individuals in this pedigree carried a nondeletional α-thalassemia gene mutation (Figure 4).

To detect the four common deletional types of α-thalassemia, we used single-tube multiplex PCR analyses. We found the presence of an anomalous fragment in II-2 and II-3, who were also positive for -α^{SEA} and -α^{3.7} alleles, as well as in III-1 and III-2 who were also positive for -α^{3.7} and -α^{4.2} alleles (Figure 1). Then, the αααanti4.2 junction fragment detection assay demonstrated that I-1, II-2, III-1, III-2, II-3, and III-3 were positive for the αααanti4.2 allele (Figure 2), which implied that these individuals were carriers of HKαα thalassemia gene. The subsequent two-round nested PCR proved that I-1, II-2, III-1, II-3, and III-3 were carriers of the HKαα allele. Taken together, the genotypes of the patients are listed as follows: I-1 positive for both HKαα/-α^{3.7} and β^{41-42/βN}, II-2 with -α^{SEA}/αα, II-1 with -α^{4.2}/αα, II-2 with HKαα/-SEA, II-3 with HKαα/-SEA, II-4 with αααanti4.2, II-5 with both-α^{3.7}/αα and β^{41-42/βN}, II-6 with normal ααα/αα, III-1 with HKαα/-α^{4.2}, III-2 with HKαα/-α^{4.2}, and III-3 with an uncertain genotype.
3.3 | Correlation of genotypes to phenotypes in the pedigree of 11 Individuals with HKαα thalassemia

Patients II-2, III-1, and II-3 with only HKαα/-SEA or HKαα/-α4.2 presented α-thalassemia trait. I-1, who was the carrier of both HKαα/-α3.7 and β41-42/βN, showed a typical β-thalassemia trait characterized by increased HbA2 (6.4%) and microcytic hypochromic anemia with mild reduction in mean cell volume (MCV), mean cell hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Besides, the genotype of the unborn fetus was HKαα/-α4.2, and its phenotype was theoretically identical to that of III-1 who shares the same thalassemia genotype with the unborn fetus (Table 1, Figure 6).

3.4 | HKαα gene sequence

The two-round nested PCR proved II-3, III-1, S1, S2, S3, and S4 are carriers of HKαα (Figure 5). Sequence analyses were performed. HKαα gene sequence data were provided in supplemental data. Specifically, sequence analysis showed that the sequences of the HKαα allele of II-3, III-1, S1, S2, S3, and S4 were highly similar. We cut this sequence into three segments: -α3.7 allele, αααanti4.2 allele, and the special allele about 802 base pairs, except the sequences we failed in detecting. The comparison between the gene sequence of the whole human genome and the special allele of 802 base pairs indicated that this 802 base pairs could be divided into three regions: the upstream region shared the same sequence with that on alpha globin gene ranging from 171,116 to 171,235, the downstream region was consistent with that on alpha globin gene ranging from 171,223 to 171,882, and astride them is the sequence we failed in detecting (NCBI Reference Sequence: Ng_0000016.10) (Figure 7).

4 | DISCUSSION

The human hemoglobin is a tetramer protein comprising a pair of α globin chains and a pair of β globin chains, encoded by the α globin genes located on the chromosome 16p13.3 and β globin genes located on the chromosome 11p15.3, respectively. α-thalassemia, in which the -α3.7, -α4.2, and --SEA deletion are commonly detected in south China, mainly arises from the deletion of α-globin genes. The α-globin gene sequence on the α-gene cluster can be described as: 5'-ζ-ψζ1-ψα2-ψα1-α2-α1-θ-3', in which both α1 and α2 genes including X, Y, and Z hemoglobin boxes are embedded within a homologous region. The -α3.7 gene type is caused by a reciprocal recombination between Z segments, and thereby contributes to a functional
α gene and an α-triplication. Similarly, the recombination between X boxes can lead to the \(-\alpha^{4.2}\) gene type (Harteveld & Higgs, 2010). The HK\(\alpha\alpha\) allele contains an \(\alpha_1\)-\(\alpha_2\) fusion gene \(-\alpha^{3.7}\) and an intact \(\alpha_2\) gene (Wu et al., 2015a). Although some mechanisms have been proposed to explain the origination of the HK allele (Wang et al., 2005), there is no certain conclusion for it yet.

As confirmed in the two-round nested PCR, I -1 was a carrier of HK\(\alpha\alpha\) thalassemia. Thus, the negative result revealed by four deletional α-thalassemia detection assays could be explained by the possibility that the \(\alpha_2\) gene band serving as the reminder of HK\(\alpha\alpha\) allele was concealed by the normal \(\alpha\) band. Hence, theoretically, there could be three possible genotypes of I -1 including HK\(\alpha\alpha\)/\(\alpha\alpha\), HK\(\alpha\alpha\)/\(-\alpha^{3.7}\), and HK\(\alpha\alpha\)/HK\(\alpha\alpha\). As shown in Figure 6, II -2, II -3, III -1, III -2, and III -3 were all positive for HK\(\alpha\alpha\) allele. Although this genotype includes both \(\alpha\alpha\alpha^{anti4.2}\) and \(-\alpha^{3.7}\), it is quite impossible that \(\alpha\alpha\alpha^{anti4.2}\) and \(-\alpha^{3.7}\) are inherited separately. Furthermore, the sequencing results showed that the HK\(\alpha\alpha\) allele in S1, S2, S3, S4, II -3, and III -1 are quite consistent with each other, indicating that HK\(\alpha\alpha\) allele is highly stable as a fusion gene. As II -4 is a heterozygote of \(-\alpha^{3.7}\) deletion and \(-\text{SEA}\) deletion, the genotype of II -2 is \(-\text{SEA}/\alpha\alpha\). As the \(\alpha\alpha\alpha^{anti4.2}\) and \(-\alpha^{3.7}\) mutations in HK\(\alpha\alpha\) allele are not possible to inherit separately, it could be deduced that I -1 is the carrier of \(-\alpha^{3.7}\) allele. Therefore, the genotype of I -1 could be HK\(\alpha\alpha\)/\(-\alpha^{3.7}\). Similarly, the genotype of III -3 relies on the determination of II -3’s spouse’s genotype. Our future research will focus on distinguishing HK\(\alpha\alpha\)/\(\alpha\alpha\), HK\(\alpha\alpha\)/\(-\alpha^{3.7}\), and HK\(\alpha\alpha\)/HK\(\alpha\alpha\).
The two-round nested PCR is a reliable method to detect the presence of HKαα allele, and the pedigree analysis should be employed to determine the precise genotype. As the carrier rate of HKαα allele in Guangxi is 0.07% (Shang et al., 2013), and it is prone to be misdiagnosed as -α 3.7/αα mutation, the actual carrier rate of HKαα allele in Guangxi may be much higher. We suggest more attention should be paid to the diagnosis of HKαα thalassemia. Accordingly, ααα anti4.2 allele detection assay should be performed once a patient is identified positive for -α 3.7 deletional type. Furthermore, to identify whether the ααα anti4.2 and -α 3.7 are present on the same or two different α genes, the two-round nested PCR should be performed if the presence of ααα anti4.2 allele is confirmed.

β-Thalassemia is characterized by microcytic hypochromic anemia as well as increased HbA2. Either blood routine examination or hemoglobin electrophoresis is hitherto the main method for its screening. Thus, the patients with both HKαα thalassemia and β-thalassemia are prone to be diagnosed as β-thalassemia only by routine tests. Typical β-thalassemia minor may interfere with the diagnosis of HKαα thalassemia. For example, in the pedigree shown in Table 1, I-1 is positive for both HKαα/ααα anti4.2 and β41-42/βN. Its routine blood test revealed microcytic hypochromic anemia with MCV 64.9 fl, MCH 19.2 pg, MCHC 296 g/L, and an increase in HbA2(6.4%). However, the possibility of HKαα thalassemia would not be usually considered based on these testing results.

Since there is no change in the abundance of the α gene in HKαα allele (Shang et al., 2013; Wang et al., 2005; Wu et al., 2015b). The clinical presentation of II-3 and II-2 is much milder than that of II-4 as shown in Table 1. II-3, who was positive for HKαα/α3.7/αα, is associated with minor thalassemia symptoms with red blood cell 5.85 × 1012, HGB 123 g/L, MCV 70 fl, MCH 21 pg, and MCHC 296 g/L. Similarly, II-2, with the same genotype as II-3, presents minor thalassemia features with RBC 6.98 × 1012, HGB 139 g/L, MCV 64.6 fl, MCH 19.9 pg, and MCHC 308 g/L. However, II-4, who is positive for -α 3.7/αα, shows the symptoms of HbH disease with RBC 5.38 × 1012, HGB 90 g/L, MCV 58.6 fl, MCH 16.7 pg, and MCHC 286 g/L. Finally, the unborn fetus is unlikely to suffer from any deleterious effects though its genotype was HKαα/α1.2, which is identical to that of III-1 (Table 1). In addition, based on the fact that there is no change in the abundance of α gene in HKαα allele (Wu et al., 2015c), the clinic symptoms of the unborn fetus could also be predicted to be similar to those of individuals with -α 1.2/αα.

The sequencing results confirmed that HKαα allele in II-3, III-1, S1, S2, S3, and S4 are quite similar, which strongly indicates that individuals with HKαα thalassemia inherit the allele from the same ancestor since III-1, S1, S2, S3, and S4 were not genetically related. Although this opinion was also proposed by Shang et al. (2013), we are the first to describe the sequence of HKαα allele and to offer the convincing evidence.

As is mentioned above, the sequenced HKαα allele can be divided into three regions: ααα anti4.2 allele, -α 3.7 allele, and between them is the upstream region which shares the same sequence with that on alpha globin gene ranging from 171,116 to 171,235, the downstream region which is consistent with that on alpha globin gene ranging from 171,223 to 171,882 (NCBI Reference Sequence: Ng_0000016.10) and astride them are the sequences we failed in detecting. This implies a complicated formation mechanism of HKαα allele and points out the need for further investigation.

Although the two-round nested PCR is an effective method to identify HKαα allele, it is time-consuming and cannot be commonly used in clinical diagnosis. As we have already revealed the sequence of HKαα precisely, we will continue to analyze its sequence and try to discover unique base pairs as its marker, so that we can simplify the detection of HKαα thalassemia.

In conclusion, a rarely huge pedigree of HKαα thalassemia is reported and an effective prenatal counseling is offered to this family. Furthermore, ααα anti4.2 detection assay is once again proposed to screen for HKαα allele in the patients positive for -α 3.7 mutation by our current studies. The relationship between genotypes and phenotypes of HKαα thalassemia was also analyzed here for clinicians to avoid the possible misdiagnosis. More importantly, our study for the first time repored the sequence of the HKαα allele, the evidence of the same ancestor with HKαα thalassemia and enriched the composition, as well as the formation mechanism of HKαα allele. These findings have significantly advanced our understanding of HKαα thalassemia and immediately provided a novel potential diagnosis and prenatal counseling approach for HKαα thalassemia.

**CONTRIBUTORSHIP STATEMENT**

Chunjiang Zhu, Haiqing Zheng, Dan Zeng, Donglan Yu, and Qiao Feng collected the samples. Wenjuan Wang and Chunjiang Zhu designed the research, conducted the research, and analyzed the research results. Wenjuan Wang wrote the paper. Chunjiang Zhu, Wenjuan Wang, Yang Xia, and Yuzhong Yang revised the paper. Linbin Jiang provided the technical support.

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**CONFLICT OF INTEREST**

The authors declare no competing financial interests.
DATA AVAILABILITY STATEMENT
All data involved in this manuscript are available.

ORCID
Wenjuan Wang https://orcid.org/0000-0002-6634-3650

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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