Molecular characterization and clinical presentation of HKαα and anti-HKαα alleles in southern Chinese subjects


The HKαα allele is a rearrangement occurring in the α-globin gene cluster containing both the -α3.7 and ααα anti4.2 unequal crossover junctions. The anti-HKαα allele is the reciprocal product containing both the -α4.2 and ααα anti3.7 unequal crossover junctions, which had been predicted but had not been detected previously. The phenotypic feature and population frequency of these two unusual alleles were not described. We report the identification of nine individuals carrying the HKαα allele and two individuals carrying the anti-HKαα allele in southern China and describe their phenotype and haplotype data. The molecular structures of HKαα allele and anti-HKαα allele were confirmed by two-round nested polymerase chain reaction assay. The mechanism of origin of both alleles is related to probably simultaneous double crossover. Heterozygotes of HKαα or anti-HKαα allele show a normal hematological phenotype. Finally, we report the carrier rates of these both alleles in the Guangxi Zhuang Autonomous Region of southern China, namely, ~0.07% for the HKαα allele and ~0.02% for the anti-HKαα allele.

Conflict of interest
Authors report no conflicts of interest.

α-Thalassemia is one of the most common inherited disorders worldwide (1). It occurs mainly in the tropical and sub-tropical regions of the world, including southern China, where the carrier rate of α-thalassemia is 8.53% in Guangdong (2) and 17.55% in Guangxi (3). α-Thalassemia is due to a deletional or non-deletional mutation occurring in the human α-globin cluster. This gene cluster is arranged as follows: 5′-α2-Ψα1-Ψα2-Ψα1-α2-α1-α3′ (4). The α2 and α1 genes have a similar structure, comprising three homologous segments (the X, Y, and Z boxes) that are punctuated by non-homologous regions (I, II, and III) (5).

In the southern China population, -α3SEA, -α3.7, and -α4.2 are the most common α-thalassemia mutations (2, 3, 6). Other α-thalassemia deletional mutations such as -α11.1 (7) and -α27.6 (8) and the α-globin gene triplexion (ααα anti3.7 and ααα anti4.2) have also been detected (8, 9). Deletional or triplicated alleles are the products of either homologous or non-homologous recombination. Common causes of unequal crossover that occur between the α2 and α1 globin genes generates single α-globin gene deletions (-α3.7 and -α4.2) and their reciprocal triplicated alleles (ααα anti3.7 and ααα anti4.2) that can be screened by Gap-PCR (10). In addition, more complex crossover events occur in this cluster, such as the quadruplicated allele (αααα) (11–13), ‘patchwork’ α2 and α1 genes (α212 and α121) (14) and the HKαα allele (15, 16). The HKαα allele is a rearrangement containing both the -α3.7 and ααα anti4.2 unequal crossover junctions, and has been reported only twice (15, 16). Information regarding the detailed phenotypic and population genetic elements of the
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HKαα allele are sparse. Furthermore, the anti-HKαα allele, a reciprocal product of the HKαα allele, has been previously predicted but not proven (15). The anti-HKαα allele is a rearrangement containing both the -α3.7 and αααα3.7 unequal crossover junctions. Here, we report nine individuals carrying the HKαα allele and two individuals carrying the anti-HKαα allele, and describe their phenotype and haplotype data. To our knowledge, this is the first report of the anti-HKαα allele and only the third report of the HKαα allele. The functional effect and origin of mechanism of these alleles is discussed, and the carrier rate of both alleles in Guangxi population is reported.

Materials and methods

Samples and hematologic analysis

Cases 2, 8, 9, and 11 (Table 1) were diagnosed with genotype of HbH disease (-α3.7 / - SEA or -α4.2 / - SEA) during routine α-thalassemia examination in their local hospitals, but did not exhibit symptoms of HbH disease. They were referred to our laboratory for further investigation. The pedigree of the family of case 2 is shown in Fig. 1a. Samples of cases 3–7 and 11 were screened from our stored samples. All individuals tested were the local people of Guangxi province in southern China. All subjects gave written informed consent.

Fresh peripheral blood were collected and hematologic data were determined by automated cell counting (Model Sysmex F-820; Sysmex Co. Ltd, Kobe, Japan). The levels of HbA2 were analyzed on the Bio-Rad Variant II HPLC system (HPLC, VARIANT™, Bio-Rad, Hercules, CA94547).

Molecular diagnosis

Genomic DNA was extracted using standard phenol/chloroform methods. The seven-deletion multiplex polymerase chain reaction (PCR) assay for common α-thalassemia mutations was performed as previously described methods (10). The two-round nested PCR strategy to confirm HKαα allele was carried out as described previously (15) but with some modification of the primers. The first round PCR primers were: L-anti4.2F (5’-CCCTTGACCCGGCCCTTCTGGTC-3’; NG_000006.1 34525 → 34547) and L-3.7R (5’-CTCTAAAGCACTCTAGGGTCCAGCG-3’; NG_000006.1 36107 → 36128) for -α4.2 allele and only the third report of the HKαα allele. The primers used in the second round nested PCR are labeled in Fig. 2.

Total cellular RNA was isolated using Trizol reagent (Gibco BRL, Gaithersburg, MD). Complementary DNA (cDNA) synthesis was performed using the First Strand cDNA Synthesis Kit (Toyobo Co., Ltd., Osaka, Japan). mRNA levels of both the mutant and wild-type α-globin alleles were measured using SYBR Green-based relative quantitative reverse transcriptase (RT)-PCR and β-globin served as a control for assessment of equivalent RNA loading as in our previous protocol (8). Three independent tests were conducted for each sample with four different α-globin genotypes (I1, I2, I3, and a normal person) in order to calculate the mean mRNA concentration.

Single-nucleotide polymorphisms (SNPs) in the α-globin cluster (NG_000006.1, 43058 bp) including 26719G/C(rs2858935), 27606C/A(novel), 29599A/G(rs2541675), 31921T/C(rs2974771), 33004C/T(rs2541669), 38757T/C(rs3760046), and 42405C/T(rs1203834) were analyzed in this study. The detection of SNPs was carried out by SnaiPhost assay using an Applied Biosystems Multiplex kit (Invitrogen, Shanghai, China). All seven SNPs were used to infer the haplotype using the PHASE 2.1.1 program.

Carrier rate analysis

In our previous study of the frequency of hemoglobinopathies among Guangxi populations, 5789 blood samples were obtained (3) and screened for α-thalassemia. Among these samples, 273 -α3.7 carriers and 91 -α4.2 carriers had been identified according to previous results (3). Since individuals carrying the HKαα allele must be positive for the -α3.7 mutation in routine thalassemia mutation analysis, we performed two-round nested PCR around the HKαα allele in 273 -α3.7 carriers. A similar strategy was used to screen for anti-HKαα allele. This resulted in cases 4–7 and case 10 identified with the HKαα allele and the anti-HKαα allele, respectively.

Results

Molecular characterization of HKαα and anti-HK allele

In the seven-deletional multiplex-PCR assay, cases 2, 8, and 9 were identified as positive for the -α3.7, - SEA, and the normal allele and case 11 was identified as positive for the -α4.2, - SEA, and the normal allele (Fig. 1b). A subsequent analysis showed that cases 2, 8, and 9 were also positive for the ααααα3.7 allele and case 11
was positive for the $\alpha\alpha^{\text{anti}3.7}$ allele (data not shown). On the basis of these results and those from previous reports (15, 16), we deduced that cases 2, 8, and 9 might carry HK$\alpha$ allele and case 11 might carry anti-HK allele. The same two-round nested PCR strategy used to confirm HK$\alpha$ allele was carried out. As expected, the first-round PCR generated a $\sim$4.0 kb PCR amplicon, and the $-\alpha^{3.7}$, and $\alpha\alpha^{\text{anti}4.2}$ junction fragments were observed in the second round of PCR in cases 2, 8, and 9 (Fig. 1c). The anti-HK$\alpha$ allele was confirmed using a similar two-round nested PCR strategy. A $\sim$4.3 kb fragment was obtained in the first round of PCR, and the $-\alpha^{4.2}$ and $\alpha\alpha^{\text{anti}3.7}$ junction fragments were detected in the second round of PCR (Fig. 1d). Other samples were shown by the same method to carry HK$\alpha$ and anti-HK$\alpha$ alleles (Fig. 1). The genotypes of all samples are summarized in Table 1. Cases 3–7 were classified as heterozygotes of HK$\alpha$ allele by two-round nested multiplex-PCR but were identified as heterozygotes of the HK$\alpha$ allele by two-round nested PCR. The $-\alpha^{4.2}$ and $\alpha\alpha^{\text{anti}3.7}$ junction fragments were detected in the second round of PCR. Carriers of HK$\alpha$ allele were identified as positive for anti-HK$\alpha$ allele by two-round nested multiplex-PCR but were identified as a heterozygote of the anti-HK$\alpha$ allele by two-round nested PCR.

### Phenotypic features of HK$\alpha$ and anti-HK allele

Table 1 summarizes the hematological data of samples from HK$\alpha$ allele or anti-HK$\alpha$ allele individuals. Samples with the HK$\alpha$/HK$\alpha$ genotype (cases 1 and 4–7) or the anti-HK$\alpha$/HK$\alpha$ genotype (case 10) displayed normal hematological phenotype. Case 3 is heterozygous for HK$\alpha$ and $\beta$-thalassemia, and exhibited symptoms of the $\beta$-thalassemia trait with reduced mean cell volume (MCV), mean cell hemoglobin (MCH), and increased HbA$_2$ level. Individuals with HK$\alpha$/- _SEA_ genotype (cases 2, 8, and 9) or anti-HK$\alpha$/- _SEA_ genotype (case 11) presented with typical $\alpha$-thalassemia trait symptoms, similar to individuals previously reported (3). The slightly lower Hb levels of cases 8 and 9 are possibly due to pregnancy. To verify the functional consequences of the HK$\alpha$ genotype, we measured the $\alpha/\beta$ mRNA expression level ratio in family members of case 2 using quantitative RT-PCR. When the normal control ($\alpha\alpha/\alpha\alpha$) was defined as 1.0, the mean relative $\alpha$-globin mRNA level was as follows: 1.01 ± 0.189 in the I1 (HK$\alpha$/HK$\alpha$), 0.45 ± 0.028 in II2 (HK$\alpha$/- _SEA_), and 0.51 ± 0.063 in II3 (alk/alk _SEA_). This result demonstrates that the $\alpha$-globin mRNA level of the HK$\alpha$ allele heterozygotes is similar to that of normal individuals. cDNAs from samples of individuals carrying the anti-HK$\alpha$ allele were not available.

### Carrier rate of HK$\alpha$ and anti-HK$\alpha$ allele in Guangxi population

Among the 5789 school children previously collected in Guangxi for a molecular epidemiological survey of hemoglobinopathies (3), four subjects (cases 4–7) were identified as positive for HK$\alpha$ allele and one subject (case 10) was positive for anti-HK$\alpha$ allele. The carrier rate in the Guangxi population was determined to be $\sim$0.07% (4/5789) for the HK$\alpha$ allele and $\sim$0.02% (1/5789) for the anti-HK$\alpha$ allele.

We next investigated the haplotype data of the HK$\alpha$ allele. Seven samples (cases 1 and 4–9) came from different cities of Guangxi and they had no genetic relationship, SNP analysis demonstrated that the haplotype of all chromosomes carrying the HK$\alpha$ allele was identical. The HK$\alpha$ allele was discovered to be linked to the haplotype GCATCTC.

### Discussion

Here, we report nine individuals with the HK$\alpha$ allele. In addition to those genotypes reported in previous cases (15, 16), our cases include a new genotype,
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Fig. 1. Pedigree of the Guangxi family, molecular characterization of the HKαα allele and anti-HKαα allele by polymerase chain reaction (PCR) assay. (a) Pedigree of the Guangxi family. (b) Results of the seven-deletional multiplex-PCR assay. The -α3.7, -αSEA junction fragments and α2 gene were detected in cases 2, 8, and 9. The -α4.2 and α2 gene were detected in cases 1, 3–7. The -α4.2, -αSEA junction fragments and α2 gene were detected in case 11. LIS1, positive control fragment. (c) Results of the two-round nested PCR assay to detect the HKαα allele. The first round PCR generated a fragment of ∼4.0 kb in all samples (top panel). In the -α3.7 nested PCR, a fragment of ∼2.0 kb was observed only in cases 1–9 (middle panel). A similar result is observed in the ααanti4.2 nested PCR, while a fragment of ∼3.0 kb is observed in the same nine individuals (bottom panel). (d) Results of the two-round nested PCR assay to detect the anti-HKαα allele. The first round PCR generated a fragment of ∼4.3 kb in all samples (top panel). In the ααanti3.7 nested PCR, a fragment of ∼2.2 kb was observed only in cases 10 and 11 (middle panel). A similar result is observed in the -α4.2 nested PCR, while a fragment of ∼1.6 kb is observed in the same two individuals (bottom panel).

Fig. 2. Mechanism of origin of the HKαα allele and anti-HKαα allele. Simultaneous double crossover generates both alleles, one crossover between the X2 and X1 boxes and the other between the Z2 and Z1 boxes. The primers used in the two-round nested polymerase chain reaction are indicated by arrows (the scale is not correct).

heterozygosity for HKαα without β-thalassemia. We also found two individuals with the anti-HKαα allele. The genotype of one is anti-HKαα/-αSEA, and that of the other genotype is anti-HKαα/αα. According to the hematological data of our cases (Table 1) and those of previous cases, individuals with the HKαα/αα or anti-HKαα/αα genotypes are asymptomatic, while individuals with the HKαα/-/αSEA or anti-HKαα/-/αSEA genotypes present with typical α-thalassemia symptoms as individuals with αα/-/αSEA genotype. Individuals with the HKαα/aα and β17/βN genotypes presented with β-thalassemia trait symptoms. No evident clinical impact was found between both alleles and the wild-type αa allele. Real-time PCR analysis failed to detect differences in α-globin mRNA levels between the HKαα/aα and αa/aα genotypes. These results indicate therefore that the HKαα or anti-HKαα alleles contain two functional α-globin genes and should be regarded as ‘silent alleles’. In addition, the HKαα allele was found to be linked to a certain haplotype GCATCTC (Table 1). The same geographical and haplotype backgrounds of these carriers have strongly indicated that all the HKαα alleles we detected should arise from a same rearrangement occasion. However, among 38 normal subjects in southern Chinese people, 10 cases were found carry this haplotype (data not shown), which indicated that GCATCTC is a common haplotype and could not be used as mark of HKαα allele. Because Guangxi has a very high frequency of α-thalassemia, particularly in the local minority populations (3, 17), we deduced that polymorphism in the α-globin globin cluster linked to
α-thalassemia determinants such as HKαα allele was not a rare phenomenon in Guangxi people.

Several mechanisms of origin have been proposed for the HKαα allele, but have not been proven (15). Our results showed that several unrelated carriers of the HKαα allele from different region of Guangxi probably have the same haplotype background (Table 1), strongly suggesting that these individuals might have inherited the HKαα allele from the same ancestor, or that the HKαα allele might be a ‘younger’ allele originating more recently. It also suggested that these HKαα alleles should arise from the same mechanism. The most likely mechanism for generation of the HKαα allele was initially thought to be unequal crossover between the X1 box of a wild-type allele and the X2 box of a -α3.7 allele, generating the HKαα derivative and its reciprocal -α4.2 derivative (15). However, as we have detected the anti-HKαα allele in this study, we propose that an alternative mechanism is simultaneous double crossover between misaligned X and Z boxes generating the HKαα derivative and its reciprocal anti-HKαα derivative (Fig. 2).

Routine PCR-based assays for α-thalassemia common deletional mutation have been widely used in local hospitals in southern China for α-thalassemia diagnosis. Since this routine analysis includes diagnosis of -SEA, -α4.2, and -α2.0, but not of αααα3.7 and ααααα4.2, individuals with HKαα/αα or anti HKαα/αα genotype will be diagnosed as -α3.7/αα or -α4.2/αα. This will result in inaccurate prenatal diagnosis in the case of a couple in whom the genotype of one individual is HKαα/αα or anti-HKαα/αα, and that of the other is αα/ -SEA. Considering the carrier rate of HKαα (0.07%) and anti-HKαα (0.02%) in Guangxi population, the potential for misdiagnosis cannot be ignored, and the current diagnostic strategy should be modified accordingly in order to improve accuracy either in the clinic or in large-scale population screening. In the case of clinical screening, PCR-based testing for triplication should be included during routine analysis. If a sample is positive for both -α3.7 and αααααα3.7, or positive for both -α4.2 and αααααα4.2, two-round nested PCR should be performed for further confirmation. In the case of large-scale population screening, two-round nested PCR should be performed in the samples classified as carrying -α3.7 or -α4.2 by routine α-thalassemia deletional mutation analysis. In addition, the non-correlation of genotype data with phenotype data could suggest the misdiagnosis of genetic tests. For example, individuals with HKαα/-SEA or anti-HKαα/-SEA genotype will be diagnosed as -α3.7/-SEA or -α4.2/-SEA, but they will have α-thalassemia trait hematological data (Hb > 100 g/l) instead of Hb H disease hematological data (Hb < 100 g/l). Therefore, the importance of phenotype analysis should be stressed in α-thalassemia diagnosis in order to decrease the misdiagnosis caused by using molecular methods only.

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