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Original Article

A rare α-thalassemia deletion, -α$^{27.6}$, is identified from three Chinese families in Fujian province

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Abstract: Alpha (α)-thalassemia (thal) is a common single-gene genetic disease in southern China, which may cause Hb Bart's hydrops fetalis and Hb H disease. In α$^+$ thal, one of the α genes is inactivated, due either to a deletion (-α) such as the rightward deletion (-α$^{3.7}$) and leftward deletion (-α$^{4.2}$), or to another form of mutation (α^{T}α), such as the Hb Constant Spring (α^{CS}α). In this study, three probands from three Chinese families in Fujian Province showed HbH disease traits, while their common genotypes of α-thal were --^{SEA}/--^{SEA} at a routine analysis. In doing so, we further found a rare 27.6 kb deletion on the α-globin gene cluster in the three probands by MLPA (multiplex ligation-dependent probe amplification) and Sanger DNA sequencing, and its breakpoints were detected to lie between coordinates 9079 and 36718 with a total of 27,640 nucleotides deletion. The accurate genotypes of the three probands were -α$^{27.6}$/--^{SEA}, which led to a very mild hemoglobin H (HbH) disease phenotype with low MCV, MCH, and HbA2 levels. Phenotypic analysis on the heterozygote of this deletion revealed it as α$^+$ mutation. In conclusion, we report a rare α-thal deletion, -α$^{27.6}$, in three Chinese families from Fujian Province. Our study extends the spectrum of the α-thal mutation in the Chinese population. It is necessary to increase awareness of the rare α-thal mutation and to increase its detection, which will prove valuable in genetic counseling and prenatal diagnosis in Fujian Province.

Keywords: α-thal, α-globin, deletion, -α$^{27.6}$, HbH

Introduction

Thalassemia (thal), characterized by a quantitative reduction of the α- or β-globin chains, is a common, single-gene genetic disease in southern China, especially in Guangxi, Guangdong and Fujian provinces [1, 2]. Approximately 80 ~90% of all α-thal is caused by genomic deletions involving the α-globin gene cluster on chromosome 16p13.3 [3, 4]. The α-globin gene cluster, in which the order is HS40-ζ2-Ψζ1-Ψα2-Ψα1-α2-α1-θ, consists of a major regulatory element (HS-40), α genes (α1and α2), an embryonic gene (ζ2), pseudo genes (Ψζ1, Ψα2 and Ψα1), and θ [5]. Usually, the α-thal pathogenic genotypes are divided into deletion types and non-deletion types. The common deletions in α-globin gene clusters in China are --^{SEA}, -α$^{3.7}$, -α$^{4.2}$, and --^{THAI}, while the common non-deletions are CS, QS, and WS [1, 6].

There are two functional α genes (α α) in one chromosome, and two types of mutations are defined: α^0 and α$^+$ thal. In α$^+$ thal, one of the α genes is inactivated, due either to a deletion (-α), such as the rightward deletion (-α$^{3.7}$) or the leftward deletion (-α$^{4.2}$), or to another form of mutation (α^{T}α), such as the Hb Constant Spring (α^{CS}α). In α^0 thal, both α genes on one chromosome are deleted or otherwise inactivated (--), such as the Southeast Asian deletion (--^{SEA}). Besides the most common deletions, a large variety of less frequently occurring α-thal deletions have been found in different populations [7, 8]. More than 40 different types have been described up to now. Among them, 10 types were found in China. Recently, α-thal genotypes of a deletion type which causes α$^+$ thal have been discovered throughout the world, including -α$^{16.6}$, -α$^{7.9}$, (α)α$^{5.3}$, -α$^{3.5}$, -α$^{2.7}$ and -α$^{2.4}$ [9-12].

Here, we identified a rare gross deletion (-α$^{27.6}$) in α-globin gene cluster from three Chinese families. We detected the details of the breakpoints of this deletion using Multiplex Ligation-dependent Probe Amplification (MLPA) and

Table 1. The clinical characteristics of three probands

Group	Sex	Age (years)	Hb (g/dL)	MCV (fL)	MCH (pg)	HbA2 (%)
Proband 1	Female	39	9.6	51.3	15.2	1.5
Proband 2	Male	6	8.12	53.2	19.8	1.6
Proband 3	Female	30	7.8	60.6	16.7	1.5

Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; HbA2: human hemoglobin, alpha 2.

Sanger DNA sequencing. The present study is, to the best of our knowledge, the first to report on the -α27.6 deletion from three Chinese families in Fujian Province. Our study extends the spectrum of the α-thal mutation in the Chinese population.

Materials and methods

Subjects

The current study was conducted between April 2015 and December 2015 at Fujian Key Laboratory for Prenatal Diagnosis and Birth Defect (Fuzhou, China). The three probands were from three Chinese families in Fujian Province of the People's Republic of China, and each showed a mild anemia. The clinical characteristics of probands are shown in **Table 1**. The study was approved by the Ethic Committees of Fujian Provincial Maternity and Children's Hospital (Fuzhou, China). Consent forms were signed by all patients and the research was conducted in accordance with the regulations of Declaration of Helsinki.

Hematological analysis

A fasting venous blood sample from each participant was obtained and taken in EDTA-contained tubes (Qiagen Inc., Valencia, CA, USA). The hematologic data were determined by automated cell counting (XS-800i; SysmexCo. Ltd., Japan). The levels of HbA, A2, and HbF were analyzed on the Bio-Rad Variant II HPLC system (HPLC, VARIAN™, Bio-Rad, USA). The diagnosis of α-thal is based on microcytosis (mean corpuscular volume (MCV) 80.0 fL, mean corpuscular Hb (MCH) 27.0 pg and normal lower Hb A2 level (2.5%).

DNA isolation

5 ml of peripheral blood in each sample was collected in our hospital. DNA was extracted from the peripheral blood leukocytes using a commercially available DNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted genomic DNA was quantified by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) to ensure that the DNA concentration was >100 ng/μl and that the optical densities were at 260/280 nm of 1.8~2.0. The genomic DNA was then stored at -80°C for further experiments.

Gap-polymerase chain reaction (gap-PCR)

Gap-PCR was used to screen for the most common deletional mutations of a-thal (--SEA, -α3.7, -α4.2, and --THAI) observed in the Chinese population. Gap-PCR conditions were performed as follows: each 50 mL reaction contained 200 mM of each dNTP, 1.5 mM MgCl2, 1xQ-solution (Qiagen GmbH), 2.5 U HotStarTaq DNA polymerase in a supplied reaction buffer (Qiagen GmbH), 100 ng of genomic DNA, and primers at various concentrations. Reactions were conducted in a T3 thermal cycler (Biometra GmbH, Germany), with an initial 15 min. denaturation at 96°C, followed by 30 cycles at 95°C denaturation for 30 seconds, 60°C annealing for 90°C seconds, and extension at 72°C for 2 min and 15 seconds. A final extension at 72°C for 5 min. 1.5% agarose gel electrophoresis was applied to detect PCR amplified results.

Reverse dot-blot hybridization (RDBH)

RDBH was used for the three non-deletional types of α-thal mutations (αQSα/, αCSα/, and αWSα/) and 17 known β-globin gene mutations {codons 41/42 (-TCTT), IVS-II-654 (C>T), _28 (A>G), codons 71/72 (tA), codon 17 (A>T), Hb E [β26 (B8) Glu-Lys, GAG>AAG or codon 26 (G>A)], codon 31 (–C), codons 27/28 (tC), codon 43 (G>T), _32 (C>A),_29 (A>G), _30 (T>C), codons 14/15 (tG), Cap t40 to t43 (-AAAC), initiation codon (T>G), IVS-I-1 (G>T) and IVS-I-5 (G>T). Briefly, the membrane-based DNA array was pre-incubated with a hybridization buffer at a hybridization temperature for 2 h and subsequently incubated in a hybridization buffer with the addition of 2 ng μ/L denatured PCR products at hybridization temperature. After removing the redundant single-strand products by washing in a series of buffers, the membrane strips were incubated with Ab-Dig conjugated with alkaline phosphatase. After washing with a

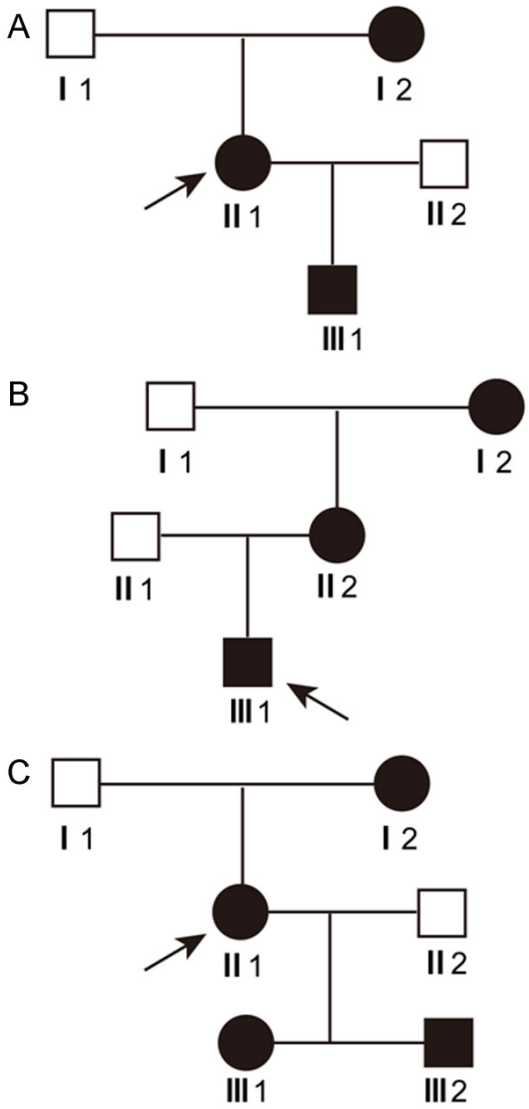

Figure 1. Pedigree of the three Chinese families. A: family 1; B: family 2; C: family 3. The arrows represent the probands.

series of washing buffers, the strips were incubated with NBT and BCIP to produce colored dots.

Multiplex ligation dependent probe amplification (MLPA) assay

An MLPA assay was performed to detect the unknown deletion in the α-globin gene cluster using the SALSA MLPA kit (MRC-Holland, the Netherlands) according to the manufacturer's instructions. The MRC-Coffalyser v.9.4 software (MRC-Holland, the Netherlands) was used as an analysis tool for the normalization of the MLPA data. The primers around the 5' and 3' breakpoints were designed: F1 (5'-AGATCTG AGGTGGCACACAAGCATG-3') and Ry (5'-CTAA-GCCCCAAGTCATGGACTCACAGT-3'). PCR amplified across the deletion following PCR conditions: hotstart of 15 min at 95°C, 35 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 65°C and elongation for 3 min at 72°C, followed by a final elongation step of 10 min at 72°C. The specific breakpoint fragment of 27.6 kb was estimated by agarose gel electrophoresis and identified by Sanger DNA sequencing (Invitrogen, CA, USA).

Statistical analysis

SPSS software version 19.0 (SPSS, Chicago, IL, USA) was applied for statistical analysis. Data were represented as the means ± standard deviation (SD). A value of $P<0.05$ was considered to indicate a statistically significant difference. One-way ANOVA (One-way analysis of variance) or Student's t-test was used to analyze the differences between groups.

Results

The hematological features of the three Chinese families in Fujian province

The pedigrees of the three Chinese families are shown in **Figure 1**. The hematological features of the three Chinese families are presented in **Table 2**. The three probands showed the HbH disease trait, but no HbH or Hb Bart's band was detected. In family 1, the proband, her mother, and son presented with microcytic hypochromic parameters with low MCV, MCH and HbA2 levels, while her husband and father showed no abnormalities. In family 2, the proband, his grandmother, and mother presented with microcytic hypochromic parameters with low MCV, MCH and HbA2 levels, while his grandfather and father showed no abnormalities. In family 3, the proband, her mother, daughter, and son presented with microcytic hypochromic parameters with low MCV, MCH and HbA2 levels, while her father and husband showed no abnormalities. The hematologic data of the probands in the three Chinese families indicated that they were HbH carriers.

Genotypic features of the three Chinese families

RDBH was used for the three non-deletional types of α-thal mutations and β-thal mutations.

Table 2. The hematological features of three Chinese families

Group	Member	Age (year)	Hb (g/dL)	MCV (Ll)	MCH (pg)	HbA2 (%)	HbF (%)
Family 1	Father I1	63	14.4	83.8	28.9	2.4	0.1
	Mother I2	62	11.0	69.5	22.4	2.2	0.3
	Proband II1	39	9.6	51.3	15.2	1.5	0.2
	Husband II2	41	16.3	91.7	30.9	2.7	0.2
	Son III1	16	12.7	68.6	21	2.4	0.2
Family 2	Grandfather I1	57	12.5	84.8	27.5	3.1	0.3
	Grandmother I2	52	13.9	77.7	20.8	2.3	0.4
	Father II1	36	13.8	82.1	28.1	3.3	0.3
	Mother II2	30	11.5	68.2	21.7	2.9	0.3
	Proband III1	6	81.2	53.2	19.8	1.6	0.4
Family 3	Father I1	54	13.9	82.7	29.8	2.9	0.3
	Mother I2	52	14.8	66.1	21.6	2.4	0.5
	Proband II1	30	7.8	60.6	16.7	1.5	0.3
	Husband II2	36	13.8	82.1	28.1	3.3	0.3
	Daughter III1	7	10.7	61.3	18.8	2.7	0.3
	Son III2	1.5	11.9	65.3	19.8	2.6	0.2

Table 3. The genotypic features of the three Chinese families

Group	Member	Genetype	
		α-thal	β-thal
Family 1	Father I1	$--^{SEA}$/αα	N/N
	Mother I2	$-\alpha^{27.6}$/αα	N/N
	Husband II1	αα/αα	N/N
	Proband II2	$-\alpha^{27.6}/--^{SEA}$	N/N
	Son III1	$--^{SEA}$/αα	N/N
Family 2	Grandfather I1	αα/αα	N/N
	Grandmother I2	$--^{SEA}$/αα	N/N
	Father II1	$-\alpha^{27.6}$/αα	N/N
	Mother II2	$--^{SEA}$/αα	N/N
	Proband III1	$-\alpha^{27.6}/--^{SEA}$	N/N
Family 3	Father I1	$--^{SEA}$/αα	N/N
	Mother I2	$-\alpha^{27.6}$/αα	N/N
	Proband II1	$-\alpha^{27.6}/--^{SEA}$	N/N
	Husband II2	αα/αα	N/N
	Daughter III1	$--^{SEA}$/αα	N/N
	Son III2	$--^{SEA}$/αα	N/N

N/N: normal.

The results showed that the non-deletional mutation of the α-thal and β-thal mutations was not found in all members of the three Chinese families. Gap-PCR was used to screen for the most common deletional mutations of a-thal in the three Chinese families. **Table 3** summarizes the genotypic data obtained from the probands and their families. The three probands showed the HbH disease trait, while their common genotypes of α-thal were $--^{SEA}/--^{SEA}$ at routine analysis, suggesting an unknown deletion exists in α-globin gene cluster. These probands inherited this deletion from their father, and their offspring also inherited this deletion.

Identification a rare deletion (-27.6 kb) in the three Chinese families

Based on the MLPA result (**Figure 2**), we identified a new 27.6 kb deletion combined with a common $--^{SEA}$ mutation existing in the three probands. The position of the -27.6 kb deletion combined with the $--^{SEA}$ deletion was shown between probe 346 and probe 400, while the $--^{SEA}$ deletion was shown between probe 184 and probe 400. The 5' breakpoint of this gross deletion was localized between probe 346 and probe 364, a region about 5.7 kb. The 3' breakpoint was between probe 226 and probe 337, a region about 0.6 kb.

The character of -α27.6 deletion in the α-globin cluster

Then, we applied 27.6F1/27.6Ry primers to obtain the PCR product of deletion in α-globin cluster from the three probands. The product was detected by Sanger DNA sequencing (Invitrogen, CA, USA). The results showed that the 27.6 kb deletion was verified in the α-globin cluster from the three probands. A comparison with the normal sequence showed that the deletion was 27,640 bp in length, with the 5' breakpoint lay in 9079 and the 3' breakpoint in 36718 (**Figure 3**). The breakpoints were found to be joined with an insertion sequence of six nucleotides: AATCCC. Furthermore, the sequence of the 27.6 kb deletion was blasted with the standard sequence at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The results showed that the sequence of 27.6 kb deletion was consistent with the standard sequence (**Figure 4**).

Discussion

The frequency of α-thal in southern China is high. Molecular epidemiology has revealed that

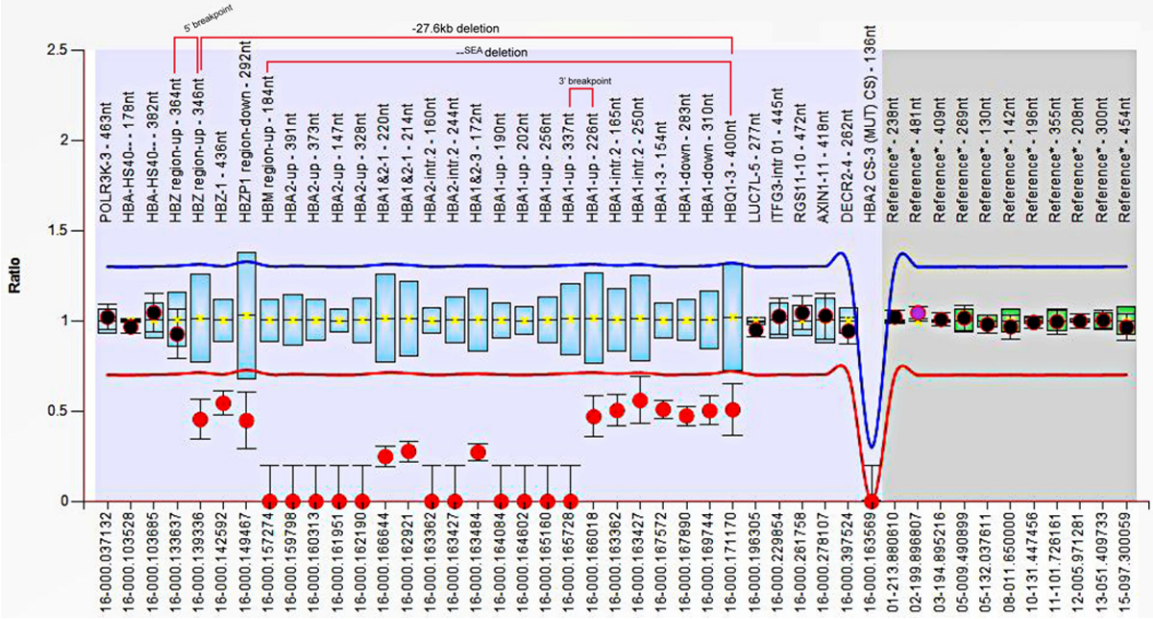

Figure 2. The α-globin gene cluster shows the 5' and 3' breakpoint regions of -27.6 kb deletion and the --^{SEA} deletion delimited by the multiplex ligation dependent probe amplification (MLPA) assay.

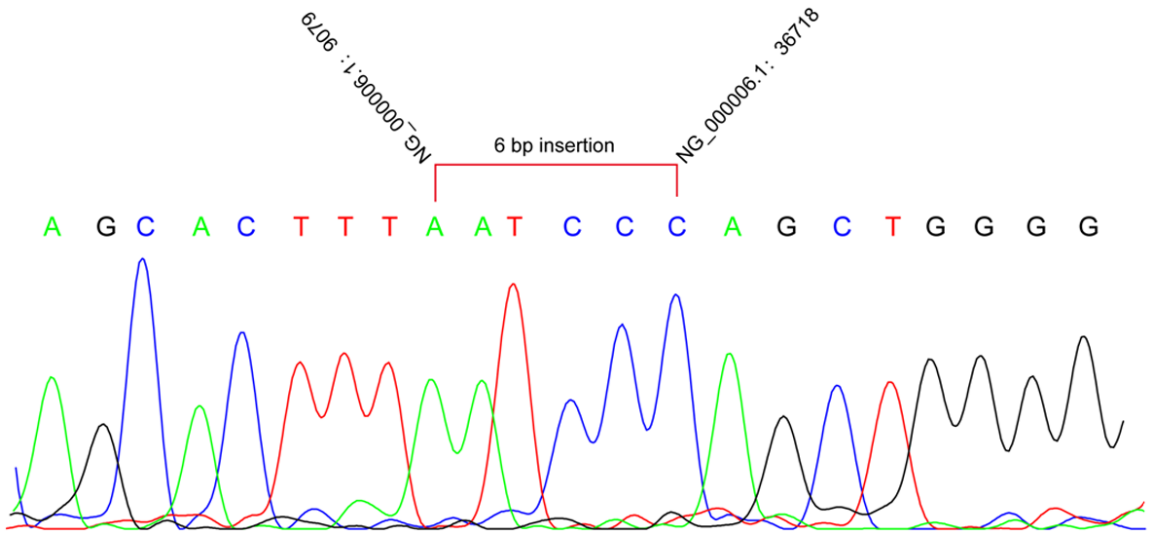

Figure 3. Characterization of the breakpoints of deletion in the α-globin cluster from the three probands by Sanger DNA sequencing. The GenBank coordinates of the two nucleotides flanking the deletion are indicated, as well as the six nucleotides that were inserted during the recombination events that led to this deletion. The deletion size is about 27,640 bp.

α-thal in most of the Chinese population is caused by three common deletion mutations (--^{SEA}, -α$^{3.7}$ and -α$^{4.2}$) and one common point mutation (α^{CS}α). Gap-PCR was applied to detect deletional mutations causing α-thal [13-15], including --^{SEA}/, -α$^{3.7}$/, -α$^{4.2}$/and --^{THAI}, so other deletions might have been missed and may be undiagnosed [16]. Ending with Hb H disease or Hb Bart's hydrops fetalis in the offspring was possible when combined with other combined with α^{0}-thal allele [7, 17]. Clinical features and molecular analysis in patients with HbH disease from different populations have revealed that the non-deletional type usually causes more severe disease than the deletional type does [16, 18, 19]. The identification and clinical

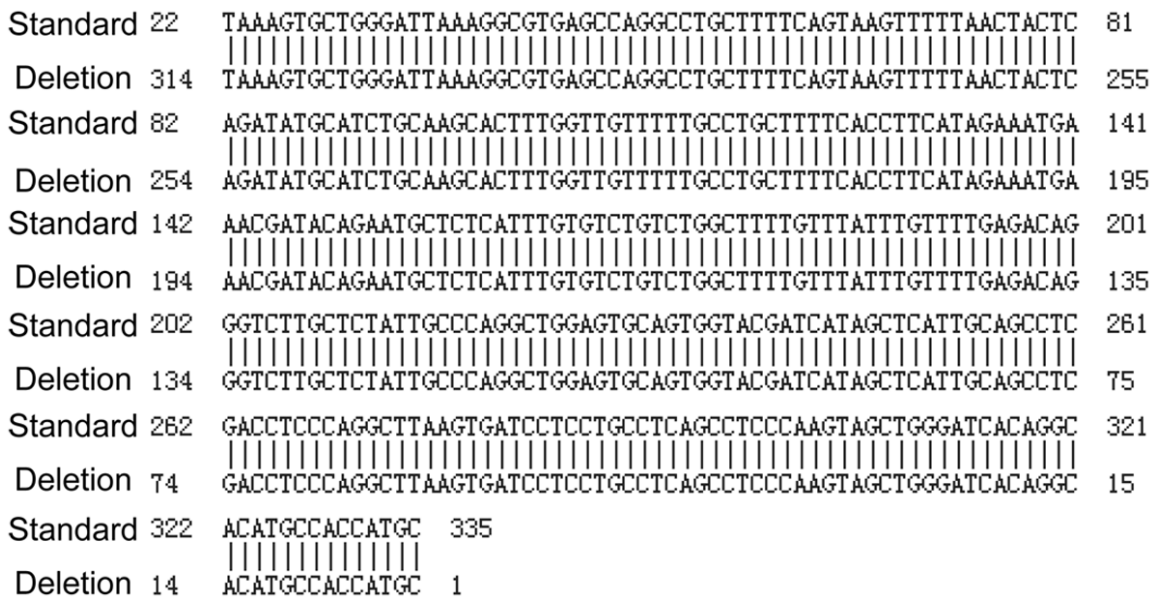

Figure 4. The sequence of 27.6 kb deletion was blasted with the standard sequence.

genetics analysis of rare or new α-thal mutations are very important areas of current molecular pathology research.

In this study, three probands from three Chinese families in Fujian Province showed the HbH disease trait, while their common genotype of α-thal were $--^{SEA}/--^{SEA}$, which we determined using gap-PCR and reverse dot-blot hybridization. Hb Bart's hydrops fetalis is a severe result of α-thal, and offspring with this disease usually die in the 30th to 40th week of pregnancy, or are aborted, or they die a few hours after being born, missing four active α genes [20]. Patients with HbH disease have only one active α gene (--/-α), and they have moderately severe but variable anemia. A person having two α genes (-α/-α or --/αα) shows only a mild and sometimes asymptomatic form of the α-thal trait, whereas silent carriers (αα/-α) are generally normal. So, a chromosome bearing only one α gene deletion (-α) is often neglected until it combines with another chromosome lacking both α genes (--), which results in HbH disease. The hematologic data of three probands indicated that they carried HbH and have only one common $--^{SEA}$ mutation inherited from their father, suggesting that an unknown and new defect might exist in the α-globin gene cluster.

To identify this deletion, MPLA and Sanger DNA sequencing were applied. The MLPA results showed that the $-\alpha^{27.6}$ deletion removes the ζ2, Ψζ1, Ψα2, Ψα1, and α2 genes, yet α1 was complete, described as α^{+}-thal. Our data showed that the 5' breakpoint in this new deletion was located in nt9078, between HS-40 and HBZ, and the 3'breakpoint located in nt36716, between HBA2 and HBA1. After calculating, the deleted region was about 27638 kb, including HBZ, HBZP, HBA2P, HBA1P and HBA2. The sequence of the 27.6 kb deletion was also blasted with the standard sequence at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The results showed that the sequence of the 27.6 kb deletion was consistent with the standard sequence. All our data verified that the genotype of the three probands were $-\alpha^{27.6}/--^{SEA}$.

According to Mendel's law, the $-\alpha^{27.6}$ deletion in these probands is inherited from the parents. In the three Chinese families, the $-\alpha^{27.6}$ deletion in the three probands was inherited from the fathers. Although the phenotype of $-\alpha^{27.6}/\alpha\alpha$ was normal or on the low border, when in combination with α^{0}-thal allele, there was a 25% risk of Hb H disease in the offspring, so it was important to diagnose this deletion.

In conclusion, we report a rare α-thal deletion, $-\alpha^{27.6}$, in three Chinese families from Fujian Province. Our study extends the spectrum of α-thal mutation in the Chinese population. It is necessary to the awareness and detection of this rare α-thal mutation, which is necessary for effective genetic counseling and prenatal diagnosis in Fujian Province.

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Disclosure of conflict of interest

None.

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