Identification of a new β-thalassaemia variant Term CD+32(HBB: c.32A>C) in two Chinese families

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ABSTRACT

Aims β-Thalassaemia is an inherited blood disorder caused by mutations in the β-globin gene cluster. Molecular characterisation of β-thalassaemia is essential for its diagnosis and management. More and more rare and novel mutations have been reported.

Methods Two Chinese families with β-thalassaemia from Fujian Province were recruited in this study. The phenotypes of the probands were confirmed through haematological analysis. Routine molecular analysis of thalassaemia was employed to identify the common mutations of thalassaemia. The rare and novel mutations were detected by direct DNA sequencing.

Results In family 1, the proband, a Chinese woman aged 31 years, showed elevated level of haemoglobin A2 (HbA2). No common mutations associated with β-thalassaemia were detected, whereas a rare mutation Term CD+32(HBB: c.32A>C) was identified through DNA sequencing. Subsequent investigation of the β-thalassaemia mutation in her family showed that her mother, her brother as well as her nephew also carried this mutation. In addition, both the proband's husband and her son carrying the rare $--^{THAI}$ mutation exhibited decreased levels of MCH, MCH and HbA2. In family 2, the proband, a child aged 1 year, showed elevated level of HbA2, but had no common mutations of β-thalassaemia. The proband was identified carrying the mutation Term CD+32(HBB: c.32A>C), which was inherited from his mother.

Conclusions In this study, we first report a rare β-thalassaemia mutation in Fujian Province, Southeast China. Moreover, our study also identified this rare mutation in humans. This finding has helped broaden the spectrum of β-thalassaemia mutations in our region and suggested that this rare mutation may be more prevalent in the Chinese population.

INTRODUCTION

Thalassaemia is a genetic disorder caused by human globin gene synthesis disorders, of which α-thalassaemia and β-thalassaemia are the most common genotypes.[1] They are common in people of Mediterranean countries, the Middle East, the Indian subcontinent, Southeast Asia and Southern China.[2–4] α-Thalassaemia is mainly caused by α-globin gene deletions and Asian deletion ($--^{SEA}$), right deletion ($-\alpha^{3.7}$) and left deletion ($-\alpha^{4.2}$) are the top three deletions found responsible for α-thalassaemia.[5] β-Thalassaemia is mainly caused by β-globin gene mutations, 17 common mutations have been found to be associated with β-thalassaemia in the Chinese population.[6–8] Regular β-thalassaemia carriers in clinical studies undergo haemoglobin A2 (HbA2) and β-globin gene mutation screening via reverse dot-blot hybridisation (RDB).[9] More than 900 genomic alterations of β-globin and over 300 mutations of the β-globin gene resulting in β-thalassaemia have been described.[10] In the Chinese population, nearly 50 different mutations have been found and the number is continuously increasing.[11–18] Multiple ligation-dependent probe amplification or next-generation sequencing (NGS) technologies can be employed to characterise unknown mutations at the molecular level.[19]

In this study, two Chinese families were found to have a rare heterogynous β-thalassaemia mutation Term CD+32 (HBB: c.32A>C) by direct β-globin gene sequencing. This mutation was inherited from the proband's mother and was also present in proband's brother and proband's nephew in family 1. In addition, a rare α-thalassaemia deletion ($--^{THAI}/\alpha\alpha$) was detected in the proband's husband and son. In family 2, the rare mutation was first identified in a child aged 1 year, who inherited the mutation from his mother.

MATERIALS AND METHODS

Subjects

Two Chinese families from Southeast China were recruited in this study. The probands came from Quanzhou City, Fujian Province, Southeast China. The proband from family 1 was a woman aged 31 years of Han nationality. The proband from family 2 was a male child aged 1 year of Han nationality. The probands underwent haematological screening and showed lower levels of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and elevated HbA2 level. Peripheral blood samples from the two families were collected and stored for further investigation. All subjects in our study denied a history of blood transfusion.

Haematological analysis

A routine blood analysis was performed using an automated cell counter (Sysmex XS-1000i; Sysmex, Kobe, Japan) to analyse the levels of MCV and MCH in the two Chinese families. MCV <82 fL and/or MCH <27 pg was suspected as thalassaemia carriers. The levels of HbA, HbA2 and HbF in the two Chinese families were detected by haemoglobin capillary electrophoresis (Sebia, Evry Cedex, France). HbA2 >3.4% or HbA2 <2.6% or HbF >2.0% or abnormal haemoglobin zone indicated abnormal haemoglobin results and was suspected as thalassaemia carriers.

Molecular analysis

Genomic DNA of the two Chinese families was obtained at Ruibao Biological using an automatic nucleic acid extractor. The deletional mutation analysis of α-thalassaemia (-$\alpha^{3.7}$, -$\alpha^{4.2}$, --^{SEA}) was performed using gap-PCR (Yaneng Biological Technology, Shenzhen). The PCR-RDB technology was used to detect the non-deletion α-thalassaemia (Hb ConstantSpring, Hb QuongSze and Hb Westmead) and 17 common mutations associated with β-thalassaemia in the Chinese population.[6–8] Six α-thalassaemia genotype-screening kits (Yaneng Biological Technology) were used to detect the rare α-thalassaemia mutations: --^{THAI}, -$\alpha^{27.6}$, HKαα, fusion gene, ααα$^{anti4.2}$ and ααα$^{anti3.7}$. Three β-thalassaemia genotype-screening kits (Yaneng Biological Technology) were used to detect Taiwanese, $^{G}\gamma^{+}(^{A}\gamma\delta\beta)^{0}$, SEA-HPFH for suspected rare β-thalassaemia deletions.

DNA sequencing and analysis

We designed specific primers according to the known DNA sequences around the HBB gene to perform PCR. The primer sequences were as follow: P1: CCAAGGACAGGTACGGCTGTCATC, P2: GCATATGCATCAGGGGCTGTTG. All primers were synthesised by Invitrogen (Shanghai). PCR detection system: 2 mM dNTPs 2 μL, 5× buffer 4 μL, 25 mM $MgCl_2$ 2 μL, Taq enzyme 1 U, 10 μM primers 0.5 μL each, template 2 μL, plus ultra-pure water to 20 μL. Amplification conditions: 95°C for 5 min; 95°C for 30 s, 60°C for 30 s, 72°C for 120 s, 35 cycles; 72°C for 5 min. Electrophoresis conditions: 1% Agar, 100 V, 40 min; 3 μL amplification products were tested by electrophoresis. The remaining amplification products were purified by TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (Takara Biomedical Technology, Beijing, China) according to the instruction of the kit. The purification of the products was done by sequencing reaction. The sequencing reaction system: BigDye 2 μL, Seq Buffer 1 μL, 10 μM primer P1 or P2 0.5 μL, ultra-pure water 4.5 μL, purified products 2 μL. The sequencing reaction conditions: 96°C for 1 min; 96°C for 10 s, 50°C for 5 s, 60°C for 240 s, 25 cycles; 4°C hold. The sequencing reaction products were purified and then added 10 μL Hi-Di to sequence by using Applied Biosystems 3500 Dx. The peak map was analysed and compared with the standard sequence (NG_000007) in GenBank to determine the mutation site.

RESULTS

Haematological analysis

The haematological test results of the proband and her family members of family 1 are shown in table 1. We assumed that the proband, proband's mother and proband's brother as well as the proband's nephew could be β-thalassaemia carriers based on the increased levels of HbA2 and decreased levels of MCV and MCH. In addition, the proband's husband and son were assumed to be α-thalassaemia carriers according to the decreased levels of HbA2, MCV and MCH. The proband's father in family 1 showed decreased level of HbA2, while the levels of MCV and MCH were normal. The haematological test results of the proband and his family members of family 2 are shown in table 2. The proband and proband's mother had β-thalassaemia traits with increased levels of HbA2, and decreased levels of MCV and MCH.

Table 2 The haematological and molecular analysis of the proband and his family members in family 2

Parameters	Proband	Mother	Father
Sex-age	M-1	F-33	M-33
RBC (10^{12}/L)	5.69	5.36	4.98
Hb (g/L)	83	114	147
MCV (fL)	51	65	90
MCH (pg)	14.6	21.3	30.1
HbA (%)	89.4	94.1	97.0
HbA2 (%)	4.0	4.7	3.0
HbF (%)	6.6	1.2	0
α-Genotype	αα/αα	αα/αα	αα/αα
β-Genotype	$\beta^{Term\ CD+32}/\beta^{N}$	$\beta^{Term\ CD+32}/\beta^{N}$	β^{N}/β^{N}

Hb, haemoglobin; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; RBC, red blood cell.

Mutation analysis of the thalassaemia genes

Three common α-thalassaemia deletions were detected by Gap-PCR in the two Chinese families. Only an 1826 bp band, representing the normal gene, appeared in the two families. The Gap-PCR and PCR-RDB results showed no common mutations and deletions were found in the entire two families. However, three members of family 1 (the proband's father, proband's husband and proband's son) had α-thalassaemia traits with the decreased level of HbA2. Then, six α-thalassaemia genotyping kits were used to detect --^{THAI}, -$\alpha^{27.6}$, HKαα, fusion gene,[20] ααα$^{anti4.2}$ and ααα$^{anti3.7}$ in the three members of family 1. Both the proband's husband and son in family 1 had the --^{THAI} mutation, while proband's father showed none of these six thalassaemia gene mutations. The subsequent follow-up showed that the proband's father in family 1 had chronic renal failure, which can explain the decreased level of haematological results. As shown in figure 1, the genotypes of three members in family 1

Table 1 The haematological and molecular analysis of the proband and her family members in family 1

Parameters	Proband	Husband	Son	Father	Mother	Brother	Nephew
Sex-age	F-31	M-31	M-7	M-57	F-53	M-32	M-4
RBC (10^{12}/L)	5.52	6.4	5.19	2.26	6.12	6.32	6.15
Hb (g/L)	114	147	113	67	125	127	118
MCV (fL)	65.2	71.3	63.2	91.6	64.5	62.8	57.9
MCH (pg)	20.7	23	19.8	29.6	20.4	20.1	19.2
HbA (%)	94.4	97.5	97.4	97.7	93.6	94.1	87.3
HbA2 (%)	4.9	2.5	2.6	2.3	5.3	5.0	5.1
HbF (%)	0.7	0	0	0	1.1	0.9	7.6
α-Genotype	αα/αα	--^{THAI}/αα	--^{THAI}/αα	αα/αα	αα/αα	αα/αα	αα/αα
β-Genotype	$\beta^{Term\ CD+32}/\beta^{N}$	β^{N}/β^{N}	β^{N}/β^{N}	β^{N}/β^{N}	$\beta^{Term\ CD+32}/\beta^{N}$	$\beta^{Term\ CD+32}/\beta^{N}$	$\beta^{Term\ CD+32}/\beta^{N}$

Hb, haemoglobin; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; RBC, red blood cell.

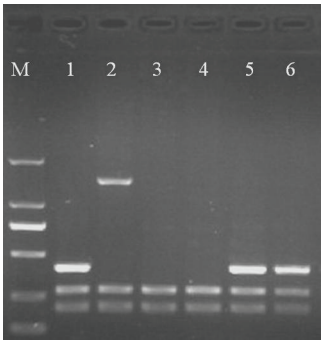

Figure 1 The detection results of six α-thalassemia genotyping kits in family 1. M: DL2000 DNA marker. Line 1: positive control of $--^{THAI}$. Line 2: positive control of $-\alpha^{27.6}$. Line 3: negative control. Line 4: the proband's father. Line 5: the proband's husband. Line 6: the proband's son. The positive control of $--^{THAI}$, $-\alpha^{27.6}$ and the negative control showed the corresponding band. Both the proband's husband and son had the $--^{THAI}$ mutation, while proband's father showed none of these six thalassaemia gene mutations.

were $--^{THAI}/\alpha\alpha$, β^N/β^N for the proband's husband and her son, $\alpha\alpha/\alpha\alpha$, β^N/β^N for the proband's father.

PCR-RDB was used to detect the common pathogenic mutations, but no common mutations for β-thalassaemia were found in the two families, which led us to further investigate the causes underlying the probands and their family members' symptoms exhibiting increased levels of HbA2.

DNA sequencing

The haematological and molecular analysis results indicated that the probands and their family members may have a rare or novel β-thalassaemia mutation. Subsequently, β-globin gene was amplified by PCR and the product was then analysed by direct DNA sequencing in the suspected subjects. As shown in figure 2, a rare mutation Term CD+32 (HBB: c.32A>C) was identified in the proband of family 1, who has a mutation of an A→C substitution at nucleotide position +32 from the stop codon TAA in the 3' untranslated region region of the human β-globin gene and has been reported first and only reported in a Chinese family.[21] The same mutation was also present in the proband's mother and her brother, as well as her nephew, exhibiting increased levels of HbA2 and decreased levels of MCV and MCH. Therefore, with the thalassaemia genotype for the proband, her mother, her brother and her nephew can be described as $(\alpha\alpha/\alpha\alpha, \beta^{Term\ CD+32}/\beta^N)$. As shown in figure 2, the proband of family 2 carried the same mutation, which was inherited from his mother.

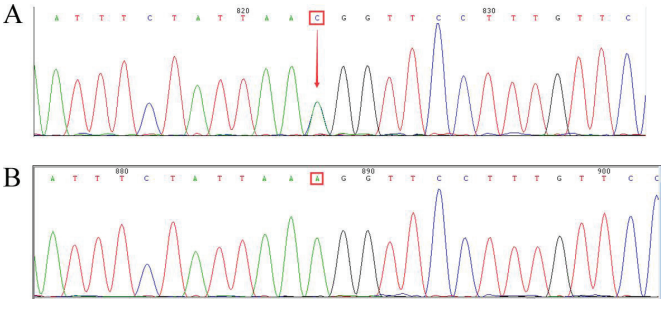

Figure 2 Sequencing analysis of the HBB gene. HBB gene sequence chromatograms of the probands and their family members. (A) The rare mutation (HBB: c.32A>C) was identified through sequencing analysis and the peak map was compared with the standard sequence (NG_000007) in GenBank. (B) The negative control.

DISCUSSION

β-Thalassaemia is a hereditary haemolytic anaemia caused by a decrease or deficiency in β-globin chain synthesis due to deletion or deficiency of the β-globin gene. Among them, two groups have been classified according to their severity. One is β^0-thalassaemia, which results in the complete absence of the β chain, and the other is β^+-thalassaemia, which induces a reduction in β chain expression.[22] Thalassaemia is widespread in Southern China, including Fujian Province. The carrying rate of β-thalassaemia in Fujian Province is about 1.32%.[23] Currently, more and more new β-thalassaemia mutations have been discovered.

Blood routine and haemoglobin electrophoresis are commonly performed for thalassaemia screening, and then molecular analysis of thalassaemia is performed in subjects with a positive screening test. In the screening of thalassaemia, analysis of HbA2 levels is extremely sensitive to the detection of β-thalassaemia and hence is widely used.[24] In the routine molecular analysis of thalassaemia, Gap-PCR is used to detect the deletional thalassaemia mutations, and PCR-RDB is used to detect common thalassaemia mutations, but not rare and novel mutations.

In this study, the results of thalassaemia screening in the probands showed a typical small cell hypopigmentation anaemia, and haemoglobin electrophoresis showed increased level of HbA2, which is indicative of β-thalassaemia trait. However, no common thalassaemia gene mutations were detected in the probands. The rare mutation Term CD+32 (HBB: c.32A>C) found in the two families has been reported for the first time in Fujian Province, Southeast China by directing DNA sequence. The molecular analysis and DNA sequencing of family 1 showed that the rare β-thalassaemia mutation was inherited from the proband's mother, and the proband's brother and nephew also carried the mutation. In addition, the thalassaemia screening of proband's husband in family 1 showed small cell hypopigmentation anaemia, with decreased level of HbA2, which is indicative of α-thalassaemia trait. Routine molecular analysis of thalassaemia and rare thalassaemia gene detection showed that his genotype was: $--^{THAI}/\alpha\alpha$, and inherited to his son. The molecular analysis and DNA sequencing of family 2 showed that the same mutation was inherited from the proband's mother with decreased level of MCV, MCH and elevated level of HbA2 HBB: c. 32A>C mutation located at the 32nd base of the termination codon, which belongs to the RNA processing mutation that causes β^+-thalassaemia. At present, this mutation has been the first and only reported in a family in Guangdong Province, South China.[21] However, the proband of family 2 carrying the same mutation showed more serious anaemia symptoms. Further analysis of his iron metabolism showed that both ferritin (3.0 μg/mL) and serum iron (3.85 μmol/L) were reduced, and transferrin (4.0 g/L) was slightly increased. These results indicated that the proband may also had iron deficiency anaemia, which may lead to more serious anaemia symptoms.

Therefore, NGS should be performed to screen for β-globin gene mutations and avoid misdiagnosis for those cases that had high level of HbA2 in haemoglobin electrophoresis screening but have no common thalassaemia gene mutation. In addition, family 1 was found to carry a rare deletional α-thalassaemia ($--^{THAI}$), which has been reported and showed an increasing trend,[25] but the routine molecular analysis of thalassaemia does not cover this genotype. So, this rare type of $--^{THAI}$ mutation might be considered to be included in the further test to avoid

misdiagnosis and reduce the birth of HbH disease and Bart's hydrops fetalis.

CONCLUSIONS

We identified a rare mutation HBB: c.32A>C, which was first reported in Fujian Province, Southeast China. Our study also identified this rare β-thalassaemia mutation in humans. Additionally, we found that family 1 also carried a rare deletional α-thalassaemia (--^{THAI}). This finding has helped broaden the spectrum of β-thalassaemia mutations in our region and suggested that this rare mutation may be more prevalent in the Chinese population, which can help improve clinical practice in the diagnosis and disease management of thalassaemia.

Take home messages

- A rare mutation HBB: c.32A>C was identified in humans.
- This rare β-thalassaemia mutation was first reported in Fujian Province, Southeast China.
- This rare mutation was found to be present in two Chinese families with six carriers and may be more prevalent in the Chinese population.

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