A novel frameshift mutation (+G) at codons 15/16 in a \(\beta^0\) thalassaemia gene results in a significant reduction of \(\beta\) globin mRNA values

Q-H Mo, X-R Li, C-F Li, Y-L He, X-M Xu

Aims: To identify a novel \(\beta\) globin gene mutation found in a Chinese family, and also to assess its functional consequences.

Methods: Haematological analysis was performed on all family members. The 23 common mutations of \(\beta\) thalassaemia found in Chinese populations were detected by means of a reverse dot blot method. Direct DNA sequencing of polymerase chain reaction (PCR) amplified complete \(\beta\) globin gene was carried out to identify the novel mutation. A real time, one step reverse transcription PCR assay was used to measure \(\beta\) globin mRNA in the reticulocytes of heterozygous patients.

Results: A novel frameshift mutation—an insertion of G between codons 15 and 16 in a homonucleotide run of four guanines—was determined, which generates a new premature chain terminator at the 22nd codon. Relative quantitative analysis of the \(\beta\) globin mRNA in heterozygous subjects demonstrated a 39.83% reduction compared normal controls.

Conclusions: The significantly lower amounts of \(\beta\) globin mRNA found in mutation carriers is probably caused by the rapid nonsense mediated degradation of the mutant mRNA. These data, combined with haematological analysis, suggest that this novel mutation of CDs15/16 (+G) results in a \(\beta^0\) thalassaemia phenotype.

Beta thalassaemia is a highly heterogeneous inherited disorder distributed worldwide. According to the regularly updated online database of human haemoglobin variants and thalassaemias on the Globin Gene Server website (http://globin.cse.psu.edu/), more than 200 different mutations have been reported to date, and each ethnic population has its own cluster of common mutations. Most \(\beta\) thalassaemias are caused by a point mutation, minor deletion, or insertion in the \(\beta\) globin gene, which result in reduced or lack of synthesis of the haemoglobin \(\beta\) globin chain.

"Searching for and identifying new mutations is a constant priority in population screening, genetic counselling, and prenatal diagnosis of thalassaemia".

In south China, \(\beta\) thalassaemia is a common disease with high carrier prevalence. For instance, Guangdong Province, located in the southernmost part of mainland China, has a carrier prevalence of 2.54% according to our current investigations. Searching for and identifying new mutations is a constant priority in population screening, genetic counselling, and prenatal diagnosis of thalassaemia.

\(\beta\) globin mutations are usually found by means of a reverse dot blot method. We developed a new, one step reverse transcription PCR assay to measure \(\beta\) globin mRNA in the reticulocytes of heterozygous patients.

Materials and Methods

Patients

The proband was a male infant aged 2 years 8 months with transfusion dependent \(\beta\) thalassaemia major. Since July 2002, when he was diagnosed at the age of 8 months, he had been given 11 blood transfusions. At present, there was no obvious splenomegaly. Both parents, who originated from Guangzhou City, Guangdong Province in south China, had classic \(\beta\) thalassaemia trait with reduced mean corpuscular volume, reduced mean corpuscular haemoglobin, and a raised haemoglobin A2 concentration (table 1). The 23 common mutations of the \(\beta\) globin gene found in Chinese populations were investigated using a RDB method established in our laboratory. Only a single mutation at \(\beta^{+}28(G\rightarrow T)\) was detected in the proband, which was inherited from his father. Next, we collected peripheral blood samples from eight family members in three generations and obtained the family’s consent to undertake our study.

Phenotypic analysis and mutation identification

Haematological analysis was performed by automated cell counting (Model Sysmex F-820; Sysmex Co Ltd, Kobe, Japan) and haemoglobin electrophoresis was carried out with a REP system (Helena Laboratory, Beaumont, Texas, USA). Genomic DNA was extracted by a standard phenol/chloroform method from leucocytes in peripheral blood.

Mutation analysis of the \(\beta\) globin gene was performed by direct DNA sequencing of the amplified PCR product using an extended reverse dot blot (RDB) method.

Abbreviations: C\textsubscript{T}, threshold value; PCR, polymerase chain reaction; PTC, premature termination codon; RDB, reverse dot blot; RT, reverse transcription.
ABI Prism 377 automated DNA sequencer. The sequences of the primers for amplifying the full length human \(\beta\) globin gene of 1833 bp were: 5'-AACTCCTAAGCCAGTGCCAGAAGAGC-3' (forward) and 5'-ATGCACTGACCTCCCACATTCCT-3' (reverse).

Genotyping by RDB assay

Two new probes used in the RDB assay for rapidly genotyping the novel mutation were designed using the Oligo 6.31 software (Molecular Biology Insights Inc, Cascade, Colorado, USA). The probe for detecting the normal allele is 5'-NH2-CCCTGTGGGGCAAGGTG-3' and the probe for detecting the mutant allele is 5'-NH2-CCCTGTGGGGGCAAGGT-3'. The membrane strips were prepared as described previously, with minor modifications. Allele specific hybridisation and colour development were carried out using Zhang's method.

Study of mRNA values

The Purescript RNA isolation kit (Gentra Systems Inc, Minneapolis, Minnesota, USA) was used to prepare purified total RNA from whole blood. Real time quantitative RT-PCR was carried out immediately by means of a real time one step RNA PCR kit (TaKaRa Biotechnology (Dalian) Co Ltd, Dalian, China) with 48 ng of total RNA and 5 pmol of gene specific primers in a 25 \(\mu\)l reaction volume. The primers for amplification of the \(\beta\) globin mRNA were 5'-CAACGCACGAGCAGACCTTCA-3' (forward) and 5'-AACCTTTAATAGAATTCGACAGC-3' (reverse).

Table 1 Haematological data and \(\beta\) thalassaemia genotype of the proband and his family

<table>
<thead>
<tr>
<th>Family members</th>
<th>Sex</th>
<th>Age</th>
<th>RBC ((10^9))</th>
<th>Hb (g/l)</th>
<th>MCV (f)</th>
<th>MCH (pg)</th>
<th>RDW (%)</th>
<th>HbA (%)</th>
<th>HbA2 (%)</th>
<th>HbF (%)</th>
<th>Genotype</th>
</tr>
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<tr>
<td>Proband</td>
<td>Female</td>
<td>2 y 8 m</td>
<td>3.62</td>
<td>78</td>
<td>21.5</td>
<td>0.354</td>
<td>82.89</td>
<td>2.83</td>
<td>14.27</td>
<td>-28/15-16</td>
<td>82.89</td>
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<tr>
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<td>33</td>
<td>5.75</td>
<td>127</td>
<td>22.1</td>
<td>0.175</td>
<td>93.85</td>
<td>6.16</td>
<td>0</td>
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<td>93.85</td>
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<tr>
<td>Mother</td>
<td>Female</td>
<td>32</td>
<td>5.16</td>
<td>98</td>
<td>19.0</td>
<td>0.175</td>
<td>94.7</td>
<td>5.30</td>
<td>0</td>
<td>15-16/N</td>
<td>94.7</td>
</tr>
<tr>
<td>Grandfather</td>
<td>Male</td>
<td>67</td>
<td>6.72</td>
<td>134</td>
<td>19.9</td>
<td>0.159</td>
<td>94.46</td>
<td>5.54</td>
<td>0</td>
<td>15-16/N</td>
<td>94.46</td>
</tr>
<tr>
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<td>Female</td>
<td>56</td>
<td>4.25</td>
<td>126</td>
<td>29.6</td>
<td>0.128</td>
<td>97.72</td>
<td>5.54</td>
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</tr>
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<td>8.25</td>
<td>118</td>
<td>28.0</td>
<td>0.130</td>
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<tr>
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<td>28</td>
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<td>2.75</td>
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<td>97.25</td>
</tr>
<tr>
<td>Mother's brother 2</td>
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<td>4.50</td>
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<td>2.27</td>
<td>0</td>
<td>N/N</td>
<td>97.73</td>
</tr>
</tbody>
</table>

The proband's peripheral blood was sampled on the 7th day after a blood transfusion.

- 28, 28 (A→G) mutation; 15-16, CDs 15/16(G) mutation; Hb, haemoglobin; MCH, mean cell haemoglobin; MCV, mean cell volume; N, normal; RBC, red blood cells; RDW, red cell distribution width.

Figure 1 (A) Mutant sequence of the sense strand. The inserted base (G) and the generated premature termination codon of TGA are marked in red and underlined. This one base insertion mutation results in numerous ambiguities (peaks overlapping, indicated by N) immediately after it occurs in the sequencing chromatogram of a heterozygote. (B) Mutant sequence of the antisense strand.
and twofold serial dilutions of quantified total RNA starting from normal controls. In the relative standard curve method, was generated by plotting the C_T (threshold cycle value) of the relative amounts of normal and mutant subjects were normalised with the sample. The relative quantities of \( \beta \) globin mRNA between normal and mutant subjects were normalised with the sample. The relative quantities of \( \beta \) globin mRNA were 14.65 (0.09) (n = 2) for heterozygous subjects carrying the CD15/16 (+G) mutation, 13.93 (0.28) (n = 5) for patients with \( \beta^+ \) thalassemia, especially for prenatal diagnosis, which may be required by the proband’s parents in future pregnancies.

To evaluate the functional effect of this novel PTC mutation, real time quantitative RT-PCR and the relative standard curve method were used to analyse the amounts of \( \beta \) globin mRNA isolated from reticulocytes and nucleated red blood cells. The generated linear regression equations describing the relative standard curves were \( y = -3.8243x + 28.5954 \) \( (R^2 = 0.999) \) for \( \alpha \) mRNA and \( y = -8.2483x + 28.3954 \) \( (R^2 = 0.999) \) for \( \beta \) mRNA. After normalisation with these equations, the calculated mean (SD) \( C_T \) values of \( \beta \) globin mRNA were 14.65 (0.09) (n = 2) for heterozygous subjects carrying the CD15/16 (+G) mutation, 13.93 (0.28) (n = 5) for patients with \( \beta^+ \) thalassemia, and 12.83 (0.48) (n = 8) for normal individuals (fig 3A). Using these mean \( C_T \) values, the relative amounts of \( \beta \) globin mRNA for the two patient groups compared with the normal control group can then be assessed (fig 3B). These results showed significantly lower amounts of \( \beta \) globin mRNA in the patient groups—the proband’s mother and grandfather had a 39.83% reduction in \( \beta \) mRNA concentration and the five \( \beta^+ \) thalassaemia heterozygotes had a 26.44% reduction compared with the normal controls.

"Nonsense mediated mRNA decay is a surveillance mechanism by which cells recognise and degrade mRNAs containing premature translation termination codons"

Molecular diagnosis of \( \beta \) thalassaemia using the RDB method has been performed in our laboratory for 12 years. Here, we extended this method for genotyping this new mutation by using a pair of preferentially selected probes. Figure 2 shows the results of the RDB test for the novel mutation. Under routine experimental conditions, the normal and mutant probes showed high specificity for detecting their corresponding complementary sequences. This is a convenient method for the molecular detection of \( \beta \) thalassaemia, especially for prenatal diagnosis, which may be required by the proband’s parents in future pregnancies.

The relative standard curve method was used to measure the relative amounts of \( \beta \) globin mRNA in the patients and normal controls. In the relative standard curve method, twofold serial dilutions of quantified total RNA starting from 192 ng to 12 ng were used to construct the standard curve for \( \beta \) and \( \alpha \) mRNA. A five point linear relative standard curve was generated by plotting the \( C_T \) (threshold cycle value) of each standard (\( y \) axis) against the logarithm of the quantity of total RNA (\( x \) axis). The reduction in \( \beta \) globin mRNA values was determined by comparing the mean \( C_T \) values of the two patient groups and the normal control group with the standard curve.

RESULTS AND DISCUSSION

Table 1 shows the results of the haematological examination of the proband and his family members. Using the haematological data, the proband’s parents and his grandfather were classified as typical \( \beta \) thalassaemia heterozygotes, with reduced mean cell volume (56.4–68.9 fL) and raised haemoglobin A2 values (5.30–6.16%).

Direct DNA sequencing analysis of the proband and his mother and grandfather determined a novel mutation—a single nucleotide insertion of guanine between codons 15 and 16 within a mutation hot spot region of the \( \beta \) globin gene, which resulted in a frameshift and premature termination at codon 22 (fig 1A). Sequencing of the antisense strands confirmed the novel insertion mutation (fig 1B). Table 1 also shows the genotypes of the proband and his family members. The DNA sequence around the mutation site is a highly GC rich region. A homonucleotide run of four guanines was changed into five guanines as a result of this one base insertion mutation. This DNA sequence feature strongly suggests that the most likely mechanism to account for the mutation is DNA replication slippage according to a recent study. 8–10

A nonsense mediated mRNA decay pathway probably plays an important role in degrading the nonsense transcripts. 8–10 Nonsense mediated mRNA decay is a surveillance mechanism by which cells recognise and degrade mRNAs containing premature translation termination codons. A recent study that focused on the decay of a PTC containing \( \beta \) globin mRNA species in human cells showed that the PTC destabilised the mRNA and greatly decreased its half life from > 16 hours to
Take home messages

- We describe a novel CDs15/16 (+G) mutation, which results in a premature stop codon and a \( \beta^b \) thalassemia phenotype
- The significantly decreased amounts of \( \beta \) globin mRNA found in mutation carriers is probably caused by the rapid nonsense mediated degradation of the mutant mRNA
- The modified reverse dot blot method described could be used for prenatal diagnosis

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