

ORIGINAL ARTICLE

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

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

J Clin Pathol 2005;58:923–926. doi: 10.1136/jcp.2004.025296

See end of article for authors' affiliations

Correspondence to:
Dr X-M Xu, Department of Medical Genetics, Southern Medical University, Tonghe 510515, Guangzhou, Guangdong, P.R. China; gzxuxm@pub.guangzhou.gd.cn; moqihua@fimmu.com

Accepted for publication 25 January 2005

Aims: To identify a novel β 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 β 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 β globin gene was carried out to identify the novel mutation. A real time, one step reverse transcription PCR assay was used to measure β 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 β globin mRNA in heterozygous subjects demonstrated a 39.83% reduction compared normal controls.

Conclusions: The significantly lower amounts of β 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 β^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.¹ Most β thalassaemias are caused by a point mutation, minor deletion, or insertion in the β globin gene, which result in reduced or lack of synthesis of the haemoglobin β 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, β 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.² Searching for and identifying new mutations is a constant priority in population screening, genetic counselling, and prenatal diagnosis of thalassaemia. Frameshift mutations of the β globin gene frequently generate a new premature termination codon (PTC), which makes it impossible to synthesise normal functional protein and results in β^0 thalassaemia.¹ Here, we report a novel frameshift mutation (codons 15/16, +G; TGG GGC→TGG G GC) in a Chinese family. This one base insertion mutation creates a nonsense terminator at codon 22 (TGA), immediately downstream.

To assess the functional effect of this PTC mutation, quantitative analysis of β globin mRNA was performed in heterozygous patients by means of a real time, one step reverse transcription polymerase chain reaction (RT-PCR)

assay. In addition, for the purposes of prenatal diagnosis, an extended reverse dot blot (RDB) method was developed for the accurate detection of this novel mutation.

MATERIALS AND METHODS

Patients

The proband was a male infant aged 2 years 8 months with transfusion dependent β 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 β thalassaemia trait with reduced mean corpuscular volume, reduced mean corpuscular haemoglobin, and a raised haemoglobin A_2 concentration (table 1). The 23 common mutations of the β globin gene found in Chinese populations were investigated using a RDB method established in our laboratory. Only a single mutation at -28(A→G) 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 β globin gene was performed by direct DNA sequencing of the amplified PCR product using an

Abbreviations: C_T , threshold value; PCR, polymerase chain reaction; PTC, premature termination codon; RDB, reverse dot blot; RT, reverse transcription

Table 1 Haematological data and β thalassaemia genotype of the proband and his family

Family members								
	Proband	Father	Mother	Grandfather	Grandmother	Maternal aunt	Mother's brother 1	Mother's brother 2
Sex	Female	Male	Female	Male	Female	Female	Male	Male
Age	2 y 8 m	33	32	56	56	34	30	28
RBC ($\times 10^{12}$)	3.62	5.75	5.16	6.72	4.25	4.22	5.33	4.50
Hb (g/l)	78	127	98	134	126	118	156	134
MCV (fl)	66.0	68.9	56.4	59.1	84.2	82.0	83.7	84.0
MCH (pg)	21.5	22.1	19.0	19.9	29.6	28.0	29.3	29.8
RDW (%)	0.354	0.175	0.175	0.159	0.128	0.130	0.125	0.119
HbA (%)	82.89	93.85	94.7	94.46	97.72	97.03	97.25	97.73
HbA2 (%)	2.83	6.16	5.30	5.54	2.28	2.97	2.75	2.27
HbF (%)	14.27	0	0	0	0	0	0	0
Genotype	-28/15-16	-28/N	15-16/N	15-16/N	N/N	N/N	N/N	N/N

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

-28, -28 (A→G) mutation; 15-16, CD515/16(+G) mutation; Hb, haemoglobin; MCH, mean cell haemoglobin; MCV, mean cell volume; N, normal; RBC, red blood cells; RDW, red cell distribution width.

ABI Prism 377 automated DNA sequencer. The sequences of the primers for amplifying the full length human β globin gene of 1833 bp were: 5'- AACTCCTAAGCCAGTGCCAGA AGAGC-3' (forward) and 5'-ATGCACTGACCTCCACATTC CCT-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- CCTGTGGGGGCAAGGT-3'. The membrane strips were prepared as described previously, with minor modifications.³ Allele specific hybridisation and colour development were carried out using Zhang's method.⁴

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 μ l reaction volume. The primers for amplification of the β globin mRNA were 5'-CAAGGGCA CCTTTGCCACA-3' (forward) and 5'-AACCTTTAATAGAAAT TGGACAGC-3' (reverse), and those for the α globin mRNA were 5'-GCACGCGCACAAGCTTCGG-3' (forward) and 5'-CAGCACGGTGTCACAGAAGCC-3' (reverse). SYBR Green I purchased from OPE Technology Development Company (Shanghai, China) was selected as the fluorescence monitoring chemistry.

PCR reactions were run on a Rotor-Gene 3000 quantitative thermal cycler (Corbett Research, Sydney, New South Wales, Australia) with data acquisition at an annealing temperature of 58°C on the SYBR channel (excitation at 470 nm, detection at 510 nm). After a holding step at 50°C for seven minutes to synthesise the first strand cDNA, thermal cycling was initiated with a first denaturation step of one minute and

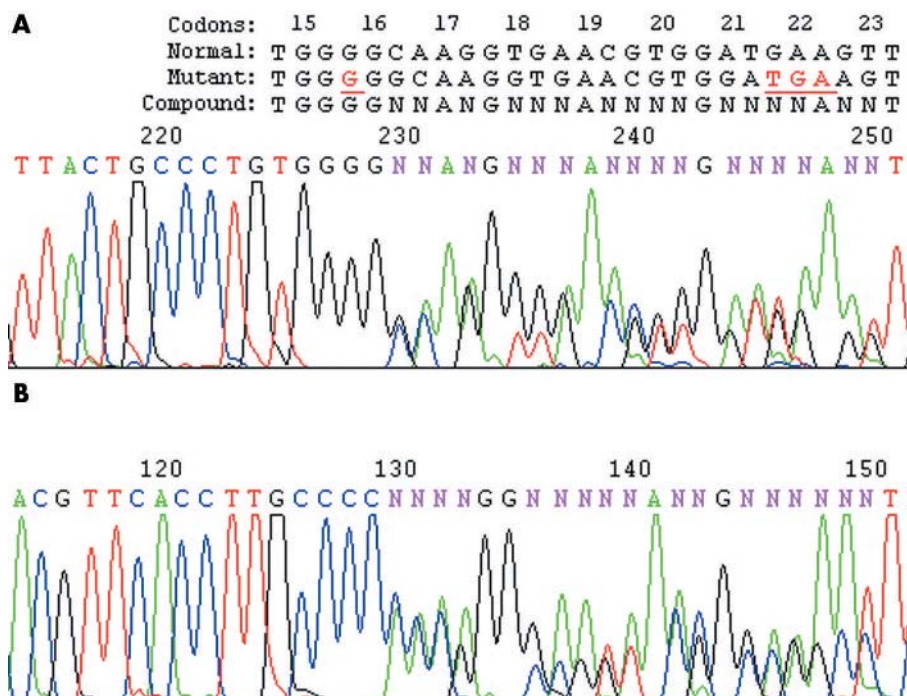


Figure 1 (A) Mutant sequence of the sense strand. The inserted base (G) and the generated premature termination codon of TGA are marked in red and underlined. This one base insertion mutation results in numerous ambiguities (peaks overlapping, indicated by N) immediately after it occurs in the sequencing chromatogram of a heterozygote. (B) Mutant sequence of the antisense strand.

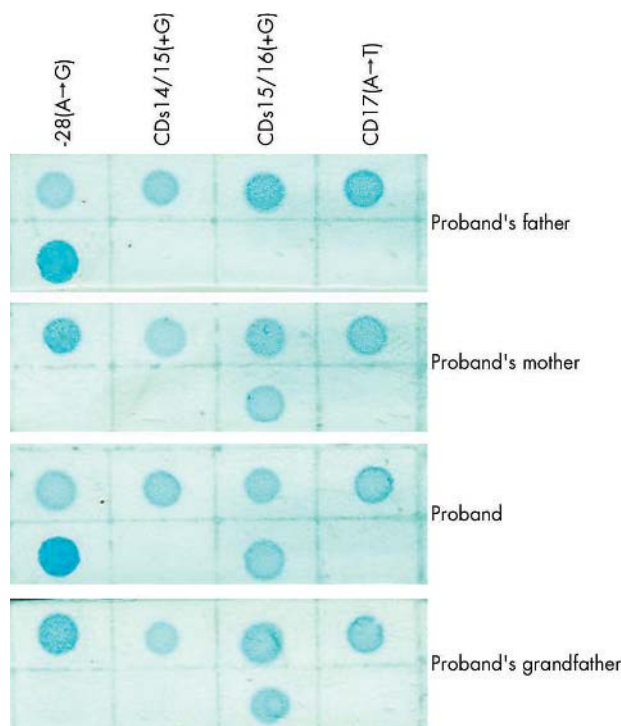


Figure 2 Reverse dot blot assay for detecting the novel CD15/16(+G) mutation. The normal probes are at the top and the mutant probes are at the bottom of each filter strip. The mutations represented by each probe are listed above, and the subjects tested are shown on the right.

30 seconds at 95°C, followed by 40 cycles of 95°C for 15 seconds, 58°C for 17 seconds, and 72°C for 15 seconds. After amplification, a melting curve analysis was performed using a ramping rate of 0.5°C/five seconds for 60–99°C to verify the specificity and identity of the PCR products.

Eight normal individuals and five patients with β^+ thalassaemia trait (four with $-28(A \rightarrow G)/N$ and one with IVS2-654 $(C \rightarrow T)/N$) were selected for the control groups (each with a normal α globin gene). The α globin mRNA was analysed simultaneously in all subjects as an internal control. All samples were tested in triplicate and subsequent calculations were done using the means. Amplification of α and β globin mRNA was carried out in separate tubes using the same amount of total RNA retrieved from the same sample. The relative quantities of β globin mRNA between normal and mutant subjects were normalised with the α globin mRNA to compensate for variations in input RNA amounts.

The relative standard curve method was used to measure the relative amounts of β 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 β and α 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 β 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 β thalassaemia heterozygotes,

with reduced mean cell volume (56.4–68.9 fL) and raised haemoglobin A_2 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 β 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.^{5 6}

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

Molecular diagnosis of β 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 β thalassaemia, 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 β globin mRNA isolated from reticulocytes and nucleated red blood cells. The generated linear regression equations describing the relative standard curves were $y = -3.4924x + 21.6465$ ($R^2 = 0.999$) for α mRNA and $y = -8.2483x + 28.5954$ ($R^2 = 0.999$) for β mRNA. After normalisation with these equations, the calculated mean (SD) C_T values of β 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 β^+ thalassaemia trait, and 12.83 (0.48) ($n = 8$) for normal individuals (fig 3A). Using these mean C_T values, the relative amounts of β 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 β globin mRNA in the patient groups—the proband's mother and grandfather had a 39.83% reduction in β mRNA concentration and the five β^+ thalassaemia heterozygotes had a 26.44% reduction compared with the normal individuals. Therefore, the resultant thalassaemic phenotype of this PTC mutation (CD15/16 +G) should be β^0 . In fact, this interesting phenomenon of greatly reduced amounts of PTC mutated β globin mRNA was reported as early as 1979.⁷ 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 β globin mRNA species in human cells showed that the PTC destabilised the mRNA and greatly decreased its half life from > 16 hours to

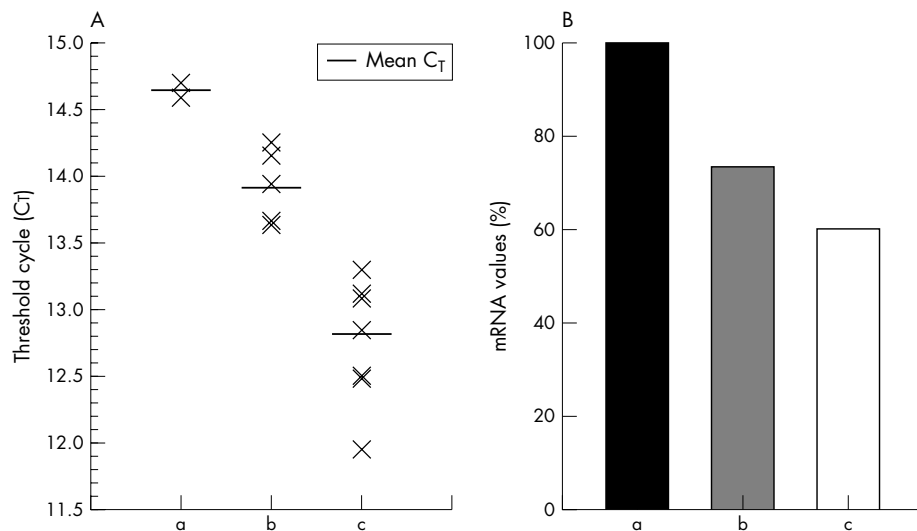


Figure 3 (A) Comparison of the threshold cycle value (C_T) of (a) two heterozygous subjects with the novel CD515/16(+G) frameshift mutation, (b) five patients with β⁺ thalassaemia trait, and (c) eight normal individuals. The proband with β thalassaemia major (-28/CD515/16) was excluded from the study of β globin mRNA values for heterozygous patients. All statistical analyses were performed using the SPSS10.0 software package for mean, standard deviation, and ANOVA with the Student-Newman-Keuls test. Significant differences were found (p < 0.05) between the three groups by pairwise multiple comparison and as a whole. (B) Relative quantitative analysis of β globin mRNA. (a) Normal individuals, (b) patients with β⁺ thalassaemia trait, and (c) heterozygotes with the CD515/16 mutation show 100%, 73.56%, and 60.17% overall β globin mRNA values, respectively.

Take home messages

- We describe a novel CD515/16 (+G) mutation, which results in a premature stop codon and a β⁰ thalassaemia phenotype
- The significantly decreased amounts of β 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

three hours.¹¹ That study could explain our findings of almost complete elimination of mutant β globin mRNA in heterozygous subjects, as assessed by real time RT-PCR analysis. Therefore, most of the remaining mRNA should be wild-type transcripts. The novel frameshift mutation of CD515/16 (+G) in a β⁰ thalassaemia gene reported here would be a valuable model for studying the mechanisms of nonsense mediated mRNA decay.

ACKNOWLEDGEMENTS

This study was supported in part by the Fund of the National Key Basic Research Developments Program of the Ministry of Science and Technology, PR China (2001CB510308), the National Natural Science Foundation of China (30270736, to Q-H Mo), and the Fund of Science and Technology Program of Guangdong Province (2002B30504).

Authors' affiliations

Q-H Mo, X-R Li, X-M Xu, Department of Medical Genetics, Southern Medical University, Guangzhou 510515, Guangdong Province, PR China

C-F Li, Y-L He, Department of Paediatrics of Nanfang Hospital, Southern Medical University

The family members gave their consent for this study to be published

REFERENCES

- 1 Weatherall DJ, Clegg JB. *The thalassaemia syndromes*. Oxford: Blackwell Sciences, 2001.
- 2 Xu XM, Zhou YQ, Luo GX, et al. The prevalence and spectrum of α- and β-thalassaemia in Guangdong Province: implications for the future health burden and population screening. *J Clin Pathol* 2004;**57**:517-22.
- 3 Zhang Y, Coyne MY, Will SG, et al. Single-base mutational analysis of cancer and genetic diseases using membrane bound modified oligonucleotides. *Nucleic Acids Res* 1991;**19**:3929-33.
- 4 Zhang JZ, Xu XM, Ma WF, et al. Reverse dot blot analysis: a rapid prenatal diagnostic approach for β-thalassaemia mutations in Chinese. *Chin Sci Bull* 1994;**39**:1659-62.
- 5 Cooper DN, Krawczak M. Mechanisms of insertional mutagenesis in human genes causing genetic disease. *Hum Genet* 1991;**87**:409-15.
- 6 Gragg H, Harfe BD, Jinks-Robertson S. Base composition of mononucleotide runs affects DNA polymerase slippage and removal of frameshift intermediates by mismatch repair in *Saccharomyces cerevisiae*. *Mol Cell Biol* 2002;**22**:8756-62.
- 7 Chang JC, Kan YW. Beta 0 thalassaemia, a nonsense mutation in man. *Proc Natl Acad Sci U S A* 1979;**76**:2886-9.
- 8 Muhlrad D, Parker R. Premature translational termination triggers mRNA decapping. *Nature* 1994;**370**:578-81.
- 9 Hentze MW, Kulozik AE. A perfect message: RNA surveillance and nonsense-mediated decay. *Cell* 1999;**96**:307-10.
- 10 Wagner E, Lykke-Andersen J. mRNA surveillance: the perfect persist. *J Cell Sci* 2002;**115**:3033-8.
- 11 Couttet P, Grange T. Premature termination codons enhance mRNA decapping in human cells. *Nucleic Acids Res* 2004;**32**:488-94.



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

Q-H Mo, X-R Li, C-F Li, et al.

J Clin Pathol 2005 58: 923-926
doi: 10.1136/jcp.2004.025296

Updated information and services can be found at:
<http://jcp.bmj.com/content/58/9/923.full.html>

	<i>These include:</i>
References	This article cites 10 articles, 6 of which can be accessed free at: http://jcp.bmj.com/content/58/9/923.full.html#ref-list-1
	Article cited in: http://jcp.bmj.com/content/58/9/923.full.html#related-urls
Email alerting service	Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections	Articles on similar topics can be found in the following collections Molecular genetics (253 articles)
--------------------------	---

Notes

To request permissions go to:
<http://group.bmj.com/group/rights-licensing/permissions>

To order reprints go to:
<http://journals.bmj.com/cgi/reprintform>

To subscribe to BMJ go to:
<http://group.bmj.com/subscribe/>