SHORT COMMUNICATION

IDENTIFICATION OF A NOVEL MUTATION IN THE β-GLOBIN GENE 3′ UNTRANSLATED REGION [+1,506 (A>C)] IN A JAPANESE MALE WITH A HETEROZYGOUS β-THALASSEMIA PHENOTYPE

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β-Thalassemia (β-thal) is characterized by the absent or reduced production of β-globin chains. The precise molecular lesion that causes decreased β-globin synthesis in β+-thal is difficult to predict when mutations occur in the locus control region (LCR), the promoter, the introns or 3′ untranslated regions (3′ UTRs). Among them, the role of the 3′ UTR of β-globin gene in mRNA stability is poorly understood, mainly due to very few cases that have mutations in this region. So far, only three mutations have been reported in the 3′ UTR of β-globin gene. Although, it is speculated that some of these reported mutations could be associated with mRNA stability, the precise molecular basis still remains unclear. We report here a novel mutation in the β-globin gene 3′ UTR [+1,506 (A>C)] in a 31-year-old Japanese male with hematological parameters suggestive of heterozygous β-thal. Further functional studies on this novel mutation reported here, may help in understanding of the regulation and expression of the β-globin gene and its products.

Keywords β-Thalassemia (β-thal), Novel mutation, 3′ Untranslated region (3′UTR)

β-Thalassemia (β-thal) is a genetically heterogeneous group of disorders characterized by the absent or reduced production of β-globin chains. The free α-globin chains accumulate and form inclusions that damage the cell membrane and cause ineffective erythropoiesis. Reduced production
of β-globin chains is either due to decreased synthesis of β-mRNA or due to decreased stability of β-mRNA secondary to mutations in exons, splice site consensus sequences or untranslated regions (UTRs). There are relatively less cases that have mutations in the 3'UTR of the β-globin gene, which prevented us from understanding the role of this region in mRNA stability. To the best of our knowledge, only three mutations have previously been reported in the 3'UTR (1,2). Studies conducted on these previously described mutations have not contributed much for the understanding of β-mRNA stability (3).

We report here a novel point mutation in the β-globin gene [+1,506 (A>C); 32 bases downstream from the termination codon of the β-globin gene in the 3'UTR] in a 31-year-old Japanese male with hematological parameters suggestive of heterozygous β-thal. Further functional studies on this novel mutation may help in our understanding of the regulation and expression of the β-globin gene and its products, thereby contributing to the understanding of the 3'UTR and the mechanism of β-globin mRNA stability.

**DNA and RNA Isolation**

DNA was extracted from whole blood using the standard phenol/chloroform method (4). RNA extraction was done using TRIZOL Reagent (Invitrogen, Tokyo, Japan) as per the protocol described in the kit. The concentration of genomic DNA and RNA was measured by spectrophotometer (NanoDrop® ND-2000; Thermo Fisher Scientific K.K., Yokohama, Japan). cDNA synthesis using reverse transcription reaction: purified RNAs (0.1μg) were reverse transcribed using oligo(dT) primers as per the protocol described in PrimeScript™ RT reagent kit (perfect real time, TaKaRa Bio Inc., Otsu, Shiga, Japan).

**β-Globin Gene and cDNA Analyses**

Whole β-globin gene, including promoter regions and the 3'UTR was amplified by polymerase chain reaction (PCR) using βF and βD primers (Table 1). The PCR reagents were from the TaKaRa Ex Taq® Kit (TaKaRa Bio Inc.). The PCR amplification was performed with an ASTEC thermal cycler PC-707 (ASTEC, Fukuoka, Japan). Cycling conditions were as follows: initial denaturation (94°C, 3 min.) followed by 30 cycles of denaturation (94°C, 40 seconds), annealing (60°C, 1 min.), extension (72°C, 2 min.) and final extension (72°C, 5 min.). The purified cDNA was amplified by β57 and β53-2 (Table 1) as primers under the condition of initial denaturation (94°C, 3 min.), 30 cycles of denaturation (94°C, 30 seconds), annealing (63°C, 30 seconds), extension (72°C, 30 seconds) and final extension (72°C, 1 min.).
TABLE 1 Primers Used for DNA and cDNA Direct Sequencing and Semi-Quantification of mRNA

| Primer          | Sequence                     |
|-----------------|------------------------------|
| βF              | 5’-AGT AGC AAT TTG TAC TGA TGG TAT GG-3’ |
| βD              | 5’-TTT CCC AAG GTT TGA ACT AGC TCT T-3’ |
| β57             | 5’-TCT GTG TAG TCC TGA TGG TGT TAT GG-3’ |
| β33-2           | 5’-CCA GTT TAG TAT TGG TAC GGA TTA GGG A-3’ |
| Q1506F          | 5’-CCC TGG CCC ACA AGT ATC ACT A-3’ |
| Q1506r          | 5’-CCC TTC ATA ATA TCC CCC AGT TT-3’ |
| N1506A probe    | 5’-VIC-AAT TTC TAT TAA AGG TTC CTT TG-MGB-3’ |
| N1506C probe    | 5’-FAM-FTT TAT TAA CGG TTC CTT TG-MGB-3’ |

DNA Sequencing

The PCR fragments were purified then directly sequenced by using the BigDye® Terminator cycle sequencing kit version 1.1 on ABI PRISM™ 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan). The same primers as mentioned above (Table 1) were used for the sequencing analysis.

Hydrolysis Probe Method (Semi-Quantification of mRNA)

The purified cDNA is semi-quantititated by a hydrolysis probe method using the TaqMan MGB SNP Kit (Applied Biosystems). The PCR amplifications were carried out on a Light Cycler™ (Roche Diagnostics K.K., Tokyo, Japan) using LightCycler® 480 Probes Master reagent (Roche Diagnostics). Q1506F and Q1506r were used as primers and N1506A and N1506C were used as probes (Table 1). Cycling conditions were as follows: initial denaturation (95°C, 10 min.), followed by 55 cycles of denaturation (95°C, 10 seconds), annealing (60°C, 30 seconds), extension (72°C, 1 second) and cooling (40°C, 10 seconds).

The patient, a 31-year-old Japanese male, was referred to us from a regional hospital for further evaluation of microcytosis. An automated hematology analyzer (XE-2100; Sysmex, Hyogo, Japan) was used for complete blood count. This patient had mild anemia (Hb 11.8 g/dL) with a normal RBC count (5.54 × 10^12/L) and microcytosis (MCV 66.9 flL). His serum ferritin level was normal (147 μg/L). These results prompted further hemoglobinopathy screening tests that showed elevated Hb A2 (5.2%) (5), normal Hb F level (0.7%) and prolonged glycerol lysis time (GLT 50, 79 seconds; normal reference range: 35–55 seconds) (6).

His genomic DNA was sequenced using specific primers to amplify the β-globin gene (−500 to +2000 bp). This analysis revealed a rare point mutation in the 3’UTR that has never before been published in the literature (Figure 1). This novel point mutation was identified in the 3’UTR [+1,506 (A>C) or 32 bases downstream of the termination codon of the β-globin gene]. There was no sequence abnormality in other parts of the β-globin gene. We confirmed that the mutation was not a polymorphism by the
A New Mutation in the β-Globin 3′ UTR (1,506 A>C)

Figure 1: Direct sequencing of the β-globin gene. A heterozygous state for a novel 3′UTR mutation [+1,506 (A>C)] was noted.

Figure 2: The sequencing results of the genomic DNA and cDNA of the patient. The genomic DNA has A and C at position +1,506 that correspond to wild and mutant alleles, respectively, while cDNA has only a wild allele A.

sequencing of more than 1,000 normal alleles. His cDNA construct prepared through reverse transcription showed only A (wild type) at position +1506, while genomic DNA had A/C at position +1506 (Figure 2). These findings were confirmed by the hydrolysis probe method (Figure 3).

Genotype-phenotype correlation is not always straightforward in β-thal, as several genetic factors play a role to ameliorate or worsen the β-thal
FIGURE 3 The result for the semi-quantification analysis of patient’s mRNA. The normal (1), patient (2) and negative control (water) (3), were monitored. When the wild allele-specific probe was used (upper light), fluorescence was obtained in normal (1) and patient (2). However, when the mutant-specific probe was employed (lower right), no fluorescence appeared in both normal (1) and patient (2). This result demonstrated the absence of mutant mRNA in the patient.

phenotype. Reduced production of β-globin chains is predictable when mutations occur in β-globin gene exons, splice site consensus sequences or promoter regions.

With few exceptions (7–9), stability elements are positioned in the 3′UTR where they are protected from disruption. In this context, it was widely anticipated that 3′UTR mutations were likely to impact β-globin mRNA levels by adversely affecting its stability. This stability is determined by many proteins that are known to affect mRNA stability and protein expression (10–13) through mechanisms that involve mRNA capping, polyadenylation and 3′ end processing.

Three earlier mutations have been reported in the 3′UTR. One such mutation is +1,480 (C>G) in PTB (polypyrimidine tract-binding protein)
A New Mutation in the β-Globin 3′ UTR (1,506 A>C)

binding sites (11). Although it reduces the 3′ cleavage activity (11), it presents as silent β-thal (14). The second reported mutation in the 3′ UTR is a 13 nucleotide deletion (+1,565 to +1,577) (15–17). This deletion has been reported to inhibit formation of mature mRNA from pre-mRNA. However, mature mRNA involving this deletion is reported to be stable (16). This is also predicted to affect transfer of mRNA from nucleus to cytoplasm. The third mutation reported in the literature is a point mutation (T>C) at position +1,570 (18,19). Although its precise mechanism is unclear, it is presumed to affect destabilization of β-globin mRNA.

From the above, it appears that all the previously described 3′ UTR mutations have not yet contributed much for the understanding of mRNA stability. We found a novel 3′ UTR mutation at +1,506 from the β-globin Cap site. The mRNA semi-quantification that was carried out in our case showed a decreased amount of the mutant mRNA. We postulate that this could be due to destabilization of mRNA, probably caused by inhibition of stabilizing protein binding, or inhibition of the step that results in mature mRNA from pre-mRNA, as noted in an earlier study (16).

To the best of our knowledge, there is no description in the published literature, of any protein that is likely to interact with a region around +1,506, where we found the 3′ UTR mutation (Figure 4). Finding of more such 3′ UTR mutations is therefore essential and paves the way for further studies to delineate a better understanding of molecular mechanisms of β-globin mRNA stability and the pathophysiology of β-thal disease.

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.
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