Aberrant mRNA Spliced in a β^0-Thalassemic Gene Having a G → A Mutation at βIVS II-1

Teruo HARANO, Keiko HARANO* and Ne WIN* *

Department of Biochemistry, Kawasaki Medical School, Kurashiki 701-0192, Japan
**Department of Clinical Nutrition, Faculty of Medical Professions, Kawasaki University of Medical Welfare, Kurashiki 701-0193, Japan*
***Pathology Research Division, Department of Medical Research (Lower Myanmar), Yangon, Union of Myanmar*

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ABSTRACT. mRNA extracted from β^0-thalassemia patient with a βIVS II-1G → A mutation, who had been diagnosed by hemoglobin biosynthesis and molecular analysis, was amplified by use of specific oligonucleotide primer set. Sequencing of the PCR product demonstrated that mRNA from DNA possessing G → A mutation at βIVS II-1 seemed to be an aberrant mRNA spliced at the site of dinucleotide GT at βIVS II-48-49 instead of GT at βIVS II-1-2. The mRNA produced from the mutant β-globin gene was inserted 47bp between the exons 2 and 3, which was expected to be unstable in the nucleus, and the globin chain translated from such mRNA might be shorter than the normal chain because the nonsense sequence (TGA) at the 7th codon in the inserted 47 bp was created. Thus, the production of an aberrant mRNA due to this nucleotide change (G → A) at βIVS II-1 was thought to be the cause of β^0-thalassemia.

Key words: β^0-thalassemia — mRNA — PCR — sequencing — missplicing

β-Thalassemia (β-Thal) is a hereditary disorder characterized by reduced synthesis of the β-globin chain of adult hemoglobin A (Hb A: $\alpha_2\beta_2$). This condition may be further subdivided into β^0-and β^+-Thal, on the basis of amount of β-globin chain produced from mRNA derived from patient's DNA.[1,2]

Although Thal has been believed to be rare disease in Japan, more than 35 different types of β-Thal were identified until the end of 2000.[3,4] Among these, four mutations, −31 Cap A → G, Codon (CD) 90 GAG → TAG (nonsense codon), β intervening sequence (IVS) II-1 G → A, and βIVS II-654 C → T, covered 60% of Japanese β-Thal patients, and these and four additional mutations, including CD 41/42 TTCTTT → TT, initiation CD ATG → ATA, AGG, ATC, ACG, GTG, and CD 127/128 CAGGCT → CCT (Hb Gunma [β127-128Gln-Ala → Pro]), account for 80%. These β-Thal mutations are thought to be common Thals in Japan, and different from those in the neighbor countries, Korea, China, Thai, and so on.[4]

In this study, the structure of β-thalassemic mRNA collected from a patient with heterozygous β-Thal for βIVS II-1G → A mutation, one of the common β-Thal mutations in Japan, was examined.

原野昭雄，原野恵子，Ne Win

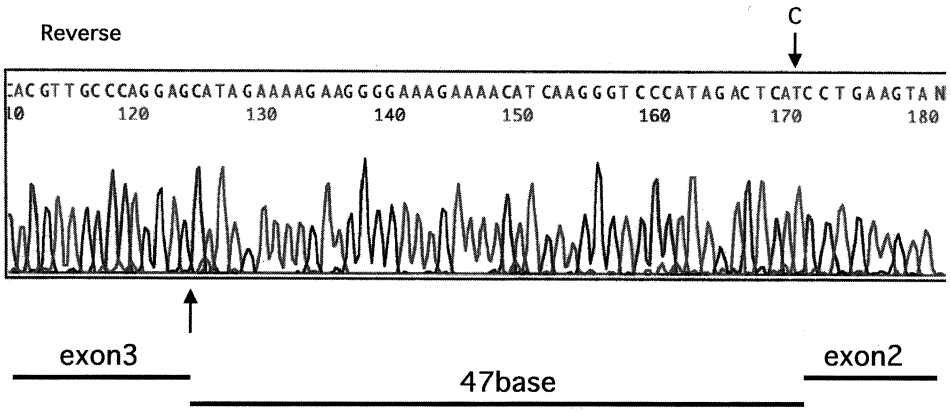
MATERIALS AND METHOD

Case Report: A 52-year-old Japanese male with microcytic hypochromia was diagnosed β^0-Thal heterozygote by Hb biosynthesis and molecular analyses.[5,6] At the time of examination, his hematological findings were: RBC 5.73×10^{12}/L, Hb 12.0 g/dL, PCV 0.377 L/L, MCV 65.8 fL, MCH 20.9 pg, MCHC 31.8 g/dL, total Bil. 0.3 mg/dL, serum iron 54 μg/dL, TIBC 282 μg/dL, Hb A_2 4.91%, Hb F 1.8%. Target cells and anisocytes were observed on the blood smear. Hb instability by isopropanol precipitation test was done and it was negative, The ratio (β/α) of the β-globin to the α-globin in the biosynthesized Hb was 0.52, being depressed biosynthesis of the β-globin chain in the reticulocytes. DNA extracted from the white cells was amplified by use of specific primer set covered from the 5' promotor region to the poly A binding site of the β-globin gene. PCR product was sequenced by the method of direct sequencing using ABI Prism: BigDye Terminator Cycle Sequencing Ready Reaction Kits (Japan PE Biosystems, Chiba, Japan) according to the procedure recommended by manufacturer.[7] Mutation was identified as a nucleotide substitution of G → A at βIVS II-1, a β^0-Thal heterozygote.

Analysis of Structure of mRNA: mRNA collected from patient's peripheral blood by use of QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech Japan, Shinjuku, Tokyo, Japan) was converted to cDNA by reverse transcriptase using First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech Japan, Shinjuku, Tokyo, Japan). These procedures were done according to the manuals recommended by manufacturers.[8,9] PCR reaction was done in a MJ mini cycler (Funakoshi Chemicals Co., Tokyo, Japan) with 30 cycles of denaturation at 95°C for 1 min., annealing at 55°C for 30 sec., and elongation at 72°C for 2 min., in the mixture of 5 μL of 10×PCR buffer, 1 μL of forward primer (20 pmol/L solution), 1 μL of reverseward primer (20 pmol/L solution), 1 μL of cDNA solution (ca. 50 ng), 1 μL of Taq polymerase (1 unit) and 41 μL of distilled water, and covered with 75 μL of mineral oil. Synthesized oligonucleotides used here were: 5'-GGATCCTGAGAACTTCAGGA-3', matching to the mutant mRNA (cDNA), as forward primer and 5'-GCAGAATCCAGATGCTCAAGG-3' as reverse ward primer. The size of PCR product was about 2 kb. After reaction, PCR product was collected as precipitates in isopropanol, disolved in 10 μL of TE (10 mmol/L Tris-1 mmol/L EDTA, pH 7.4) solution and subjected to electrophoresis on 1% agarose gel. After electrophoresis and staining with ethidium bromide, the proper size (about 2 kb) DNA fragment was collected by use of Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad Laboratories, Hercules, CA, USA).[10] The DNA was reversely sequenced from 3' site in normal by the direct method as described above.

RESULTS

Agarose gel electrophoresis of first-PCR product prepared from cDNA by using of the specific primer corresponding to mutant sequence showed no products. However, the second PCR product to the first PCR reactant in the same way revealed about 2 kb DNA fragment on the gel, corresponding to the



g

5'·····CCTGAGAACTTCAGG atgagtctatgggacccttgatgttttctttcccc

ttcttttctatgCTCCTGG GCAACGTGCTGGTCTGTGTGCTGGCCCAT·····3'

Fig 1. Reverse sequencing of PCR product amplified by use of oligonucleotide primer corresponding to the mutant gene (βIVS II-1 G → A).

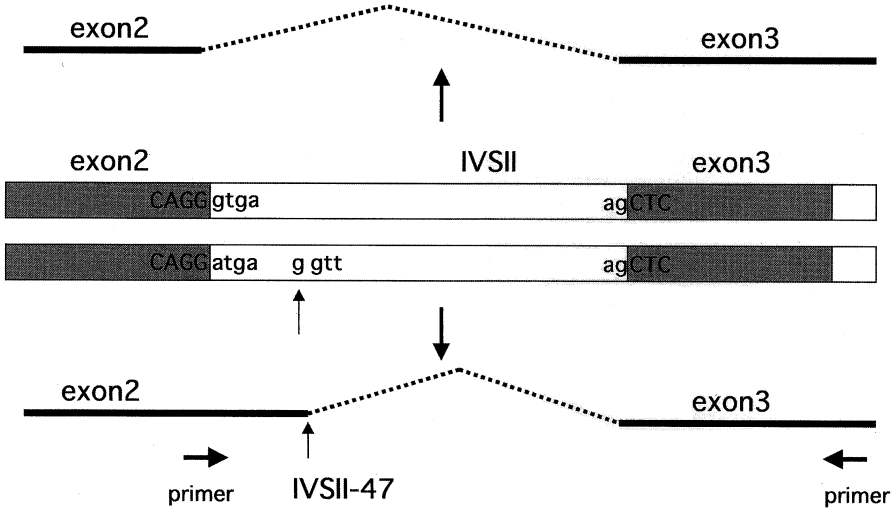


Fig 2. Structure and transcription of the normal and β^0-globin genes. Upper: Sequence around junctions between the exon 2 and IVS II, and IVS II and exon 3 in the normal gene, and mRNA sequence removed by splicing is indicated by caret. Bottom: Sequence arround junctions between the exon 2 and IVS II, and IVS II and exon 3, and mRNA including the sequence from 1 to 47 of IVS II, present in the dominant β^0-Thal gene transcript, is indicated by caret.

expected fragment of cDNA having mutation of G → A at βIVS II-1. This DNA fragment was extracted from gel and subjected to direct-sequencing from

the reverse (3') site. The result revealed the insertion of 47 mer nucleotides between exons 2 and 3, suggesting that the splice resulting in the mutant gene occurred between dinucleotide GT at βIVS II-48-49, and dinucleotide AG at βIVS II-849-850, normal splicing acceptor site (Fig 1, 2). In this case, βIVS II-48-49 GT dinucleotide acted as a cryptic splicing donor site.

DISCUSSION

In the course of the synthesis of Hb molecule, it is very important to be synthesized the normal messenger RNA (mRNA) in vivo through the course of the transcription of DNA to produce transcript, precursor mRNA (pre-mRNA), and processing to matured mRNA from pre-mRNA. The dinucleotides of GT at the 5' end site (donor site) and AG at the 3' end site (acceptor site) of the intervening sequence (IVS) play a very important role in the course of the production of matured mRNA from pre-mRNA, i.e. splicing IVS after binding 7-methylguanosine to the Cap site, 5' end of the transcripted pre-mRNA, and poly adenyl group to the poly A binding site, 3' end of the pre-mRNA. The dinucleotide sequences, GT and AG, are called "Consensus Sequence". They are important sequences conserved at the ends of 5' and 3' of IVS in all of mammalian genes.[11,12] If one base of these nucleotides changes to other base, the splicing of IVS does not occur between these dinucleotides (GT/AG) but produces an aberrant mRNA, which may be unstable and a longer or shorter than the normal mRNA. In the case of β-globin gene with βIVS II-1G → A, the normal mRNA is not produced by missplicing due to the nucleotide mutation (Fig 1, 2). As oligonucleotide sequence of the forward PCR primer to amplify cDNA derived from β^0-Thal gene used here corresponded to that of the mutant gene, the detected PCR product had 47 mer longer than the normal (Fig 2). The globin translated from this abnormal mRNA may be of at least 18 amino acids longer than the normal 146 amino acids. However, in this additional DNA length the nonsense codon (TAG) is present at the 7th position from 5' end of IVS II and subsequently produces the shroter β-globin chain (110 amino acid chain).

In this study, the sequence of product amplified by use of PCR primer corresponding to the β^0-thalassemic mutant gene (βIVS II-1G → A) was examined and detected the presence of aberrant mRNA adding of 47 bp. However, due to the mutation of one base of consensus dinucleotide GT at the splicing donor site, it is also possible that other GT sequence site(s) to be as a cyptic splice donor site, e.g. splicing between the GT sequence at the 5' site of βIVS I and the AG sequence at the 3' site of IVS II.[13] This process will make a shorter mRNA composing of exons 1 and 2. In the case of β^+-Thal with βIVS II-654 C → T, the GT dinucleotide at βIVS II-653-654 created by this mutation acts as a cryptic splice donor site to the dinucleotide AG at 3' site βIVS II-849-850 (normal accepter site of βIVS II) and the dinucleotide AG does as a cyptic acceptor site at βIVS II-578-579 to the dinucleotide GT at βIVS II-1-2 (the normal donor site) to produce an aberrant mRNA adding 73 bp between exons 2 and 3.[14–16] As it is possible to occur the normal splicing between the normal splicing donor and acceptor sites to produce the normal mRNA which translates the normal globin chain, β-Thal mutation with βIVS II-654 C → T is thought to be β^+-Thal. There are various types of β^0-Thal or

β^+-Thal according to the position and the kind of mutated nucleotide. Additionally, revealing an aberrant mRNA in the PCR product of the second reaction might suggest its trace amount and very unstablility in the peripheral blood.

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