A Case Report of Diagnosis of α-thalassemia-2

Teruo HARANO, Keiko HARANO*, Sung-Im CHO 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*
***Department of Clinical Pathology, Seoul National University Hospital, Seoul 110-744, Korea*
****Pathology Research Division, Department of Medical Research, 5 Ziwaka Road, 11191, Yangon, Union of Myanmar*

Accepted for publication on February 3, 2001

ABSTRACT. An apparently healthy 31-year old Japanese man was presented with mild hypochromia and low grade microcytosis during a medical check-up. The MCV and MCH values were low and serum iron level was also decreased. Remaining hematological and biochemical examinations revealed normal. With the provisional diagnosis of thalassemia trait, either β or α, further investigations were done for confirmatory documents. Isoelectric focusing and DEAE-HPLC showed no abnormalities. The β/α ratio of globin biosynthesis was also normal. DNA analysis by the PCR method using special primer sets was positive with $-\alpha^{3.7}$ (α-thal-2) and final confirmation was documented by direct sequencing of PCR products where deletion site was detected. Here we highlighted that, β/α ratio may not be changed in some form of $-\alpha^{3.7}$ (α-thal-2) patient. DNA analysis is mainstay of investigation to attain the accurate diagnosis in α-thal syndromes.

Key words: α-thalassemia — α-thalassemia-2 — hematology — Hb biosynthesis — DNA analysis

Thalassemia (thal) is a series of hereditary disorder characterized by impaired synthesis of globin chains resulting in hypochromic microcytic types of anemia.[1] Thus α-thal results from reduced rate of synthesis of α-globin chain and β-thal from that of β-chain and leads to changes in β/α ratio. There are four α-globin genes and account the known forms of α-thal representing the presence of one, two, three, or four α-thal genes, i.e. silent carrier, α-thal-2 responsible for milder defect, has $-\alpha/\alpha\alpha$ (-: indicates the deletion of one of two α-genes arranged on one chromosome), α-thal trait, α-thal-1 responsible for a marked deficiency, has $--/\alpha\alpha$ or $-\alpha/-\alpha$, Hb H disease, including about 5% of Hb H (β_4) in the total hemoglobin (Hb) and revealing the presence of specific Hb H inclusion body, has $--/-\alpha$, and hydrops fetalis, causing stillbirth, has --/--.[1] Generally, the α-thal syndromes are more difficult to characterize and study in comparison to β-thal syndromes. The carrier state for the α-thal-2 or α-thal-1 may be impossible to identify: hematologic changes are subtle or absent, the Hb electrophoretic pattern is

原野昭雄, 原野恵子, Sung-Im Cho, Ne Win

normal, and the concentration of the minor Hb components are within the normal range. Adult silent carrier or α-thal-2 has no abnormality in Hb findings and ordinarily also normal clinical condition. Hematological changes are usually normal with the exception of occasional mild microcytosis. In α-thal-1, low grade red cell microcytosis and hypochromia with mild anisopoikilocytosis may be seen, but anemia is absent or very mild.

Here we report how a case with the clinical suspicion of thal has finally diagnosed accurately after running a battery of investigations and thorough analysis of the laboratory results.

The hematological and biochemical investigations by ordinary methods were done in an apparently healthy 31-year-old Japanese man. His peripheral blood film examination was apparently normal apart from a mild hypochromia and low grade microcytosis. Neither poikiocytes, target cells, nucleated cells nor other abnormal cells were seen. Hematological findings were RBC 6.22×10^{12}/L (normal=$4.4\text{-}5.5\times 10^{12}$), total Hb concentration 14.4 g/dL (normal=13.5-17.5), hematocrit 45.9% (normal=39-52), MCV 73.8 fL (normal=87-103), MCH 23.3 pg (normal=29-35), reticulocyte count 1.0% (normal=< 1.5), serum iron 43 μg/dL (normal=80-150), and TIBC 289 μg/dL (normal=267-435). All of these hematological and biochemical findings, except low serum Fe level, had favoured the possiblity of the diagnosis of thal trait, either β or α.

Then, Hb analysis by isoelectric focusing (pH rangae: 6-9)[2] and high performance liquid chromatography (HPLC) on DEAE-5PW column (7.5×75 mm, Tosoh Co. Ltd., Tokyo, Japan)[3] were done. No abnormal Hb was detected and Hb A_2 content (2.65%) and Hb F content (<1%) were also found to be normal (normal Hb A_2 and Hb F are 2.2-3.5% and <1.5%, respectively). Hb instability test (isopropanol precipitation test)[4] was also performed and it was negative. So the findings through these special investigations done so far gave no information yet.

Further, Hb biosynthesis was carried out incubating the reticulocytes in an amino acid culture medium containing ^{3}H-leucine at 37°C for two hours.[5] After incubation and removal of the excess ^{3}H-leucine by washing with saline, red cells are collected and lysed by adding 0.1% saponin solution to get the biosynthesized Hb hemolysate. Then, globin was collected from the hemolysate by HCl-acetone treatment and chromatographed on CM-cellulose column (CM-52, Whatman Paper Co., Maidstone, Kent, UK; column size=0.8×10 cm) using an eluent with a cationic gradient (Na^+: 7.5-32.5 mmol/L), to separate it into α- and β-globin chains[6] Cationic Na^+ gradient was prepared by mixing 50 mL of 8 mol/L urea-phosphate buffer (pH 6.85) containing 7.5 mmol/L Na^+ with that containing 32.5 mmol/L Na^+ concentration. The chromatogram thus obtained was shown as Fig 1. Radioactivities incorporated into the β-globin chain was 12,600 dpm and that of α-globin chain was 12,700 dpm. So β/α globin chain synthesis ratio was 0.99 (normal=0.9-1.2) and there was no diagnostic information again.

Then we moved further with PCR-based DNA mutation analysis. DNA was extracted from leucocytes of perpheral blood by phenol-chloroform method.[7] Aliquot of 0.1 μg genomic DNA was amplified by the use of thermostable DNA polymerase (HotStarTaq DNA polymerase, Qiagen Ltd. Co., Tokyo, Japan) and three different special primer sets which were complementary to normal ($\alpha\alpha$), $-\alpha^{3.7}$ (α-thal-2) and $--^{SEA}$ (α-thal-1) sequence.[8]

A 30 cycles of denaturation (at 94°C for 1 min), annealing (at 56°C for 30 sec), and extention (at 72°C for 2 min) was accomplished in the thermal cycler. The amplified DNA products were electrophoresed in 0.8% agarose or 5% polyacrylamide gel and then photographed (Fig 2). The PCR was positive with the primers of normal and α-thal-2 and was negative with that of α-thal -1. These PCR findings lead the case to be an α-thal-2 having $-\alpha^{3.7}/\alpha\alpha$ genotype.

The amplified DNA products of α-thal-2 gene was further analyzed by direct sequencing to determine and confirm the region where deletion had occurred. Method of BigDye terminator cycle sequencing (BigDye Terminator Cycle Sequencing Ready Reaction Kits, PE Applied Biosystems, Chiba, Japan) was applied and synthetic oligonucleotides were used according to the instruction by manufacturer. As shown in Fig 3, most of nucleotide sequences of the IVS 2 were derived from the α1-gene (i.e. G at nt. 55 ; GGGCCCTC at nts. 115-121 ; C at nt. 126 ; and the presence of Apa I site at nts. 114-119 GGGCCC). Moreover, the sequence of the 3' flanking region was also identified to that of α1-gene. Since the sequence of exon 1, IVS 1, and exon 2 is common in both gene, there is the crossing over of the sequence in the region of upstream of 5' site of IVS 2 in between them. This is usually seen in $-\alpha^{3.7}$ I form.[8,9]

Finally, we come to an end for an establishment of an accurate diagnosis of a case with the hematological features of thal trait. Since from the very beginning, we have excluded the possibility of iron deficiency anemia for two reasons : (a) microcytosis cannot be expected in iron deficiency anemia with a milder degree of reduction of serum concentration (43 μg/dL) and (b) increased TIBC is one of the characteristic features of iron deficiency anemia.

Deletion of a single α-thal gene is betrayed by neither clinical nor hematologic abnormality. The silent carrier α-thal state is inferred from findings of family studies in which a propositus has a symptomatic α-thal syndrome. Reticulocytes from adults presumed to have the silent carrier state on the basis of family studies synthesized less α- than β-globin. In any given individual, however, the β/α synthetic ratio may fall within the normal range. Measurement of the β/α-globin mRNA ratio distinguishes more clearly between individuals with one, two, three, or four functional α-gene. This measurement and gene mapping may afford the reliable means for precise identification.

It has been well known that most of Japanese α-thal are due to the deleteion of the α-globin gene ; the nucleotide mutation is very rare. The α-thal-2 (deletion of 3.7 kb from the sequence of 5'-α2-α1-3' arranged on chromosome) has a genotype of $-\alpha^{3.7}/\alpha\alpha$, and α-thal-1 (the deletion of about 18.5 kb including the whole sequence of 5'-α2-α1-3') has another genotype of $--^{SEA}/\alpha\alpha$, the Southeast Asian type, which is common in SEA area and also can be seen in Japan.[10,11] The α-thal in Japan, particularly α-thal-2, having the genotype of $-\alpha/\alpha\alpha$ can be found in about 2% of the population[12,13] and described as $-\alpha^{3.7}$ I form. Therefore, the homozygotes for α-thal-2 as a carrier of phenotype α-thal-1 ($-\alpha/-\alpha$ or $-\alpha^{3.7}/-\alpha^{3.7}$) can only occasionally be observed.[14] In addition, most of the carriers of phenotype of α-thal-1 have genotype $--^{SEA}/\alpha\alpha$. Thus, Hb H genotype rarely discovered in Japan is much consistent with $-\alpha^{3.7}/--^{SEA}$.[11-14]

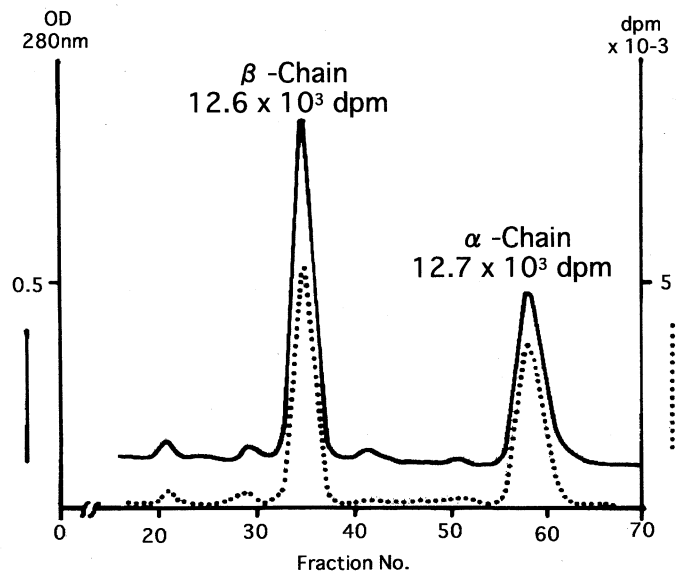

Fig 1. Urea CM-52 cellulose column chromatographic separation of the globin obtained from biosynthesized Hb. The numbers indicate the radioactivities (total dpm) incorporated into the relevant chains.

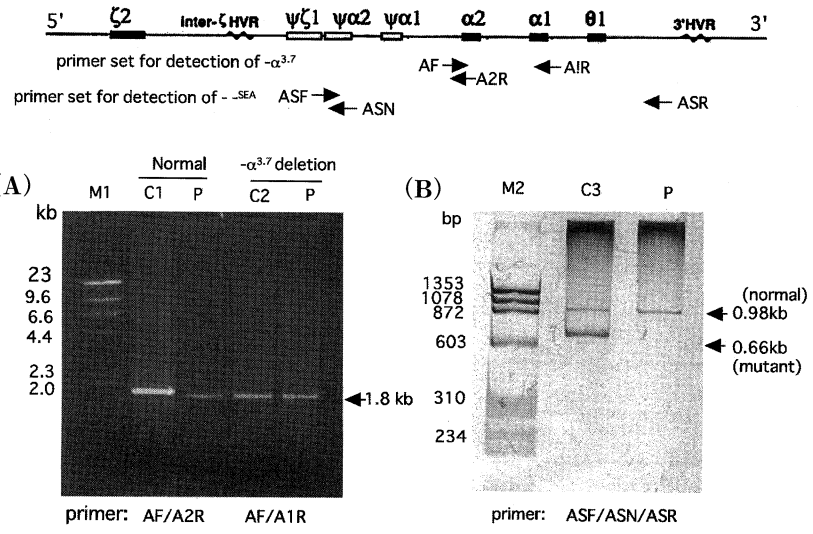

Fig 2. The location of three defferent special primer sets to detect the normal ($\alpha\alpha$), α-thal -2 gene ($-\alpha^{3.7}$) and α-thal-1 gene ($--^{SEA}$) (Top).

A: Electrophoresis on a 0.8% agarose gel of PCR products obtained by use of primer set to detect the $-\alpha^{3.7}$ thal gene, which was visualized by staining with ethidium bromide. M1: Molecular weight marker. C1: Normal control with $\alpha\alpha$ gene. C2: Control with $-\alpha^{3.7}$ thal gene. P: Patient.

B: Electrophoresis on a 5% polyacrylamide gel of PCR products obtained by use of primer set to detect the $--^{SEA}$ thal gene, which was visualized by silver-staining. M2: Molecular weight marker. C3: Control with normal gene and $--^{SEA}$ thal gene. P: Patient.

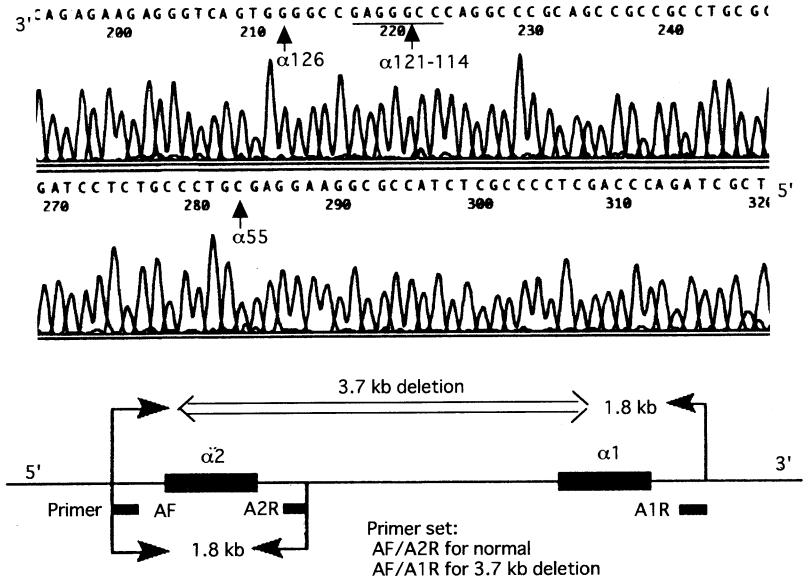

Fig 3. Reverse sequencing of the αIVS 2 region of PCR product prepared by use of special primer set to detect the $-\alpha^{3.7}$ thal gene by the method of BigDye terminator cycle sequencing (Top) and the region deleted from the arrangement of 5'-α2-α1-3' (Bottom).

In-depth and extensive studies are worthy to be conducted in the future, particularly on this type of α-thal-2 of "rightward" deletion, provided family members who are obligate carriers of α-thal gene must be included. The well known clinical heterogeneity of the thal syndrome is a reflection of the great heterogeneity of mutations affecting the globin gene.

ACKNOWLEDGMENT

This study is partly supported by a Research Project Grant from Kawasaki Medical School (12-105) and partly by a Grant-in-Aid (12672259) from the Ministry of Education, Science, Culture and Sports, Japan.

REFERENCES

1) Bann HF, Forget BG: Hemoglobin: Molecular, Genetic and Clinical Aspects. Philadelphia, W B Saunders, 1986, pp169-350
2) Harano T, Harano K, Koide T, Okada M, Ueda S, Shibata S: Mass screening method of abnormal hemoglobin by isoelectric focusing. Jpn J Clin Path **28**: 149-152, 1980
3) Harano T, Harano K, Katsuki T: Hb Yaizu [β79 (EF3) Asp-→ Asn]: A new β chain variant found in a Japanese female. Hemoglobin **19**: 21-25, 1995
4) Carrell RW, Kay R: A simple method for detection of unstable haemoglobins. Brit J Haematol **23**: 615-619, 1972
5) Harano T, Ueda S: Biosynthetic and analytical method of hemoglobin-A new technique for the diagnosis of thalassemia. Jpn J Clin Path **29**: 61-70, 1981
6) Clegg JB, Naughton MA, Weatherall DJ: Abnormal human haemoglobins, separation and characterization of the α and β chains by chromatography, and determination of two new variants, Hb Chesapeake and Hb J Bangkok. J Mol Biol **19**: 91-108, 1966
7) Poncz M, Solowiejezyk D, Harpel B, Mory Y, Schwartz E, Surrey S: Construction of human gene libraries from small amounts of peripheral blood: Analysis of β-like globin

genes. Hemoglobin **6**: 27-36, 1982
8) Baysal E, Huisman THJ: Detection of common deletional α-thalassemia-2 determinants by PCR. Am J Hematol **46**: 208-213, 1994
9) Higgs DR, Hill AVS, Bowden DK, Weatherall DJ, Clegg JB: Independent recombination events between the duplicated human α globin genes; implications for their concerted evolution. Nucleic Acids Res **12**: 6965-6977, 1984
10) Stephen H, Embury SH, Lebo RV, Andre M, Dozy AM, Kan YW: Organization of the α-globin genes in the Chinese α-thalassemia syndromes. J Clin Invest **63**: 1307-1310, 1979
11) Harano T, Harano K, Ueda S, Nagao T, Mori T: The molecular basis of Hb H disease in a Japanese girl. Act Haematol Jpn **52**: 1128-1136, 1989
12) Shimizu K, Harano T, Miwa S, Ohba Y, Harano K, Huisman THJ: Arrangements of α and γ globin genes in Japanese newborn babies. Acta Haematol Jpn **48**: 491-491, 1985
13) Harano T, Wada Y, Miyaji T, Harano K, Hayashi A, Ueda S, Ohba Y: Diversity of globin gene arrangement in Japanese newborn babies. Acta Haematol Jpn **50**: 419-419, 1987
14) Harano T: The present state of thalassemias and hemoglobinopathies in Japan. XIIth International Symposium on Technical Innovations in Laboratory Hematology, Abstract p46, in Kobe, 1999