β^0 -Thalassemia due to a Nonsense Mutation at $\beta 90$ (GAG→TAG) in Human Hemoglobin Gene

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We studied a patient with severe anemia and jaundice who exhibited a high hemoglobin A₁ (HbA₁) level secondary to an increase in HbF despite normal glucose tolerance. The red blood cells showed anisocytosis, poikilocytosis and polychromasia; target cells, Howell-Jolly bodies, Heints bodies and punctate basophilia were observed. No defect or reduction in activity was observed in 19 red cell enzymes. A family history of similar anemia in the patient's daughter and cousins on the mother's side indicated an involvement of genetic factors. Gene cloning and DNA analysis showed that the condition is a new type of β^0 -thalassemia caused by a nonsense mutation (GAG→TAG) in codon 90 of the β -globin gene.

Key words: β^0 -thalassemia, Nonsense mutation, Codon 90, Gene cloning, DNA analysis

HbA₁ and HbA_{1c} levels have been studied increasingly as indices of long-term blood sugar control in diabetic patients (1) and as aids in the early detection of diabetes (2). As a result, many types of hemoglobinopathy have been found by routine clinical evaluation of the HbA_{1c} level (3). We encountered a patient with severe anemia and jaundice in whom the HbA₁ level was abnormally high at 20.1% due to an increase in HbF despite the absence of glucose intolerance. By a detailed examination this condition was identified as β^0 -thalassemia caused by a nonsense mutation (GAG → TAG) in codon 90 of the β -globin gene.

PATIENT AND METHODS

Patient (Record: November, 1980)

The patient was a 42-year-old female with a history of jaundice at the age of 13 and untreated anemia at the age of 19. She received a transfusion after giving birth to a child at the age of 24, and subsequently demonstrated severe jaundice and

anemia. She underwent splenectomy at the age of 27 with a diagnosis of hemolytic anemia, but this diagnosis had not been confirmed. She also underwent cholecystectomy due to gall stones at the age of 39. At the time of the examination, the patient still showed jaundice and anemia and complained of mild malaise. There was a history of one abortion. She was 154 cm tall and weighed 48 kg.

On physical examination, there were no marked abnormalities except for anemia and jaundice, functional systolic murmur, and hepatomegaly.

Laboratory data included RBC $263 \times 10^4/\mu\text{l}$, Hb 6.8 g/dl, Ht 26.0%, MCV 99 fl, and MCH 26 pg, indicating severe anemia. The total nucleated cell count including erythroblasts and leukocytes was $44,700/\mu\text{l}$; reticulocytes were increased to 32.5%. Total bilirubin was high at 4.5 mg/dl (direct 1.3 mg/dl, indirect 3.2 mg/dl), but transaminase levels were normal. However, liver biopsy suggested hemosiderosis. Renal function and electrolyte levels were normal. The results of direct and indirect

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Coomb's tests were both negative. Other findings were: Fe 287 $\mu\text{g}/\text{dl}$, Cu 81 $\mu\text{g}/\text{dl}$, Mg 2.0 mEq/l, haptoglobin 10 mg/dl or less, vitamin B12 600 pg/ml, folic acid 2.3 ng/ml, total iron binding capacity (TIBC) 247.8 $\mu\text{g}/\text{dl}$, unsaturated iron binding capacity (UIBC) 33.6 $\mu\text{g}/\text{dl}$, red cell iron (^{59}Fe) utilization 78.7%, plasma iron disappearance rate (PID) $T_{1/2}$ 48 minutes. The HbA₁ level determined with Quik-Sep (Isolab Inc., Ohio, USA) was 20.1%, and the HbA_{1a} + b and HbA_{1c} levels determined according to the method of Trivelli et al (macro-column method) (4) were 4.33% and 15.24%, respectively. These high values were, however, inconsistent with the results of 50g-OGTT (plasma glucose; 0'–76, 30'–105, 60'–135, 90'–71, 120'–70 mg/dl).

The patient's family consisted of herself, her husband, and a daughter, who was found to have jaundice and anemia from about the age of six. Her father and two brothers were alive, but her mother had died of pancreatic cancer with jaundice at the age of 47. Jaundice had been noted in 2 of her cousins on her mother's side (Fig. 1).

Methods

Blood was collected using EDTA-2Na and heparin as anticoagulants, and used for the following evaluations.

Red cell morphology: The morphology of red blood cells was studied under light and scanning electron microscopy using the peripheral blood and bone marrow aspirate from the iliac crest.

Hemoglobin analysis: Abnormal hemoglobin was examined by isoelectric focusing. HbA₂ was determined by cellulose-acetate membrane electrophoresis. HbF was measured by Betke's alkali denaturation method (5).

Erythrocyte degeneration tests: Isopropanol tests

Fig. 1. Pedigree of patient with β^0 -thalassemia trait.

●; proband ○; female □; male
●■; female and male with β^0 -thalassemia trait
●/□; deceased

(6) were performed.

Erythrocyte fragility tests: Osmotic fragility tests against various concentrations of NaCl were performed according to the method of Parpart et al (7).

Red cell enzymes: The activities of 19 red cell enzymes were examined.

Hb synthesis: Hb synthesis was induced by allowing reticulocytes to incorporate ^3H -Leu in a synthetic medium. The hemolysate was treated with HCl-acetone to obtain globin, and α - and β -globins were separated by CM-cellulose (CM-52; Whatman Co., New Jersey, USA) column chromatography (eluant: 8M urea-sodium phosphate buffer, pH 6.8; Na ion concentration 5 mM to 35 mM). The radioisotope uptake was evaluated in each fraction.

DNA analysis: DNA was extracted from leukocytes of the patient by the method of Poncz et al (8). Genomic Southern-blot-hybridization was performed as previously described (9). The probe used here was the Bam HI-Eco RI fragment (0.9 Kb) containing the intervening sequence (IVS) II of the β -globin gene which was radiolabeled by the procedure of nick translation. A combinant library was constructed by ligating Hind III-digested genomic DNA fragment (ca. 7.5 Kb) to Charon 28 which had been digested with Hind III and subsequently dephosphorylated. The ligated mixture was packaged in the phage particles using the in vitro packaging extract (Packagene, Promega Biotech, Co., USA). Recombinant phage was screened using the β -IVS II probe. A 2.8 Kb Sph I-Pst I fragment was subjected to subcloning to M13mp18 phage and to nucleotide sequencing (Dideoxy method) using a universal primer as well as several synthetic primers.

RESULTS

Red blood cells showed anisocytosis, poikilocytosis, and polychromasia (Fig. 2A, Fig. 2B); target cells, Howell-Jolly bodies, Heintz bodies, and punctate basophilia were observed. On bone marrow puncture, the nucleated cell count was $44.9 \times 10^4/\mu\text{l}$, and M/E ratio was 0.15; an increase in the cell count in erythrocytic series was observed. The HbF and HbA₂ levels were elevated at 14.1% and 4.84%, respectively. No bands of abnormal hemoglobin were observed by cellulose-acetate membrane electrophoresis or isoelectric focusing (pH

Fig. 2A. The morphology of red blood cells studied under light microscopy (peripheral blood, May-Grünwald-Giemsa stain)

Fig. 2B. The morphology of red blood cells studied under scanning electron microscopy (peripheral blood)

6.7–7.7).

During the erythrocyte degeneration tests, gradual precipitation was observed from immediately after the initiation of the depletion of distilled water, and osmotic fragility tests using various concentrations of saline showed an increase in resistance of the erythrocyte membrane. No defect or reduction of activity was observed in the following 19 red cell enzymes examined: hexokinase, glucosephosphate isomerase, phosphofructokinase, aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, monophosphoglyceromutase, enolase, pyruvate kinase, lactate dehydrogenase, glucose 6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, glutathione reductase, glutathione peroxidase, adenylate kinase, adenosine deaminase, acetylcholinesterase, and pyrimidine 5'-nucleotidase.

Analysis of Hb synthesis showed suppression of synthesis of β -globin as compared with α -globin (β/α ratio = 0.35) (Fig. 3). The findings resulting from the digest of the DNA with several restriction endonucleases and hybridization with a β -IVS II probe did not show a deletion over at least 100 bp

in the region surrounded and within the β -globin gene, and gave the presence of polymorphism at the Ava II site in the β -globin gene. Two types of β -globin gene clones possessing plus and minus polymorphisms at Ava II site were obtained from about 5×10^5 gene library. Nucleotide sequencing of the relevant clones from the 5' flanking region

Fig. 3. The measurement of radioisotope uptake in α - and β -globins after hemoglobin synthesis induced by allowing reticulocytes to incorporate ^3H -Leu in a synthetic medium.

○-○ optical density ●-● ^3H -Leu uptake (total activity; β -globin 215×10^3 dpm α -globin 620×10^3 dpm β/α 0.35).

Fig. 4. Autoradiographs of portion of the sequencing gels showing the partial nucleotide sequence of both normal and mutant alleles for this case in the area of the point mutation. Nonsense mutation (GAG $\rightarrow$ TAG) at codon 90 is shown. (Ter), termination codon.

through the 3' flanking region of the gene was carried out. There was no abnormal sequence in the clone possessing plus Ava II polymorphism, but another clone showed that the first nucleotide G of the codon GAG encoding 90th amino acid (Glu) in the Exon II segment of the β -globin gene was substituted with T, forming the termination codon TAG (Fig. 4).

DISCUSSION

In this patient the high HbA₁ level, despite the absence of glucose intolerance, prompted closer examination. The macro-column method using Bio-Rex 70 showed that the increased HbF influenced HbA_{1a} + b and HbA_{1c} fractions.

Characteristics of clinical features in this patient were: 1) hemolytic anemia with primary manifestations of anemia and jaundice (especially an increase in indirect bilirubin), 2) a family history of similar anemia in the patient's daughter and cousins on the mother's side, indicating an involvement of genetic factors, 3) exclusion of autoimmune diseases and abnormalities in red cell enzymes, and 4) morpho-

logical abnormalities of erythrocytes, increases in HbA₂ and HbF, and the appearance of Howell-Jolly bodies and Heintz bodies. Therefore, suspecting unstable hemoglobin or thalassemia, we carried out amino acid analysis and DNA gene analysis of hemoglobin, which demonstrated a nonsense mutation (GAG $\rightarrow$ TAG) in codon 90 of β -globin gene. However, due to the precipitation which gradually increased from immediately after the start of distilled water depletion, erythrocyte degeneration tests and amino acid analysis were difficult to perform. In fact, the identification of amino acid abnormalities was impossible in the hemolysate after removal of the sediment.

Variations of β^0 -thalassemia due to nonsense mutations such as substitution of AAG with stop codon TAG at β^{17} (10), substitution of GAG with TAG at β^{39} (11) and substitution of GAG with TAG at β^{43} (12) have been reported. Deletions have also been shown to cause β^0 -thalassemia in a Turkish patient (11) and in Indian patients in the areas of Sind and Gujarat (13). More recently, β^0 -thalassemia due to a nonsense mutation in codon 121 was reported (14). However, there have been no reports of a nonsense mutation with the substitution of GAG with stop codon TAG at β^{90} as was observed in the present case. This patient was a native of the place of her present residence (Wakayama), but there have been no reported instances of β^0 -thalassemia in or near this region.

Regional screening of β^0 -thalassemia of the same type as this patient is anticipated. In this patient, who exhibited a heterozygous mutation, the β chain is considered to be complete as far as the 89th amino acid, but to lack the 92nd amino acid (histidine) which connects with Fe of heme. Therefore, the molecule, which is incomplete as hemoglobin, is considered to have been present as a very labile peptide.

As a matter of convenience, we propose that this type of β^0 -thalassemia should be named β^0 -thalassemia Wakayama, since thalassemia identical to the type found in this case has not been reported in other regions.

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