β-Thalassemia Unlinked to the β-Globin Gene in an English Family

By S.L. Thein, W.G. Wood, S.N. Wickramasinghe, and M.C. Galvin

An inherited hypochromic microcytic anemia transmitted in an autosomal manner has been observed in three generations of an English family. Affected members had the hallmarks of homozygous β-thalassemia, ie, elevated levels of hemoglobin A2 and imbalanced globin chain synthesis. However, despite extensive sequence analysis, no mutations could be found in or around the β-globin genes of either the propositus or two other affected members from two different generations. Linkage analysis using restriction fragment length polymorphisms in the β-globin gene cluster clearly showed that the gene responsible for the β-thalassemia phenotype segregates independently of the β-gene complex. Therefore, this condition represents a novel form of the disease.

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The β-thalassemias are a heterogeneous group of inherited anemias resulting from reduced or absent expression of the β-globin gene. They are characterized in the heterozygous state by hypochromic microcytic red blood cells (RBCs), an increase in the proportion of the minor adult hemoglobin (Hb A2), and an imbalanced ratio of α- to β-globin chain synthesis. More than 130 β-thalassemia alleles have been characterized by DNA sequence analysis, and all are because of mutations within the β-globin gene itself or its immediate flanking regions. This catalogue of defects, including promoter mutations, splice junction defects, frameshift, and nonsense mutants, has been a model for other recessive single-gene defects.

In rare instances, sequencing of the β-globin gene has failed to identify a mutation responsible for the β-thalassemia phenotype. In these cases it has been suggested that the defect may lie in the β-globin locus control region (LCR), the major regulator of the β-globin cluster, which lies 50 to 60 kb upstream of the gene itself. Deletions of this region that leave the β globin intact are known to inactivate the β gene. Therefore, in all cases the proposed genetic determinant is thought to lie in cis to the β-globin gene.

Analysis of the rare β-thalassemia cases from temperate, nonmalarial regions has provided a rich source of isolated mutations, frequently ones with unusual defects. Here, we investigate an English family in which the β-thalassemia phenotype segregates independently of the β-globin gene cluster, representing a novel form of the disease.

MATERIALS AND METHODS

Subjects. The pedigree of an English family of Anglo-Saxon origin is shown in Fig 1. Several members from three generations had evidence of heterozygous β-thalassemia.

Hematologic analysis. Blood and bone marrow samples were taken as part of a clinical study. Blood samples were collected with EDTA as anticoagulant, and hematologic data were obtained using an automated cell counter. The percentage of Hb A2 was quantitated by electrophoresis and spectrophotometry after cellulose acetate electrophoresis at pH 8.6. In vitro synthesis of globin chains was determined after incubating peripheral reticulocytes with [3H]-leucine for 1 hour and after separating the labeled globins on carboxymethyl-cellulose chromatography.

Electron microscopy of the bone marrow. Marrow (from III-7, Fig 1 and Table 1) was aspirated from the iliac crest, and a few marrow fragments were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer. The fixed fragments were processed for transmission electron microscopy as described earlier.

DNA analysis. DNA was extracted from peripheral blood leukocytes and analyzed by Southern blotting as previously described. Five micrograms of DNA was digested to completion with various restriction endonucleases, and the fragments were separated by electrophoresis in 0.8% agarose gels. Seven restriction fragment length polymorphisms (RFLPs) in the β-globin gene cluster, HindIII-α, HindIII-α2, HindIII-α, HindIII-β, HindIII-β2, Avall-β, and BamHI-β, were studied, and the β-haplotypes were determined. The probes used in analysis of the RFLPs in the β-globin cluster include (1) BamHI/EcoRI β-globin gene, (2) HindIII 3.2 α-globin gene, (3) BglII/Xbal β-globin gene, and (4) PstI 4.4 β-globin gene. All DNA probes were radiolabeled with 32P-deoxy-cytidine triphosphate (CTP) by the random priming method (Mega-prime, Amersham, UK). The number of α-globin genes was determined by hybridizing BamHI and BglII-digested DNA with α- and β-globin gene probes, respectively. The 3' α-hypervariable region (HVR) was analyzed by hybridizing HindIII-digested DNA with the 3' α-HVR insert (4 kb) from pα3' HVR64 obtained by PstI/Xbal digest.

DNA amplification and sequence analysis. The β-globin genes were enzymatically amplified by the polymerase chain reaction (PCR) using a DNA thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) and primers API and AP2, which flank the β gene. Primers API and AP2 direct the amplification of a 2.6-kb fragment encompassing the whole of the β-globin gene from 860 bp upstream of the cap site to 320 bp downstream from the termination codon. Amplification was performed in a 100 μL reaction volume containing 1 μg genomic DNA, 0.2 mmol/L each of d-guanosine triphosphate, deoxythymidine triphosphate (dCTP), dATP, and d-adenosine triphosphate, 10 pmol each of primer API and AP2, and 2.5 U of Taq DNA polymerase in a buffer containing 50 mmol/L KCI, 10 mmol/L Tris-HCl, pH 8.3, at 20°C, and 2.5 mmol/L MgCl2. The mixture was overlaid with liquid paraffin. After an initial denaturation of 2 minutes at 94°C, annealing of 2 minutes at 55°C, and extension of 3 minutes at 72°C, 30 cycles of PCR were performed under the following conditions: 94°C (0.5 minute), 55°C (2 minutes), 72°C (5 minutes); the last extension reaction at 72°C was prolonged to 10 minutes. After amplification, 5 μL of each reaction...
Fig 1. Pedigree of the English family showing nonlinkage of the β-thalassemia phenotype with the β- and α-globin gene clusters. The β-gene cluster is represented by the β-haplotypes, which are denoted by the presence (+) or absence (−) of the 7 polymorphic restriction enzyme sites (see Fig 3 for details of sites). The alleles detected by 3α HVR are represented by (a) through (n).

was electrophoresed in 1.0% agarose gel. In the PCR, either primer AP1 or AP2 was 5′-biotinylated. The biotinylated single stranded (ss) DNA was isolated after incubation of the PCR product with 0.15 mol/L NaOH using magnetic beads (Dynabeads M-280 Streptavidin) and a magnetic particle concentrator (MPC-E; Dynal, UK, Wirral, UK) as described. The ss DNA derived from the PCR product was directly sequenced using Sequenase (US Biochemicals, Cleveland, OH) and a panel of sequencing primers. A total sequence of 2400 nucleotides was determined using sequencing primers S1 to S12. S1 through S6 were used to sequence biotinylated AP2-primed ssDNA and S7 through S12 to sequence biotinylated AP1-primed ssDNA. The sequences 5′ through 3′ of these primers are as follows: AP1, TCC AGG CAG AAA CAG TTA GAT G; AP2, GGC ATA GGC ATC AGG GCT G; S1, AGC ACA TTG ATT TGT ATT TAT TC; S2, CGA TCT TCA ATA TGG TTA C; S3, GAT GAA GTT GGT GGT GAG GCC; S4, TAG AAT GGG AAA CAG AAT GA; S5, CAA TGT ATG CCT CTI TGC AC; S6, GTC TGT GTG CTG GCC CAT C; S7, CAG TTT AGT GTG ACT TA; S8, CCA GCC TTA TCC CAA CCA T; S9, CAT TAC ACT TTA ACC CAT A; S10, CAT TCG TCT GTT TCC CAT CT T; S11, CAA AGG ACT CAA AGA ACC TC; S12, CAA CTT CAT CCA CTG TCA CC.

DNA fingerprint analysis. Genomic DNA was digested with the enzyme HinfI, Southern blotted, and hybridized with a panel of 7 human minisatellite probes (MS1, MS2, MS29, MS31, MS43, MS51, and pM3).18

RESULTS

Case report. The propositus was a 57-year-old English man who presented with a stroke. A routine blood count showed a normal level of Hb with persistent hypochromia and microcytosis of the RBCs. He had no splenomegaly and was not jaundiced. Peripheral blood smears showed a mild poikilocytosis and targeting of the hypochromic and microcytic RBCs. Reticulocytes were not increased. Investigations to delineate the cause of the persistent hypochromic microcytosis included a bone marrow examination that showed a normocellular marrow with increased reticuloendothelial iron, and increased siderotic granules that were randomly distributed within the RBC precursors. No ring sideroblasts were seen. The serum ferritin was increased at 480 μg/mL, whereas serum iron and the total iron binding capacity (TIBC) were within normal limits at 23.7 μmol/L and 49 μmol/L, respectively. Hb analysis showed the level of Hb A2 to be marginally elevated at 3.1% and showed a normal Hb F level of 0.4%. The in vitro α/β-globin chain
synthesis ratio was increased at 1.8. These results are consistent with a diagnosis of heterozygous β-thalassemia.

Electron microscopy. The majority of the erythroblasts were ultrastructurally normal (Fig 2A). In particular, they did not display any of the features of congenital dyserythropoietic anemia (CDA) types I, II, or III. Intracytoplasmic inclusions resembling precipitated α-globin chains were not found. Rare late erythroblast and marrow reticulocyte profiles displayed a few iron-laden mitochondria that were sometimes enclosed within autophagic vacuoles (Fig 2B and C). The bone marrow macrophages occasionally contained a phagocytosed late erythroblast at various stages of degradation (Fig 2D). Some of the phagocytosed erythroblasts had aggregates of electron-dense granular material within their cytoplasm, similar to those seen in mildly affected patients with β-thalassemia trait.

Family studies. Hematologic studies of family members were performed, and the results are shown in Table 1. An inherited anemia, segregating as a single autosomal gene defect in a Mendelian fashion, is transmitted in three generations of this family. Affected members were all distinguishable from normal family members by the thalassemic changes in their blood films that showed poikilocytosis and targeting of the RBCs. These changes were more prominent in some affected family members (III-7 and IV-5) than in others. They have a similar pattern of mild anemia with hypochromic microcytic RBCs, and discriminant function analyses were supportive of a diagnosis of thalassemia trait (Table 1). Furthermore, all affected members have slightly increased Hb A2 levels and imbalanced α/β-chain synthesis ratios. In 5 cases, including the proband, the globin synthesis ratios were measured on two separate occasions. The differential diagnoses of this inherited anemia include iron deficiency, sideroblastic anemia, or CDA. These were excluded by a normal serum iron, TIBC, and ferritin in all members (except II-6 who has severe iron deficiency due to ulcerative colitis). There were no ring sideroblasts in the bone marrow on light and electron microscopy, nor were there ultrastructural abnormalities indicative of dyserythropoiesis in the majority of the erythroid precursors in the bone marrow.

Sequence analysis of the β-globin genes. The β-globin genes in the propositus (II-3) and in 2 other affected family members (III-7 and IV-5) of 2 generations (Fig 1) were enzymatically amplified by PCR and directly sequenced by the dideoxy chain termination method. A total sequence from each β gene of 2,400 nucleotides was determined from 630 bp upstream of the mRNA cap site of the β-globin gene to 290 bp downstream of the termination site including the whole β-globin gene with its 3 exons and 2 introns. Sequences were determined on both DNA strands. However, no nucleotide changes other than known polymorphisms were

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The ratio MCV/RBC has been proposed by Mentzer as a useful index of differentiating iron deficiency anemia from thalassemia trait. In II-3, II-5, III-4, III-7, III-9, IV-4, and IV-5, these values were all less than 14 supportive of heterozygous β-thalassemia. Their corresponding DFs (MCV – [5 x Hb] – RBC), another discriminant function proposed by England and Fraser were -7.84, 4.83, -0.41, -0.51, 6.74, 4.72, and 4.03, values again supportive of a diagnosis of heterozygous β-thalassemia. In contrast, individual II-6, who has iron deficiency anemia, has an MCV/RBC ratio of 14.3 and DF of more than 6.0 at 11.07.

Abbreviation: ND, not done.
Family member IV-5 was heterozygous for the \((-T + ATA)\) rearrangement at the \(-530\) region upstream of the \(\beta\) gene,\(^{19}\) but II-3 (propositus) and IV-7 have the normally reported sequence variation at this position.

**Analysis of the \(\beta\)- and \(\alpha\)-globin gene clusters.** Seven RFLPs in the \(\beta\)-globin gene cluster HindIII-\(\alpha\), HindIII-\(\psi\gamma\), HindIII-\(\psi\beta\), HindIII-\(\psi\beta\), Avall-\(\beta\), and BamHI-\(\beta\), were studied, and the \(\beta\)-cluster haplotypes were determined\(^4\) (Fig 3). Heterozygosity for these sites in the propositus and several affected members (II-5, IV-4, and IV-5) indicates the absence of a major gene deletion or rearrangement within the cluster (Fig 1). Furthermore, analysis of the \(\beta\)-haplotypes clearly showed that the gene responsible for \(\beta\)-thalassemia does not behave as an allele of the \(\beta\)-gene cluster. Inspection of Fig 1 shows that if the gene responsible for \(\beta\)-thalassemia were allelic to the \(\beta\) gene, it must be carried on a chromosome with either the C or D haplotype. Of the affected family members, 5 have the D haplotype, 2 have the C haplotype, and 1 has both. The \(\beta\)-haplotypes in the affected individual in generation I must be either AD or BC. Because the 2 haplotypes differ at 5 separate RFLP sites, multiple recombination or gene conversion events would be needed to convert haplotype C to D or vice versa in the affected individual in generation I and individual II-3; this seems extremely unlikely.

The number of \(\alpha\)-globin genes was determined by hybridizing BamHI- and BglII-digested DNA with \(\alpha\)- and \(\zeta\)-globin gene probes, respectively. The \(\alpha\)-genotype is normal (\(\alpha\alpha/\alpha\alpha\)) in all members of the family. Using the \(3'\) \(\alpha\)-globin HVR as a marker for the \(\alpha\)-globin gene cluster,\(^{26}\) we showed that the disorder segregates independently of the \(\alpha\)-gene complex (Fig 1), thus excluding the possibility that the imbal-
β-THALASSEMIA UNLINKED TO THE β-GLOBIN GENE

The phenotype of the affected members of this family is β-thalassemia trait with microcytosis (mean corpuscular volume [MCV] = 71.1 fl), hypochromia (mean corpuscular hemoglobin [MCH] = 23.0 pg), globin chain imbalance, and a raised level of Hb A₂ (mean = 3.8%). Although the Hb A₂ levels are somewhat lower than those usually seen in people with typical β-thalassemia trait (mean 5.0%), they are clearly increased above normal levels (mean 2.5%). Globin chain imbalance is the major characteristic of the thalassemias, and the values seen here (1.6 to 2.0) are typical of β-thalassemia trait. The only other disorders in which chain imbalance has been reported are some myeloproliferative/leukemic conditions and some cases of CDA. There was no evidence for any inherited leukemia condition in this family, and CDA was excluded by electron microscopy.

The occurrence of individual III-7 of iron-laden mitochondria in a rare late erythroblast and marrow reticulocyte and of phagocytosed erythroblasts within an occasional macrophage are, presumably, a consequence of chain imbalance. These abnormalities have been reported in β-thalassemia traits and in subjects with HbE. Therefore, we are left with what appears to be a bona fide β-thalassemia disorder in which the gene responsible is not allelic to the β-globin gene, and is totally unlinked to the β-gene complex. A similar situation was reported previously but, subsequently, turned out to be caused by a recombination event. We think this occurrence is most unlikely in the family in our study because multiple recombination events or multiple gene conversions would be required. A further possible example of a β-thalassemia gene unlinked to the β-globin complex has recently been reported in a family that has several members with thalassemia intermedia in which only one β-thalassemia allele could be detected. However, it is not clear if the unusual severity in these cases is caused by an additional thalassemia defect.

What is likely to be the nature of a gene that appears to specifically downregulate expression of the β-globin gene? The defect could affect transcription of the β gene itself, or the mechanism of downregulation could be posttranscriptional. An imbalance of α:β mRNA would support a transcriptional defect; such studies are underway. Furthermore, because the defect is unlinked to the β cluster, both β-globin genes should be equally affected. However, sequence analysis in 3 affected members did not show any polymorphisms within the coding regions of the β genes that would distinguish the mRNA from the two alleles in this family.

The regulation of the β-globin gene has been studied in some detail. The human β-globin locus is composed of 5 linked functional β-like-globin genes, 5'-e, 0γ, 5'γ, δ, and β-3', that span ~50 kb upstream of the ɛ-globin gene, is the major regulatory region that controls the transcription of the β-globin domain (Fig 3). A similar regulatory region (HS-40) is located 40 kb upstream of the ɛ-gene complex and controls the transcription of the embryonic γ2-gene and the two α-genes, α2 and α1.

The expression of the human α- and β-globin genes requires the interaction of various erythroid restricted (GATA-1, NFE-2, and others) and ubiquitous transcription factors with critical areas of the complexes, including the LCR and HS-40 element, as well as the promoters of the various individual genes. As yet, no transacting factors have been characterized that are either developmental stage specific or distinguish between the α and β complexes. However, there are several situations in which expression of one or other globin genes is selectively affected.

The hemopoietic cell line, K562, expresses all of the globin genes except β. The K562 β-globin genes function normally when transferred to other erythroid cells, but no expression is seen when normal β-globin genes are transferred into K562 cells. It seems probable that K562 cells either lack a positive factor or express a negative regulatory factor that appears to specifically repress the β-globin gene.
Several families have been described in which there is hereditary persistence of fetal Hb resulting in increased γ-globin gene expression in adults but in which the gene responsible is clearly not linked to the β-globin cluster. The function of such a gene remains unknown. It is not clear if it affects γ gene expression directly or indirectly, and the mode of inheritance is not obvious. Nevertheless, these conditions demonstrate that there are genes that can apparently affect a specific globin gene.

Most cases of α-thalassemia are caused by mutations of α-globin genes. However, several individuals have now been described in whom α-thalassemia occurs as an acquired disorder associated with myelodysplasia. These individuals, predominantly elderly and previously healthy men, acquire an almost complete repression of α-globin gene expression. In all the cases that have been studied in detail, the α-globin complex is intact, but there is a severe reduction in α-globin mRNA and α-globin chain synthesis. Thus, the molecular basis for this syndrome appears to be an acquired defect in transcription of α-globin genes, but the precise mechanism and its relationship to myelodysplasia is unknown. A similar situation has also been reported in a case of myelodysplasia associated with β-thalassemia.

Another unusual type of α-thalassemia is associated with mental retardation (ATR-X syndrome). Molecular analysis has shown that the α-globin complex is intact and that the α genes are normal. Linkage analysis shows that the phenotype of α-thalassemia/mental retardation not only segregates independently of the α-globin complex but also is clearly inherited as an X-linked recessive disorder. The molecular bases of the ATR-X syndrome and the acquired α-thalassemia associated with myelodysplasia, at present, are unknown, but both these conditions show a selective effect on the α-globin gene complex.

It is not known whether the abnormality in these conditions lies in a trans-acting factor that directly interacts with one or other of the globin genes, or whether the effect is indirect but still specific for only one of the genes. The defect in the family in this report may be similar to those described above. It represents the first well-characterized family with β-thalassemia not linked to the β-globin cluster.

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Beta-thalassemia unlinked to the beta-globin gene in an English family

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