Characterization of a Spontaneous Mutation in $\beta$-Thalassemia Associated With Advanced Paternal Age

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We characterized the molecular defect in a Swiss patient with a spontaneous $\beta$-thalassemia mutation. Cloning and DNA sequencing of her $\beta$-globin gene revealed a new frameshift mutation due to a single nucleotide deletion at codon 84 of the $\beta$-globin gene. Restriction site polymorphism showed that the mutation arose on her paternal chromosome. Direct sequencing of a polymerase chain reaction amplified DNA segment showed absence of the lesion in both alleles of her father's $\beta$-globin gene and confirmed the spontaneous nature of this mutation.

The inherited disorder of globin synthesis, $\beta$-thalassemia, predominantly affects Mediterranean and Asian populations. The mutation is usually inherited from a parent and segregates as an autosomal recessive Mendelian trait. More than 60 different molecular lesions have been shown to cause $\beta$-thalassemia. Occasionally, $\beta$-thalassemia arises spontaneously; to date, three such cases have been described. The first report was a case of $\beta$-thalassemia trait in a Swiss woman whose parents and three siblings had no evidence of $\beta$-thalassemia. The two other cases of spontaneous mutation were reported in a northern European and a Greek family, and their molecular lesions have been characterized. We have now determined the molecular lesion in the original Swiss family and describe a hitherto unreported frameshift mutation.

MATERIALS AND METHODS

Subjects. The clinical and hematologic data on this Swiss family have been previously described. The proband is a 23-year-old female with heterozygous $\beta$-thalassemia (hemoglobin [Hb] A2 = 5.4%, Hb F = 1.8%, $\beta/\alpha = 0.34$); her father and mother were 45 and 44 years old, respectively, at the time of her birth. Neither her parents nor her three siblings show any evidence of $\beta$-thalassemia. Analysis of blood and serum types, isozymes, and HLA markers showed $<1$ in 10,000 chance that the father was not the biologic father.

DNA extraction and Southern blotting. DNA was extracted from peripheral blood leukocytes (PBLs) by standard methods. Genomic DNA was digested with the restriction endonuclease AvaII, fractionated by agarose gel electrophoresis and transferred to a nitrocellulose membrane. The blot was hybridized with an IVS-2 $\beta$-globin probe as previously described. Southern blotting was also performed with the insulin gene probe after digestion with RsaI.

Molecular cloning and DNA sequencing. Genomic DNA 100 ug from the proband was restricted with HindIII and fractionated on a 10% to 40% sucrose gradient. A partial DNA library was constructed in the bacteriophage vector A2001 (Stratagene, San Diego) from the sucrose fraction containing the 7.8-kilobase (kb) HindIII fragments. The library was screened with an $\alpha$-2P $\beta$-globin probe, and recombinant clones were isolated and purified. The location of the mutation in the cloned $\beta$-globin gene was detected by the RNase cleavage method, as previously described. DNA sequencing was performed by the dideoxy chain termination method after subcloning in M13 mp18.

Polymerase chain reaction and DNA sequencing. Genomic DNA 1 ug was amplified by PCR using the DNA polymerase from Thermus aquaticus as previously described. The two oligonucleotide primers, 5' TCTGCTATTGGTCTATT 3' and 5' GCCAT-CACTAAAGGCACCG 3', were situated at position 100 of the first intervening sequence and at codon 68 of the $\beta$-globin gene, respectively. The amplified 165-base pair (bp) segment of the $\beta$-globin gene was purified by polyacrylamide gel electrophoresis (PAGE) and subjected to double-stranded dideoxy DNA sequencing with the upstream PCR primer as sequencing primer in the presence of $\alpha$-32P dCTP and the modified T7 DNA polymerase (Sequenase, United States Biochemicals, Cleveland).

RESULTS

Insulin gene polymorphism. Studies of the variable region 5' to the insulin gene revealed that the father was homozygous for a 2.5-kb band and the mother was homozygous for a 4.2-kb band. The proband inherited both bands. This finding reinforces the probability of paternity in this family.

Molecular analysis of the proband $\beta$-globin genes. Southern blot analysis after AvaII digestion of the $\beta$-globin gene indicated that the father is homozygous for the 2.0-kb and the mother for the 2.2-kb AvaII fragments. The proband was heterozygous for the 2.0- and 2.2-kb fragments and therefore inherited the former from the father and the latter from the mother. Recombinant bacteriophage clones harboring the 7.8-kb $\beta$-globin gene containing HindIII fragment were isolated from the proband, and their parental origin was determined by AvaII digestion. RNase cleavage analysis of the clone with the 2.0-kb AvaII polymorphism showed a break in the SP6-synthesized RNA at the position corresponding to exon 2. The 1.8-kb BamHI fragment from each parental chromosome was subcloned in M13mp18 and subjected to DNA sequencing. A single bp deletion involving a G nucleotide at codon 64 in exon 2 of the $\beta$-globin gene was identified only in the recombinant DNA clone with the 2.0-kb AvaII fragment inherited from the father (Fig 1).
Molecular analysis of paternal β-globin genes. Since the mutation in the proband lies on the paternal chromosome, we ascertained the spontaneous nature of this mutation by direct DNA sequencing of the same region in the father, a normal control, one of the three siblings, as well as the proband. The region around codon 64 was amplified by PCR as a 165-bp segment. DNA sequence analysis was normal in all samples except the sample of the proband in which, following the first G of codon 64, we found an overlapping DNA sequence pattern typical of a mixture of two DNA sequences following a single base deletion12 (Fig 1). Direct sequencing clearly showed the absence of the single base deletion at codon 64 in the father and the hematologically normal sibling and confirmed the spontaneous nature of the mutation.

DISCUSSION

We characterized the spontaneous mutation in a Swiss individual heterozygous for β-thalassemia. The lesion is caused by a deletion of a guanine base at codon 64 (normal sequence GGC) in the second exon of the β-globin gene which shifts the reading frame and causes premature termination at codon 88. The molecular basis for the small deletion probably involves a looping out of the template during DNA replication. Such an event has been shown to occur in AT-rich region13. This new mutation has not been described previously in any population.

Two spontaneously occurring β-thalassemia lesions have already been characterized. We reported a case in a northern European family in which a C to T transition caused a nonsense mutation at codon 39.3 Surprisingly, this mutation was identical to a common hereditary form found in the Mediterranean area,4 suggesting that this region of the β-globin gene may be a hotspot for mutation. The other case, reported in a Greek family,4 resulted from a nonsense mutation at codon 121. This homozygous β-thalassemic individual inherited a known β-thalassemia allele from his mother, while both of his father's β-globin alleles were normal.

The father in the present case was 45 years old when the proband was born. Advanced paternal age predisposes to spontaneous mutation, presumably because of mutation in the sperm DNA.6 It is interesting that the mutation in the Greek case of spontaneous β-thalassemia also arose on a paternal chromosome.4 In the other case, restriction site polymorphisms at the β-globin gene cluster were not informative for identifying the parental origins of the mutation. Thus, spontaneous mutations in β-thalassemia appear to follow a pattern similar to that of unstable Hbs and Hb M, in which advanced paternal age was also a predisposing factor.16

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