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Differentiation of homozygous hemoglobin E from compound heterozygous hemoglobin E–β^0-thalassemia by hemoglobin E mutation analysis

John P. Johnson, MD, Elliot Vichinsky, MD, Deborah Hurst, MD, Anne Camber, BS, Bertram Lubin, MD, and Elaine Louie, PhD

From the Divisions of Medical Genetics and Hematology, Children's Hospital Oakland, Oakland, California

Objectives: To facilitate the differential diagnosis of hemoglobin FE in newborn infants (homozygous hemoglobin E vs hemoglobin E–β^0-thalassemia).
Methods: The β-globin gene in DNA from infants found to have hemoglobin FE in the California newborn screening program was amplified by the polymerase chain reaction, and the product was digested with *MnlI*, which fails to cut the product when the hemoglobin E mutation is present. When both amplified alleles fail to be cut, homozygous EE is diagnosed. If only one allele is cut, a β-globin allele without the E mutation is present *(non-E)*, which is most likely a gene with a β^0-thalassemia mutation.
Results: Samples from 18 infants revealed an *EE* genotype, and from two samples a *non-E/E* genotype was determined. Clinical examination of these two patients confirmed a diagnosis of hemoglobin E–β^0-thalassemia. An independent clinical diagnosis agreed with DNA analysis for all 17 of the 20 infants for whom follow-up and family studies were available. The DNA results were obtained within a week, but the clinical diagnoses often could not be resolved unequivocally for months.
Conclusions: The direct analysis of patient DNA samples for the hemoglobin E mutation allowed rapid and accurate diagnosis in this sample of infants with hemoglobin FE on the newborn screen. This rapid discriminatory test should reduce cost and simplify the diagnostic approach for these patients, which currently consists of expensive and lengthy follow-up until clinical data and family studies result in a diagnosis. (J PEDIATR 1992;120:775-9)

The Asian population has a major genetic burden of hemoglobin disorders, many of which cause mild anemia in the carrier, including α-thalassemia, β-thalassemia, and hemoglobin E. Hemoglobin E is the most common structural hemoglobin variant in this population. In some Southeast Asian groups the carrier frequency for hemoglobin E is as high as 55%.[1] Therefore hemoglobin E is frequently detected by newborn hemoglobin screening programs in regions with large Asian populations.

PCR	Polymerase chain reaction

Carriers of this mutation (hemoglobin E trait) have minimal hematologic manifestations except for microcytosis.

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Reprint requests: John P. Johnson, MD, Division of Medical Genetics, Children's Hospital Oakland, 747 52nd St., Oakland, CA 94609.

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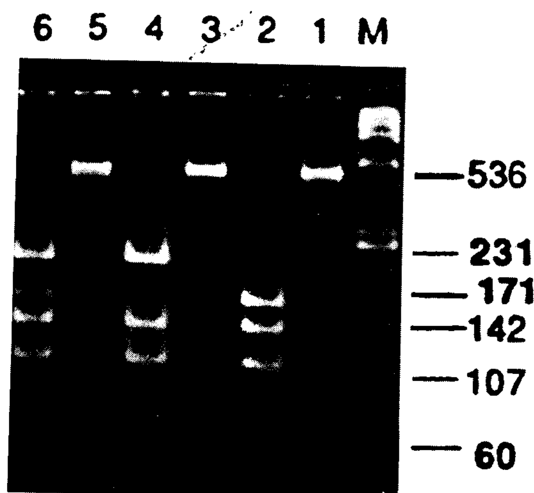

Figure. Ethidium bromide–stained 5% NuSieve gel showing paired uncut (536 base pairs) amplification products of patient DNA samples *(lanes 1, 3, 5)* and *Mnl* I digestions of the same amplification products *(lanes 2, 4, 6)*. *Lanes 1 and 2* are from a normal person (*non-E/non-E* genotype, 171 and 60 base pairs). *Lanes 3 and 4* are from a homozygous hemoglobin E patient (*EE* genotype, 231 base pairs). *Lanes 5 and 6* are from a patient with compound heterozygous hemoglobin E–β^0-thalassemia or from a person with the hemoglobin E trait (*non-E/E* genotype, 231, 171, and 60 base pairs). Other bands, of sizes 142, 107, 21, 21, and 15 base pairs, are common to all β-globin E–region PCR products digested with *Mnl* I and serve as a control for *Mnl* I enzyme activity. Lane *M* is a size standard (ϕX174/*Hae* III). The PCR program used in these amplifications is as follows: denaturing 1 minute at 94° C, annealing 2 minutes at 55° C, and extension 3 minutes at 72° C, repeated for 30 cycles.

Although the laboratory manifestations are slightly more abnormal, individuals with homozygous hemoglobin E do not have significant clinical disease and are hematologically similar to those with β-thalassemia trait.[2] In patients who have compound heterozygous hemoglobin E trait and β^0-thalassemia trait, hemoglobin E–β^0-thalassemia results; these patients can have serious hemolytic anemia and ineffective erythropoiesis (thalassemia intermedia), and are often dependent on transfusions.[1] The carrier frequency of β-thalassemia approaches 5% in the Southeast Asian population, and the majority of these thalassemia mutations produce a nonfunctional (β^0) gene.[3] Therefore about 5% of newborn infants who inherit a hemoglobin E mutation will also inherit a thalassemia mutation from the other parent, and hemoglobin E–β^0-thalassemia will develop.

A goal of the California Newborn Screening Program is to identify hemoglobin E–β^0-thalassemia cases as soon as possible to minimize the growth failure, infections, and severe anemia that can develop in infancy. Infants with either homozygous hemoglobin E or hemoglobin E–β^0-thalassemia will have a hemoglobin pattern of FE. We used a simple and reliable direct DNA test in infants with hemoglobin FE to distinguish homozygous hemoglobin E from heterozygous hemoglobin E–β^0-thalassemia.

METHODS

Hematologic analysis. Whole peripheral blood samples from 20 patients with a neonatal screening pattern of hemoglobin FE were provided by the state. Children's Hospital Oakland Hemoglobinopathy Laboratory performs confirmatory testing on positive newborn screening results, using electrophoresis or thin-layer isoelectric focusing; all 20 specimens were analyzed at this reference laboratory.

The separation of hemoglobins F, A, and E was done by electrophoretic techniques, including thin-layer isoelectric focusing (pH 6.0 to 8.0)[4] and cellulose acetate electrophoresis (pH 8.5).[5] In certain cases, confirmation was done by citrate agar electrophoresis (pH 6.0)[6] for C, E, and O-like hemoglobins. Family studies were attempted for all cases of confirmed hemoglobin FE. When both parents were available, complete blood cell count, erythrocytic indexes, smear, hemoglobin electrophoresis, and, when appropriate, studies to exclude β-thalassemia trait were performed.

DNA analysis. For this study the DNA analysis was done independently and without knowledge of the hematologic analysis. Peripheral blood samples were obtained from the infants at many different ages and stages of follow-up evaluation.

The hemoglobin E mutation is a single base substitution (G → A) of codon 26 in the β-globin gene. To detect the hemoglobin E mutation, we processed blood samples in a standard manner to isolate DNA[7] and performed polymerase chain reaction.[8,9] With the use of KM29 and RS42 primers flanking the hemoglobin E mutation,[10] an amplification product of 536 base pairs was produced from the patient's DNA. The PCR product was digested with the restriction enzyme *Mnl* I, which recognizes either CCTC $(N)_7$ or $(N)_7$ GAGG. The hemoglobin E mutation (*G*AGG → *A*AGG) abolishes a normal *Mnl* I site,[11] which results in a fragment of abnormal size (larger) on an ethidium bromide–stained 5% NuSieve agarose gel (FMC BioProducts, Rockland, Me.) after electrophoresis (Figure). If the patient has no E mutation, the entire PCR product is digested to completion, resulting in several smaller fragments. If the patient is homozygous for the E mutation, the larger, uncut product is present, but other *Mnl* I sites are present in the PCR product, and these are cut to generate many small fragments, which provides a useful internal control for function of the restriction enzyme. A patient with one hemoglobin E mutation and one non-E–containing β-globin gene has the larger aberrant E fragment, the smaller non-E

Table. Results of hemoglobin screening, DNA analysis, and clinical diagnosis and follow-up for 20 newborn infants with hemoglobin FE and 5 control subjects

	Hematologic family studies	Clinical decision based on hematology	DNA analysis	Parental confirmation (DNA)	Clinical decision; DNA
Patient No.					
1	FA: AE; MO: AE	No follow-up; presumed EE	E/E	FA: non-E/E; MO: non-E/E	No follow-up; EE
2	FA: AE; MO: AE	No follow-up; presumed EE	E/E	FA: non-E/E; MO: non-E/E	No follow-up; EE
3	FA: E; MO: AE	No follow-up; presumed EE	E/E	FA: E/E; MO: non-E/E	No follow-up; EE
4	FA: AE; MO: AE	No follow-up; presumed EE	E/E	FA: non-E/E; MO: non-E/E	No follow-up; EE
5	FA: AE; MO: E	No follow-up; presumed EE	E/E	FA: non-E/E; MO: E/E	No follow-up; EE
6	FA: E; MO: E	No follow-up; presumed EE	E/E	FA: E/E; MO: E/E	No follow-up; EE
7	FA: AE; MO: E	No follow-up; presumed EE	E/E	FA: non-E/E; MO: E/E	No follow-up; EE
8	FA: AE; MO: AE	No follow-up; presumed EE	E/E	FA: non-E/E; MO: non-E/E	No follow-up; EE
9	FA: N/A; MO: AE	Need follow-up	E/E	FA: N/A; MO: non-E/E	No follow-up; EE
10	FA: N/A; MO: E	Need follow-up	E/E	FA: N/A; MO: E/E	No follow-up; EE
11	FA: N/A; MO: AE	Need follow-up	E/E	FA: N/A; MO: non-E/E	No follow-up; EE
12	FA: N/A; MO: E	Need follow-up	E/E	FA: N/A; MO: E/E	No follow-up; EE
13	FA: N/A; MO: AE	Need follow-up	E/E	FA: N/A; MO: non-E/E	No follow-up; EE
14	FA: N/A; MO: AE	Need follow-up	E/E	FA: N/A; MO: non-E/E	No follow-up; EE
15	FA: N/A; MO: AE	Need follow-up	E/E	N/A	No follow-up; EE
16	N/A	Need follow-up	E/E	N/A	No follow-up; EE
17	FA: N/A; MO: AE	Need follow-up	E/E	N/A	No follow-up; EE
18	*FA: β-thalassemia trait; MO:AE*	*Need follow-up; β-thalassemia/E*	*Non-E/E*	*N/A*	*Need follow-up; non-E/E*
19	*N/A*	*Need follow-up*	*Non-E/E*	*N/A*	*Need follow-up; non-E/E*
20 (EF)	N/A	Need follow-up	E/E	N/A	No follow-up; EE
Control subject No.					
21	AE		Non-E/E	N/A	
22	AE		Non-E/E	N/A	
23	β-Thalassemia/E		Non-E/E	N/A	
24	β-Thalassemia/E		Non-E/E	N/A	
25	β-Thalassemia major		Non-E/non-E	N/A	

F, A, E, Hemoglobins; *FA,* father; *MO,* mother; *N/A,* not available or applicable; *EE,* DNA result showing the E mutation on both chromosomes; *E/non-E,* DNA result showing the E mutation on only one chromosome.

fragment, and the control fragments. This pattern is seen in patients with hemoglobin E–β^0-thalassemia.

RESULTS

Hemoglobin analysis. Hemoglobin electrophoresis revealed 19 of the 20 FE screen samples to have hemoglobin FE, and one to have hemoglobin EF. We were able to obtain blood samples from both parents for complete hematologic evaluations for only nine patients (Table). A clinical diagnosis based on the hemoglobin FE pattern and family studies resulted in a diagnosis of homozygous hemoglobin E in eight cases, and of hemoglobin E–β^0-thalassemia in the other case. Only the last patient required further hematologic evaluation. In 11 of the cases the diagnosis could not be established by family studies because at least one of the parents was not available.

Because homozygous hemoglobin E cannot be reliably distinguished from hemoglobin E–β^0-thalassemia in the newborn infant by using standard hematologic methods, the 11 patients for whom family studies could not be accomplished were reevaluated at 3-month intervals until a diagnosis could be established. A diagnosis was reached for eight of these infants; continuing follow-up was planned for the others.

DNA analysis. Analysis of DNA was performed within a week of receipt of blood samples; the result provided for each sample is listed in the Table. In the patient with hemoglobin EF and in 17 of 19 with hemoglobin FE, an *EE* genotype was detected. Two had *non-E/E* genotypes; both were confirmed to have hemoglobin E–β^0-thalassemia by hematologic studies of the parents. For one of these patients, the diagnosis was made as a result of the DNA study before the family studies could be obtained.

Five other samples were used as control subjects for the interpretation of DNA results. All had the expected results with DNA analysis. Two patients with hemoglobin AE (hemoglobin E trait) and two with hemoglobin E–β^0-thalassemia had *non-E/E* genotypes, and one patient with

β-thalassemia major had a *non-E/non-E* genotype. For eight neonatal samples for which both parents were later available for confirmatory DNA analysis, the results were consistent with the hematologic diagnosis (Table).

Correlation of hematologic and DNA findings. Of the 20 patients with an FE hemoglobin pattern at birth, 15 of 18 predicted to have homozygous hemoglobin E (genotype *EE* with DNA analysis) have had this diagnosis confirmed by follow-up family, clinical, and hematologic studies. Because of the EE DNA diagnostic information, 10 infants for whom repeated follow-up to reach a diagnosis (parents were unavailable) was planned, did not require follow-up. As mentioned above, a clinical diagnosis based on family studies and follow-up visits has not been reached for three of these infants. For the two remaining study patients, *non-E/E* genotypes were detected and the diagnosis of hemoglobin E–β^0-thalassemia has been confirmed. In one patient this genotype provided a presumptive diagnosis of hemoglobin E–β^0-thalassemia before this diagnosis was reached clinically. Therefore, even in this retrospective, blind study, an important clinical diagnosis was provided by DNA analysis.

DISCUSSION

Hemoglobin analysis was added to the California Newborn Screening Program in the ethnic and genetic context provided by a large population of Southeast Asians. The program is detecting a significant number of newborn infants with hemoglobin FAE, who are considered carriers of the mutation and who will have an AE pattern (hemoglobin E trait) after the disappearance of the fetal hemoglobin. Many infants are also found to have hemoglobin FE. These patients either have two copies of the E mutation (homozygous hemoglobin E, genotype *EE*) and will eventually have only hemoglobin E, or they have one copy producing E hemoglobin and a second, defective β-globin gene, most often with a β^0-thalassemia mutation (hemoglobin E–β^0-thalassemia, genotype *E/non-E*). As in homozygous infants, in these latter patients hemoglobin A is not present. It is essential to distinguish EE homozygotes from those with hemoglobin E–β^0-thalassemia because the clinical outcomes are radically different. In addition, this information is important for couples who have had a child with hemoglobin E–β^0-thalassemia and who can then be offered prenatal diagnosis for future pregnancies.

The current medical follow-up to establish a definitive diagnosis for newborn infants with hemoglobin FE is not ideal. Those with homozygous EE cannot be distinguished clinically or hematologically from those with hemoglobin E–β^0-thalassemia unless the family is studied.[12] If both parents are found to have hemoglobin E, either being homozygous or having the hemoglobin E trait, a presumptive diagnosis of homozygous hemoglobin E is made for the infant. If both biologic parents are correctly identified and tested, and if one parent carries an E mutation and the other has the β^0-thalassemia trait, their infant with hemoglobin FE must be heterozygous for the E mutation and for β^0-thalassemia.

Our study illustrates several drawbacks to this standard diagnostic approach for newborn infants with hemoglobin FE. The first is that diagnosis in the baby depends on information from the parents. Significant parental involvement, including an examination and laboratory testing, is necessary to ensure that the baby is not at risk. Many evaluations may be required before the diagnosis can be confirmed. Second, if both parents are unavailable for study, the baby must be followed for an extended time until a diagnosis can be made. Finally, correct assignment of paternity is critical.

Because of the ethnic derivation of the population having infants with hemoglobin FE, language and cultural barriers have made extended follow-up evaluations extremely difficult.[13] The experience in our screening program is that the father is often unavailable for testing. In most cases the mother is found to have the hemoglobin E trait, and the fact that information is missing from the father makes immediate diagnosis of the infant's status impossible.

The simple β-globin gene analysis that we propose, in conjunction with hemoglobin electrophoresis, yields an accurate diagnosis and distinguishes infants with homozygous hemoglobin E from those with hemoglobin E–β^0-thalassemia. The diagnosis is established directly, obviating the need for hematologic evaluations of both parents to verify either the hemoglobin E trait or β^0-thalassemia carrier status and to identify the baby's *EE* genotype indirectly (by inference). Although we used peripheral blood samples for this study, the blood spots on filter paper obtained by the screening program can be used for DNA analysis.[14-18] If this method is reliable, it will facilitate diagnosis for some newborn infants. Patients found to have hemoglobin E–β^0-thalassemia can be further evaluated regarding the specific thalassemia mutation present, but there are many such mutations,[19, 20] and this analysis adds significant complexity and expense. However, linkage analysis using β-globin gene region polymorphisms[21] is available for prenatal diagnosis within affected families.

There are two potential pitfalls to the DNA test we propose. One is nonamplification of one of the β-globin alleles during the PCR reaction. This could occur with a primer mismatch caused by a polymorphism at the 3′ end of one of the primers in the patient's genomic DNA, or because of a large deletion within the primer annealing region on one of the β-globin alleles. If such selective nonamplification of

a β-globin gene occurs, the result could be misinterpreted as an *EE* rather than a *non-E/E* genotype. Because of the low frequency of reported mutations and deletions in the KM29 and RS42 primer-binding regions in Southeast Asian populations,[19, 20] this should not be a common problem.

Another error in DNA test interpretation could occur if the patient has a unique mutation within the *Mnl* I recognition site that eliminates the enzyme cut and would be misinterpreted as a hemoglobin E mutation. Although this situation has been reported,[22] it is a rare exception that could lead to the identification of a homozygous *EE* genotype when the true genotype is *non-E/E*. However, hemoglobin electrophoresis will distinguish these mutant hemoglobins.

For the reasons noted above, a DNA diagnosis of hemoglobin EE mutation status should be verified with hematologic studies at the age of 9 to 12 months, at which time only hemoglobin E should be present and mild anemia, microcytosis (a 30% reduction in mean corpuscular volume), and a characteristic blood smear should be found.[3]

State authorities estimate that 150 newborn infants with hemoglobin FE will be identified annually within the Asian population of California. The program screened 610,000 infants in the first 13 months and identified 143 with hemoglobin FE. Assuming, as in our study, that about half of these patients require prolonged follow-up to establish a clinical diagnosis, we estimated that the annual cost of the conventional diagnostic approach to California's health care system would be approximately $87,000, as opposed to $21,000 for the proposed DNA approach.

These preliminary data indicate that a genetic approach to diagnosis of newborn infants with hemoglobin FE should be successful on two levels. One is that a rapid assessment of the patient's hemoglobin E mutation status is provided. The other is that significant financial savings may be achieved by eliminating some of the clinic visits necessary to make a conventional clinical diagnosis.

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