Detection of the Most Common Mutations Causing β -Thalassemia in Mediterraneans Using a Multiplex Amplification Refractory Mutation System (MARMS)

Paolo Fortina,¹⁻⁴ Giulia Dotti,^{2,4} Rebecca Conant,^{2,4} Gregory Monokian,^{2,4} Teresa Parrella,¹⁻³ Wendy Hitchcock,^{2,4} Eric Rappaport,^{1,3} Elias Schwartz,^{1,3,5} and Saul Surrey^{1,3,5}

¹Division of Hematology, ²Molecular Biology Diagnostics Unit, ³Departments of Pediatrics, ⁴Pathology and Clinical Laboratories and ⁵Human Genetics, The Children's Hospital of Philadelphia, The University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

A rapid, simple, cost-effective, non-radioactive method for detection of the most common mutations causing β -thalassemia in Mediterranean people has been developed by combining multiplexing with the amplification refractory system. This approach, the multiplex amplification refractory mutation system (MARMS), provides an easy assay for direct detection of normal and mutant β -globin genes in homozygotes and heterozygotes. The strategy involves multiplex PCR of four of the five regions of interest within the β -globin gene in a single reaction containing a common oligoprimmer and either the normal or mutant oligonucleotides corresponding to IVS-1 nucleotide 1 or IVS-1 nucleotide 6, IVS-1 nucleotide 110, codon 39, and IVS-2 nucleotide 1 regions. Primers are chosen so that the sizes of the four PCR products differ, thereby facilitating detection on agarose gels following amplification. Patient samples are primed with either four normal or four mutant oligonucleotide mixtures and the common oligoprimmer, and PCR products run in parallel on gels to detect band presence or absence. This approach simplifies mutation detection and shows promise for automation employing fluorescent-tagged primers.

β -Thalassemia is a heterogeneous genetic disease associated with defective expression of the β -chain of human hemoglobin (Hb).⁽¹⁾ Its distribution encompasses the Mediterranean basin, including North Africa and the Middle East, as well as China, India, and Southeast Asia.⁽²⁾ More than 100 mutations have been defined that lead to aberrant expression of the β -globin gene in humans.⁽³⁾ Of the several different types, the IVS-1 nucleotide 1, IVS-1 nucleotide 6, codon 39, IVS-1 nucleotide 110, IVS-2 nucleotide 1, and IVS-2 nucleotide 745 changes are the most common mutations among Mediterraneans and account for approximately 92% of the β -thalassemia defects in this area.⁽⁴⁾ A wide variety of strategies and techniques are currently available to detect mutations⁽⁵⁻⁸⁾; however, none appears ideal for rapid screening of a large number of samples. The most important issues are detection of as many mutations as possible, cost effectiveness, rapidity and accuracy in obtaining the diagnosis, and, eventually, the ability to handle large numbers of samples by automated methods. Methodology should also be simple enough to be used in clinical laboratories. A cost-effective, simple, rapid test that specifically detects the five prevalent mutations in the Mediterranean basin would be of major benefit, and could be applied to common mutations in other population groups, as well. To address some of these issues, we have attempted to develop an efficient muta-

tion scanning method by combining the technique of the amplification refractory mutation system (ARMS)-PCR⁽⁹⁻¹³⁾ and multiplexing,⁽¹⁴⁾ in which various regions of the β -globin gene are amplified in the same tube, allowing analysis of many loci at one time. This approach, which greatly simplifies the diagnosis of the most common Mediterranean β -thalassemia mutations, also holds promise for use in a multiplex, ARMS-PCR fluorescence-tagged gene amplification⁽¹⁵⁾ strategy that may further lead to complete automation, thereby reducing reagent cost and templates required without increasing the risk of missed or erroneous diagnoses.

MATERIALS AND METHODS

DNA samples were obtained from normal controls and patients that were either homozygous or heterozygous for the common Mediterranean mutations. In addition, 1 individual was heterozygous for Hb Lepore. Genomic DNA was extracted using previously described protocols⁽¹⁶⁾ or with an automated extractor (ABI, Foster City, CA). In addition, samples were also kindly provided by Dr. C. Camaschella (University of Turin, Italy). The five common mutations previously described in Mediterraneans⁽⁴⁾ were studied. Genotypes (Table 1) were confirmed either by DNA sequence analysis using a PCR-based, cycle-sequencing approach employing a laser-activated, fluorescence-emission DNA sequencer⁽¹⁷⁻¹⁹⁾

TABLE 1 β -Thalassemia Genotypes

Sample	Genotype
RD15	N/IVS-1 nucleotide 110
RD16	N/Lepore
KN47	N/IVS-1 nucleotide 5
AN47	N/IVS-1 nucleotide 6
WN47	IVS-1 nucleotide 5/IVS-1 nucleotide 6
10 Ped	Codon 39/IVS-1 nucleotide 6
23 Ped	IVS-1 nucleotide 110/IVS-1 nucleotide 110
17	IVS-1 nucleotide 6/IVS-1 nucleotide 6
27	IVS-2 nucleotide 745/IVS-1 nucleotide 6
520	IVS-1 nucleotide 110/IVS-1 nucleotide 6
575	N/IVS-1 nucleotide 1
577	Codon 39/IVS-1 nucleotide 1
579	N/Codon 39
634	-29/Codon 24
698	N/IVS-2 nucleotide 1
744	N/IVS-1 nucleotide 110
779	N/IVS-1 nucleotide 6
813	N/IVS-2 nucleotide 745
897	IVS-1 nucleotide 1/IVS-1 nucleotide 1
984	Codon 39/codon 39
999	Codon 39/IVS-1 nucleotide 110

or by denaturing gradient gel electrophoresis.^(7,8) Oligoprimers for either PCR or DNA sequence analysis were synthesized on a 380B DNA Synthesizer (ABI) by the phosphoramidite method at 0.2 μ mole scale with (2-O-cyano-ethyl)-phosphoramidites,⁽²⁰⁾ and were then purified.

Normal and mutant PCR primers differed in that their terminal 3' nucleotide corresponded to either normal or mutant sequence. An additional mismatched residue was also incorporated

at 3 nucleotides in from the 3' nucleotide⁽¹²⁾ in both normal and mutant primers to ensure selective amplifications (Table 2). Each of the primers was tested with normal and mutant DNA samples under stringent PCR conditions to ensure that selective amplification occurred with either normal or mutant primer. The strategy for multiplex generation of differently sized PCR products was accomplished using a single common 5' primer located at -109 bp upstream of the β -globin gene, as illustrated in Figure 1. Sequence of this primer was identical to the normal sequence.⁽²¹⁾ Screening for the five mutations involved four separate PCR reactions. Mixtures contained the common upstream primer and either four normal (A-N) or four mutant primer pairs (A-M) (Table 2) for the IVS-1 nucleotide 1, IVS-1 nucleotide 110, codon 39, and IVS-2 nucleotide 1 sequences. Screening for the IVS-1 nucleotide 6 mutation was done in two separate PCR reactions (with normal or mutant primer) because detection of the IVS 1 nucleotide 1 and IVS-1 nucleotide 6 mutations was not possible in the same lane on electrophoresis. Normal and mutant PCR products are readily scored by the presence or absence of the correctly sized band following electrophoresis on agarose gels.

PCR was performed according to the method previously described.⁽²²⁾ Reaction mixtures (50 μ l) contained 1 μ g of genomic DNA, 100 μ M of each dNTP, the common upstream primer (β -CRP1) and either four normal or four mutant primer sets in a buffer containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 0.01% (wt/vol) gelatin. The mixture was heated at 95°C for 5 min to

denature the DNA, and then quickly chilled on ice. *Taq* DNA polymerase (4 units) was added before overlaying the samples with 50 μ l of mineral oil. The samples were then subjected to 25 cycles on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) with denaturation at 93°C for 1 min and combined annealing and extension at 66°C for 2 min. The last cycle had a 3-min extension at 66°C. Approximately 20% of the PCR product was then analyzed following electrophoresis on a 4% (wt/vol) agarose (NuSieve GTG) gel.

RESULTS AND DISCUSSION

Selective amplification of normal sequences with normal primers is shown in Figure 2. Our results show that separate amplifications using normal primers for each of the five regions in normal DNA give single bands of expected size (lanes 2, 4, 6, 8, and 10). Multiplex PCR using normal primers for four of the five regions in normal DNA also yields the expected combined band pattern (lanes 12 and 14), whereas no corresponding amplification products were detected with mutant primers (lanes 3, 5, 7, 9, and 11). These results show that normal primers can be multiplexed in the same tube, and that mutant primers will not prime normal DNA under these conditions. Twenty-one different genotypes were tested (Table 1). Amplification products were generated in the multiplex reactions only with the appropriate normal and mutant primers as shown in Figure 3. Using MARMS, it was possible to confirm all of the common β -thalassemia mutations present in 17 of

TABLE 2 Oligonucleotide Primers Used for Mutation Detection by MARMS

Primers	Coordinates	Sequence	pmoles
IVS-1 nucleotide 1-N	+143 to +172	5'-TTAAACCTGTCTTGTAAACCTTGATACGAAC-3'	90
IVS-1 nucleotide 1-M		5'-TTAAACCTGTCTTGTAAACCTTGATACGAAT-3'	90
IVS-1 nucleotide 6-N	+149 to +178	5'-TCTCCTTAAACCTGTCTTGTAAACCTTCATA-3'	10
IVS-1 nucleotide 6-M		5'-TCTCCTTAAACCTGTCTTGTAAACCTTCATG-3'	10
IVS-1 nucleotide 110-N	+253 to +282	5'-ACCAGCAGCCTAAGGGTGGGAAAATAGTCC-3'	20
IVS-1 nucleotide 110-M		5'-ACCAGCAGCCTAAGGGTGGGAAAATAGTCT-3'	20
Codon 39-N	+298 to +327	5'-CAGATCCCCAAAGGACTCAAAGAACCTGTG-3'	10
Codon 39-M		5'-CAGATCCCCAAAGGACTCAAAGAACCTGTA-3'	10
IVS-2 nucleotide 1-N	+496 to +525	5'-AAGAAAACATCAAGGGTCCCATAGACTGAC-3'	90
IVS-2 nucleotide 1-M		5'-AAGAAAACATCAAGGGTCCCATAGACTGAT-3'	90
β -CRP1	-109 to -89	5'-ACCTCACCTGTGGAGCCAC-3'	90

Coordinates are given relative to the cap site (+1) of the β -globin gene, with "N" indicating normal and "M" mutant primers, respectively. β -CRP1 refers to the common primer (Fig. 1). Bold letters identify 3' mutation and second base change at 3 nucleotides in from the 3' nucleotide.

FIGURE 1 Strategy for multiplex amplification of the areas encompassing the most common β -thalassemia mutations in Mediterraneans. Approximate location of five common β -thalassemia mutations is indicated ($\blacktriangle$) within the β -globin gene. Multiplex amplification is accomplished using a common upstream primer (β -CRP1) and a mixture of either the four normal or four mutant primer sets (Table 2) in each PCR reaction. Size of expected PCR products in base pairs is also shown.

FIGURE 2 PCR amplification of normal DNA in regions encompassing the most common Mediterranean β -thalassemia mutations. Amplifications were done with DNA from normal controls using normal primers (lanes 2, 4, 6, 8, and 10) or mutant primers for the same regions (lanes 3, 5, 7, 9, and 11). Multiplex amplification of four regions in normal DNA encompassing IVS-2 nucleotide (nt) 1, codon 39, IVS-1 nucleotide 110, and IVS-1 nucleotide 6 with normal primers (lane 12) or mutant primers (lane 13) is also shown. Lanes 14 and 15 show the same pattern of multiplex allele-specific PCR using the IVS-1 nucleotide 1 normal and mutant primers, respectively. PCR products are sized relative to markers generated from a *Hae*III digest of ϕ X174 Rf DNA (lane 1). Arrows show location of each PCR product using indicated primers.

FIGURE 3 Detection of common Mediterranean β -thalassemia mutations by MARMS. All amplifications were done with either normal (N) or mutant (M) primer sets corresponding to the IVS-1 nucleotide 1, IVS-2 nucleotide 1, IVS-1 nucleotide 6, codon 39, and IVS-1 nucleotide 110 regions of the human β -globin gene. Multiplex amplification was done using separate reactions containing a mixture of either the normal or corresponding mutant primer sets with DNA from a heterozygote for the IVS-2 nucleotide 1 mutation (lanes 2 and 3), DNA from a heterozygote for the IVS-1 nucleotide 1 mutation (lanes 4 and 5), DNA from a compound heterozygote for the codon 39 and IVS-1 nucleotide 1 mutations (lanes 6 and 7), DNA from a compound heterozygote for -29 and codon 24 mutations (lanes 8 and 9), respectively. PCR products are sized relative to markers generated from a *Hae*III digest of ϕ X174 Rf DNA (lane 1).

the 21 samples studied. The other four had one or more nucleotide -29, codon 24, IVS-1 nucleotide 5, IVS-2 nucleotide 745, and Lepore mutations.

Although the multiplex PCR assay provides a rapid way to screen numerous patients for the five common mutations, care must be taken to consider the clinical data in interpreting genotype results for some individuals. The clinical data are most important in resolving the diagnosis of a patient who appears to be heterozygous for one of the five common mutations screened for in the multiplex assay. For example, in sample 27, the five normal primers produce normal amplification products, while the mutant primer set only yields a 256-bp fragment, consistent with heterozygosity for the IVS-1 nucleotide 6 mutation. However, the patient's clinical data suggested a severe form of β -thalassemia, and therefore, further testing was required to delineate the molecular basis for this patient's phenotype. DNA sequence analysis of amplified DNA from this patient confirmed the presence of an additional mutation at position IVS-2 nucleotide 745. An additional concern is that of the absence of any PCR product using the multiplexed mutant primer set. This result could be due to either the existence of other β -thalassemia mutations not screened for, or alternatively, the result of inappropriate priming in the reaction. Therefore, it is advisable if no signal is present with the mutant set, that a primer pair from a different locus be included with the mutant primer set to recheck the results and to serve as an internal control for PCR amplifications. It is also of interest that only full-length PCR products of the expected size were seen in the multiplex reactions, even though the primers for the smaller PCR products are nested within the largest PCR product. Therefore, formation of heteroduplexes in the multiplex reaction does not appear to be of major concern, since generation of these species is probably minimized by the repeated annealing and denaturation steps as well as by the strand-displacement activity of *Taq* polymerase during PCR amplification. These results demonstrate selective multiplex amplification of normal and mutant alleles for the common mutations in Mediterraneans, and show promise for a rapid, cost-effective, automated, fluorescence-based, allele-specific MARMS assay for mutation screening.

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