Clinical Evaluation of a Reverse Hybridization Assay for the Molecular Detection of Twelve MEFV Gene Mutations, Dimitri Tchernitchko,1 Marie Legendre,1 Andrée Delahaye,1 Cécile Cazenave,1 Florence Niel,1 Michel Goossens,1,2 Serge Anselm,3 and Emmanuelle Girodon1,4 (ServicedeBiochimieetdeGénétiqueMoléculaire,HôpitalHenriMondor,AP-HP,94010Créteil,France;INSERMU468GénétiqueMoléculaireetPhysiopathologie,94010Créteil,France;*thetwofirstauthorscontributedequallytothiswork;†addressexcorrespondencetothisauthorat:ServicedeBiochimieetdeGénétiqueMoléculaire–INSERMU468,HôpitalHenriMondor,51,AvenueduMaréchaldeLattetattassin,94010Créteil,France;fax33-1-4981-2842,e-mailemmanuelle.girodon@im3.inserm.fr)

Familial Mediterranean fever (FMF) is an autosomal-recessive disorder (MIM 249100) characterized by recurrent attacks of fever and serositis, affecting principally Sephardic Jewish, Armenian, Arab, and Turkish populations. Early diagnosis is important to initiate colchicine therapy, which prevents the occurrence of attacks and of renal amyloidosis, the major complication of the disease. The identification of MEFV (1, 2), the gene responsible for the disease, allowed the use of an early molecular test of diagnostic value for FMF patients (3), which negates the needs for unnecessary invasive investigations. The MEFV gene, located on chromosome 16p13.3, contains 10 exons and encodes the marenostin/pyrin protein, a molecule acting as a regulator of the proinflammatory interleukin-1-dependent pathway and of the apoptosis mediated by the apoptosis-associated Speck-like protein containing a CARD (ASC) (4, 5), and which belongs to the death domain-fold family (6).

More than 40 different FMF-associated mutations have been described to date (7), the most frequent ones being located in exon 10. Indeed, the M694V, V726A, M680I, and M694I mutations account for 65–95% of FMF alleles depending on the ethnic origin of the patient (8). E148Q is a frequent sequence variation situated in exon 2, but its involvement in the development of the disease remains controversial (9). The molecular diagnosis of FMF is based on various methods, including tedious and time-consuming scanning techniques such as denaturing gradient gel electrophoresis (DGGE) (3) or direct sequencing (10). Restriction enzyme analysis enables the detection of known mutations but still requires multiple DNA amplifications. An in-house amplification refractory mutation system (ARMS) has been developed (11), which belongs to the death domain-fold family (6).

References

17. Delahaye,1 Ce´cle´deBiochimieetdeGe´n´etiqueMol´eculaire,94010Cr´et´eil,France;*the two first authors contributed equally to this work;†addressexcorrespondencetothisauthorat:ServicedeBiochimieetdeG´en´etiqueMol´eculaire–INSERMU468,H´opitalHenriMondor,51,AvenueduMar´echaldeLattetattassin,94010Cr´et´eil,France;fax33-1-4981-2842,e-mailemmanuelle.girodon@im3.inserm.fr)
and 10 [M680I (G>C), M680I (G>A), I692del, M694V, M694I, K695R, V726A, A744S, and R761H] can be simultaneously screened for by a reverse hybridization procedure.

The assay includes four successive steps for which reagents are provided: (a) DNA isolation from blood samples; (b) in vitro multiplex amplification reaction; (c) hybridization of amplification products to a test strip comprising a staining control, 8 wild-type-, and 12 mutant-specific immobilized oligonucleotide probes; and (d) detection of bound biotinylated sequences by streptavidin–alkaline phosphatase and color substrate. According to the protocol recommended by the manufacturer, DNA is extracted from 100 μL of whole blood (EDTA or citrate) to obtain 150 μL of a 35–80 ng/μL DNA solution, in a procedure that requires ~80 min. Five microliters of DNA template are amplified by a multiplex PCR in a single tube, requiring ~2.5 h. The products from the 20-μL PCR reaction may be checked by visualizing four amplicons of 206, 236, 295, and 318 bp on a 3% agarose, ethidium bromide-stained electrophoresis gel. The hybridization can be performed either in a water bath with a shaking platform (50 rpm) and temperature adjustable to 45 °C, which requires ~3 h, or in an automatic incubator, which requires ~2.5 h.

After hybridization at 45 °C, stringent wash at 45 °C, and color development at room temperature, the results are interpreted with a coding table. In theory, the presence of only the wild-type signals corresponds to the absence of the 12 tested mutations, but it does not exclude the existence of one or two rare mutations. Heterozygous genotypes produce hybridization to mutant probe(s). One of the wild-type lines disappears in the case of a homozygous mutant or of a compound heterozygote for neighboring mutations, e.g., M694V and M964I (Fig. 1) or M680I (G>C) and M680I (G>A). Staining intensities of positive lines may vary; however, this is of no significance for interpretation of the results.

We successively assessed the reverse hybridization procedure in terms of its specificity and sensitivity, the influence of temperature, the practicability of the assay if performed manually or with use of an automated incubator, and the efficacy of the DNA extraction protocol provided with the reagent set in comparison with our in-house phenol–chloroform extraction. Amplifications were conducted on an Applied Biosystems thermocycler 9700, using the protocol recommended by the manufacturer, and hybridizations were performed manually or in an AutoLIPA™ automated incubator (Innogenetics), which can process up to 30 strips simultaneously.

Fig. 1. Representative hybridization patterns obtained with the FMF StripAssay.
Each strip comprises a staining control, 12 mutant oligonucleotide probes, and 8 corresponding wild-type probes. Strips A1–A4 were incubated with N/N, M694V/N, M694V/M694V, and M680I (G>C)/E148Q PCR products, respectively; strip A5 was incubated with a sample without DNA. Strips B1–B5 demonstrate the influence of the incubation temperature on the sensitivity of the method: they were incubated with a M694V/M694I PCR product at 43, 44, 45, 46, and 47 °C, respectively.
We evaluated the specificity and the sensitivity of the reverse hybridization technique by testing 65 DNA samples from FMF patients (n = 32), carriers (n = 24), or control individuals who did not carry any of the 12 mutations (n = 9). Written informed consent for genetic studies was given by the patients and the healthy controls. DNA samples were obtained by a classic phenol-chloroform extraction and were successfully amplified. Reverse hybridization was performed according to an automated procedure. All samples had been previously characterized in our laboratory by the reference method, i.e., DGGE analysis of exons 10, 5, 3, and 2 and their intron-flanking regions, followed by restriction enzyme analysis or direct sequencing (3). The genotypes identified by both techniques are indicated in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol49/issue11/. With regard to the mutations screened by the FMF StripA<sup>away</sup>, concordance with the reference method was 100%.

To evaluate potential interferences leading to erroneous interpretation attributable to sequence variations located at primer or probe binding sequences, we tested DNA samples possessing mutations or polymorphisms situated in analyzed regions but differing from the 12 mutations detected by the assay (Table 1 in the online Data Supplement). As expected, we found no nonspecific hybridization to mutant probes with the following sequence variations situated in exons 2 (G138G, E148V, A165A, E167D, and R202Q), 3 (R354W and R408Q), 5 (E474E, Q476Q, and D510D), and 10 (S675N, S683S, A701A, S703S, F706P, F721F, and E723D). However, in the absence of compound heterozygous (such as E148Q/E148V) or homozygous samples containing those variants, we could not assess the affinity of these alleles for the corresponding wild-type oligonucleotides immobilized on the strips and the possible repercussion on the annealing of PCR primers. The occurrence of such pitfalls should be considered for any technique by screening laboratories.

We also evaluated the convenience of using the manual method required strict attention to the protocol, especially concerning hybridization temperature. The use of an automated incubator facilitated temperature management and yielded substantial saving of time, but it consumed more reagent.

We also assessed the DNA extraction protocol with the reagents included with the assay. Blood samples from 31 consecutive FMF patients and unaffected relatives were divided into two sets, and DNA was extracted in parallel and analyzed by two procedures: (a) phenol-chloroform extraction and DGGE analysis; and (b) DNA extraction and reverse hybridization analysis using the FMF StripA<sup>away</sup> protocols. Tested genotypes were M694V/M694V (n = 3), M694V/V726A (n = 2), M694V/K695R (n = 2), M694I/V726A (n = 1), V726A/F479L (n = 1), M694V/N (n = 8), V726A/N (n = 4), M694I/N (n = 2), and N/N (n = 8). Using the extraction method included with the assay, we obtained DNA concentrations ranging from 33 to 72 μg/L (35–80 μg/L was the estimated yield from the manufacturer). The yield of the DNA extraction protocol included in the assay allows repeated tests from a limited blood amount (100 μL). We successfully amplified all of the DNA samples that had been extracted by a phenol–chloroform method and those obtained with the reagents provided with the assay. The results of the two procedures were fully concordant.

Because FMF lacks both specific clinical symptoms and biochemical abnormalities and is particularly frequent in some Mediterranean populations, the identification of two allelic MEFV mutations is of utmost diagnostic value (3). The availability of a test to detect the most frequent MEFV gene mutations simultaneously should prompt molecular genetics laboratories to investigate patients with recurrent fever on a routine basis. The FMF StripA<sup>away</sup> is technically suitable for use in any diagnostic laboratory, allows the simple and rapid screening of up to 30 samples when an automated incubator is used, and can detect the most frequent MEFV mutated alleles in 1 working day. The assay gives reliable results as long as incubation recommendations are followed, especially for temperature, which can be controlled best by the use of an automated incubator. The reverse hybridization assay does not detect rare mutations; nevertheless, considering the mutated alleles characterized in our laboratory over the past 3 years, the use of the reverse-hybridization method would have detected 99.3% of the MEFV mutations characterized by the DGGE strategy for exons 2, 3, 5, and 10 (Table 1). Identification of polymorphisms by DGGE still appears useful for segregation studies in families in which the patients present with a FMF or FMF-like phenotype, possibly allowing ruling-out of involvement of the MEFV locus in the occurrence of the disease. The high cost of the FMF StripA<sup>away</sup> may be a limitation ($850–$950 for 20 tests) especially when making use of an automatic incubator, which further raises its cost, but its use provides savings in time.
Table 1. Allele frequencies of MEFV mutations detected in our laboratory among FMF patients who fulfilled the diagnosis criteria according to Livneh et al. (12).a

<table>
<thead>
<tr>
<th>Sequence variation</th>
<th>No. of alleles</th>
<th>Percentage of mutated alleles</th>
<th>Cumulative frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>M694V</td>
<td>568</td>
<td>62.2</td>
<td>62.2</td>
</tr>
<tr>
<td>V726A</td>
<td>133</td>
<td>14.7</td>
<td>76.9</td>
</tr>
<tr>
<td>M680I (G&gt;C)</td>
<td>75</td>
<td>8.2</td>
<td>85.1</td>
</tr>
<tr>
<td>E148Q</td>
<td>46</td>
<td>5.0</td>
<td>90.1</td>
</tr>
<tr>
<td>M694I</td>
<td>34</td>
<td>3.7</td>
<td>93.8</td>
</tr>
<tr>
<td>F479L</td>
<td>21</td>
<td>2.3</td>
<td>96.1</td>
</tr>
<tr>
<td>R761H</td>
<td>20</td>
<td>2.2</td>
<td>98.3</td>
</tr>
<tr>
<td>K695R</td>
<td>4</td>
<td>0.4</td>
<td>98.7</td>
</tr>
<tr>
<td>A744S</td>
<td>2</td>
<td>0.2</td>
<td>98.9</td>
</tr>
<tr>
<td>P369S</td>
<td>2</td>
<td>0.2</td>
<td>99.1</td>
</tr>
<tr>
<td>M680I (G&gt;A)</td>
<td>1</td>
<td>0.1</td>
<td>99.2</td>
</tr>
<tr>
<td>I692del</td>
<td>1</td>
<td>0.1</td>
<td>99.3</td>
</tr>
<tr>
<td>Other</td>
<td>6</td>
<td>0.7</td>
<td>100</td>
</tr>
<tr>
<td>No identified mutation</td>
<td>883</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1796</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The study group included Armenians (38.9%), Sephardim (22.8%), Arabs (11.5%), Turks (7.5%), Ashkenazim (0.5%), individuals of heterogeneous at-risk origin (2.4%), and individuals from classically non-at-risk origin (15.7%).

b Mutations detected by the FMF StrIп™ assay are in bold.

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References


12. Simple Sequence-specific-Primer-PCR Method To Identify the Three Main Apolipoprotein E Haplotypes, Panagiotis Pantelidis,1* Michelle Lambert-Hamill,2 and Anthony S. Wierzbicki2 (1 Pan-Pathology Molecular Diagnostics Laboratory and 2 Department of Chemical Pathology, St. Thomas’ Hospital, Lambeth Palace Road, London SE1 7EH, UK; * address correspondence to this author at: Pan-Pathology Molecular Diagnostics Laboratory, Department of Infection, 5th Floor North Wing, St. Thomas’ Hospital, Lambeth Palace Road, London, SE1 7EH, UK; fax 44-20-79280730, e-mail panagiotis.pantelidis@gstt.sthames.nhs.uk)

Apolipoprotein E (ApoE) is involved in the binding, internalization, and catabolism of lipoprotein particles. ApoE is secreted by macrophages and hepatocytes and serves as a ligand for the LDL (Apo B/E) receptor and on hepatic tissues for the specific ApoE type 1 and 2 receptors for triglyceride-rich remnants (1, 2). The gene coding for ApoE is located on chromosome 19q13.32 and contains several polymorphic loci. The three common isoforms of ApoE, termed E2, E3, and E4, are defined by two single-nucleotide polymorphisms (SNPs) at positions 2059(T/C) and 2197(C/T) of the gene, which are located in the codons that code for amino acids 112 and 158 of the mature protein. The presence of a T allele at position 2059 (2059-T) of the gene defines a cysteine at position 112 (Cys112), whereas 2059-C defines an arginine for the same position (Arg112). Similarly, 2197-C on the gene defines an arginine at position 158 (Arg158), whereas 2197-T codes for cysteine for the same position (Cys158). ApoE3 (Cys112/Arg158) is the most common of the three isoforms found. The ApoE2 isoform Cys112/Cys158 is associated with remnant hyperlipidemia because this isoform has a lower affinity for ApoE receptors. The ApoE4 (Arg112/Arg158) isoform is associated with increased hepatic synthesis of VLDL and is a risk factor for Alzheimer disease and other neurologic diseases. Other rare isoforms are also found and are associated with mixed hyperlipidemia (1).

Several methods have been used for genotyping the three major ApoE isoforms. The problems inherent in phenotyping methods have led to the gradual adoption of genotyping methods for determination of ApoE isoforms in most laboratories (3). By far the commonest method has been PCR amplification followed by restriction fragment length polymorphism (RFLP) analysis. Although many variations of this methodology have been published, the original method used HhaI restriction endonuclease, which cleaves at the CGGC sequence that encodes