Oligonucleotide ligation assay for rapid and sensitive identification of carriers of a missense mutation in the cardiac β-myosin heavy chain gene causing hypertrophic cardiomyopathy in an Indian family

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The identification of mutations or sequence polymorphisms in defined segments of genomic DNA is achieved by a variety of molecular methods. The present study illustrates the value of testing for known point mutations in the human cardiac β-myosin heavy chain (MYH7) associated with familial hypertrophic cardiomyopathy (HCM) by the oligonucleotide ligation assay (OLA). We have adapted OLA for the assessment of a G → T transversion in codon 712$^{Arg–Leu}$ of the β-myosin heavy chain gene, which caused HCM in a previously reported Indian family. This mutation was originally detected by scanning all exons of the *MYH7* gene of the index patient by SSCP (single strand conformation polymorphism) and direct DNA sequencing. Carriers of the mutation in the family were determined by OLA (and in parallel by restriction analysis). We conclude that the OLA procedure can be utilized in a nonisotopic format as a simple and rapid procedure to distinguish between 'mutation carriers' and 'non-mutation carriers' in families with known mutations.

HYPERTROPHIC cardiomyopathy (HCM) is a primary disease of the myocardium with an autosomal dominant pattern of inheritance, which is clinically variable and genetically heterogeneous[1–3]. It is characterized clinically by a broad spectrum of symptoms, including palpitation, dyspnea, chest pain, syncope and arrhythmia. The disease can be caused by a mutation in one of the following genes, all encoding proteins of the cardiac sarcomere: β-myosin heavy chain[4], α-tropomyosin[5], myosin-binding protein-C[6], cardiac troponin-T[5], cardiac troponin-I[7], essential and regulatory light chain of myosin[8], cardiac α-actin[9] and possibly titin[10]. The most frequently affected genes are those coding for β-myosin heavy chain, troponin T and myosin-binding protein-C. The prevalence of MYH7 mutations alone in HCM families is probably > 30%. Recently, we have reported a novel missense mutation in codon 712$^{Arg–Leu}$ of the β-*MHC* gene (cDNA position 2221 G → T) which is responsible for HCM with incomplete penetrance in an Indian family with a reported history of premature sudden death[11]. The identification of HCM-related mutations should help to improve knowledge on causes and mechanisms of the disorder. It is also of clinical importance since it bears relevance to counselling of families or carriers at risk, in particular with risk assessment of individuals disposed to HCM.

Genetic typing involves the identification of a disease-related mutation in a defined segment of genomic DNA by any one of a variety of molecular methods. Different strategies of genetic typing have been developed. Predominantly, they are based on PCR amplification of genomic or mRNA/cDNA sequences followed by restriction, heteroduplex analyses of various types, probing of single-strand conformation analysis or direct DNA sequencing. We have used and demonstrate here, allele-specific ligation of diagnostic oligonucleotides arranged head-to-tail along the DNA of interest, allowing ligation if oligonucleotides match perfectly, but not if a mismatch exists at the site of mutation. This procedure has previously been described as oligonucleotide ligation assay (OLA)[12]. We have used it to monitor the presence or absence of a missense mutation (G → T) in the *MYH7* codon 712$^{Arg–Leu}$ in the genomic DNA of relatives of our Indian HCM proband (family code: MT).

Blood samples were obtained with informed consent from the proband and relatives of family MT[11]. For control studies, normal healthy individuals were also analysed. High-molecular weight DNA was extracted from venous blood lymphocytes of patients and control persons by using a QIAamp Blood Kit (Qiagen).

Primers were obtained from MWG-Biotech (Germany) on the basis of reported nucleotide sequences of the β-myosin heavy chain gene (*MYH7*)[13]. The exon 19 of the β-myosin heavy chain gene was PCR amplified using DNA from the index patient and his relatives. Cycling conditions (Hybaid Omnigene cycler) were 55°C/72°C/94°C for annealing, extension and denaturation, respectively. PCR primer sequences for exon 19 were 5′-TGA CTT GCT AAG ATT ACA AGC-3′ (forward) and 5′-ATC CCA TTC CCA TCA GGG CAG-3′ (reverse).

Three oligonucleotide probes were used for the OLA detection of the missense mutation (G → T) in codon 712 of the *MYH7* gene. One probe was common to both alleles (the reporter probe) and two probes were allele-specific, discriminating between wild type and mutant alleles, respectively. The common probe placed immediately downstream of the mutation site was 5′-phosphorylated and carried a Cy5 label at its 3′-end. The allele-specific probes varied in length by 3 bases. The OLA reaction was conducted as described previously[14]. Briefly, all OLA reactions were carried out in

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20 µl volumes containing 100 mM KCl, 20 mM *Tris*-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM NAD+, 200 fmoles of each oligo probe, 2 µl of PCR-amplified exon 19 DNA and 2 units of thermostable *Thermus aquaticus* DNA ligase (NEB). Linear amplification of product was achieved by 15 cycles of 94°C for 2 min, 55°C for 3 min in a Hybaid Omnigene thermocycler. For analysis, 2 µl OLA products were mixed with 4 µl of a 5 : 1 mixture of deionized formamide and 50 mM EDTA containing Cy5-labelled oligonucleotide size markers (30, 50 and 100 nt long). DNA was denatured at 94°C for 2 min, cooled on ice and loaded onto the gel. OLA products and size markers were separated in standard denaturing 8% PAA-urea gels on an ALF*express* sequencer. Cy5 fluorescence signals were evaluated using the ALFwin analyser V1.02 software (Pharmacia Biotech).

Oligonucleotide probes (5′ → 3′) – Wildtype: 5′OH-AGGAAAGGCTTCCCCAACC**G** (20 bases). Mutation specific: 5′OH-TGCAGGAAAGGCTTCCCCAACC**T** (23 bases). Common reporter probe: 5′P-CATCCT-CTACGGGGACTTCC-Cy5 (20 bases).

A restriction cleavage site (SfaN I) suitable to identify mutation carriers was identified using the DNASIS V2.0 program. To monitor cleavage versus non-cleavage, exon 19 PCR products were digested for 2 h at 37°C with 1 U of SfaN I enzyme (New England Biolab). The digestion products were separated in 6% polyacrylamide gel and visualized by silver staining.

The three-generation pedigree of family MT with 9 living first-degree relatives is depicted in Figure 1. The proband (indicated by the arrow) was a 33-year-old man who was symptomatic for the last six years with NYHA class II exertional dyspnea. ECG and echocardiogram analyses fulfiled conventional diagnostic criteria of HCM[15]. The proband's mother (I : 2, 68 years) and two brothers (II : 3, 38 years; II : 6, 32 years) were also symptomatic. A younger brother of the mother died suddenly at age 40 years (I : 4) due to cardiac problems, but no details were reported. Six of 9 members were mutation carriers, including a boy (age 7) in generation III (son of II : 4). Four were symptomatic, one was unassessed (II : 2) and one was asymptomatic (III : 1). To summarize, penetrance was probably incomplete and age-dependent. Clinical courses were moderate to severe[11].

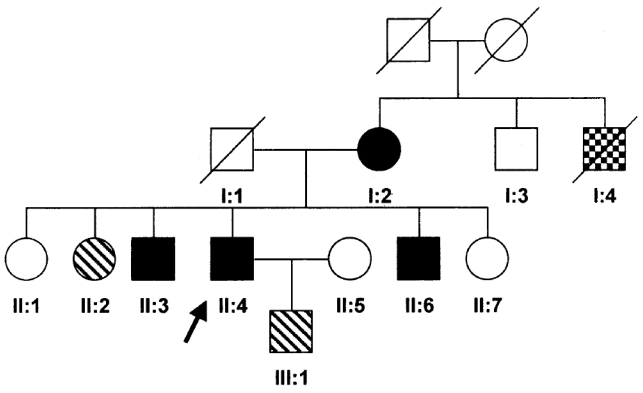

Figure 1. Pedigree of family MT. Squares and circles indicate male and female members, respectively. Open, closed and slashed symbols indicate unaffected, affected and deceased members, respectively. The stippled symbol designates one case of sudden cardiac death (no details reported). The two hatched symbols indicate one clinically unassessed (II:2) and one asymptomatic (7-year-old) carrier of the mutation. The arrow points to the proband.

The monitoring of the HCM-associated missense mutation in codon 712 by the OLA test required three probes: a common reporter probe (20 bases long) and two allele-specific probes. The common probe was 5′-phosphorylated and carried a Cy5 fluorescence label at its 3′-end. The two allelic probes differed in sequence at their 3′-ends: they had either a G (wild type, 20 bases long) or a T (mutant, 23 bases long) in that position. Upon annealing to their respective template strands and subsequent ligation, the wild type probe combination gave rise to a 40-base long product, whereas the mutant combination resulted in a 43-base long ligation product. The wild type-specific probe was unable to promote ligation on a mutant template and vice versa. Thus the appearance of a 43-base long product was indicative of the mutation. Since mutation carriers were heterozygous, they were recognized by the formation of a double peak at 40 and 43 bases. The non-carriers had only one peak at 40 bases. The underlying constellation is schematically depicted in Figure 2.

The results of the OLA tests with DNA of family MT members is shown in Figure 3 *a*. The upper line represents a homozygous normal proband (non-carrier) and the lower line, a heterozygous mutant carrier. Unligated oligonucleotides (20 or 23 bases) are seen on the far left side. Ligation products of 40 bases (single peak, upper line) or of 40 and 43 bases (double peak, lower line) are positioned between 30 and 50 base length markers. The analysis with homozygous DNA from 4 non-carriers of the family (I : 3, II : 1, II : 5 and II : 7) yielded single peaks at 40 bases, whereas heterozygous DNA from 6 mutation carriers (I : 2, II : 2, II : 3, II : 4 II : 6 and III : 1) gave rise to double peaks at 40 and 43 bases (Figure 3 *b*).

Restriction site analysis was based on the elimination of a SfaNI site as a consequence of the G → T transversion in the mutant allele. With amplified exon 19 DNA (289 bp) of carriers, a 110-bp fragment was obtained which was not seen in wildtype DNA. Instead, this fragment was cleaved into two segments, 84 and 26 bp long. In the DNA of carriers, roughly half of the DNA was resistant to cleavage at this site. The restriction patterns (Figure 4) were concordant with the single- and double-peak distribution of the OLA test in Figure 3 *b*.

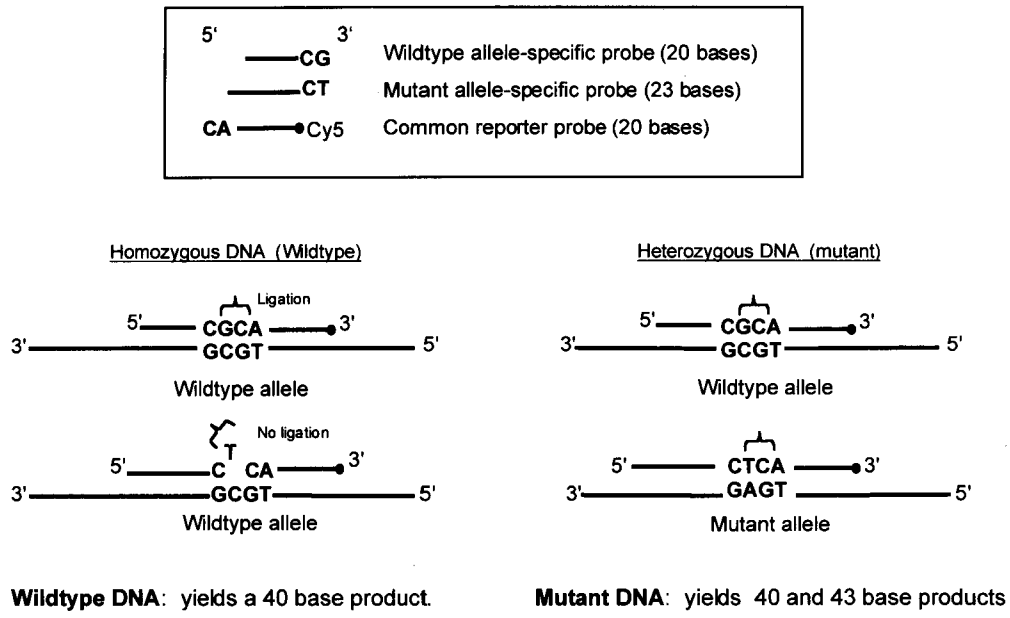

Figure 2. OLA principle – each OLA locus is interrogated by three probes. The competing allelic probes are complementary to either the normal or mutant non-coding strand. The differentiating nucleotides, G or T, are located at the 3′-ends of the allele-specific probes which differ in length by 3 bases. The common reporter probe is positioned immediately adjacent to the respective allelic oligonucleotides. The fluorescent Cy-5 tag is attached to the 3′-end of the reporter probe. Ligation products obtained with wild type and mutant alleles are distinguished by a 3-base length difference.

The molecular analysis of mutations responsible for inherited cardiovascular disease in general and for HCM in particular, has become a major topic in basic cardiological research. Respective investigations, although conducted worldwide, vary in different countries with respect to extent and intensity. Most of currently available data about HCM have been obtained in the US, in some European countries and in Japan. In India, research of this kind is still in an early stage. We propose that studies in India might add significantly to these activities by broadening the scope of genetic and clinical epidemiology of this disease. Although clinical experience in diagnosis and therapy has been established in a number of Indian tertiary clinical centres, no systematic efforts, to our knowledge, have been undertaken to investigate inherited causes or prevalence of HCM. We assume that the size and ethnic diversity of populations in conjunction with cultural habits of frequent consanguineous marriages may afford an improved understanding of a number of genetic aspects of HCM such as the modes of transmission, the relative frequencies of different genes or the role of genetic modifiers which may vary depending on ethnic background. Transmission of HCM is generally considered to be dominant. Very few reports exist indicating that recessive mutations may also be encountered[16,17]. There is no a priori reason to exclude the possibility that recessive mutations contribute to the overall incidence of HCM.

The genetic analysis depends on methods to recognize HCM-mutation carriers. In a previously unidentified patient or family, the first step is the determination of the gene and the mutation within the gene responsible for the disorder. The mutation may be detected by either of two strategies. One relies – after thorough clinical investigation of all family members – on first, genomic localization of a disease gene (through marker linkage or haplotype analysis) and then on a detailed study of the suspected gene. The second strategy rests on the direct investigation of candidate *HCM* genes in the genomic DNA of a proband. This approach does not depend on the recruitment of the entire family. It requires, however, information about disease gene sequences and protocols for the rapid assessment of sequence changes. Since eight *HCM* genes (with known sequences) have been identified and since fast analytical protocols are increasingly available, the candidate gene approach is gradually becoming the method of choice in most institutions engaged in the molecular diagnosis of HCM causes. In our case (family MT), the candidate gene analysis was done with the DNA of a single proband and family members were subsequently investigated by probing genomic DNA for the absence or presence of a G–T base exchange in codon 712 of exon 19 of the β-MHC gene found in the proband[11].

For carrier identification we have used the OLA procedure which relies on ligation of juxtapositioned oligonucleotides annealed to PCR-amplified DNA

templates. This assay combines the sensitivity of target sequence amplification by PCR and the specificity of DNA ligase which acts only if oligonucleotides match perfectly with their template strands. This requirement is strict and guarantees that false positive ligation products do not compromise the analysis. The conversion of mutated oligonucleotides into ligation products is indicative of the mutation in the template. Advantages in practical terms are (i) template strands can be used without the requirement of processing or purification; and (ii) data are obtained in a non-isotopic format in a simple electrophoretic mobility test. We wish to emphasize that the OLA method can easily be adapted to low-budget protocols. The advanced equipment (ALF express sequencer) used in this study can be replaced by straightforward and cheap standard gel electrophoresis.

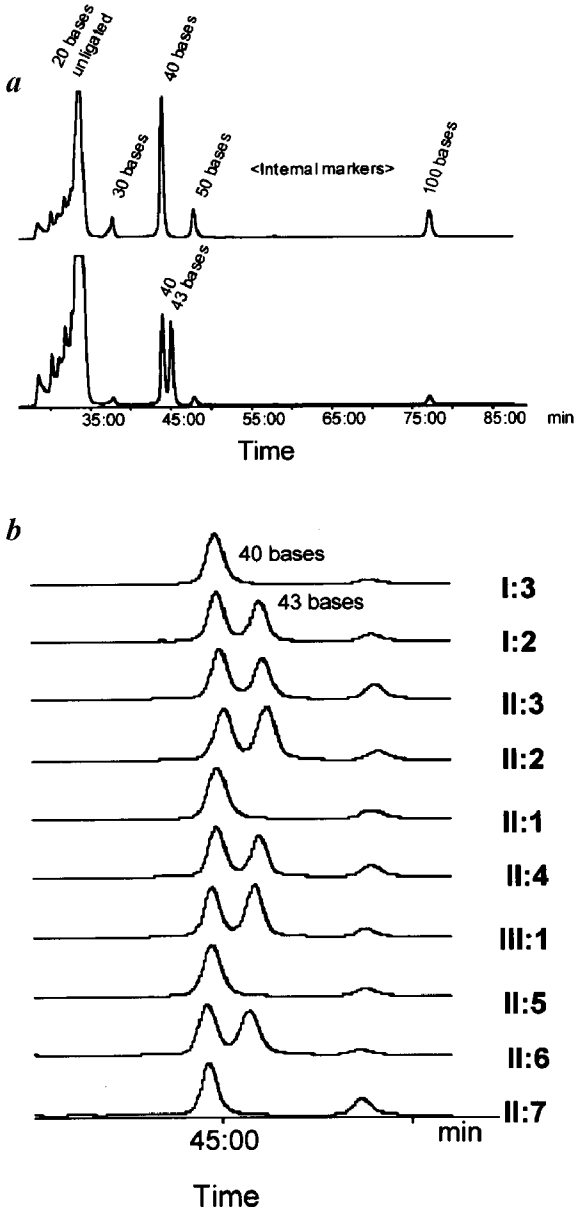

Figure 3. Electrophoretic separation of OLA ligation products. ***a***, Upper line depicts the ligation product (40 bases) obtained with DNA of an unaffected individual. Lower line shows a double peak (40 and 43 bases) from a heterozygous affected person. Peaks at the left side represent unligated probe. Cy5-labelled internal size markers (30, 50 and 100 bases) are indicated. ***b***, OLA results obtained with DNA of 10 members of family MT. Unaffected individuals have one ligation peak, and affected members have two peaks. (Right) Position in the pedigree.

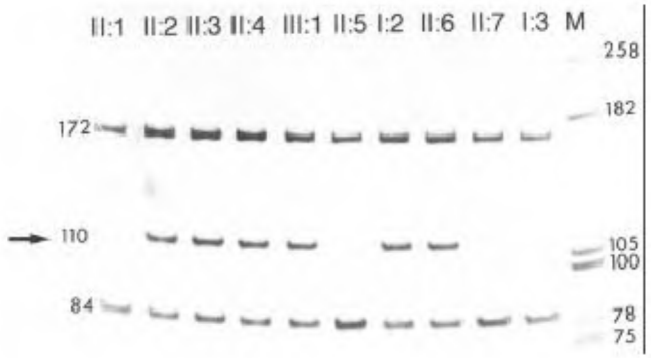

Figure 4. Cleavage of exon 19 DNA with SfaNI endonuclease. The G → T transversion in codon 712 of the β-myosin heavy chain gene is accompanied by the loss of a SfaNI cleavage site. The length of the PCR product is 289 bp. Wild type DNA has 3 and mutant DNA has 2 SfaNI sites. A 110-bp fragment obtained with the mutant product is cleaved into 84 and 26 bp fragments with wild type DNA as substrate. Smaller restriction fragments (26 and 7 bp) obtained from wild type alleles are not visualized.

To corroborate the mutation analyses, we have compared the OLA data with the SfaNI restriction enzyme digestion applied in the original report[11]. A disadvantage of the restriction approach is its ambiguity with respect to changes within the recognition site (usually 4 to 6 bp long). In most instances several distinct changes within that site will alter the DNA cleavage pattern. This is particularly relevant to the β-MHC gene, since different mutations can affect the same codon, e.g. in codon 403 (refs 4, 18 and 19), 719 (refs 20 and 21) and 741 (refs 22 and 23). To obtain an accurate diagnosis, sequencing or the use of multiple restriction enzymes may be required[24]. In addition, not all mutations are recognized by restriction enzymes.

In summary, we have demonstrated that the OLA method can be used for the rapid identification of mutation carriers in families once a mutation has been assessed in the index patient. It is anticipated that the determination of a mutation carrier status, in particular, in pre-clinical or early stages of the disease may in the future become beneficial in terms of improved clinical management. The goal of genetic investigations in families is ultimately the systematic evaluation of mutations and their phenotypic consequences through comparison of clinical courses and outcomes not only in patients of one family, but in many different families. Knowledge of natural disease histories in relation to causative mutations should help to improve our understanding of

HCM and to develop preventive measures or therapies suitable to cope with functional impairments and risks associated with HCM.

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Activation of the human serum complement cascade by insecticides

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In view of the importance of complement system in the initiation, regulation and end effects of immune responses and understanding of immune system as one of the targets for the toxic effects of insecticides, we tested benzene hexachloride (BHC) and malathion for their effects on human serum complement using C3 activation as a test parameter. The methodology used was cross-immunoelectrophoresis. Both the insecticides used at different doses (1 to 100 ppm) activated C3, the third component of the complement. This is a finding pertaining to interaction of these insecticides with the immune system. The activation was through alternative pathway. This was confirmed by blocking the classical pathway by EDTA and EGTA. The validity of this strategy was confirmed by subjecting serum to similar treatments using aggregated IgG and zymosan, the activators of classical and alternative pathways, respectively. This study also suggests that C3 activation may serve as an effective test parameter to assess immunointeractions of insecticides.

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INSECTICIDE poisoning is an important cause of worldwide morbidity and mortality. Ninety-five per cent of fatal insecticide poisoning occurs in developing countries[1]. Among the third-world countries, India is the biggest consumer of insecticides[2]. The residual nature of organochlorine and neurotoxicity of organophosphate is well known. The toxic effects in the biological system are due to homicidal, accidental and chronic exposure to humans[3,4]. Toxicological research has indicated that the immune system is a potential 'target organ' for toxic damage by insecticides[5]. There are recommendations for a range of immune function tests, but if segregated individually, they may not lead to any concrete evidence. Many of these chemicals appear to cause immunosuppression[6]. Further studies are required to understand the interaction of insecticides with the immune system and to narrow down the test parameter[7].

Complement proteins are increasingly being recognized as the key components of the immune response network[8–10]. This system consists of proteolytic zymogens and regulatory proteins. The zymogens get activated as a cascade, either through calcium and antibody-dependent classical pathway or through antibody-independent and Mg^{2+}-dependent alternative pathway. C3 is central to the system, which initiates the alternative pathway and is the merging point for the classical and alternative pathways. On activation, chemotactic anaphylatoxins C3a and C5a along with opsonins C3b and C4b are generated. These peptides may bring injury to self, if generated in excessive