Letters to the Editor

Mutations in *cis* can confound genotype-phenotype correlations in hypertrophic cardiomyopathy

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EDITOR—Familial hypertrophic cardiomyopathy (FHC) is an autosomal dominant disease with a wide range of clinical features; a "benign" condition in some families, it can cause a high incidence of sudden death in others. FHC is caused by mutations in at least nine genes encoding sarcomeric proteins.[1] The gene most commonly implicated in causing FHC is that encoding the β-MHC protein. Over 60 missense mutations have been described in the *β-MHC* gene.[1] The mutations identified cluster in exons 3-27 of this 40 exon gene; these encode the functionally important ATP, actin, essential and regulatory light chain binding sites. Based on analysis of clinical features in genotyped families (grouped by mutation), broad genotype-phenotype correlations have been proposed for individual mutations. Such analyses have shown that certain mutations of *β-MHC*, for example Arg403Gln and Arg453Cys, produce a "malignant" phenotype associated with a high incidence of sudden death.[2] Others, such as Val606Met[2] and Leu908Val,[3] tend to behave in a "benign" fashion. However, a minority of families harbouring previously reported "benign" mutations show a greater than expected incidence of adverse events.[3 4]

Classical genetic studies in model organisms have shown that "second hits" in a single gene can modify an abnormal phenotype.[5] Double mutations in *cis*, that is, in the same copy of a gene, have been postulated as a possible mechanism accounting for discrepancies in genotype-phenotype studies. For example, double mutations have been described in the enzyme cystathione beta-synthase causing particularly severe homocystinuria.[6] It is also possible that double mutations of disease genes may not be as rare as one might expect. A comprehensive mutation screen of 44 patients with cystic fibrosis found four had inherited one double mutant allele.[7] All evidence points to mutated β-MHC genes acting in a dominant negative fashion, that is, the "poison polypeptide" hypothesis.[8 9] This model easily accommodates double mutations as phenotypic modifiers in FHC, with the "second hit" either further compromising, or improving, the function of the mutant protein within the sarcomere.

Mutation detection in FHC is complicated by its molecular heterogeneity. Mutations can be found efficiently, however, using a combination of linkage analysis where possible and TMHA. We have recently begun a comprehensive mutation screen of *β-MHC* to investigate inconsistencies in genotype-phenotype correlation.

Methods and results

Family members were ascertained through our clinical practice and evaluated by physical examination, ECG, and echocardiography, allowing the diagnosis of familial hypertrophic cardiomyopathy (MIM 192600) to be made in those affected. All participants gave informed consent and local ethical committee approval was granted. Findings in members of family A at presentation are shown in table 1 and fig 1. II.9 declined participation, but is an obligate carrier based on his position in the pedigree.

There were three instances of sudden death. III.5 died aged 17 years and II.6 died aged 30 years. I.1 died suddenly aged 60 years with

Table 1 Clinical details of affected members of family A

	I.1	*II.2*	*II.3*	*II.6*	*II.7*	*III.3*	*III.5*
Age/age at death	Sudden death aged 60	29	45	Sudden death aged 30	37	8	Sudden death aged 17
Symptoms					Exertional dyspnoea, NYHA II, dizzy spells	Dizzy spells	
Signs		ESM from age 12	ESM		ESM		
ECG			QRS axis +180°, ST/T changes SLI 33		QRS axis −60° SLI 33	SLI 36.5	
Max LV wall thickness (mm)	Heart weight 540 g	15.4	17.2		25.4	10.5*	
SAM/gradient			Incomplete SAM		Incomplete SAM		

*>99th centile for age/body weight/body surface area.
SLI = Sokolov-Lyon Index; ESM = ejection systolic murmur; SAM = systolic anterior movement of mitral valve.

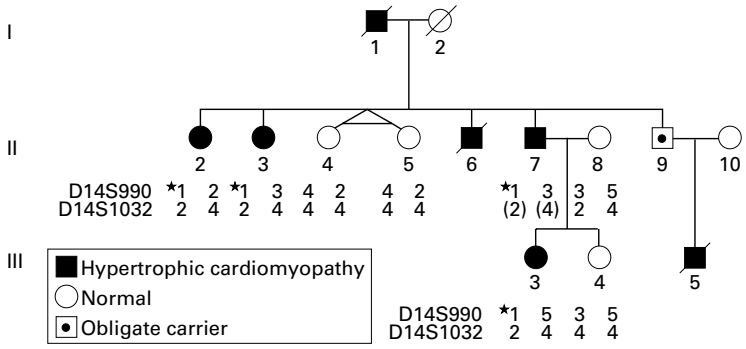

Figure 1 Pedigree of family A. D14S990 and D14S1032 are microsatellite markers. The highlighted haplotype (*) segregates with the disease. Because affected subjects share only one haplotype it can be deduced that both mutations in β-MHC lie on the same parental chromosome. All subjects available for genetic analysis and shown as being affected by hypertrophic cardiomyopathy were positive on analysis for both mutations of β-myosin heavy chain. Conversely all unaffected subjects were negative for both mutations.

clear necropsy evidence of FHC (heart weight 540 g) but also pathological features of coronary artery disease.

DNA was extracted from peripheral lymphocytes and linkage analysis was performed using flanking microsatellite markers.[10] Analyses in family A were consistent with a *β-MHC* causative mutation (fig 1). Intronic primers were designed flanking each exon from 3-27 of *β-MHC*. DNA from two affected subjects (II.2, II.7) was amplified using high fidelity polymerases and "touchdown" PCR.[11] Mobile phase gradients and melting temperatures for TMHA of each amplimer were calculated using the Wavemaker™ software package. Analysis of the PCR products using a DHPLC apparatus (Transgenomic Wave™) showed heteroduplex formation, indicative of heterozygous variants, in exon 3 in one subject and in exons 16 and 20 in both.[12] The exon 3 variant is a common polymorphism that we have seen in approximately 20% of normal chromosomes. Sequence analysis of this variant (T275C) did not predict an amino acid change. Haplotype analysis indicates that the two other variants are present on the same disease associated parental chromosome (fig 1). The presence of both variants in all available affected subjects was confirmed by DHPLC.

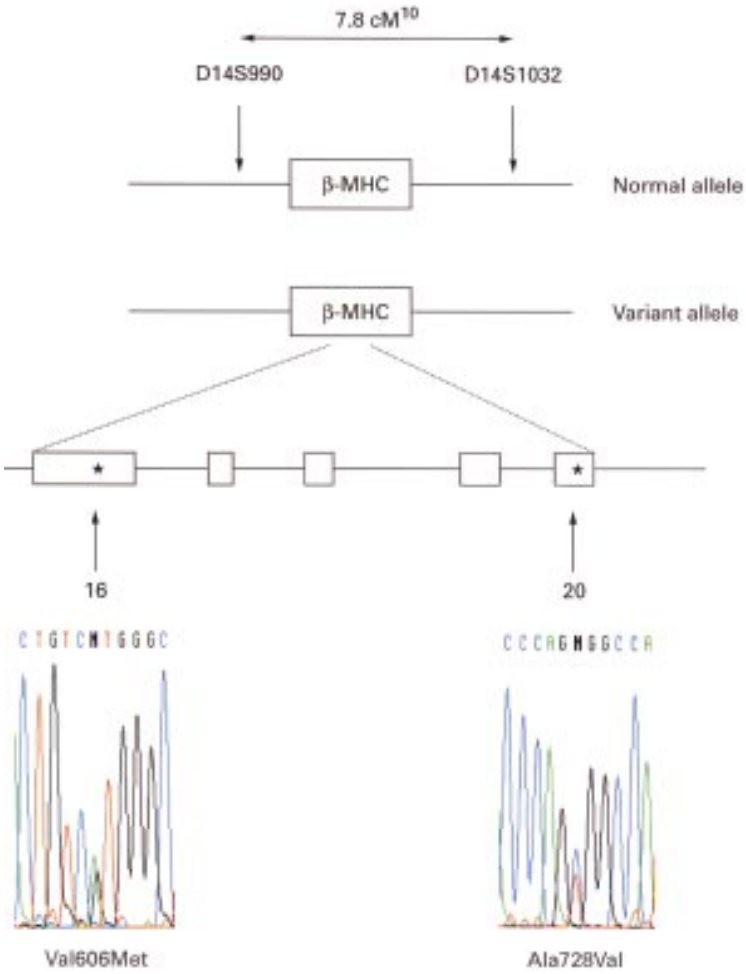

Figure 2 Diagram showing the flanking microsatellite markers, exons 16 to 20 of the disease allele of β-MHC in family A, and DNA sequence showing the mutations (see text for details).

Sequencing of these PCR products on an ABI377 showed a G>A transition resulting in the previously described Val606Met mutation in exon 16 and a C>T transition resulting in an Ala728Val mutation in exon 20 (fig 2). The Ala728Val introduces an *Msc*I site. Restriction enzyme analysis of 200 control chromosomes excluded the possibility that the mutation was a common polymorphism (data not shown). The alanine residue at this position has been conserved in diverse myosin isoforms from *Drosophila* to man and lies close to the essential light chain interface. Therefore, we predict that both the Val606Met and Ala728Val mutations are pathogenic.

Discussion

Previous studies in four families suggest that Val606Met is a benign mutation.[2 13] This clinical impression was supported by recent in vitro analyses.[14] The disease in family A, however, has produced two instances of early sudden death (fig 1, II.6 and III.5) and contributed to the death of a further person (I.1) in eight known affected subjects. In addition, two affected subjects (II.2, III.3) presented with symptoms or signs in childhood. We propose that this adverse natural history reflects the Ala728Val mutation acting in concert with the Val606Met mutation. Because the mutations are expected to affect both actin and myosin light chain interactions, they are likely to have a cumulative detrimental effect on myosin function. If, in screening this gene, we had discontinued our analysis on discovery of the Val606Met substitution, used low sensitivity techniques, or typed only for known mutations,[3] the Ala728Val mutation would have remained undetected and the severe phenotype would have been wrongly attributed to the Val606Met mutation. Until the frequency of double mutations in FHC is known, great care is needed in establishing genotype-phenotype correlations and in genetic analysis based on typing known mutations.

Examples of two mutations within an FHC family have been described, but these have not been found through further analysis of the same allele. The second mutation in these examples has been either non-pathogenic,[8] situated in a different FHC disease gene, that

is, leading to double heterozygosity,[15] or identical by descent in consanguineous families.[16] Although such double heterozygous and autozygous subjects are of interest, the two mutations are unlikely to cosegregate in many members of a family. Therefore, these phenomena may contribute to discordant phenotypes in particular subjects with FHC, but will not have a systematic impact on genotype-phenotype correlation. A similar argument pertains to the much discussed "genetic background" effect as an explanation for families who have discordant phenotypes. A constellation of unlinked genes affecting the phenotype of a dominant disorder are unlikely to cosegregate with the disease causing allele for more than one or two meioses. Only if such unlinked disease modifiers are much more common in one population than another, and hence are continually reintroduced into certain family "gene pools", could they have a consistent effect on disease phenotype. In contrast, two (or more) tightly linked mutations will continue to be inherited together and so will confound genotype-phenotype correlations based on either mutation alone, even in very large families.

Analysis of FHC families who do not match the expected phenotype of their known mutation could provide further examples of "double mutations" having a deleterious, or even conceivably a beneficial effect. Until such studies are performed it is impossible to state how common disease modifiers in *cis* will be. However, it is notable that the contractile protein genes are large targets for mutation, with FHC causing mutations arising over extensive genomic regions; thus, double mutations may be relatively frequent. Our data do not indicate how this compound allele evolved. Empirically, it is perhaps most likely that the mutations arose independently; indeed, examples of the Val606Met mutation have arisen before as independent events,[17] and such relatively mild alleles would be more likely to persist longer in the population, increasing the likelihood of a "second hit" in that gene.

We conclude that genotype-phenotype correlations in FHC can be confounded by the presence of more than one mutation in a single copy of a disease gene. Genotype-phenotype correlations have become increasingly important in the study of disease. In addition to helping formulate a prognosis for the individual patient, they allow us to define prognostic groups which may be used, for example, to assess treatment. Analysis of genotyped patient groups allows us to understand better the functional impact of the mutated protein and gain an understanding of the pathogenic mechanisms of the disease. This study illustrates how such analyses could be misleading if a comprehensive analysis of the gene in question has not been undertaken.

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Identification of a large rearrangement of the *BRCA1* gene using colour bar code on combed DNA in an American breast/ovarian cancer family previously studied by direct sequencing

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EDITOR—Linkage data suggest that *BRCA1* and *BRCA2* gene alterations account for the majority of hereditary breast/ovarian cancer cases.[1] Though comprehensive screening of the *BRCA1* gene has been attempted on a number of occasions, only two thirds of expected mutations have been detected so far.[1–3] Most efforts have relied on standard PCR techniques, including direct sequencing, single strand conformation polymorphism (SSCP) analysis, heteroduplex analysis (HDA), denaturing gradient gel electrophoresis (DGGE), and protein truncation testing (PTT), with a focus on point and small mutations.[2] The relatively low rate of detection of *BRCA1* gene mutations may be because of the existence of large rearrangements, which are not detected by such approaches. Supporting this hypothesis, a number of large rearrangements, ranging from 0.5 to 23.8 kb and spanning the entire *BRCA1* gene, have recently been detected by Southern blotting, analysis of *BRCA1* lymphocyte transcripts, and long range PCR.[4–14] In most cases, the characterised rearrangements are the result of unequal recombination events between Alu sequences, which cover 41.5% of *BRCA1* introns.[15]

Here we report the identification, using colour bar coding on combed DNA, of a previously undescribed large rearrangement of the *BRCA1* gene in an American breast/ovarian cancer family with ancestors from France and Germany (fig 1).

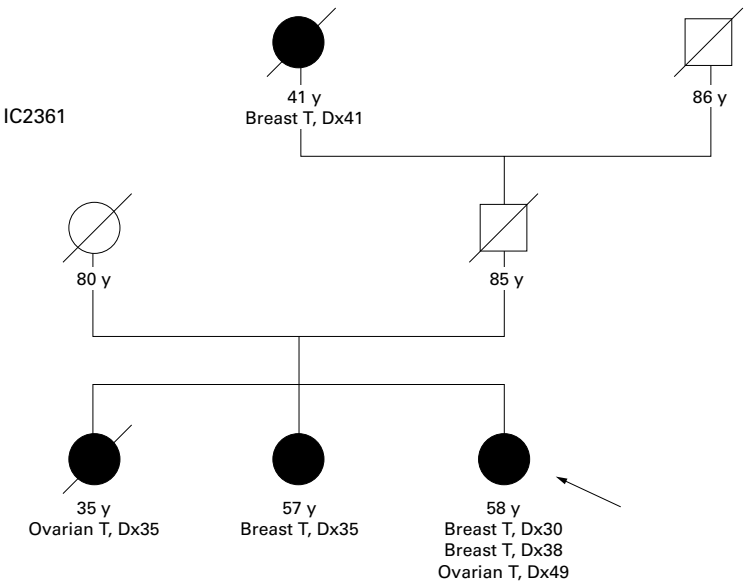

Figure 1 Pedigree of American family IC2361. Current ages or ages at death are indicated below each symbol. Tumour locations (T) and ages at diagnosis (Dx) are reported. The arrow indicates the patient studied.

Material and methods

The index case was diagnosed with breast cancer at the age of 30 and ovarian cancer at the age of 49. She had one sister with breast cancer diagnosed at the age of 35, another sister with ovarian cancer diagnosed at the age of 35, and a paternal grandmother with breast cancer diagnosed at the age of 41. The index case was referred to Cedars-Sinai Medical Center (Los Angeles, USA) for a genetic consultation. She elected to participate in *BRCA* gene testing, as she hoped to characterise her apparent genetic susceptibility so that her daughter could know her own status with greater certainty. No *BRCA1* or *BRCA2* gene mutation was identified by direct DNA sequencing (BRACAnalysis™, Myriad Genetic Laboratories Inc, Salt Lake City, USA). Because the a priori likelihood of carrying a *BRCA* gene mutation was high, the case was referred to our laboratory to search for large rearrangements in the *BRCA1* gene (family quoted IC2361).

The strategy for the detection of large rearrangements developed in our laboratory is based on a full four colour bar code of the *BRCA1* region on combed DNA.[16] Combing relies on homogeneous stretching of DNA molecules at a constant rate of 2 kb/μm.[17] Fluorescence in situ hybridisation (FISH) is then performed on combed DNA.[18] The probes used include a PAC covering the whole *BRCA1* region and long range (LR) PCR products (6.5 to 10 kb long) covering a number of exons, therefore bar coding the PAC. We have optimised the *BRCA1* bar code reported by Gad *et al*[16] by adding new LR products to allow for the detection of rearrangements as small as 2 kb. Finally, in addition to the PAC, a complex bar code of the *BRCA1* region was designed with seven probes (fig 2). This approach allows for a panoramic view of the *BRCA1* gene and its flanking regions.

Germline DNA was extracted from a lymphoblastoid cell line, with a step in agarose blocks in order to preserve its integrity. After combing of the DNA on silanised surfaces[17 18] and FISH with the set of probes, microscope screening was then performed.

Results

With a few fields of microscopic view, a number of full signals without LR13-15 probes were