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Mitochondrial DNA in idiopathic cardiomyopathy

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Aims To investigate the frequency of pathogenic mitochondrial DNA mutations in idiopathic cardiomyopathy.

Methods and Results We investigated the occurrence of seven previously reported pathogenic mitochondrial DNA point mutations in 52 patients with idiopathic dilated cardiomyopathy (blood n=33, myocardium n=19), 10 patients with hypertrophic cardiomyopathy (blood n=7, myocardium n=3), 67 controls with ischaemic heart disease (blood n=53, myocardium n=14) and eight controls with no overt cardiac disease (blood n=4, myocardium n=4). Total DNA or cell lysates were studied by polymerase chain reaction amplification and restriction fragment length polymorphism analysis for the identification of the following mitochondrial DNA point mutations: A3243G, A3252G, A3260G, A4269G, A8344G, T8993G/C and T9997C. None of these point mutations were detected in the blood or myocardium of any of the individuals with dilated or hypertrophic cardiomyopathy or in the controls. In addition we investigated the occurrence of major deletions of mitochondrial DNA in eight patients with dilated cardiomyopathy (myocardium n=7, skeletal muscle n=1), three patients with ischaemic heart disease (myocardium n=3) and one control myocardium by Southern blot analysis. Deletions were not detected in any of the patients.

Conclusion The results suggest that although these mutations are known to be associated with specific cardiomyopathies, they are not a common feature of idiopathic cardiomyopathy.
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Key Words: Idiopathic dilated cardiomyopathy, hypertrophic cardiomyopathy, ischaemic heart disease, mitochondrial DNA, mitochondria.

Introduction

The cardiomyopathies are a clinically and genetically heterogeneous group of cardiac diseases in which the myocardium is primarily involved. Three distinct categories of cardiomyopathy can be classified by functional and pathological criteria; hypertrophic cardiomyopathy, dilated cardiomyopathy and restrictive cardiomyopathy. It has been proposed that approximately 45–50% of familial hypertrophic cardiomyopathy cases are due to mutations in genes encoding contractile proteins of the sarcomere including; β-cardiac myosin heavy chain gene, cardiac troponin T gene and α-tropomyosin gene[1]. Familial hypertrophic cardiomyopathy is inherited as an autosomal dominant trait although mutations may arise de novo, giving rise to sporadic cases of the disease[2]. It has also been estimated that genetic mutations underlie the pathogenesis of 20–25% of cases of idiopathic dilated cardiomyopathy[3]. Mutations in genes encoding enzymes involved in β-oxidation of fatty acids, including acyl coenzyme A (CoA) dehydrogenase and carnitine palmitoyltransferase II, can cause recessive forms of dilated cardiomyopathy[4] and an X-linked dilated cardiomyopathy has been associated with deletions in dystrophin, the gene responsible for skeletal and cardiac myopathies in Duchenne and Becker muscular dystrophies[5]. In addition to genetic factors, environmental toxins, metabolic abnormalities, inflammatory or infectious diseases, autoimmune abnormalities and neuromuscular disorders are also known contributory factors to the disease[6].

Although the causes of a number of cardiomyopathies are now recognised, many cases of primary dilated cardiomyopathy or hypertrophic cardiomyopathy remain unexplained. It is now known that cardiomyopathy can occur as a result of point mutations in the highly conserved transfer RNA genes of mitochondrial DNA. The mitochondrial genome, exclusively inherited through the maternal line, is a 16·5 kilobase (kb), circular, double stranded molecule that encodes two

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Table 1 Clinical characteristics of patients

Disease	Blood				Myocardium				Quadriceps			
	Patient no.	Age (y)	Sex		Patient no.	Age (y)	Sex		Patient no.	Age (y)	Sex	
			M	F			M	F			M	F
IDCM												
sporadic	1–27	59 ± 11	20	7	34–52	47 ± 12	15	4	27	58		1
familial	28–33	36 ± 14	3	3								
HCM	1–7	66 ± 8	2	5	8–11	49 ± 8	1	2				
IHD	1–53	64 ± 9	45	8	54–67	53 ± 6	13	1				
Controls	1–4	64 ± 8	1	3	5–8	56 ± 14	1	3				

IDCM = idiopathic dilated cardiomyopathy, HCM = hypertrophic cardiomyopathy, IHD = ischaemic heart disease. Age in years is expressed as mean ± standard deviation.

ribosomal RNAs, 22 transfer RNAs and 13 proteins involved in the mitochondrial respiratory chain and oxidative phosphorylation system. The oxidative phosphorylation system is fundamental to the synthesis of cellular ATP and consequently pathogenic mutations of mitochondrial DNA are believed to cause disease by impairing the function of the mitochondrial respiratory chain and oxidative phosphorylation system, thereby decreasing cellular ATP synthesis. Mitochondrial DNA point mutations have been identified in a broad spectrum of clinical phenotypes. The majority of these are associated with neurological disorders such as the mitochondrial myopathies and encephalomyopathies, although a number of mitochondrial DNA point mutations have been reported in patients with cardiomyopathy, either alone or as part of a multisystem disorder[7–15]. We have analysed seven of these mutations, including the most common mitochondrial DNA mutations and those previously linked to cardiomyopathy, to determine their prevalence in idiopathic cardiomyopathy. The positions of the point mutations within the mitochondrial genome and their main associated clinical phenotypes are: A3243G mitochondrial encephalomyopathy with lactic acidosis and stroke like episodes (MELAS); A3252G mitochondrial encephalomyopathy with diabetes mellitus; A3260G maternally inherited adult onset myopathy with cardiomyopathy; A4269G mitochondrial encephalomyopathy and progressive intractable cardiac failure; A8344G myoclonic epilepsy with ragged red fibres (MERRF); T8993G/C neurological muscle weakness, ataxia and retinitis pigmentosa (NARP); also Leigh's syndrome and hypertrophic cardiomyopathy and T9997C maternally inherited hypertrophic cardiomyopathy.

Methods

Patients

Patient details are given in Table 1. The diagnosis of patients was determined following cardiac catheterization. At the time of sample collection, all patients were receiving optimum therapy as judged on clinical grounds. Treatments involved combinations of diuretics as well as a wide spectrum of other drugs including angiotensin converting enzyme (ACE) inhibitors, digoxin, nitrates, inotropes, calcium antagonists, beta blockers and anticoagulants.

Blood samples were obtained from patients presenting at the Royal Brompton Hospital with idiopathic dilated cardiomyopathy of varying severity; 43% were New York Heart Association (NYHA) I, 40% were NYHA II, 13% were NYHA III and 4% were NYHA IV. The mean ejection fraction in the idiopathic dilated cardiomyopathy group was 29 ± 12% (range 10–55%), as estimated from the angiogram, echocardiogram or radionuclide test. Six of the idiopathic dilated cardiomyopathy samples were from patients with a familial history; the pedigrees suggested autosomal dominant inheritance. In order to correct for any mutagenic effect of failing myocardial function and oxidative stress on mitochondrial DNA, we selected a group of patients with ischaemic heart disease and heart failure, of various degrees of severity; 12% were NYHA I, 46% were NYHA II, 36% were NYHA III and 6% were NYHA IV. The mean ejection fraction was 31 ± 15% (range 8–65%). Of the 93 patients from whom blood was obtained, 71% were being treated with diuretics and 62% were taking ACE inhibitors.

Cardiac samples from 19 idiopathic dilated cardiomyopathy, three hypertrophic cardiomyopathy and 14 ischaemic heart disease patients, obtained from the Royal Brompton Hospital and St George's Hospital, were taken at the time of heart transplant. These patients all had severe heart failure (NYHA III–IV). Of the 36 patients, all were being treated with combinations of diuretics, 81% were taking ACE inhibitors and 31% were taking digoxin.

Four control cardiac samples were obtained post mortem from subjects with no pre-existing cardiac disease, within 72 h of death. Skeletal muscle was obtained from one patient with idiopathic dilated cardiomyopathy undergoing biopsy at the Royal Free Hospital.

Blood

Lysed white blood cells, from 5–10 ml of whole blood, were digested with proteinase K and DNA was extracted with phenol/chloroform according to standard methods[16], or 200 μl of whole blood was lysed in 1 ml double distilled H_2O and centrifuged at 2200 ***g*** for 5 min. Lysis was repeated twice. The pellet was resuspended in 600 µl of TE buffer (Tris-HCl 10mM, EDTA 1 mM, pH 8·0) and centrifuged at 15000 ***g*** for 5 min. This step was repeated twice. The resulting pellet was resuspended in 200 µl lysis buffer (KCl 50 mM, Tris-HCl 20 mM, $MgCl_2$ 2·5 mM, Tween 20 (0·45% v/v), Nonidet P40 (0·45% v/v) pH 8·3), and incubated with 0·8 mg proteinase K (Sigma) at 55 °C for 20 min. One hundred microlitres of sterile double distilled H_2O was added and the reaction terminated by heating at 90 °C for 10 min.

Cardiac tissue

Cardiac tissue samples were homogenised in 2 ml extraction buffer (75 mM NaCl, 50 mM EDTA(K^+) using a glass hand-held homogeniser. One hundred microlitres of 0·1% SDS and 4 mg proteinase K were added. The homogenates were incubated at 56 °C for 3 h, a further 4 mg of proteinase K was added and the digestion was continued overnight at 37 °C. Samples were then subjected to standard phenol/chloroform extraction[16].

Polymerase chain reaction

One hundred nanograms of total DNA or 10 µl cell lysis product was used for polymerase chain reaction amplification. Polymerase chain reaction was carried out in a final volume of 50 µl containing 10 mM Tris-HCl (pH 9·0 at 25 °C), 50 mM KCl, 0·1% Triton X-100, 1·5 mM $MgCl_2$, 0·2 mM of each dNTP (Pharmacia) and 1 µM of each primer (R&D Systems), and overlaid with 80 µl of liquid paraffin. Polymerase chain reaction was carried out in a Hybaid Omnigene thermal cycler. Samples were initially incubated at 96 °C for 4 min prior to the addition of 1·25 units of *Taq* DNA Polymerase (Promega U.K.) at 72 °C. The nucleotide sequence of primers and amplification conditions were as described previously; A3260G[8], A4269G[9], T9997C[11], A3252G[17], T8993G/C[18], and A8344G[19]. To detect the A3243G mutation, primers L(forward)2928-2947, H(reverse)3558-3539 were used. Denaturation, primer annealing and extension times were 1 min at 94 °C, 55 °C and 72 °C respectively, which were repeated for 30 cycles. All amplifications were followed by a final extension at 72 °C for 10 min.

Restriction fragment length polymorphism analysis of the polymerase chain reaction products was used to identify specific point mutations. Ten microlitres of polymerase chain reaction product was incubated with 2–5 units of the appropriate restriction enzyme as described in the references except that *Apa*I, was used in the detection of the A3243G mutation; *Mae*I, an isochizmer of *Bfa*I, was used in the detection of the T9997C mutation; and *BstN*I, was used in the detection of the A8993G/C mutation. *BstN*I will restrict both the T8993G and T8993C mutation. Restriction enzyme conditions were as outlined by the manufacturers (Promega UK, Stratagene, Boehringer Mannheim). Digested products were separated by agarose gel electrophoresis (1·2% agarose for the A3243G mutation (Sigma), 2% agarose for the T8993G/C mutation (Sigma) and 4% metaphor agarose for the A3252G, A3260G, A4269G, A8344G, T9997C mutations (Flowgen)) and visualized under UV light.

Positive controls

Positive controls were not available for mutations at A3260G, A4269G, A3252G and T9997C. In order to ensure confidence in the results obtained, controls were established for each of these assays by amplifying a separate region of the mitochondrial DNA which contained the same restriction endonuclease site as the mutation under investigation. Primer pairs used in the generation of control polymerase chain reaction products and their restriction fragment lengths obtained were as follows: A3260G, L8171-8190 and H8386-8345, restriction with *Xmn*I yielded fragments of 163bp and 53bp; A4269G, L8171-8190 and H8726-8707, restriction with *Ssp*I yielded 278bp and 278bp; A3252G, L6782-6801 and H8386-8345, restriction with *Acc*I yielded 630bp, 548bp, 230bp, 180bp and 18bp; T9997C, L4116-4135 and H4299-4270, restriction with *Mae*I yielded 101bp and 83bp. Controls were examined under the same amplification conditions as the samples.

Southern blot analysis

For Southern blot analysis, 3 µg of total DNA was digested with 10 units of *Pvu*II (Promega, U.K.) for 3 h at 37 °C. Digested DNA was separated on a 0·8% agarose gel and transferred onto a nylon membrane (Hybond-N, Amersham, U.K.). The blot was hybridized with mitochondrial DNA probe extracted from human placenta[20]. Probe labelling, hybridization and development conditions were as specified in the enhanced chemiluminescence (ECL) protocol (Amersham International).

Results

We were unable to detect any of the seven previously described pathological mitochondrial DNA point mutations in 52 patients with idiopathic dilated cardiomyopathy (blood samples n=33, myocardium n=19), 10 patients with hypertrophic cardiomyopathy (blood

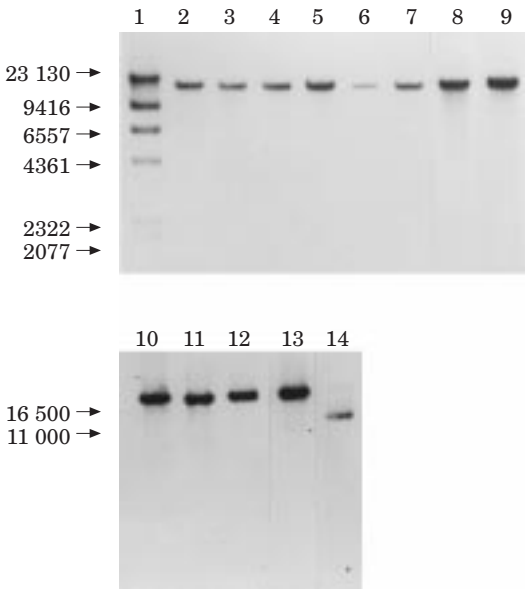

Figure 1 **Southern blot analysis of mitochondrial DNA. Lambda Hind III marker (lane 1). DNA extracted from myocardium of sporadic idiopathic dilated cardiomyopathy patients (lanes 2, 6, 7, 10, 11, 12, 13) and skeletal muscle of idiopathic dilated cardiomyopathy patient number 27 (lane 9). DNA extracted from myocardium of ischaemic heart disease patients (lanes 3, 4, 5). DNA extracted from control myocardium (lane 8) and, as a positive control, DNA extracted from a patient with mitochondrial DNA deletion (lane 14).**

samples n=7, myocardium n=3), 67 patients with ischaemic heart disease (blood samples n=53, myocardium n=14) and eight controls (blood samples n=4, myocardium n=4). Southern blot analysis did not show any major deletions or rearrangements of mitochondrial DNA in eight patients with idiopathic dilated cardiomyopathy (myocardium n=7, quadriceps n=1) or three patients with ischaemic heart disease (myocardium n=3) (Fig. 1).

Discussion

The clinical phenotypes associated with the mitochondrial DNA point mutations are varied. Mutation at T9997C has been reported in hypertrophic cardiomyopathy with the possible association of Bright disease[11], while the mutations associated with cardiomyopathy at positions A3260G, A4269G, T8993G/C are related to neurological disorders including mitochondrial myopathy[8] encephalomyopathy[9] and Leigh's syndrome[12], respectively. Mutation at A3243G is commonly associated with MELAS, although recently it has been reported in cases of maternally inherited hypertrophic cardiomyopathy[21,22] and dilated cardiomyopathy[23], indicating that cardiomyopathy can be the presenting and major clinical feature of this phenotypically heterogeneous mutation. The A8344G mutation has not been reported as a direct cause of cardiomyopathy although patients with this mutation often develop cardiomyopathy as part of a multisystem disorder. This distinguishes the A8344G mutation from the actual cardiomyopathic point mutations described above. The mutation at A3252G has mainly been associated with mitochondrial encephalomyopathy although two members of the family described had cardiac involvement at death[17].

In this study we were unable to detect any of these point mutations in any of our patients and we suggest that that these point mutations are not a common feature of idiopathic cardiomyopathy and are mainly associated with cardiomyopathy when it is part of a multisystem disease. However, It is possible that mitochondrial DNA point mutations other than those analysed may be present in these patients, or that nuclear genes encoding subunits of the mitochondrial respiratory chain may be involved in the aetiology of these diseases.

Although Southern blot analysis has shown no deletions of mitochondrial DNA, this does not discount the possibility that low level deletions are present in these patients, but have not been identified due to limitations in the sensitivity of the analysis. Deletion mutations have been seen in association with cardiomyopathy. These deletions vary in position and size, with a deletion of 7436bp (between nt8637 and nt16073) the most commonly seen, although they have only been observed following polymerase chain reaction analysis and their relevance is not yet known[24,25]. However, Marin-Garcia *et al*.[26] reported that it is more likely the 7436bp mutation occurs as an effect of heart dysfunction rather than as a primary cause of cardiomyopathy. Whether deletions are a primary cause of cardiomyopathy or whether they arise as a result of oxidative stress in cardiomyopathic hearts, similar to the increase of the 4977bp common deletion in ageing tissues[27], is not yet fully understood.

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