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WHOLE MITOCHONDRIAL GENOME AMPLIFICATION REVEALS BASAL LEVEL MULTIPLE DELETIONS IN MTDNA OF PATIENTS WITH DILATED CARDIOMYOPATHY

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Summary: Basal level multiple deletions in the heart mitochondrial (mt) DNA of patients with dilated cardiomyopathy were analyzed with the recently developed long PCR technique. The amplification of the whole mitochondrial genome and the length dependent preferential amplification ensure that all existing deletions are detected simultaneously. Primer shift long PCR proved the specificity of our method in the detection of deletions. We used primers that were 15644 bp and 13765 bp apart, which comprised almost the entire mitochondrial genome, detected a total of 14 kinds of deletions in 18 out of the 40 endomyocardial biopsy samples from patients with dilated cardiomyopathy. These deletions ranged in size from 3.3 kb to 12.6 kb. The proportion of the deleted to wild-type mtDNA was calculated to be less than 9% for any of these deletions. Aged patients had a comparatively higher proportion. Deletions were rarely found in mtDNA of leukocytes from the same group of patients and of 38 additional patients, as well as in myocardia of 25 normal controls ($P < 0.01$). No deletion was found in leukocytic mtDNA of the 21 control subjects. It is therefore concluded that cardiomyopathic heart has a high frequency of mtDNA deletions. With regard to the low quantity and the cumulative nature of these deletions by aging, they could have only little pathogenic effect on the development of dilated cardiomyopathy, but rather be a sign of increasing stress of the heart promoting the damage of mtDNA.

The human mitochondrial genome is a circular 16569 base pair (bp) extra-nuclear DNA molecule that encodes 13 enzyme subunits required for oxidative metabolism, as well as 22 tRNAs and 2 rRNAs required for their translation. Each human cell has thousands of mitochondrial (mt) DNA molecules. Different tissues rely on mitochondrial energy to various extents. The heart, brain, skeletal muscle and endocrine glands have a high oxidative metabolism rate and are therefore especially dependent on mitochondrial energy. With increasing amount of mutant mtDNA, the mitochondrial energy output could drop below a threshold for the normal function of cells and tissues, and clinical manifestations may appear.

Various deletions in mtDNA have been reported as being related to different diseases, including Leber's hereditary optic neuropathy, myoclonic epilepsy and ragged red fibers, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes, progressive external ophthalmoplegia, ma-

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Abbreviations: DCM, dilated cardiomyopathy; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

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ternally inherited myopathy and cardiomyopathy, or cardiomyopathies (1-3). More than 70 different deletions in human mtDNA ranging in size from 9 bp to 10987 bp have been identified in various diseases so far. The pathogenic effect of the mutant mtDNA on different diseases is hard to be evaluated because of the heteroplasmic nature of mutations. It is believed that mtDNA heteroplasmy is important for the different spectrum of symptoms in mitochondrial genetic diseases depending on the frequency of the mutant mtDNA. Diagnostic procedures which only detect the mutation but fail to determine levels of heteroplasmy may be inadequate.

Although mtDNA deletions have been reported in patients with DCM (4), they are far from complete in covering all basal level deletions. Previously, the detection of deletions was dependent on different PCR methods and/or RFLP analyses (4-6). Both usual PCR and RFLP are either not feasible or too complicated for the simultaneous screening of different sized multiple deletions in one individual. We therefore developed a long PCR method to detect multiple deletions simultaneously in patients with DCM (7), with primers that can amplify almost the entire mitochondrial genome screening all possible large deletions in different patients and can detect the levels of heteroplasmy at the same time. Basal levels of mtDNA deletions in endomyocardial biopsy samples of patients with DCM were found. Heteroplasmic levels could also be calculated at the same time. The results provide a further insight to the understanding of the molecular pathogenesis of DCM.

MATERIALS AND METHODS

Sample preparation

Leukocytes were separated through gradient density centrifugation from peripheral blood collected from 21 normal controls and 68 patients with DCM. Left ventricular endomyocardial biopsy samples from 30 out of the 68 patients, and right ventricular endomyocardial biopsy samples from another 10 patients with DCM were collected. Normal control cardiac samples were from 25 donor hearts. Post-mortem samples of right atrium, left atrium, right ventricle, left ventricle, kidney and liver were collected from 4 patients with diseases other than DCM within 12 hours after their death. All tissue samples were snap frozen in liquid nitrogen and kept under -70°C before use.

Total DNA

Isolation of total DNA from peripheral leukocytes and cardiac biopsy samples was carried out as described previously (8, 9). Leukocytes were incubated with extraction buffer (10mM Tris-Cl, 100mM EDTA, 0.5% SDS and 20μg/ml RNAse, pH 8.0), and digested with Proteinase K. Total DNA was extracted with phenol once, precipitated with ethanol and recovered in $TE_{0.1}$ (10mM Tris-Cl, 0.1mM EDTA, pH8.0). For biopsy and autopsy samples, homogenization in extraction buffer with a glass homogenizer was carried out before digestion with Proteinase K. Further steps were the same as described above.

Primer design and analysis

Primers (Table 1) were selected from the reported mtDNA sequence (10). All primers have high annealing temperature and very high specificity. These primers enable us to amplify both the normal sized and deleted mtDNAs. Primers L3, L4, H1 and H2 were used for primer shift PCR amplification.

PCR amplifications

PCR amplification was carried out in a Hybaid Omnigene Temperature cycling system with GeneAmp XL PCR kit (Perkin Elmer). In 50 μl reaction volume, the amount of template DNA in each reaction tube was 15-30 ng. The concentration of each of the four dNTPs was at 0.2 mM, and that of both primers was 0.25 μM. In addition to a hot start with the temperature hold at 78°C for 5 min, cycling conditions were as follows: 94°C denaturation for 50 s for one cycle; then primer annealing and extension at 68°C for 8 min 15 s to 11 min 15 s, denaturation at 94°C for 15 s for 32 cycles; primer annealing and extension in the second 16 cycles was automatically increased for 20 s per cycle. Finally primer annealing at 62°C for 60 s and extension at 72°C for 15 min for one cycle.

Table 1. Primers used in long PCR experiment

Names	Locations in mtDNA	Sequences 5'3'
L1	2695-2720	GAGGCGGGCATAACACAGCAAGACGA
L2	2697-2720	GGCGGGCATAACACAGCAAGACGA
L3	5606-5326	CTCTGCATCAACTGAACGCAA
L4	5817-5835	CCTCGGAGCTGGTAAAAAG
H1	16347-16328	GGGGACGAGAAGGGATTTGA
H2	16445-16426	TTATGGGCCCGGAGCGAGGA
H3	16459-16436	GGCCCGGAGCGAGGAGAGTAGCAC
H4	1772-1749	GGCGGGCATAACACAGCAAGACGA

Primer shift PCR

For the validation of the long PCR deletion screening method, primer shift PCR was carried out with primer pairs L3-H2 and L4-H1. Due to the difference in the melting temperature of the primers, the cycling conditions are slightly different from that mentioned above. Three step temperature cycling was used in addition to the hot start: annealing at 59°C for 60 s, extension at 72°C for 6 min 15 s, and denaturation at 94°C for 15 s for a total of 32 cycles. Primer extension time in the second 16 cycles was increased for 20 s per cycle.

Analysis of the PCR products

Samples of 6-10 µl from each PCR product were analyzed in horizontal gels of 0.8% agarose with 0.5 µg/ml ethidium bromide, visualized and photographed with an uv-transilluminator. Molecular size marker was the mixture of *Lambda* DNA/Hind III digested fragments and *Phi* X 174 DNA/Hae III digested fragments.

Quantitative analysis of the deleted mtDNA

PCR products with certain mtDNA deletions were run in 4% polyacrylamide gels, then silver stained and laser scanned with an Ultrascan XL (LKB) laser densitometer. The proportion of the deleted mtDNA was integrated automatically. Percentage of the deleted mtDNA was 100 x (area of the deleted bands) / (area of the wild-type band + area of the deleted bands).

Statistical analysis

Frequency of deleted mtDNAs in different groups of subjects was compared by chi-square analysis, and continuous variables were compared by Student's t-test.

RESULTS

Both wild-type and deleted mtDNAs were amplified in one reaction.

As shown in Figure 1 a-c, a 15644 bp or a 13765 bp fragment was amplified corresponding to the normal mtDNA. The smaller fragments represented amplified mtDNAs with a certain length of deletions. The size differences between the normal fragments and the smaller fragments should be the size of deletions. A total of 14 different deletions were identified. They ranged in size from 3.3 kb to 12.6 kb (Figure 1b and 1c). As many as 6 different deletions were found in one individual.

Proportion of deleted to wild-type mtDNA.

The proportion of the deleted mtDNA to wild-type mtDNA was calculated with the Laser scanning of the results of electrophoresis, which showed that the majority was wild-type mtDNA in all cases. The highest proportion of deleted mtDNA was 9% of the total.

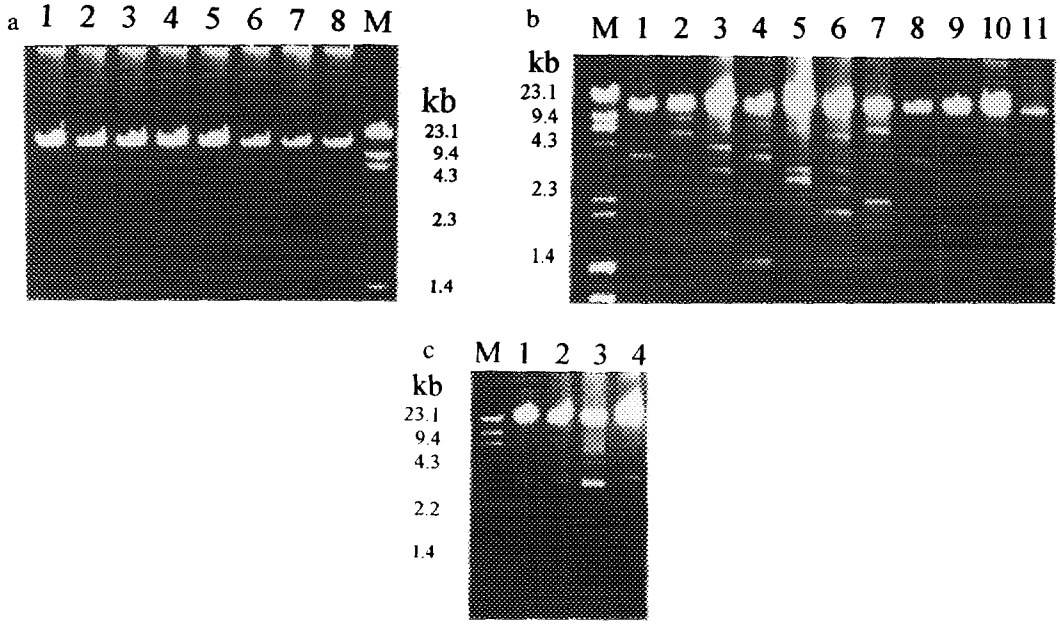

Figure 1. Major deleted mtDNAs found in patients with DCM.
a: Showed wild-type mtDNA amplified from patients' leukocytic mtDNA and normal control myocardial mtDNA.
b: The 13.7 kb band and c: the 15.6 kb band in each lane are the wild-type mtDNA. All smaller bands represent mtDNAs with certain length of deletions.

Certain mtDNA deletions exist in both the cardiomyocytes and the leukocytes of patients with DCM.

Most of the deletions were found in cardiac sample of the DCM patients (Table 2). Significant difference was found in deletion of the blood mtDNA as compared with that of the cardiac mtDNA in the same group of patients ($P < 0.01$). Deletions were rarely found in mtDNAs from leukocytes of an additional 37 patients and none in 21 of the normal controls. All the 14 kind of deletions were observed in endomyocardial biopsy samples from the left ventricle, but only 6 kind of that were observed in samples from the right ventricle of the cardiomyopathic patients. MtDNA deletions were only found in post-mortem samples of ventricles of two patients and liver of one patient. The age difference in different groups of subjects weakens the significance of the pathogenic effect of these deletions in patients with DCM because of their older age.

Validation of the method and support of the existence of deletion *in vivo*.

Primer shift PCR amplification of the regions with mtDNA deletions helps to elucidate the specificity of the method. As shown in Figure 2, primer pair L1-H3 amplified four fragments with molecular sizes of 13.7 kb, 10.4 kb, 5.4 kb and 3.3 kb, respectively, whereas the primer pair L3-H2 only amplified three fragments. The size differences between the first 3 fragments were exactly

Table 2. Frequencies of deletions of mtDNA from different samples

	DCM			Control		Post-mortem		
Deletion size (Kb)	Blood (n=68)	RV (n=10)	LV (n=30)	Blood (n=21)	LV (n=25)	RV/LV (n=4)	Kidney (n=2)	Liver (n=2)
3.3			1					1
5.0	3	2	1			2		
7.4			3		2			1
8.3		1	4			2		
9.6			1		2			
9.8			1					
10.0		2	3					
10.7			2					
11.0			1					
11.4		2	1					
11.6		1	3					
11.9			4			1		
12.1			3					
12.6		1	2					
Total:	3	9	30		4	5		2
P value:		<0.01	<0.01 (with blood) >0.05 (with RV)		<0.01(with DCM LV)			
Mean age:	51.71±11.35	43.809.01	52.4210.57	31.903.35	37.5614.26			
P value:		<0.05	>0.05 (with blood) <0.01 (with RV)	<0.01 (with DCM blood)	<0.01 (with DCM LV)			

RV: Right ventricle.
LV: Left ventricle.

the differences of the distances between the two pairs of primers, i.e., 2.9 kb. Further shift of primers to L4-H1 lead to a further shift of the product sizes. Namely, the position shift in primers lead to the size shift in amplified fragments. The 3.3 kb fragment in lane 1 may reflect a deletion with beginning or ending point outside the range of L3-H2. Therefore, it could not be amplified with this pair of primers.

Length-dependent preferential amplification allows smaller fragments to be amplified in the first place other than the wild-type mtDNA, thus ensuring all deleted mtDNAs to be amplified. The editing correction ability of the Vent DNA polymerase ensures the amplification of both the normal sized wild-type mtDNA and the deleted mtDNAs simultaneously. With regard to the predominance of wild-type mtDNA over deleted mtDNA as template in the PCR reactions, normal mtDNA is still preferentially amplified.

DISCUSSION

In previous studies (3-5), PCR showed deletions in myocardial mtDNA of patients with DCM. It was envisaged that such deletions were responsible for the pathogenesis of DCM, or at least a part of DCM, the so called mitochondrial cardiomyopathy (11). It was reported recently (12) that normal protein synthesis and mitochondrial respiration had been observed when wild-type mtDNA exceeds 10% of the total mtDNA.

In our studies, the wild-type mtDNA was accounting for at least 91% of the total mtDNA in all the subjects. That is to say that deleted mtDNA rarely reached 9% of the total mtDNA. The low pro-

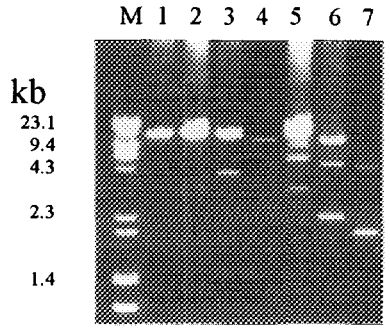

Figure 2. Primer shift PCR.
Lane M, Molecular size marker; PCR products of L1-H3 from different samples were shown in: Lane 1, normal leukocytic mtDNA; Lane 2, normal cardiac mtDNA; Lanes 3 and 5, DCM endomyocardial biopsy mtDNA. Lane 4 and Lane 6 showed the products after primer shift to L3-H2. Lane 7 showed the products of L4-H1.

portion of mtDNA deletions in patients with DCM raises the question of the pathogenic significance of these deletions. Although the number of mtDNA mutations are more frequent in patients with DCM, the existence of mtDNA deletions in patients might be a result of heart disease having affected the mtDNA. This hypothesis is further supported by findings of mtDNA deletions in myocardia of patients with various other diseases (13, 14). However, a high ratio of deleted mtDNA (235 types of deletions) had been observed in a patient with DCM (15). The amount of deleted mtDNA was up to 84% of the total mtDNA. An accidentally died 28 years old control subject had 67 types of mtDNA deletions. They amounted to 23% of the total mtDNA. This high ratio of deletions might account for the pathogenesis of cardiomyopathy in their described patient.

On the other hand, mtDNA mutations may increase with age (9, 16), either pathologically or naturally. Once the proportion of mutant mtDNA rises above a threshold, clinical manifestations appear. Usually the level of threshold is comparatively high. For example, for point mutations in patients with myoclonic epilepsy and ragged red fibers, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes, maternally inherited myopathy and cardiomyopathy, the level of mutant mtDNA is as high as 85% of the total mtDNA (16). The progressively increasing nature of mutant mtDNA in human disease suggests the need of monitoring mutations in mtDNA for suspected patients. Therefore, it is reasonable to monitor the progression of specific mitochondrial disease with the presently described method by means of monitoring the level of mtDNA damage. The whole mitochondrial genome amplification described here is feasible for large scale investigations and pre-clinical diagnosis. Carriers of clinically relevant large insertions and deletions should also be easily detected with this method.

In our study, variability of heteroplasmy was observed in the mtDNA deletion patterns in different tissues and even in different heart chambers. Deleted mtDNA genomes in blood and other tissues do not mirror the situation in heart. The tissue divergence of the mtDNA deletion patterns may reflect different levels of insult to mtDNA (e.g. free radicals) in different tissues, or reflect a different mechanism in the formation of deletions.

The mechanism of mtDNA deletions is still not well defined. Recombination via flanking direct repeats is believed to be the cause of this kind of deletion (2). Flanking direct repeats could lead to pseudo-recombination and generation of long stretches of single-stranded DNA, which may be a prerequisite to the occurrence of large deletions. Free radical damage to mtDNA was believed to be a initiator to the above process (17), and point mutations might be a basis for the occurrence of deletions (unpublished observations). Because the nucleus regulates the replication and transcription of mtDNA by providing the proteins needed for biosynthesis, it is reasonable that nucleus gene mutations might predispose the mtDNA to deletions. An autosomal locus at 10q 23.3 -24.3 was reported to be involved in the formation of mtDNA deletions (18). Nevertheless, further evidence is needed to show the exact mechanism of mtDNA deletions, and whether these deletions are disease causative or just a phenomenon of a discrete disease.

Mitochondrial DNA has a high frequency of deletions, and more than 95% deletions occur within the so called *Major Deletion Limit*, that spans two thirds length of the mitochondrial genome. The *Major Deletion Limit* is between the origins of replication sites of the light and the heavy strands (1). Deletions rarely occur across the origins of replications, or such deletions are not able to replicate and are then lost during turn-over of the mtDNA. Most of the mtDNA deletions are spontaneous (19, 20), and occur usually between two direct repeats which are potential hotspots for such mutations (2). There are one 15 bp direct repeat, four 13 bp direct repeats, sixteen 12 bp direct repeats, and numerous shorter direct repeats in the mitochondrial genome (21). It is therefore common that several deletions co-exist within one individual. Our results show that multiple deletions exist in myocardial mtDNA of patients with DCM. Since almost all possible deletions are amplified in one PCR reaction, the presently described method might be the easiest and the most suitable way for the screening of deletions in the mitochondrial genome.

The main advantage of deletion analysis by long PCR technique is its ability to amplify DNA with or without deletions within a segment of more than 30 kb length with only one pair of primers. Therefore, this method could detect any deletion existing in the 16.5 kb long whole mitochondrial genome.

In conclusion, the present study applied the newly developed long PCR technique for the first time for the screening of deletions of human genes, and confirmed the existence of mtDNA deletions in patients with DCM. The deletion pattern was different in different tissues. These deletions also occur in mtDNA from tissues other than myocardium. These observations support the hypothesis that mtDNA damage accumulate in myocardium of patients with DCM, and indicates that the mitochondrial respiratory stress increase the damage of mtDNA. The mechanism of this damage deserves to be further investigated.

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