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Detection of the 4977 Base Pair Mitochondrial DNA Deletion in Paraffin-Embedded Heart Tissue Using the Polymerase Chain Reaction—A New Method to Probe Sudden Cardiac Death Molecular Mechanisms?

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ABSTRACT: Detection of mitochondrial DNA deletions is performed in fresh or frozen material. At our institute, however, heart samples from subjects referred for autopsy are systematically processed for histologic examination (that is, paraffin-embedded). We were interested to know if mtDNA deletions can be detected in such material. Our data indicate that: 1) the most frequently observed deletion—the 4977 base pair deletion—can easily be detected in paraffin-embedded heart tissue; 2) this assay is sufficiently sensitive, since very low levels of the deletion can be found in normal heart tissue from young adults; and 3) buffered formalin appears to be the fixative of choice.

Recent literature shows that repeated episodes of ischemia result in the accumulation of mtDNA deletions in myocardial cells. Because ischemic heart disease is a major cause of sudden cardiac death, a sensitive method for the detection of mtDNA damage in myocardial cells will be an important tool to facilitate understanding of unexpected cardiac arrest mechanisms.

KEYWORDS: pathology and biology, sudden cardiac death, mitochondrial DNA, polymerase chain reaction

Somatic mitochondrial DNA (mtDNA) mutations were initially observed in heart and brain tissues from aged individuals [*1,2*]. An age-related increase in the most frequently observed mtDNA deletion—the 4977 base pair (bp) deletion—has been demonstrated in human hearts [*3*]. It has been hypothesized that a major cause of aging is the decline of mitochondrial oxidative phosphorylation due to the accumulation of somatic mutations in the mtDNA [*4*].

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Recently, high levels of mtDNA deletions have been observed in heart samples from patients with severe coronary atherosclerosis or cardiomyopathies, conditions associated with sudden cardiac death [*3,5,6*]. Thus, the detection of mtDNA mutations in pathologic material might be of value to gain some insight into mechanisms underlying unexpected cardiac arrest, of which very little is known at the molecular level.

Somatic mtDNA deletions were detected using PCR based assays with primer pairs designed to flank each specific deletion [*1,2*]. Using one of these primer pairs, PCR could generate either a large PCR product from the normal mtDNA, or a much shorter product from the deleted mtDNA. Since normal mtDNA was present in normal cells at much higher levels than deleted mtDNA, the latter could not be detected using standard PCR running parameters. It was necessary to run PCR with very short PCR cycle time to allow preferential amplification of the much shorter and rarer deleted mtDNA. Indeed, it has been estimated that about 1 among 1000 mtDNA copies was deleted in one normal human heart muscle cell [*1*].

All the previous assays for the presence of deleted mtDNA were done in fresh or frozen material. We were interested to know if mtDNA deletions could be detected in paraffin-embedded specimens, which are easily obtainable at our institute. DNA sequences including virus insertions and mutated oncogenes have been detected in paraffin-embedded material, although DNA extracted from this material was much degraded and reduced in size [*7*]. A product up to 1300 bp in length was the largest DNA fragment to have have been successfully amplified from formalin-fixed material [*8*]. Because DNA from this material did not allow amplification of larger DNA fragments, we thought that it could allow an easy amplification of the short deleted mtDNA fragment, being unhampered by competition with normal mtDNA, which would be degraded by the fixative procedure. To test this hypothesis, we analyzed different PCR assays in paraffin-embedded heart tissue samples.

Material and Methods

Samples

Heart muscle samples from 25 human cadavers (16 males and 9 females) were obtained at random, regardless of disease and cause of death. The ages of patients were 3 weeks to 92 years. Postmortem delay between death and autopsy was 4 hours to 15 days.

Preparation of Samples

Samples measuring approximately 10 × 10 × 3 mm were fixed by immersion in various fixatives (Bouin's fixative, unbuffered formalin, neutral buffered formalin). Fixation time was 24 hours to 7 days. Samples were then dehydrated, infiltrated with paraffin, and sectioned at 4 μM.

For histologic examination, sections were mounted and stained with hematoxylin and eosin.

For PCR, one unstained 4 μM thick section was collected in a microfuge tube and suspended without prior deparaffinization in 100 to 200 μL of digestion buffer (200 μg/mL proteinase K in 50 mmol/L Tris (pH 7.6) and 1 mmol/L EDTA). Samples were incubated overnight at 37°C. The proteinase K was heat activated at 95°C for 10 minutes.

Primers

Gel purified primers were purchased from Genset. The primers 1 and 2 were covering the following regions: primer 1 or MT1 (8225-8247) and primer 2 or MT4 (13707-13729) as described [*1*]. The primers 3 and 4 were complementary to the following sites: primer 3 or L820 (8201-8220) and primer 4 or H1363 (13650-13631) as described [*2*].

PCR

One 5 μL aliquot of each prepared DNA sample was separately amplified with each of the two primer pairs. Reactions were performed in 100 μL of PCR mixture containing 200 μmol/L of each dNTP (dATP, dCTP, dGTP, dTTP), 2 units of *Taq* DNA polymerase (Beckman), 50 mmol/L KCl, 10 mmol/L Tris (pH 8.3), 4 mmol/L MgCl2, and 10 pmol of each primers. This solution was overlaid with 100 μL of paraffin oil, and 40 cycles of amplification were carried out using a LEP PREM DNA amplifier.

For primers 1 and 2 the standard cycling parameters were 1 min at 95°C, 1 min at 59°C, and 2 min at 72°C. The short cycling time parameters for primers 1 and 2 were 20 s at 95°C and a single annealing and extension time of 20 s at 60°C.

For primers 3 and 4 the standard cycling parameters were 1 min at 95°C, 1 min at 42°C, and 2 min at 72°C, and the short cycling time conditions were 15 s at 95°C, 15 s at 42°C, and 120 min at 72°C.

Analysis of Amplified DNA

Ten μL aliquots from each PCR reaction were run on a 2% agarose electrophoresis gel containing ethidium bromide. Gels were visualized and photographed under UV light.

Results

In paraffin-embedded samples of heart, we could only amplify the deleted mtDNA with primers 1 and 2 using standard PCR cycling parameters (Fig. 1). No DNA was generated using primers 1 and 2 with the short cycling time conditions. With primers 3 and 4 we were unable to amplify any DNA, using standard or short cycling time parameters.

To confirm that the 520 bp fragment obtained with primers 1 and 2 in standard cycling time conditions was the expected DNA fragment generated from the deleted mtDNA, we first mapped this DNA fragment with 3 restriction enzymes (Fig. 2). As expected, the agarose gel electrophoresis was consistent with the restriction map deduced from the

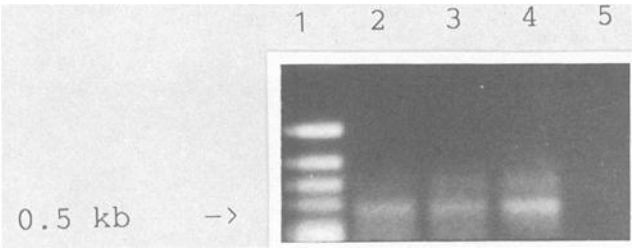

FIG. 1—*Amplification and analysis of three adult heart DNA samples. Amplification was carried out using primers 1 and 2 with 40 long cycles (1 minute at 95°C, 1 minute at 59°C, 2 minutes at 72°C). Lane 1: molecular weight markers. Lanes 2 to 4: three adult DNA samples. Lane 5: no DNA.*

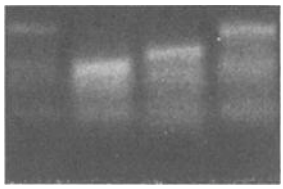

FIG. 2—*Analysis of digestion products from the 520 bp PCR product generated with primers 1 and 2. Lane 1: uncut. Lane 2: HaeIII. Lane 3: DdeI. Lane 4: RsaI.*

DNA sequence. Second, we amplified the 520 bp fragment in a second round PCR with an internal primer as described [*1*]. This hemi-nested PCR generated the expected 360 bp fragment (Fig. 3).

We then tested various heart samples for the presence of the deletion. Of these 25 samples, 22 had the deletion. All were samples from adults with age ranging from 20 to 94 years. Two adult samples were negative for the deletion. Both cases had more than one week delay between death and autopsy, and PCR to amplify the 268 bp β-globin fragment as a control was unsuccessful, suggesting that the failure to amplify the mtDNA deletion was due to extreme postmortem DNA degradation [*9*]. The newborn heart sample was also negative for the deletion, but the β-globin gene was successfully amplified in this case. All adult samples having the deletion also contained amplifiable β-globin gene.

We then tested the influence of fixatives (Fig. 4). All the above data were obtained from neutral buffered formalin-fixed material with a fixation time less than 48 h. For comparison, various adult heart samples were fixed in Bouin's fixative, unbuffered formalin and neutral buffered formalin. With Bouin's fixative no DNA was amplified either from mtDNA or from nuclear DNA as a control. With unbuffered formalin only two of six samples had amplifiable mtDNA deletion. Both samples also had amplifiable β-globin gene, which was not detected in the four other samples. With neutral buffered formalin all heart muscle DNA samples demonstrated the deletion and the β-globin gene, if the fixation time was less than 72 h. Fixation for longer periods of time resulted in the absence of deleted mtDNA and of the β-globin gene.

Discussion

Our data indicate that the 4977 bp mtDNA deletion is easily detected in paraffin-embedded material. The assay is very sensitive since low levels of the deletion in young adults can be detected.

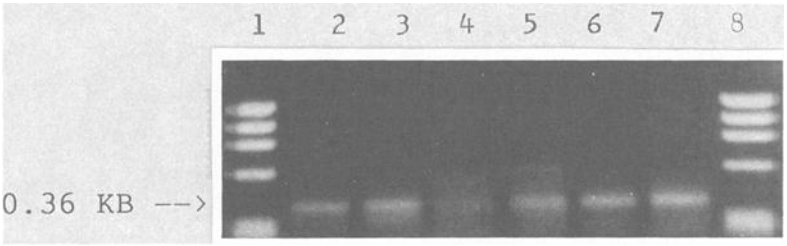

FIG. 3—*Analysis of hemi-nested amplification products. A first round of PCR was carried out using primers 1 and 2. A second round with 30 PCR cycles was carried out using an internal primer. Lane 1 and 8: molecular weight markers. Lanes 2 to 7: adult heart NA samples.*

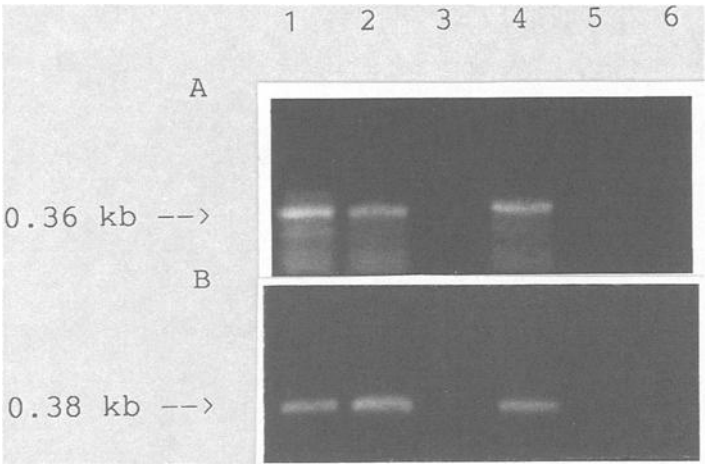

FIG. 4—*Influence of fixative type on amplification. Panel A: amplification of the deleted mtDNA. Panel B: amplification of the β-globin gene. Adult heart samples were immersed in fixative for 48 hours. Lanes 1 and 2: neutral buffered formalin. Lanes 3 and 4: unbuffered formalin. Lanes 5 and 6: Bouin's fixative.*

The low detection level is probably due to the degradation of the wild-type mtDNA by the fixation procedure. Being degraded, normal mtDNA does not compete for the primers with the deleted mtDNA. Too much degradation, however, either with Bouin's fixative, or postmortem degradation, may not allow any DNA amplification at all. In this regard, each heart sample from adults having an amplifiable β-globin gene also contained at least one amplifiable mutated mtDNA. A diploid human cell contains 2 copies of the β-globin gene, and it has been estimated that about 1 among 1000 mtDNA copies is deleted in one myocardial cell. Thus, the β-globin fragment, which is similar in size to the deletion fragment, may be considered as a reliable control. Other controls can be short fragments of the normal mtDNA, but they are far more abundant than the single copy β-globin gene.

Absent or weak PCR products may also be due to a prolonged period of fixation leading to a dramatic reduction in the yields of DNA that can be extracted. Some authors have reported that improvements in the yields of DNA from paraffin-embedded material may be produced by microwaving the sample or increasing the length of proteinase K digestion up to 5 days [*10*].

Other factors influence PCR. First, there is some variation between similar primer pairs in their ability to amplify DNA. For unknown reasons, one of the two primer pairs we tried did not amplify the deleted mtDNA, although the small size of the product it could have generated was well within the range of amplifiable DNA fragments in paraffin-embedded material. Second, with short cycling time we were not able to amplify the deleted mtDNA. It may be that the PCR process is less efficient in paraffin-embedded material compared with fresh or frozen samples, a phenomenon already observed by others [*11*].

Finally, the presence of the 4977 bp mtDNA deletion in all adult heart samples is consistent with previously reported data [*1*]. It has been suggested that increased levels of this deletion in human myocardial cells can be caused by repeated episodes of ischemia in the context of severe coronary atherosclerosis [*3*]. Since coronary atherosclerosis is the leading cause of sudden cardiac death, it would be interesting to determine deleted mtDNA levels due to ischemic damage, methodology that is being developed.

Acknowledgment

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