J Forensic Sci, Nov. 2004, Vol. 49, No. 6
Paper ID JFS2004073
Available online at: www.astm.org

Eliana Y. Polisecki,[1] M.Sc.; Laura E. Schreier,[2] Ph.D.; Julio Ravioli,[3] M.D.; and Daniel Corach,[1] Ph.D.

Common Mitochondrial DNA Deletion Associated With Sudden Natural Death in Adults

ABSTRACT: One of the most frequent causes of death in developed countries is sudden natural death (SND), which is the most common indication for medico-legal autopsies. Cardiac diseases are frequently detected among SND. Mitochondrial DNA (mtDNA) is easily damaged by reactive oxygen species, and it may cause dysfunction in tissues, leading to early events in cardiovascular disease. A specific mtDNA deletion of 4977 bp is associated to aging, myocardial dysfunction, and bioenergetic deficit. The potential link between mtDNA damage and SND has not been investigated before. Our aim was to evaluate the accumulation of the common mtDNA4977-deletion in cardiac muscle samples from autopsies of SND in adults ($n = 14$) in comparison to control samples from unnatural deaths ($n = 12$). Serial dilution-polymerase chain reaction method was performed to estimate the proportion of the total mtDNA harboring the mtDNA4977-deletion. Coefficient variation intra-assay was 8%, and inter-assay was 12%. MtDNA4977-deletion percentage was higher in samples obtained from victims of SND than in those from subjects who died of unnatural causes ($p < 0.05$). No differences in mtDNA4977-deletion were found between SND victims 39–51 years old, and no correlation was found between these samples and age, $r = 0.30$, $p = 0.29$ while it was significant among control samples, $r = 0.68$, $p < 0.05$. The association between mtDNA4977 deletion with SND victims might offer a tool to provide additional information to clarify complex SND investigations.

KEYWORDS: forensic science, sudden natural death, Mitochondrial DNA, 4977 bp mitochondrial DNA deletion, aging

One of the most frequent causes of death in developed countries is the so-called sudden natural death in adults (SND), which is the most common indication for medico-legal autopsies (1–3). Victims die in a short time period after onset of symptom, by no apparent previous causes and without clinical information that might establish the cause of death. In such situations, criminal responsibility may be suspected. Nowadays, SND is considered a syndrome constituted by a cluster of clinical events; however, the precise sequences of events that ultimately result in death are not so clear.

Data from several countries show that cardiac diseases are the most frequently detected disorders among SND, which is in accordance with the fact that more than 50% of atherosclerotic ischemic heart disease patients die by sudden cardiac death (4–7).

Mitochondria are the major sites producing reactive oxygen species (ROS) during oxidative phosphorylation. Mitochondrial DNA (mtDNA) is easily damaged by ROS because it is neither protected by histones nor by chromatin structure, and a poor antioxidant mechanism operates within the organelle. Thus, mtDNA has a shorter half-life and higher mutational rate than nuclear DNA (8). Mitochondrial damage potentially impacts a variety of cellular functions, ranging from energy production to cell signaling. This may cause dysfunction in tissues, leading to early events in cardiovascular disease (9). Cumulative mtDNA damage by ROS has been considered to be associated to the development of cardiovascular disease (10,11).

[1] Servicio de Huellas Digitales Genéticas, Cátedra de Genética y Biología Molecular, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.
[2] Departamento de Bioquímica Clínica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.
[3] Cátedra de Medicina Legal, Facultad de Medicina, Universidad de Buenos Aires, Argentina.

Received 22 Feb. 2004; and in revised form 19 June 2004; accepted 20 June 2004; published 5 Oct. 2004.

Mitochondrial deletions have been shown to increase with age and have been suggested to contribute to myocardial dysfunction (12,13). A specific mtDNA deletion of 4977 bp (mtDNA4977-deletion) occurs between two 13 bp direct repeats in the mtDNA sequence located between positions 8482:16460, resulting in the deletion of the genes encoding all or part of four polypeptides for complex I, one for complex IV, two for complex V (all of them involved in the mitochondrial oxidative phosphorylation (OXPHOS) system), and five tRNA genes. This "common" deletion accumulates preferentially in tissues of low mitotic activity and high metabolic rate such as cardiac muscle, skeletal muscle, and brain (14,15). This deletion has attracted considerable interest since it is a common cause of several degenerative mitochondrial disorders such as Kearns-Sayre or Progressive External Ophthalmoplegia syndromes (16) and also, as stated above, it contributes to the aging process, myocardial dysfunction, and bioenergetic deficit contributing to cellular pathology (17).

The potential link between mtDNA damage and SND has not been investigated before. Our aim was to evaluate the accumulation of the common mtDNA-deletion in cardiac muscle samples from victims of SND in adults. The detection of a possible association of mtDNA4977-deletion with SND in adults may contribute to clarify probable causes of SND, which in turn could be useful in medico-legal autopsies.

Materials and Methods

Samples

A set of 14 cardiac muscle tissues from consecutive victims of SND whose ages ranged from 18–70 years was tested. None of the subjects was under intensive physical activity at the moment of death. On the other hand, 12 cardiac muscle samples from consecutive corpses not affected by natural death, age range from 22–71 years, were obtained and investigated as control samples (Table 1).

TABLE 1—*Age and gender of the studied groups.*

Groups	n	Age (Mean ± SD)	Female/Male
Sudden Natural Death	14	46 ± 17.6	3/11
Unnatural Death	12	49.6 ± 16.1	4/8

Not significant.

Death causes from these samples consisted of suicides, murders, or accidents. Sudden death due to natural or unnatural causes was identified when it occurred unexpectedly, in accordance with the guidelines of the World Health Organization's system of international classification of diseases (18).

Samples were collected at the autopsy rooms at the Legal Medicine Department, Buenos Aires Province.

In order to be preserved at room temperature, samples were immersed in solid salt (sodium chloride) and transferred to our lab immediately after the autopsy.

DNA Extraction

After washing the tissue samples with distilled water, genomic DNA was isolated from approximately 250 mg of cardiac muscle by a sodium dodecyl sulfate – proteinase K digestion at 56°C overnight, and DNA was purified further by solvent extraction. Extracted DNA was quantified by spectrophotometric analysis.

PCR Amplification

A serial dilution-polymerase chain reaction method was performed. In order to estimate the proportion of total mtDNA harboring the mtDNA4977-deletion (19,20), DNA was previously digested for 2 h at 37°C with 2 U PstI and 2 U EcoRI, which cut normal DNAs at positions 9020 and 1240, respectively, within the deleted region. This procedure avoids amplification of wild-type products during PCR reaction. Serial dilution of the digested DNA was performed to obtain concentrations of 400 to 25 ng for the mtDNA 4977 amplification and of 75 to 0.1 ng for the total mtDNA amplification. The first set of primers (forward, 15978-15997—5′caccattagcacccaaagct 3′; reverse, 16421-16401 5′ttgatttcacggaggatggtg 3′) yielded a 444 bp fragment upon amplification of the HVRI region within the D-Loop not susceptible to deletion. This fragment was used for quantification of total mtDNA. If mtDNA with the 4977 bp deletion is present, the forward 8285-8304, 5′ ctcagagcccactgtaaagc3′; reverse 13595-13576, 5′ cttgtcagggaggtagcgat3′ (21) primer set yields a 389 bp fragment after amplification.

PCR was performed in a Perkin-Elmer/Cetus DNA Thermal Cycler. The reactions were carried out in a 30 μL reaction mixture containing DNA, 0.2 mM of each dNTP, 0.08 nM of each primer, 1.25 U Taq DNA polymerase (T-Plus DNA polymerase from Higway molecular Biology – Inbio – UNICEN), 50 mM KCl, 1.25 mM $MgCl_2$, 10 mM Tris-HCl (pH: 7.5), and 0.1% Triton X-100. The PCR consisted of 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C, and 1 min primer extension at 72°C for both primer sets. In all cases, DNA-free samples were run as negative controls.

Semi-Quantitative Analysis of the 4977 bp "Common" Deletion

The PCR products were separated on 2% agarose gels, run in TBE at 110 V for 50 min. DNA was stained with ethidium bromide 0.002 μg/mL and visualized by means of a UV light at 258 nm (Fig. 1*a*).

The products were detected using a Foto/Analys® Investigator - Fotodyne® Incorporated, and quantified using an Image Quant 5.1 software (Molecular Dynamics, Amersham Pharmacia Biotech).

The decline in optical density (OD) of the PCR products for each dilution series versus the amount of DNA used in the PCR reactions was fitted on a semi-logarithmic plot, according to a regression line identified by the equation $OD = a + b \times \log(\text{ng DNA})$, with $r > 0.9$ (Fig. 1*b*). Following Corral-Debrinski et al. (19), the percentage of deleted mtDNA with respect to total mtDNA was determined by the ratio of the DNA dilutions that reduced the PCR product OD of the deleted mtDNA and total mtDNA curves to the same level. To confirm the nature of the PCR product obtained from samples showing the presence of deleted mtDNA, all PCR products were sequenced by the Big Dye Terminator System (ABI310, Applied Biosystems, USA), using the Cambridge sequence (22) as reference.

Precision

Reproducibility of the method was assessed by within-run precision in 15 replicates of a single sample and between-day precision, in duplicate, throughout a 25-day study ($n = 12$). Coefficient variations were calculated as SD/mean and expressed as percentage.

Statistical Analysis

Differences between groups were tested using unpaired test-t. Correlation between variables was assessed using the Pearson correlation test. Differences were considered significant at p value less than 0.05.

Results

By sequencing analysis we confirmed a single breakage point between positions 8482 and 13460 in all samples affected, which defines the deletion of 4977bp, flanked by a 13 bp repeat. When precision of the technique applied was evaluated, within-run precision (CV%) was 8%, and between-run was 12%.

The semi-quantification of the mtDNA4977-deletion in cardiac muscles obtained from the autopsies is shown in Table 2. MtDNA4977-deletion percent was higher in samples obtained from victims of SND than in those obtained from victims of death by unnatural causes ($p < 0.05$).

When SND victims were divided into 2 subgroups according to age distribution, no differences were found in mtDNA4977-deletion percentage between victims 39–51 years old (Table 3).

TABLE 2—*mtDNA4977-deletion in sudden natural and unnatural death of cardiac muscle samples.*

	mtDNA-4977%
Sudden Natural Death	0.052 ± 0.030*
Unnatural death	0.032 ± 0.014

* $p < 0.05$.
Results are expressed as percentage, mean ± SD.

TABLE 3—*Percent of mtDNA4977-deletion in sudden natural death subgroups according to age distribution.*

Sudden Natural Death Subgroups	n	mtDNA-4977%
18–39 years	6	0.036 ± 0.012
51–69 years	8	0.059 ± 0.037

Not significant, $p = 0.15$.
Results are expressed as mean ± SD.

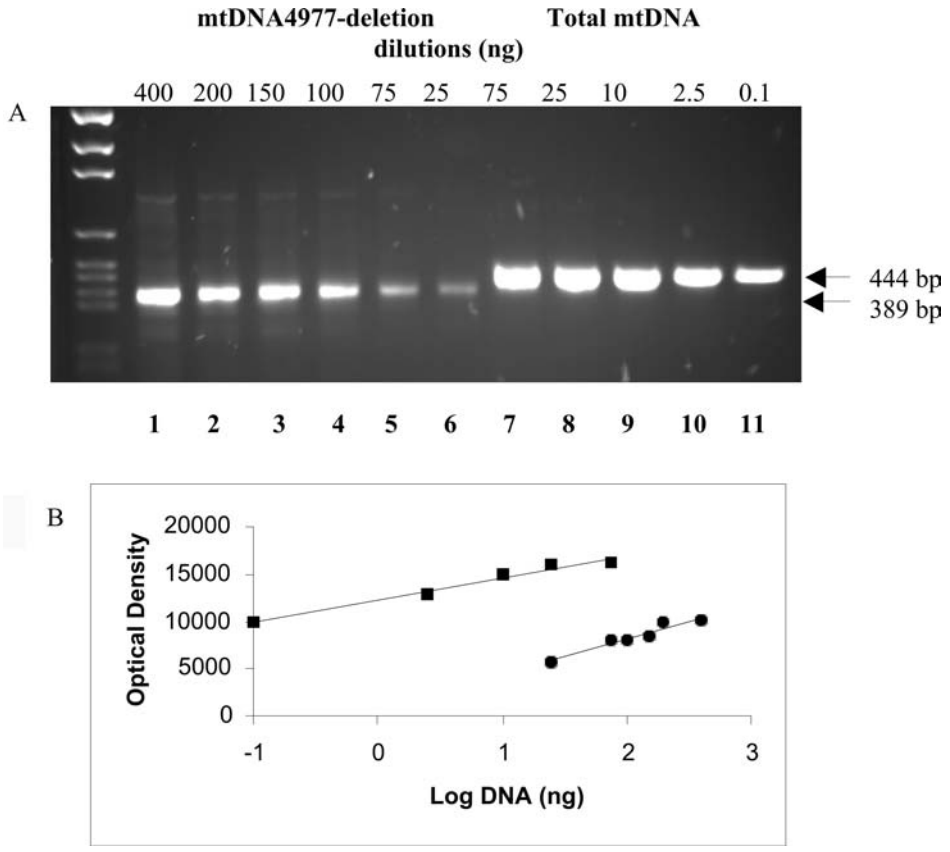

FIG. 1—*Example of semi-quantification by serial dilution-PCR of total and deleted aliquots of genome DNA obtained from cardiac muscle tissues. (A) Agarose gel 2.0%: Lanes 1–6 represent the PCR products amplified from 4977 bp deleted mtDNA serially diluted. Lanes 7–11 represent the PCR products amplified from total mtDNA serially diluted. Band intensities are related to mtDNA amounts. Details are described in Materials and Methods. (B) Graph showing the densitometric data points and fitted curves of total mtDNA (squares) and the mtDNA4977-deletion (circles). One typical experiment is shown.*

According to this, no correlation was found between these samples and age, $r = 0.30$, $p = 0.29$, while a significant association was obtained between mtDNA4977-deletion percentage from samples of victims of unnatural death and age, $r = 0.68$, $p < 0.05$.

Discussion

The aim of our investigation was to search an association between SND and an increase of mtDNA4977-deletion. No previous reports have described this association before. The accumulation of this deletion was higher in autopsy cardiac muscles samples from victims affected by SND than in samples from subjects who died by unnatural causes. Moreover, the increase of mtDNA4977-deletion in SND did not show correlation with age.

There are few data regarding DNA quantitation, since methods are either very time-consuming, imprecise, or expensive. However, we performed a DNA semiquantitation procedure that was validated by an acceptable reproducibility added to the specificity confirmed by sequencing.

Our results indicate that samples from SND have a significant increased percentage of mtDNA4977-deletion in comparison to samples from unnatural causes. It is necessary to take into account that mitochondrial diseases can be due to either a homoplasmic or a heteroplasmic mutation. In the case of a heteroplasmic mutation, the disease state develops only after a threshold level of mutation has been achieved. When the percent-mutated DNA rises above the threshold, evident symptoms will be present, and then an acute (and sometimes lethal) condition will be observed (23).

The extent of dysfunction will depend on the aerobic energy requirements of each tissue, which explains why tissues with high energy demands, such as cardiac muscle, are affected more frequently by mitochondrial defects. Faulty aerobic metabolism involving the heart may be due to defects of OXPHOS or to defects of fatty acids oxidation. Large-scale re-arrangement of mitochondrial DNA has been identified in patients with cardiomyopathy as a main clinical feature and associated with disorders of cardiac energy metabolism (24).

In addition, the increase in susceptibility of cardiac tissue to DNA damage could lead to ischemic heart disease, and this may be associated to the high incidence of SND among coronary artery disease patients (3,19). Mitochondrial DNA damage may result from ROS production in vascular tissues and may be, in turn, an early event in the initiation of atherosclerotic lesions (10).

It is noteworthy that heart failure was described in patients affected by Kearns-Sayres syndrome, a sporadic and ultimately fatal multisystem disorder, characterized by major mtDNA deletions (25). This could suggest an association between DNA damage and cardiac dysfunction.

On the other hand, other authors demonstrated that cell lines with 100% deleted mitocondrial DNAs exhibited a complete impairment of respiratory chain function and oxidative phosphorylation, presumably because of the absence of mitochondrial protein translation resulting from the loss of essential tRNA genes from the deleted mitochondrial genome (26).

It is already known that mtDNA4977-deletion increases with age due to the increase of oxidative stress (27). The relationship

between mtDNA4977-deletion and aging also was observed in this study among samples from unnatural death causes. However, no differences were found in the percentage of mtDNA4977-deletion between samples from young and old victims of SND, and no significant correlation with age was observed. The lack of correlation is attributed to the fact that samples from young victims showed high levels of such deletion, indicating that this deletion may be associated with SND, irrespective of age.

These findings have never been associated with SND before and should be confirmed in a larger sample size. It would be useful to know if this deletion is widespread outside the heart, in particular other muscle tissues, or in brain. Furthermore, it is important to note that a high proportion of mutant mtDNA must be present to produce a clinically relevant damage, however, the threshold value above which symptoms appear is unknown and must be determined. On the basis of the results obtained, a larger sample size and diverse tissue types are being investigated in our laboratory in order to develop a potential forensic marker that might shed light on SND.

Although the cause of sudden death might be due, in part, to multiple genetic effects, energy reduction, as a consequence of anomalous and fast-mutated mtDNA replication, may play a remarkable role in SND. Even though mitochondrial genome has become increasingly popular in the forensic area for individual identification, in this study the association between mtDNA 4977 bp common deletion with SND victims might offer a tool to provide additional information to clarify complex SND investigations.

Acknowledgments

This work was supported by grants from the University of Buenos Aires (B-038 to DC and B-069 to LS) and CONICET (0746 to DC).

References

1. Hirsch CS. Forensic pathology and the autopsy. Arch Pathol Lab Med 1984;108(6):484–9. [PubMed]
2. Di Mario VJ, Di Maio DJ. Natural death as viewed by the medical examiner: a review of 1000 consecutive autopsies of individuals dying of natural disease. J Forensic Sci 1991;36(1):17–24. [PubMed]
3. Escoffery CT, Shirley SE. Causes of sudden natural death in Jamaica: a medicolegal (coroner's) autopsy study from the University Hospital of the West Indies. Forensic Sci Int 2002;129:116–21. [PubMed]
4. Doolan A, Langlois N, Semsarian C. Causes of sudden cardiac death in young Australians. Med J Aust 2004;180:110–2 [PubMed]
5. Kannel WB, Gagnon DR, Cupples LA. Epidemiology of sudden coronary death: population at risk. Can J Cardiol 1990;6(10):439–44. [PubMed]
6. Shen W, Edwards WD, Hammill SC, Bailey KR, Ballard DJ, Gersh BJ. Sudden unexpected nontraumatic death in 54 young adults: a 30-year population-based study. Am J Cardiol 1995;76:148–52. [PubMed]
7. Aizaki T, Izumi T, Kurosawa T, Shoi N, Furukawa M, Kurihara K. Sudden cardiac death in japanese people aged 20–60 years- an autopsy study of 133 cases. Jpn Circ J 1997;61:1004–10. [PubMed]
8. Yakes FM, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc Natl Acad Sci USA 1997;94(2):514–9. [PubMed]
9. Knight-Lozano CA, Young CG, Burow DL, Hu Z, Uyeminami D, Pinkerton KE, et al. Cigarette smoke exposure and hypercholesterolemia increase mitochondrial damage in cardiovascular tissues. Circulation 2002;105:849–54. [PubMed]
10. Ballinge SW, Patterson C, Knight-Lozano CA, Burow DL, Conklin CA, Hu Z, et al. Mitochondrial integrity and function in atherogenesis. Circulation 2002;106:544–9. [PubMed]
11. Bogliolo M, Izzotti A, De Flora S, Carli C, Abbondandolo A, Degan P. Detection of the 4977 bp mitochondrial DNA deletion in human atherosclerotic lesions. Mutagenesis 1999;14(1):77–82. [PubMed]
12. Levitsky S, Laurikka J, Stewart RD, Campos CT, Lahey SJ, McCully JD. Mitochondrial DNA deletions in coronary artery bypass grafting patients Eur J Cardiothorac Surg 2003;24:777–84. [PubMed]
13. Ruppert V, Maisch B. Mitochondrial DNA deletions in cardiomyopathies. Herz 2000;25(3):161–7. [PubMed]
14. Cortopassi GA, Arnheim N. Detection of a specific mitochondrial DNA deletion in tissues of older humans. Nucleic Acids Res 1990;18(23):6927–33. [PubMed]
15. Liu VW, Zhang C, Nagley P. Mutations in mitochondrial DNA accumulate differentially in three different human tissues during ageing. Nucleic Acids Res 1998;26(5):1268–75. [PubMed]
16. Shoffner JM, Lott MT, Voljavec AS, Soueidan SA, Costigan DA, Wallace DC. Spontaneous Kearns-Sayre/chronic external ophthalmoplegia plus syndrome associated with a mitochondrial DNA deletion: a slip-replication model and metabolic therapy. Proc Natl Acad Sci USA 1989;86(20):7952–6. [PubMed]
17. Porteus WK, James AM, Sheard PW, Porteous CM, Packer MA, Hyslop SJ, et al. Bioenergetic consequences of accumulating the common 4977-bp mitochondrial DNA deletion. Eur J Biochem 1998;257:192–201. [PubMed]
18. World health organization, manual of the international statistical classification of diseases and related health problems. Vol. 1, 10th ed. Geneva: WHO, 1992.
19. Corral-Debrinski M, Stepien G, Shoffner JM, Lott MT, Kanter K, Wallace DC. Hypoxemia is associated with mitochondrial DNA damage and gene induction. JAMA 1991;266(13):1812–6 [PubMed]
20. Corral-Debrinski M, Horton T, Lott MT, Shoffner JM, Beal F, Wallace DC. Mitochondrial DNA deletions in human brain: regional variability and increase with advanced age. Nature Genetics 1992;2:324–9. [PubMed]
21. Lee H, Pang C, Hsu H, Wei Y. Differential accumulations of 4,977 bp deletion in mitochondrial DNA of various tissues in human ageing. Biochim Biophys Acta 1994;1226:37–46. [PubMed]
22. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature 1981;290:457–65. [PubMed]
23. Berdanier CD, Everts HB. Mitochondrial DNA in aging and degenerative disease [Review]. Mutat Res 2001;475:169–84. [PubMed]
24. Antozzi C, Zeviani M. Cardiomyopathies in disorders of oxidative metabolism. Cardiovasc Res 1997;35:184–99. [PubMed]
25. DiMauro S, Schon EA. Mitochondrial respiratory-chain diseases. N Engl J Med 2003;348:2656–68. [PubMed]
26. Tang Y, Schon EA, Wilichowski E, Vazquez-Memije ME, Davidson E, King MP. Rearrangements of human mitochondrial DNA (mtDNA): New insights into the regulation of mtDNA copy number and gene expression. Mol Biol Cell 2000;11:1471–85. [PubMed]
27. Wei Y. Oxidative stress and mitochondrial DNA mutations in human aging. [Minireview]. Exp Biol Med 1998;217(1):53–63.

Additional information and reprint requests:
Daniel Corach, Ph.D.
Servicio de Huellas Digitales Geneticas
Junin 956 (C113AAD)
Buenos Aires, Argentina