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MITOCHONDRIAL DNA^{4977} DELETION IN BRAIN OF NEWBORNS DIED AFTER INTENSIVE CARE

EDIT A. NÁDASI,[1]* B. MELEGH,[1] L. SERESS,[2] G. KOSZTOLÁNYI[1,3]**

[1] Department of Medical Genetics and Child Development, Medical Center,
[2] Central Electronmicroscopic Laboratory, Faculty of Medicine, University of Pécs
[3] MTA-PTE Clinical Genetics Research Group, Pécs, Hungary

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Mitochondrial DNA (mtDNA) deletion affecting 4977 base pairs (mtDNA^{4977}), the most common mtDNA mutation in humans, was analysed in brain specimens (frontal, temporal, and cerebellar cortices, caudate nucleus, thalamus, and hippocampus) and in other tissues (blood clot, liver, kidney, heart, and muscle) taken at autopsy of deceased neonates. mtDNA^{4977} deletion determined by polymerase chain reaction (PCR) could be demonstrated in each neonatal sample, however, quantity of mtDNA^{4977} deletion was less in the newborn samples than in those of the elderlies. Results obtained suggest that contrary to certain data mtDNA^{4977} deletion can be present in neonates. The mtDNA^{4977} deletion could be generated by perinatal hypoxia or temporary oxygen oversaturations during the intensive care of the neonates, as the mtDNA is sensitive to oxidative damage. In combination with other factors an additional causative role of mtDNA^{4977} deletion reported here cannot be ruled out in development of cerebral palsy or mental retardation of unknown origin often seen in neonates underwent neonatal intensive care procedures.

Keywords: Mitochondrial DNA deletion – ageing – brain damage – perinatal hypoxia – intensive care

INTRODUCTION

The background and pathomechanism of neurological impairments underlying child disability are poorly understood. Despite of extensive diagnostic investigations, about one-third of mental retardation cannot be identified with any biological markers [19]. The cause of brain damage is known only in one-third of children with cerebral palsy, in another one-third the damage is more or less explainable with possible mechanisms, and in the rest the reason is completely unknown [8, 21].

Human mitochondrial DNA, a 16.569 bp circular molecule coding for a significant part of the oxidative phosphorilation system (OXPHOS) is the major source of cellular energy production. mtDNA mutations can lead to mitochondrial dysfunction which may cause inherited diseases of the neuromuscular system. Studies of human

* At present: Department of Public Health and Preventive Medicine, Faculty of Medicine, University of Pécs, Pécs, Hungary
** Corresponding author; e-mail: gyorgy.kosztolanyi@aok.pte.hu

mtDNA mutations in normal adult subjects have revealed that the most common deletion (mtDNA^{4977}) accumulates with age in different regions of the brain, especially in the basal ganglia [4, 15, 17]. This raise of mtDNA^{4977} in the elderlies is detectable also in different other tissues [5, 20, 23]. Possible explanations include both endogenic (free radicals) and exogenic (environmental effects) factors [4, 22]. Hence, mtDNA^{4977} has generally been regarded, even if not without controversy [7], as the most common somatic mutation of human beings and, among others, may offer plausible explanation for the mechanism of ageing and the deterioration of brain functions in the elderlies [1, 5, 12, 14, 22].

On the base of a possible causal relationship between the acquired (somatic) mutations of mtDNA and the deterioration of the neurological functions [4, 9, 22], we decided to examine the occurrence of mtDNA^{4977} in brain samples from newborns died after treatment at the intensive care unit. According to our original assumption, in the group of former preterm infants whose brain damage is unexplainable, the neurological impairment might be associated with the raise of mtDNA deletion in different regions of the brain, since both endogenic (e.g. pre- and perinatal hypoxia, free radicals, prematurity) and environmental factors (intensive care including arteficial ventillation) may generate mtDNA mutations.

MATERIALS AND METHODS

Investigations were carried out from postmortem tissue samples collected from newborns and infants of different ages, and from adults as controls. Samples were taken during autopsies from frontal, temporal, and cerebellar cortexes, caudate nucleus, thalamus, and hippocampus. In some cases samples were also taken from blood clot, liver, kidney, heart and muscle. Samples were carefully collected by means of different tools to avoid contamination. When not used immediately, specimens were frozen to store. Data on family history, clinical investigations and autopsy were collected from case records.

DNA was isolated from tissue samples by phenolic extraction: tissue frozen in liquid nitrogen has been smudged and incubated in 1.2 ml/mg tissue digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS pH 8.0 and 0.1 mg/ml proteinase K added freshly) for 3 hours in water-bath at 37 °C. The completely digested tissue sample was purified by adding phenol/chloroform/isoamyl-alcohol (25 : 24 : 1) two times and chloroform/isoamyl-alcohol (24 : 1) once. DNA was precipitated by adding cold absolute alcohol and resuspended in sterile bidestillated water. DNA solutions were stored also frozen (–20 °C).

Hundred ng of DNA was used for PCR, determined by spectrophotometry. Amplification of intact and deleted mtDNAs were carried out according to the original (slightly modified) protocol of Soong and Arnheim [16]. In this construction, the primer for deleted mtDNA (MT1A) does not amplify the wild type one, while the primer for healthy mtDNA (MT1C) does not amplify the deleted one (Fig. 1). **MT1A**: AAT TCC CCT AAA AAT CTT TGA AAT A, **MT2**: AAC CTG TGA GGA

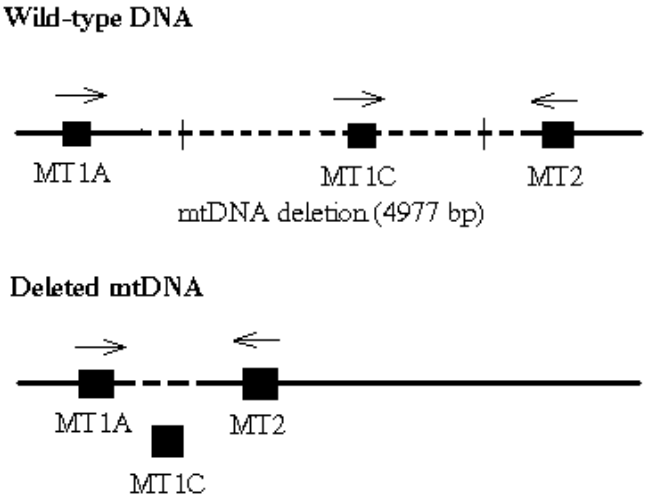

Fig. 1. Scheme showing how the mtDNA^{4977} deletion brings primer MT1A and MT2 close together to enable amplification to take place under short cycle times

AAG GTA TTC CTG C, **MT1C**: AGG CGC TAT CAC CAC TT TGT TCG. PCR (1× PCR buffer, 1–1 mM primer of each, 187.5 mM dNTP of each, 2.5 mM $MgCl_2$, 100 ng DNA, 2U of Taq in a final volume of 50 µl) was carried out in a thermocy-

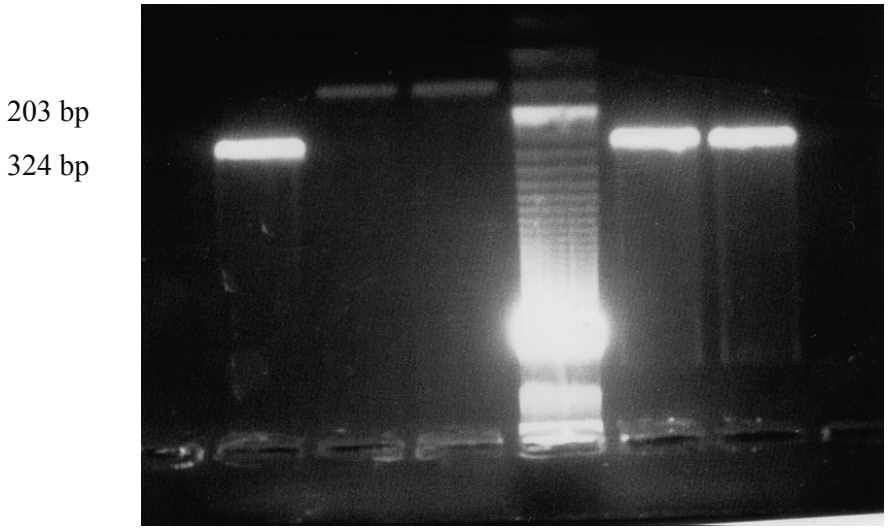

Fig. 2. Mitochondrial PCR products

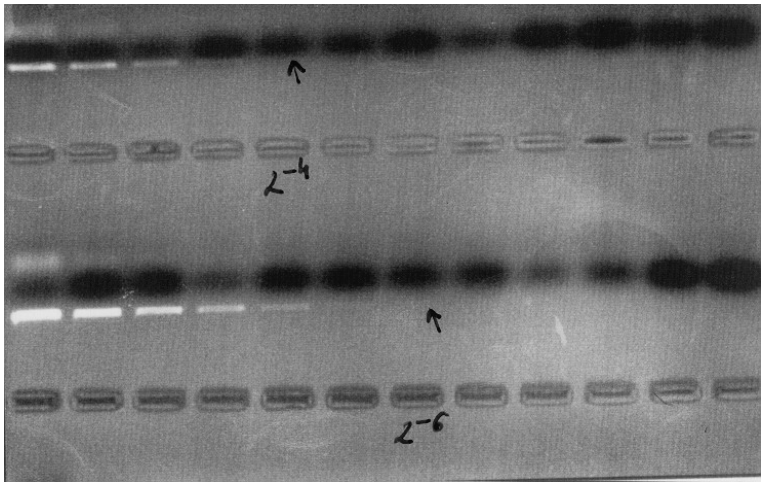

Fig. 3. Demonstration of the detection threshold method performed for estimation of the ratio of wild type and deleted mDNA on agarose gel

cler (MJR-PTC150) by 20-sec denaturation at 92 °C followed by a single annealing and extension step of 20 sec at 60 °C during 35 cycles and a final extension step of 72 °C for 3 min. Product length is 203 bp and 324 bp when copied from deleted mtDNA and wild type mtDNA respectively. Amplification was checked by electrophoresis of 5 µl of product in 2% agar gel (70 V, 50 mA, 30 min) stained with ethidium bromide (Fig. 2).

Comparison of deleted and wild type mtDNA in every single sample was executed by electrophoresing serial dilutions of the PCR products in 2% agarose gel (70 V, 50 mA, 30 min) stained with ethidium bromide. Twenty µl PCR product was taken out of the 50 µl total volume. Ten µl of this 20 ml was loaded into the first well (concentration $2^0 = 1$, dilution = 1), while the remaining 10 µl was mixed with 10 µLlbidestilled water. Ten ml of this latter mixture was loaded into the second well (concentration 2^{-1}, dilution = 1/2). After further dilutions of the remaining mixture, the concentration is 2^{-11}, dilution = 1/2048 in the last (12th) well. Deleted and wild type mtDNA from the same sample were electrophorized in the same gel.

Presence of $mtDNA^{4977}$ in our samples was expressed as a ratio of deleted and wild type mtDNA, estimated by the detection limit of electrophoretic bands on the agarose gel containing serial product dilutions as follows (Fig. 3). In wells next to each other, concentration of the PCR product always decreases by half. Considering the last well where the product is still visible (limited dilution point), the ratio of the two different products can be expressed by a relative figure. E.g., in the specimen where PCR product of deleted mtDNA disappears in the 5th well (concentration

is 2^{-4}) while PCR product of wild type mtDNA disappears in the 10th well (cc. is 2^{-9}), the ratio of wild type and deleted mtDNA amplification products is $2^{-4}/2^{-9} = 2^5 = 32$ (ratio: 32 : 1). Thus, in this sample wild type mtDNA was estimated to be 32 times more than deleted mtDNA.

RESULTS

Neither the patients themselves, nor their parents had any symptoms, clinical or laboratory findings suggesting inherited mitochondrial diseases. Gestational ages of the newborns died within the first month ranged between 30 and 37 weeks. Prior to death all infants underwent pathological conditions prone to hypoxia and all were treated with oxygen, mostly by arteficial ventillation. Drugs used during treatment of the patients were the usually administered medicaments and were applied as recommended by approved guidelines. Adult control subjects died of different malignancies, stroke and acut ischemic heart failure.

Results obtained are summarized in Tables 1 and 2. Relative figures representative of at least two experimental determinations concern the ratio of PCR product from deleted/wild type mtDNA. The lack of some data is the consequence of difficulties in taking samples from tiny brain regions still prematured in consistency of tissue as well.

Table 1
Ratio of PCR products from mtDNA^{4977} to products from wild type mtDNA in adult tissues expressed by non-quantitative relative figures

Age Sex	FK	TK	KK	NC	TH	HC	BC
35 y F	1 : 16	1 : 16	1 : 16	1 : 4	1 : 8	–	–
55 y M	1 : 8	1 : 8	1 : 16	1 : 32	1 : 16	–	1 : 64
60 y M	1 : 32	1 : 8	1 : 8	1 : 8	1 : 8	1 : 8	1 : 128
65 y M	1 : 8	1 : 16	1 : 32	1 : 16	1 : 8	1 : 8	–
70 y M	1 : 16	1 : 4	1 : 32	1 : 64	1 : 8	1 : 32	1 : 128
75 y M	1 : 16	1 : 8	1 : 16	1 : 16	1 : 16	1 : 64	–
80 y F	1 : 32	1 : 32	1 : 64	1 : 8	1 : 16	1 : 8	1 : 128
90 y M	1 : 32	1 : 16	1 : 64	1 : 32	1 : 16	1 : 32	–

Table 2
Ratio of PCR products from mtDNA^{4977} to products from wild type mtDNA in tissues taken from newborns and young infants expressed by non-quantitative relative figures

Age Sex	Cause of death	Intensive therapy	FC	TC	CC	C	TH	HC	BC	L	K	H	M
2 d F	IRDS, cardial insuff.	Box O_2	1:16	1:64	1:32	–	–	1:32	–	–	–	–	–
2 d F	Intrauterine dystrophy, IRDS	AV/O_2	1:8	1:16	1:16	1:8	1:8	–	–	–	–	–	–
2 d F	IRDS, shock, hypothermia	AV/O_2	1:16	–	–	–	–	–	1:32	1:16	1:8	1:8	–
2 d M	Fallot's tetralogy	–	1:64	1:64	1:16	–	–	–	–	–	–	–	–
3 d F	IRDS, cardial insuff.	AB, AV/O_2	1:16	1:64	1:64	–	–	1:64	–	–	–	–	–
5 d M	Bronchopneumonia	Transf, AV/O_2	1:32	1:16	1:16	1:32	–	–	–	–	–	–	–
6 d M	Intracranial hemorrhage, RDS type II	AB, AV/O_2	1:32	1:16	1:32	–	–	1:32	–	–	–	–	–
6 d M	IRDS, cardial insuff.	AB, AV/O_2, Steroids	1:16	1:8	1:16	1:8	1:8	1:16	–	1:16	1:8	1:16	1:8
7 d F	IRDS, cardial insuff.	AB, AV/O_2	1:8	1:16	1:16	1:8	1:8	1:8	–	1:8	1:8	–	–
10 d F	Patau's syndrome	Box O_2	1:32	1:16	1:64	1:64	1:64	1:16	–	–	–	–	–
14 d F	Bronchopneumonia, PBD	Transf, Ig, AB, AV/O_2	1:4	1:16	1:16	1:64	1:64	1:64	–	–	–	–	–
19 d M	Bronchopneumonia, IRDS	Transf, AB, AV/O_2	1:16	1:16	1:16	1:16	1:16	1:8	–	1:8	1:16	1:8	1:16
42 d F	PBD, atelectasia and bronchopneumonia	Transf	1:32	1:64	1:16	1:16	1:32	–	–	–	–	–	–
90 d M	Pneumonia left side	Transf, AB, AV/O_2 Box O_2	1:8	1:16	1:16	1:16	1:8	1:32	–	–	–	–	–
130 d M	Cong. heart defect	AV/O_2	1:64	1:32	1:32	1:8	1:32	1:32	–	–	–	–	–

FC: frontal cortex, TC: temporal cortex, CC: cerebellar cortex, C: caudate nucleus, TH: thalamus, HC: hippocampus, BC: blood clot, L: liver, K: kidney, H: heart, M: muscle, AB: antibiotics, AV: arteficial ventillation, Ig: immunoglobulins, PBD: persistent Botall duct

mtDNA^{4977} was present in all brain samples taken from 8 control subjects aged 35–90 years and of the 15 newborns and infants. The ratio of PCR products of deleted/wild type mtDNA seems to be lower in the infant group than in adults, however, the experimental system applied in this study is insufficient for quantitative statements. Nevertheless, relative figures relating to level of mtDNA^{4977} correlate neither to the site of sample-taking nor to the age of the patient, cause of death, or treatment. The ratio of mtDNA^{4977} was lower in blood clots than in brain samples in every single case examined.

DISCUSSION

Analysis of the mtDNA^{4977} deletion levels in brain samples from newborns and adults revealed that mtDNA damage was present in all subjects, irrespective of age, sex, cause of death and terminal treatment. Although the relative figures presenting the ratio of PCR products from mutant and wild type mtDNA may indicate a lower level of mtDNA^{4977} in newborns than in adults, the limited dilution approach performed in this study is non-quantitative and does not allow precise statement on the proportion of damaged mtDNA. Nevertheless, results are surprising, since the original suggestion that mtDNA^{4977} may play a significant role in ageing was based on experiments in which mtDNA^{4977} was found in normal adult but not fetal brain [5, 16].

To explain our findings, two alternatives are worth to consider. First, mtDNA^{4977} is present in the normal human brain already in the beginning of life. Second, mtDNA mutations in neonates may be generated by perinatal hypoxia and/or intensive care.

As concerning the first alternative, how can presence of mtDNA^{4977} detected in every newborn brain be explained, considering the generally accepted nature of this mutation as a genetic damage acquired during life? Since neither our patients whom the samples were taken from, nor their parents showed any clinical or laboratory findings which would indicate inherited mitochondrial diseases, mtDNA^{4977} found should be classified as somatic mutations. mtDNA is particularly prone to genetic damage due to the absence of protective histons and the limited capacities of its own DNA repair system. Moreover, due to its site within the inner mitochondrial membrane mtDNA is exposed to the harmful effects of free radicals formed during OXPHOS in the respiratory chain. Consequently, the mutation rate in mtDNA is higher compared to nuclear DNA, a fact which was put forward as a major factor in senescence [1, 5, 12, 22]. According to this theory, somatic accumulation of mtDNA deletions during life in post-mitotic tissues is an important contribution to ageing. Age-associated accumulation of mtDNA^{4977} was found in different parts of the brain, highest ratio being regions where congenital mitochondrial diseases occur the most expressively [4, 5, 17].

Although very few results are known from previous investigations on mtDNA^{4977} in young subjects [4, 5, 11, 17], its existence, even in the beginning of life, cannot be

excluded by these observations. The Arnheim-group *did* find deleted mtDNA in fetal samples [5, 16], although after an increased number of PCR cycles. Corral-Debrinski et al. have investigated, together with elderlies, a 24-year-old individual who also was found to have $mtDNA^{4977}$ [4]. Meisser and von Wurmb detected very low levels of $mtDNA^{4977}$ in muscle of every normal individuals investigated even below 20 years of age [11]. (Unfortunately, although Soong et al. have referred to a 4-month-old infant enrolled in their study, no results relating to this patient were given afterwards [17].) Accordingly, our results, although unexpected, are not in absolute contradiction to previous findings.

Most human cells contain hundreds of mitochondria and each mitochondrion contains multiple mtDNAs. mtDNA deletions have been proposed to result from errors in DNA replication [22]. When a mutation arises, it creates an intracellular mixture of mutant and wild type molecules, known as heteroplasmy. Mitochondria are randomly segregated to each daughter cell at the time of cell division. Therefore, if a mtDNA mutation is generated in a relatively small proportion of mitochondria in a progenitor cell, the proportion of mutant and wild type mtDNA can change through random segregation and thus be very different between cell lines.

Although most postnatal human central neurons are postmitotic and glial cells rarely divide, recent observations strongly suggest that neuronal divisions continue up to the first few postnatal months [13], and that human brain does produce new cells even in adulthood [6]. Moreover, mtDNA can turn over in nondividing cells [15, 17]. Hence, a higher level of $mtDNA^{4977}$ in post-mitotic tissues of aged individuals may result, at least partly, from a difference between youngs and elderlies in the mutation rate and in the actual mitochondrial turnover, rather than from accumulation of life-long mutation events. Our observation that the level of the PCR products from $mtDNA^{4977}$ was lower in DNA isolated from blood cells with agile division rate than in brain cells with low or no-division activity may also be the result of different segregation and/or turnover rate in these tissues. The level of mutant mtDNA differs among tissue types, being higher in postmitotic tissues such as skeletal muscle and brain, whereas lower levels are found in rapidly dividing tissues like leucocytes [2, 3]. Therefore, the role of $mtDNA^{4977}$ accumulation in ageing has to be reconsidered and its contribution to the senescence seems to be less significant than has been assumed.

The second alternative to explain our results, the causative role of perinatal pathologic condition and intensive care of the infants prior to death, is worth to consider. Most newborns enrolled in our study suffered from perinatal/postnatal hypoxia and were treated with oxygen. The importance of reactive oxygen radicals' influence on mtDNA damage has been demonstrated in patients with chronic cardiac ischemia, and the level of $mtDNA^{4977}$ in ischaemic heart is increased relative to age-matched control samples [4, 22]. Neither oxygen exposure, nor drugs usually administered for cardiac, circulatory, respiratory, antimicrobial, immunologic, etc. supports in perinatal intensive care are supposed to have mutagenic effect [10], however, this supposition is based on the results of mutagenic tests using cytogenetic endpoint analyses, and no data are known about their possible effect on the extremely sensitive mtDNA.

Although no correlation between the level of mtDNA^{4977} and the cause of death or the treatment applied could be detected, our results would suit our original assumption and the causative role of mtDNA mutations in the cerebral damage due to perinatal pathologic conditions cannot be excluded. Clinical signs of disorders with mitochondrial dysfunction include developmental delay, seizures, encephalopathy, hearing loss, myoclonic seizures, stroke-like episodes and peripheral neuropathy, all of which are frequently seen in children surviving perinatal hypoxia.

In conclusion, it could well be that mtDNA^{4977} generated in newborns' brains due to the extreme sensitivity of mtDNA to oxidative damage may play a significant role in the development of neuromuscular impairment frequently seen in surviving preterm or asphyxiated newborns who otherwise have no gross cerebral damage and the reason of their cerebral palsy is unknown. New techniques must be developed to allow analyses of the total spectrum of mtDNA damage in brain before the role that mtDNA damage plays in contributing to degenerative neurological disease and ageing can be fully evaluated.

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