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SHORT COMMUNICATION

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Detection of the age-dependent 4977 bp deletion of mitochondrial DNA

A pilot study

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Abstract In recent years a number of mitochondrial DNA (mtDNA) deletions have been detected in various tissues from individuals over 20 years of age. It has been postulated that these deletions are associated with natural aging. In order to determine whether a correlation exists between age and the amount of deleted 4977 bp mtDNA, we used two PCR reactions to study total DNA (nuclear and mitochondrial DNA) extracted from skeletal muscle (m. iliopsoas) obtained at autopsy from 93 individuals representing a wide age spectrum (range: 3 months–97 years). The primer pair L15/H15 was used to amplify a 533 bp fragment of intact mtDNA to determine the percentage of total DNA. A second PCR with the primer pair L35/H35 was then employed to amplify a 667 bp fragment of the deleted mtDNA. The amount of template DNA necessary to amplify the specific fragments of deleted mtDNA was found to decrease with age. Whereas no 4977 bp deletion could be detected in subjects under 20 years of age even with 1000 ng of total DNA, in individuals aged 21 to 30 years 1000 ng total DNA were sufficient. Only 1 ng total DNA was needed in all individuals over 70. Our results show that the 4977 bp deletion can be a useful marker of natural aging in human subjects.

Key words Mitochondrial DNA · Age dependence · Skeletal muscle · 4977 bp deletion · PCR

Introduction

Determination of age at death based on aspartic acid racemization is a reproducible method in forensic pathology and has been applied in teeth (Ritz et al. 1993), bone (Ritz et al. 1994) and rib cartilage (Pfeiffer et al. 1995). Until now no method for age determination in soft tissues is available. The analysis of the mitochondrial genome has acquired increasing importance in legal medicine (e.g. Wilson et al. 1995; Alonso et al. 1996; Lutz et al. 1996). It enables not only forensic identification of human trace material but also the investigation of specific deletions in mtDNA. These deletions occur in skeletal muscle and other soft tissues and show a marked correlation with age (Holt et al. 1988; Wei 1992).

At least 20 different mtDNA deletions have been found in various tissues (Mita et al. 1990; Linnane et al. 1992; Simonetti et al. 1992) which can be generated by free radicals formed during cell respiration in the mitochondria (Harman 1960). The mitochondrial genome is especially at risk of deletion because it contains no introns (Anderson et al. 1981) and undergoes frequent replication without the benefit of adequate repair mechanisms (Clayton et al. 1974). Moreover, mtDNA is located near the inner membrane of the mitochondria where the breakdown of enzymes produces an abundance of free radicals.

One hypothesis postulates that some of the deleted DNA fragments enter the cytoplasm through the mitochondrial membrane and are incorporated into the nuclear genome. This may lead to a change in the information content of the nuclear DNA that results in cell aging (Richter 1995).

The most common deletion is the 4977 bp deletion, which has been observed in biopsy (Simonetti et al. 1992) and autopsy material from individuals aged 20 years and older (Münscher et al. 1993; Baumer et al. 1994). This deletion is especially frequent in well-differentiated cells with a low mitotic rate, such as brain and muscle cells (Arnheim and Cortopassi 1992). While the incidence of the 4977 bp deletion in these cells has been shown to increase with age, (Corral-Debrinski et al. 1992; Lee et al. 1994), studies on liver tissue have failed to reveal a similarly consistent correlation between the 4977 bp deletion and age (Lee et al. 1994; Yen et al. 1994).

In addition to the observed association with aging, levels of mtDNA deletion are also elevated in individuals with degenerative diseases, such as Parkinson's disease (Ikebe et al. 1990) and cardiomyopathies (Li et al. 1995),

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compared to age-matched controls. The level of deletion was also found to be increased in cirrhosis of the liver (Yamamoto et al. 1992).

In order to determine whether the quantity of template DNA required to detect the specific signal of the 4977 bp deletion decreases in direct proportion to age, we studied a large series (n = 93) of individuals divided into 10-year age groups beginning at 0–10 and extending to 80 years and above.

Materials and methods

At autopsy 500 mg of skeletal muscle (m. iliopsoas) was excised from 93 selected individuals who died of an acute or periacute event (e. g. massive blood loss, acute central nervous system death) in 1995 and 1996 at the Institute of Legal Medicine of the Medical University of Lübeck. The post mortem interval prior to removal of tissue ranged from 4 h–96 h. To rule out the effects of chronic disease processes on the quantity of deleted mtDNA, individuals with systemic or degenerative disorders (e.g. cardiomyopathies, liver cirrhosis) were exempted. Cadavers exhibiting beginning or advanced putrefaction were also excluded. The iliopsoas muscle was chosen because it is barely affected by physical exercise and thus is ideally suited to minimize the possible effects of individual differences in fitness on mtDNA deletion. The subjects were divided into groups according age at death, with approximately equal numbers of individuals representing each decade of life from 0–10 to 71–80 years and over 80 years old.

To check for possible PCR inhibitors and to determine the total amount of mtDNA for subsequent amplification of the 4977 bp deletion, the 533 bp fragment of undeleted mtDNA in each sample was amplified with 1 ng total DNA. This indirect approach for comparing results in different samples was necessary since mtDNA cannot yet be quantified by a slot-blot method. The primer pair L35/H35, which span the site of the deletion, was then used to demonstrate the 4977 bp deletion. If the deletion was present, the deletion specific 667 bp signal was found, if there was no deletion, the Taq polymerase was unable to amplify the entire 5644 bp sequence between the two primers under the PCR conditions described below.

DNA extraction

Total DNA was isolated from 100 mg skeletal muscle utilizing a DNA extraction kit (Immucor, FRG) according to the manufacturer's instructions. The amount of DNA was first measured photometrically and then precisely quantified with a human-specific slot-blot technique (Gibco BRL, FRG).

PCR

Primers were synthesized by MWG Biotech (FRG) using nucleotide sequences obtained from the literature (Anderson et al. 1981). The 5-nucleotide extended primer pair (L15: nucleotide position 3304; 5′-AACATACCCATGGCCAACCTCCTAC-3′ and H15: nucleotide position 3836; 5′-GGCAGGAGTAATCAGAGGTGTTCTT-3′ was used to amplify the 533 bp fragment from undeleted mtDNA, the extended primer pair L35 (nucleotide position 8285; 5′-CTCTAGAGCCCACTGTAAAGCTAAC-3′) and H35 (nucleotide position 13928; 5′-CTAGGGTAGAATCCGAGTATGTTGGAGAA-3′) to amplify the deletion-specific 667 bp fragment.

Specific mitochondrial fragments were amplified in a 25 µl reaction mix containing 400 pM for each primer, 200 µM for each dNTP, 1 U Taq polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 8.4) and 1.2 mM $MgCl_2$. Samples were amplified in a Perkin Elmer GeneAmp thermocycler 2400 (Norwalk, USA) using 40 cycles with 40 s denaturation at 94° C, 40 s annealing at 65° C and 50 s extension at 72° C, with a final extension for 7 min at 72° C. The high annealing temperature of 65° C produced the best signal-to-noise ratio whereas lower temperatures led to an increase of unspecific amplification products.

Detection system

Of the amplification product 7 µl was separated on ultrathin polyacrylamide gels (7.5% C/2.5%T, 60 mM formate) at 1000 V/50 mA and 20 W for 45–55 min. Each run was stopped when the bromphenol blue line had advanced 8–9 cm. Visualization was done with silver staining (Allen et al. 1989).

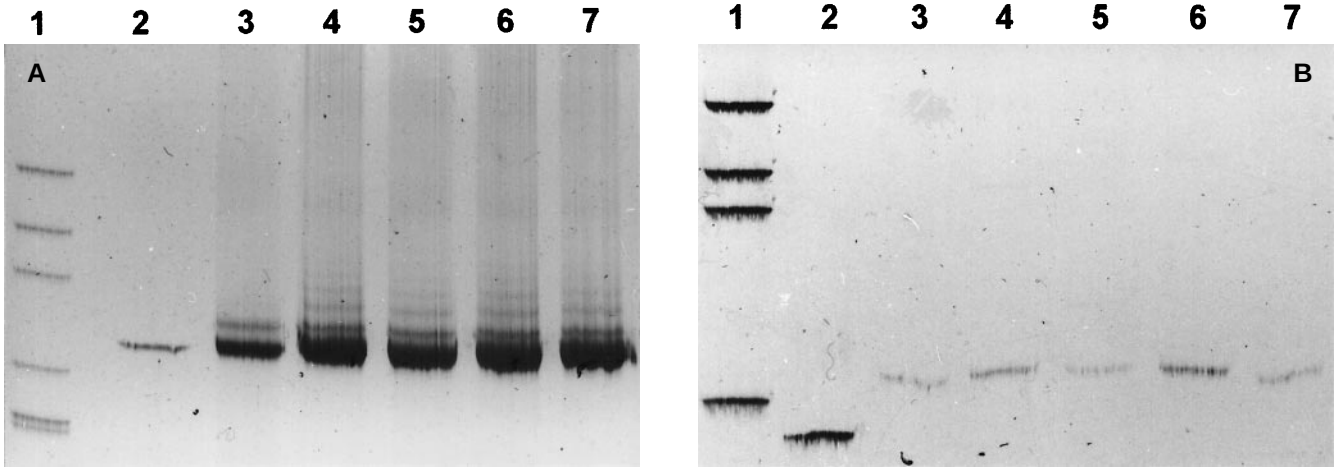

Fig. 1 A, B Amplification of mitochondrial DNA fragments from 1 ng total DNA (mitochondrial DNA and nuclear DNA) extracted from skeletal muscle tissue. Polyacrylamide gel electrophoresis (PAGE) following silver staining. **A** Amplification of the 533 bp fragment of the undeleted mtDNA. *Lane 1* PhiX174/HinfI size standard; *lane 2* 500 bp size standard (5 ng); *lanes 3–7* PCR products of the intact mitochondrial DNA from 5 individuals over 70 years of age. **B** Amplification of the 667 bp fragment of the 4977 bp deleted mtDNA. *Lane 1* Phi X174/Hae III size standard; *lane 2* 500 bp size standard (5 ng); *lanes 3–7* PCR products of the 4977 deleted mtDNA from the 5 persons shown in Fig. 1A

Table 1 Detection of the 4977 bp deleted mtDNA. Shown are the number of persons in each age group in which the listed template DNA amounts could detect the deletion specific signal. A correlation between age and template amount is evident

Group	Age in years	Number of samples	Total DNA concentration (ng)						
			1000	500	100	50	10	5	1
1	0–10	10	0	0	0	0	0	0	0
2	11–20	5	0	0	0	0	0	0	0
3	21–30	15	13	12	12	10	0	0	0
4	31–40	9	9	9	7	6	1	0	0
5	41–50	9	9	9	7	7	6	2	0
6	51–60	13	13	13	13	12	11	7	4
7	61–70	13	13	13	13	13	12	9	6
8	71–80	8	8	8	8	8	8	8	8
9	> 80	11	11	11	11	11	11	11	11

Results

The deletion-specific 667 bp fragment could be demonstrated in 75 of the 93 samples studied. The amount of template DNA required to obtain the specific signal was found to decrease with age. The 4977 bp deletion could not be demonstrated in individuals younger than 20 years old despite the application of 1000 ng total DNA. In subjects aged 20 to 30 years, the deletion was detected with 1000 ng total DNA in 13 out of 15 cases. In all individuals over 70 the specific 533 bp and 667 bp signals were obtained using only 1 ng total DNA (Fig. 1A, B). Although the borders between the age groups overlapped, a clear correlation was evident between age and the quantity of 4977 bp-deleted mtDNA (Table 1). If the same amount of total mtDNA was applied in individuals of different ages, the strength of the signal increased in direct proportion to age (Fig. 2). The prerequisite for this was that the starting amount of total mtDNA was the same in all subjects, as estimated by the previous amplification of the 533 bp fragment.

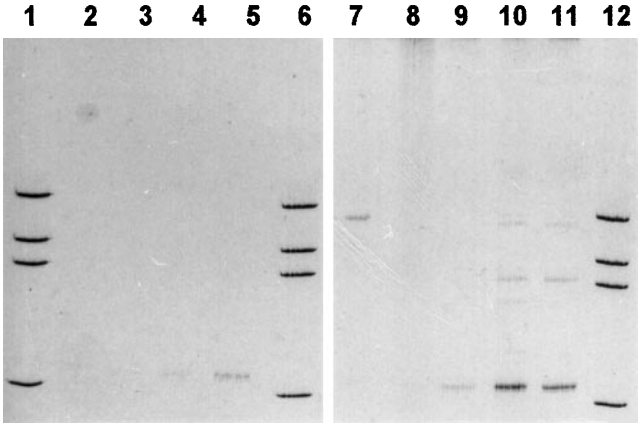

Fig. 2 Age-dependent accumulation of the 4977 bp deleted mtDNA. PAGE after silver staining. Amplification of the deletion specific 667 bp fragment according to increasing age. *Lane 1* PhiX174/HaeIII size standard; *lane 2* 10 ng total DNA from a 45-year-old subject; *lane 3* from a 54-year-old; *lane 4* from a 67-year-old; *lane 5* from an 80-year-old; *lane 6* PhiX174/HaeIII size standard; *lane 7* 100 ng total DNA from a 37-year-old subject; *lane 8* from a 45-year-old; *lane 9* from a 54-year-old; *lane 10* from a 67-year-old; *lane 11* from an 80-year-old; *lane 12* PhiX174/HaeIII size standard

Discussion

Specimens from 93 skeletal muscles (m. iliopsoas) obtained at autopsy from individuals of different ages were studied. To our knowledge, this is the first time that autopsy material from such a large number of individuals has been studied for the 4977 bp deletion; the largest previous study examined 59 muscle biopsies (Lee et al. 1994). Like other authors (Arnheim and Cortopassi 1992; Lee et al. 1994) we found a correlation between the level of 4977 bp deletion in skeletal muscle and age: the older the individual, the less template DNA needed for PCR in order to obtain the same deletion-specific fragment.

The data presented here provide a firm foundation for establishing a more precise method for determining age based on the level of deleted mtDNA. They already enable a rough estimation of age based on PCR amplification of mtDNA. A template of 1 ng total DNA demonstrating the 4977 bp deletion would suggest that the individual is over 50 years old. No detection of the 4977 bp deletion would be suggestive of an individual less than 20 years of age. It remains for future studies on specimens from individuals aged between 10 and 20 years to determine the age at which deleted mtDNA first appears.

At present only a few endogenous and exogenous factors are known which could influence the level of deleted mtDNA. Among the principal endogenous factors are the various degenerative processes, such as Parkinson's disease and cardiomyopathies in which the proportion of deleted mtDNA is elevated as compared with age-matched controls (Ikebe et al. 1990; Li et al. 1995). Other groups have detected increased deletion in persons with chronic hypoxic damage to heart muscle (Corral-Debrinski et al. 1991) and the brain (Merril et al. 1996). However, it is not yet known, however, whether the mtDNA deletion is a consequence of the underlying disease process or a cause of it. Possible distortions arising from physical exercise or from systemic disease were largely excluded in the present study. In order to determine the

physiological increase in deleted mtDNA due to cell aging alone, we only examined tissue from individuals who died of an acute or periacute event (e. g. intoxication, drowning). Additional investigations were performed to exclude a major degenerative disease if this could not be confirmed by macroscopic findings alone.

Exposure to sunlight is one exogenous factor that can drastically alter the amount of deleted mtDNA in skin. In the same individual, areas of skin exposed to sunlight can have up to 12.5% deleted mtDNA, compared to only 0.4% in unexposed areas (Pang et al. 1994). The literature contains no studies on other possible exogenous factors that could produce specific deletions in mtDNA, such as ionized rays. Neither sunlight nor ionized rays, however, played a role in our study because the m. iliopsoas is not exposed to them.

All factors capable of distorting the levels of deleted mtDNA cannot be determined until the mechanisms causing mtDNA deletion are better understood.

The samples examined here are currently undergoing more precise quantification by a kinetic PCR method. It is hoped that the data thus obtained can help to establish reference values for the increase in deleted mtDNA during physiological aging in various organs and tissues. Deviations from these age norms could then provide supplemental information for clarifying diseases or causes of death resistent to detection by morphological means alone.

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