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Ageing-associated 5 kb Deletion in Human Liver Mitochondrial DNA

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Using PCR technique and restriction mapping, we analyzed liver mitochondrial DNA(mtDNA) of 2 stillborn babies and 55 Chinese subjects from 27 to 86 years old and blood cell mtDNA from 20 subjects of various ages. An ageing-associated 4,977-bp deletion was detected between nucleotide position 8,469 and 13,447 (or between 8482 and 13460) in the liver mtDNA of older subjects. In the region containing the junction fragment, we observed a 13 bp repeat "ACCTCCCTCACCA". Moreover, the incidence of the deleted mtDNA of each of the study subjects was found to increase with age. The deletion was found in 5 out of 8 patients of the 31-40 age group and 9 out of 11 patients of the 41-50 age group, and in all the patients over 50 years old. The deletion was not observed in either the mtDNA of the liver of the stillbirth or the blood cells of subjects of all the age groups. These results support our previous contention that liver mitochondrial respiratory functions decline with age and the hypothesis that continuous accumulation of mitochondrial DNA mutation is an important contributor to ageing process.

Ageing has been defined as the progressive accumulation of changes associated with or responsible for the ever-increasing susceptibility to diseases and death which accompany advancing age (1). The free radical theory of ageing (1,2) has received much attention in light of the fact that reactive oxygen species can damage biomolecules of fundamental biological importance. Reactive oxygen species such as the superoxide radical (O_2^-),

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ABBREVIATIONS: mtDNA, mitochondrial DNA; PCR, polymerase chain reaction.

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hydrogen peroxide (H_2O_2), hydroxyl radical (OH·), and singlet oxygen can be produced by normal metabolism, irradiation, and a broad spectrum of chemical compounds (3). Moreover, mitochondria are subject to high oxidative stress and are actively involved in biological oxidations. Thus, these reactive oxygen species and semiquinone radicals may be continuously present in the mitochondria (4). It is generally recognized that mtDNA are compact naked DNA with very few, if any, introns (5). In sharp contrast to nuclear DNA, mtDNA is replicated with much higher mutation rate (6), and without proof-reading or DNA repair system (5), and is thus more vulnerable to attack by the above reactive oxygen species and free radicals (4,7). Therefore, the accumulation of these reactive oxygen species and damage to DNA molecules have been suggested and well-received to play an important role in ageing process (3,5,7,9-13).

A specific mutant mtDNA with a 4,977-bp deletion was detected in various tissues of old humans (14-16) and was not observed in fetal tissues. However, it remains unclear as to when the specific deletion in mtDNA starts to appear during the human life-span. Another important issue is that whether there is "threshold effect" of the deleted mtDNA on normal biochemical functions of the cell. To our particular interest is that what is the relationship between our previous observation that liver mitochondrial respiratory functions decline with age and the incidence (or frequency) of the specific deletion of mtDNA in human liver tissues from patients of various ages. Using a combination of polymerase chain reaction (PCR) strategies and restriction mapping, we have tried to answer these questions.

MATERIALS AND METHODS

Collection of Samples

We obtained 35 liver specimens from a previous study (17) and 22 liver specimens in the past one year and 2 stillbirth with

a total of 57 samples. The liver biopsies (0.5-1.0 g) were obtained from patients with non-liver diseases during exploratory laparotomy with the consent of patients. The patients had been screened to exclude liver diseases by clinical, histological, and biochemical criteria (SMA-32 analyses). An aliquot of 1 ml of the blood was collected from patients of various ages in sampling tubes filled with heparin.

Preparation of Human Mitochondrial DNA

Human liver mitochondria were prepared according to the procedure developed in this laboratory (18) with modifications (17). Liver mtDNA was prepared from isolated mitochondria according to the method of Palva and Palva (19). Blood cell mtDNA was prepared from whole blood according the method developed in this laboratory (20).

Oligonucleotide Primers

Two primers encompassing the target gene were chemically synthesized by Promega Corp. (Madison, WI). The nucleotide sequences of the primers L7901-L7920 and H13631-H13650 are the same as those described by Ikebe et al.(14).

Polymerase Chain Reaction (PCR)

The mtDNA (2 ng/μl from mtDNA preparation) was amplified in a 100 μl reaction mixture containing 200 μM of each dNTP, 1 μl of each primer, 0.5 μl (2.5 units) of Taq DNA polymerase (Perkin-Elmer/Cetus), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 1.5 mM $MgCl_2$. To detect the 5.77 kb PCR product from undeleted mtDNA, we performed the amplification cycle of 1 min denaturation at 94°C, 1 min annealing at 56°C, and 5 min extension at 72°C for 30 cycles in a Perkin-Elmer/Cetus thermal cycler. In order to detect the shorter and rarer deleted mtDNA molecules, the extension time was shortened to 45 sec and the rest of the cycle parameters were the same as above. The amplified fragments were analyzed electrophoretically on 0.8% agarose gel and the DNA bands were detected fluorographically after staining with ethidium bromide.

RESULTS

Human cellular mtDNA from the stillbirth, adult liver tissues and blood cells were examined by agarose-gel electrophoresis of the PCR products amplified with 2 specific pairs of primers. By using long PCR cycle time, we detected a product of 5.77 kb with or without the presence of a 773-bp fragment (Fig. 1). This result represents amplification of the region between the primers in undeleted and deleted mtDNA, respectively. When using short PCR cycle time, we only detected a product of the 773-bp fragment in the liver mtDNA's from older individuals (Fig. 2), which represents amplification of the region between the primers in the deleted mtDNA. Moreover, the intensity of this 773-bp band was

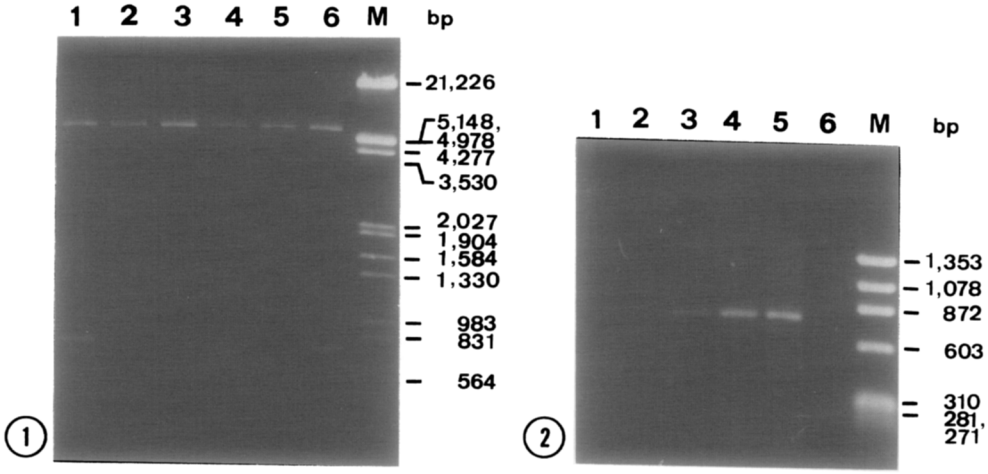

Fig. 1 Electrophoretogram of the 5.77 kb PCR fragment with and without the 4,977-bp deletion in the mtDNA from Chinese subjects of various ages.
Lane 1 and lane 2 represent the mtDNA of the liver and blood cell, respectively, of a 79-year old subject; lane 3 is the liver mtDNA of a 35-year old subject, lane 4 is the liver mtDNA of a 28-year old subject, lane 5 represents the liver mtDNA from a stillbirth, and lane 6 is the liver mtDNA of a 57-year old subject. M represents the lambda DNA digested with Hind III and EcoR I.

Fig. 2 Electrophoretogram of the specific 4,977-bp deletion in the liver mtDNA from Chinese subjects of various ages.
Lane 1 represents the blood cell mtDNA of a 79-year old subject, lane 2 is the liver mtDNA of a stillbirth, lane 3 is the liver mtDNA of a 35-year old subject, lane 4 represents the liver mtDNA of a 57-year old subject, lane 5 is the liver mtDNA of a 79-year old subject, lane 6 is the liver mtDNA of a 28-year old subject. M represents the ϕx174 DNA digested with Hae III.

found to increase with the age of the individual from whom the liver mtDNA was prepared. The 773-bp PCR product from the deleted mtDNA was mapped using 12 restriction enzymes and the results are shown in Fig. 3. In the region expected to contain the junction fragment, we observed a 13-bp repeat "ACCTCCCTCACCA". On one side of this repeat are sequences from the structural gene encoding ATP synthase subunit 8 while on the other side are sequences from the gene coding for subunit 5 of NADH dehydrogenase. Moreover, the incidence of deleted mtDNA of each individuals was found to increase with age. Among the 55 adults, 5 out of 8 in the 31-40 age group and 9 out of 11 in the 41-50 age

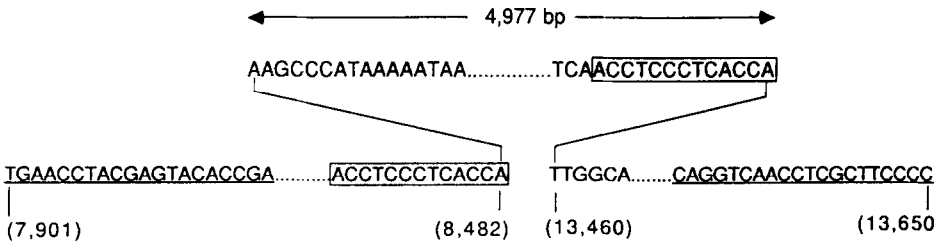

Fig. 3 The flanking sequences near the 4,977-bp deletion sites. The underlined 20-nucleotide sequences at 5' and 3' ends were primers used to amplify the DNA segment encompassing the 4,977-bp deleted region. The 13 bp direct repeats on both ends are boxed. The nucleotide sequences of the undeleted regions (773 bp) near the 13-bp direct repeats were confirmed by restriction mapping with 12 restriction endonucleases. The numbers in the map refer to the published nucleotide sequence of human mitochondrial genome (8).

group displayed the 4,977-bp deletion in their liver mtDNA, and all the 34 subjects over 50 years old exhibited the deletion (Table 1). However, this deletion was not observed in either the stillbirth or the blood samples of all the study subjects including aged populations.

DISCUSSION

Effects of ageing on the respiratory functions and oxidative phosphorylation of the mitochondria have been widely studied (17, 21-24). All the results indicate a decrease in the rate of oxygen consumption and ATP levels in the tissue cells of individ-

Table 1. Presence of the 5 kb-deleted mitochondrial DNA in Chinese subjects of different ages

Age group	Number of patients examined	Number of patients (%) with deleted mtDNA
27-30	2	0 (0)
31-40	8	5 (62.5)
41-50	11	9 (81.8)
51-60	14	14 (100.0)
61-70	14	14 (100.0)
≥ 71	6	6 (100.0)

This table shows that the specific 4,977-bp deletion in mtDNA started to appear in the individuals above 30 years old and consistently appeared in all the subjects over 50 years old.

uals with increasing age. In an animal study, Piko et al. (24) found that the frequency of the mutation (deletion/addition) rate of mtDNA in the rat and mouse was increased during ageing process. Recently, some investigators found a specific 4,977-bp deletion in the mtDNA of old humans (14-16). However, these studies did not include human subjects of intermittent ages. Therefore, we are interested to know whether the deleted mtDNA appears in the middle-age or even young population. The other question is whether the incidence of the deletion is increased with age. Our results demonstrated that the deleted mtDNA starts to appear in about 60% of the study subjects above 30 years old and consistently occurred in all the subjects over 50 years old (Table 1). The proportion of the deleted mtDNA was found to increase with the increase of age as clearly shown by the increasing intensity of the 773-bp band in older individuals (Figs. 1 and 2). However, such deleted mtDNA was not observed either in the liver tissue of the stillbirth or in the blood cells from subjects of all age groups. Moreover, all the patients examined in this study had no history of any of the known mitochondrial diseases. We thus suggest that this specific deletion is an age-dependent event. This is easily rationalized because that neither the liver tissue cells of the stillbirth nor the blood cells from subjects of all ages are "aged" cells. According to the results obtained in this study, the deleted mtDNA starts to appear in the early middle age. However, we still cannot fully explain why the mitochondrial respiratory functions gradually decrease from the middle-age to the older-age individuals. Heteroplasmy and threshold effect of mtDNA may play a very important role (22, 25-27). Since there are hundreds or thousands of mitochondria in any one of the tissue cells, the presence of mutated mtDNA may not cause significant effect on the bioenergetic functions until the mutant/wild-type ratio reaches a critical value. These

effects have been observed in inherited mitochondrial diseases such as Leber's hereditary optic neuropathy (28-30), Kearns-Sayre syndrome (31), and myoclonic epilepsy and ragged-red fiber disease (32). We thus believe that the quantity of the deleted mtDNA in the tissue cells will determine the capability and capacity of respiration and oxidative phosphorylation functions of the mitochondria in the tissue. If the quantity of the deleted mtDNA is in excess of certain percentage of total mtDNA molecules (above the threshold value), the mitochondrial respiratory functions will be significantly impaired leading to irreversible damage of the mitochondria.

It has become an emerging conception that the age-dependent decline of bioenergetic functions of the tissue cells is associated with mtDNA mutation(s) in animals and humans (14-16). In addition to the 4,977-bp deletion, we are looking for some other age-related mtDNA mutations. We believe that ageing is a highly complex biological process, and the 4,977-bp deletion of mtDNA may be only one of the many factors leading to ageing in humans.

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