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Mutations in mitochondrial DNA accumulate differentially in three different human tissues during ageing

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ABSTRACT

In 60 human tissue samples (encompassing skeletal muscle, heart and kidney) obtained from subjects aged from under 1 to 90 years, we used quantitative PCR procedures to quantify mitochondrial DNA (mtDNA) molecules carrying the 4977 bp deletion (mtDNA^{4977}) and 3243 A→G base substitution. In addition, the prevalence of multiple mtDNA deletions was assessed in a semi-quantitative manner. For all three tissues, the correlations between the accumulation of the particular mtDNA mutations and age of the subject are highly significant. However, differential extents of accumulation of the two specific mutations in the various tissues were observed. Thus, the mean abundance (percentage of mutant mtDNA out of total mtDNA) of mtDNA^{4977} in a subset of age-matched adults is substantially higher in skeletal muscle than in heart and kidney. However, the mean abundance of the 3243 A→G mutation in skeletal muscle was found to be lower than that in heart and kidney. Visualisation of arrays of PCR products arising from multiple mtDNA deletions in DNA extracted from adult skeletal muscle, was readily made after 30 cycles of PCR. By contrast, in DNA extracted from adult heart or kidney, amplification for 35 cycles of PCR was required to detect multiple mtDNA deletions. Although such multiple deletions are less abundant in heart and kidney than in skeletal muscle, in all tissue extracts there are unique patterns of bands, even from different tissues of the same subject. The differential accumulation of mtDNA^{4977}, other mtDNA deletions and the 3243 A→G mutation in the three tissues analysed presumably reflects different metabolic and senescence characteristics of these various tissues.

INTRODUCTION

A decline in mitochondrial bioenergetic function in various tissues occurs during human ageing (1,2). This decline has been ascribed to the occurrence and accumulation of mutations in mitochondrial DNA (mtDNA) (3–6). Three different types of mtDNA mutations in human ageing have been reported, namely deletions, short duplications and base substitutions. Deletions are typified by a particular deletion of 4977 bp, denoted mtDNA^{4977} (the so-called 'common' deletion) that arise from sequence excision between two directly repeated 13 bp sequences in mtDNA (7–9). Many other deletions are also encountered in ageing human tissues (10,11). A mutation representing a 260 bp tandem duplication in the D-loop region has been reported (12,13). Base substitution mutations that have been recognised to occur in tissues of senescent individuals include the A to G transition at nucleotide (nt) 3243 (denoted 3243 A→G) (14–19).

In general, it has been found that the deletion mutations of mtDNA, especially mtDNA^{4977}, occur and accumulate in various human tissues with increasing age, including skeletal muscle (7,8,16,19,20), heart (21,22), brain (23,24), lung (25) and skin (26,27). It is not well established whether different tissues do accumulate similar, or different levels of mtDNA^{4977}. A systematic study of different tissues using identical procedures is warranted. Furthermore, the relationship between the base substitutions of mtDNA and age remains unclear. Thus, concerning the occurrence of the 3243 A→G mutation in skeletal muscle, that has been studied by several laboratories, contradictory conclusions were drawn. This mtDNA mutation was reported to accumulate during ageing in human skeletal muscle (14,19,28). In contrast, Pallotti *et al*. (16) obtained data suggesting that the base substitutions at nt 3243 occur sporadically and show no age-association. In another study, the 3243 A→G mutation has been demonstrated to occur sporadically in mtDNA of ageing human skin (18). Further investigations in a wider range of tissues, as well as a greater number of samples, are therefore needed to establish if there is any general pattern in the age-relatedness of occurrence of the 3243 A→G mutation.

In the present study, we have applied quantitative PCR procedures to analyse the occurrence of mtDNA^{4977} (19) and the 3243 A→G base substitution (17) in three different human tissues, namely, skeletal muscle, heart and kidney, as a function of age. In addition, we have used a semi-quantitative PCR procedure to examine the occurrence of multiple deletions of mtDNA in these three tissues. In the quantitative PCR-based assays used, we define *incidence* as the frequency of occurrence of detectable PCR products resulting from the relevant mutation, and *abundance* as the quantified level of that mutation (expressed

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as percentage of mutant mtDNA out of total mtDNA). The results clearly indicated that both mtDNA^{4977} and molecules bearing the 3243 A→G mutation accumulate with age in each of skeletal muscle, heart and kidney. A differential accumulation of mtDNA mutations in the three tissues was observed, in terms of the quantified levels of mtDNA^{4977} (and the estimated relative abundance of multiple deletions), as well as the quantified levels of mtDNA bearing the 3243 A→G mutation.

MATERIALS AND METHODS

Human tissues and DNA

Portions of skeletal muscle, heart and kidney were obtained postmortem from human subjects. These were kindly provided by the Victorian Institute of Forensic Pathology. The study was approved by the Standing Committee on Ethics in Research on Humans of Monash University. In this study, a total of 60 tissue samples were obtained from subjects of ages ranging from 1 h to 90 year old, and comprised 22 samples of skeletal muscle, 20 of heart and 18 of kidney. Total cellular DNA was extracted from ~100 mg of each tissue sample as described (8).

Recombinant plasmids, oligonucleotide primers and PCR conditions

The recombinant plasmids used as experimental standards, the oligonucleotide primers and PCR conditions were described by Zhang *et al*. (19) for quantifying mtDNA^{4977} and by Liu *et al*. (17) for quantifying the 3243 A→G mutation. The nomenclature of primers is according to Vaillant and Nagley (29).

Detection and quantification of mtDNA^{4977}

The procedure to detect and quantify mtDNA^{4977} is detailed by Zhang *et al*. (19). Briefly, the level of mutant and total mtDNA molecules were measured using an external standard, namely pCZ21 (as reference plasmid) which contains a 773 bp segment of the mtDNA (nt 7901–13650) bearing the breakpoint of the 4977 bp deletion between nt 8470 and 13447. The 773 bp insert in pCZ21 also carries a DNA sequence common to both mutant and normal mtDNA. Two PCR tests were involved. In the first PCR, the level of total mtDNA molecules in a given input of total cellular DNA was determined, while the second PCR enabled measurement of the level of mtDNA^{4977} in the same amount of total cellular DNA. The levels of mtDNA^{4977} and total mtDNA were determined by comparing the band intensities of the PCR products from tissue DNA with those of an appropriate series of inputs of standard pCZ21 DNA. The intensities of the ethidium bromide-stained DNA bands were analysed, after laser densitometry of photographs of the gels, using a MicroComputer Imaging Device, Imaging Research Inc. The abundance of mtDNA^{4977} in the sample (expressed as the percentage of total mtDNA) is determined from the ratio of the levels of mtDNA^{4977} and total mtDNA. The sensitivity of the procedure enables the detection of levels of mtDNA^{4977} as low as 0.0001% of total mtDNA (19).

Detection of multiple mtDNA deletions in various tissues

The detection of multiple mtDNA deletions was carried out by PCR using the primer pair L7901[20] and H16540[27], whose binding sequences are 8.64 kb apart from each other on the mtDNA genome. For semi-quantitative analysis, each sample was adjusted to have the same input of total mtDNA molecules for PCR, on the basis of data obtained by the procedure outlined in the previous section (first PCR) to determine the amount of mtDNA in a given input of total cellular DNA. The conditions of amplification to detect multiple deletions are described in detail by Zhang *et al*. (10), and involved either 30 or 35 cycles of PCR.

Detection and quantification of the 3243 A→G mutation in mtDNA

The procedure based on allele-specific polymerase chain reaction (AS-PCR) is detailed by Liu *et al*. (17). Briefly, two recombinant plasmids containing a 1407 bp DNA segment (nt 2322–3728) were employed as standard DNA templates in PCR (15). pCZ1 carries the normal DNA (A at nt 3243) and pCZ2 carries the mutant DNA (G at nt 3243). The oligonucleotide primers (normal-specific and mutant-specific) used are described by Liu *et al*. (17). The AS-PCR conditions are optimised such that the mutant mtDNA, which differs from the normal mtDNA by a single base pair, is selectively amplified in a high background of normal mtDNA.

In order to carry out the quantitative AS-PCR analysis, two different types of PCR reaction were carried out (17). The first involved conventional PCR, using normal-specific primer (H3284[42]T) in order to standardise the mtDNA content in each tissue sample. The reference plasmid was pCZ1 carrying a normal mtDNA segment (A at nt 3243). The concentration of DNA in each tissue sample was adjusted such that each has the same input of mtDNA templates for the subsequent AS-PCR. The second PCR reaction (AS-PCR) involved the use of mutant-specific primer (H3284[42]C) and the reference plasmid pCZ2 carrying a mutant mtDNA segment (G at nt 3243), to detect and quantify the 3243 A→G mutation in mtDNA. To determine the abundance of the mutant mtDNA, the yields of the PCR product from the tissue DNA were compared densitometrically (as above) with those from the standards. The sensitivity of the procedure enables the detection of levels of mtDNA carrying the 3243 A→G mutation as low as 0.01% of total mtDNA (17).

Statistical analyses of correlation coefficients

Statistical analyses were carried out using the Microsoft Excel (5.0) statistical package. The correlation between the abundance of each mutation and the age of the subject was established by regression analysis, plotting the natural logarithm of the abundance as a function of age. For those samples where the mutation was not present at detectable levels, the random number generation system of Excel was used to generate arbitrary abundance values between zero and the observed limit of detection. To accommodate possible slight variations between different sets of randomly generated numbers for a particular mutation, the mean correlation coefficient r was obtained by taking the average of r values from 10 independent sets of randomly generated sub-threshold values.

RESULTS

Incidence and abundance of mtDNA^{4977} in human tissues

The incidence and abundance of mtDNA^{4977} in the three human tissues were analysed, using two PCR procedures to determine separately the total mtDNA and mutant mtDNA molecules, in a given input of total cellular DNA (19).

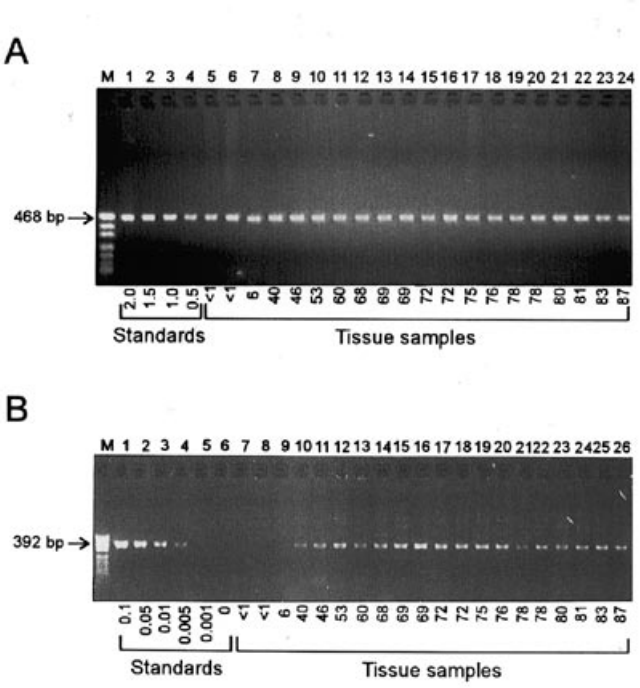

Figure 1. Quantification of mtDNA^{4977} by PCR in DNA extracted from heart tissues. (**A**) Measurement of total mtDNA content in heart samples using primers L7901[20] and H8368[25]. Plasmid pCZ21 was used as external standard, such that lanes 1–4 contain PCR products amplified from 2.0, 1.5, 1.0 and 0.5 ng, respectively, of pCZ21. Lanes 5–24 contain PCR products amplified from DNA in extracts of heart tissues from the relevant 20 subjects aged under 1–87 years (indicated underneath the gel), respectively. (**B**) Measurement of deleted mtDNA in the same set of heart DNA samples using primers L8282[24] and H13650[20]. Plasmid pCZ21 was used as external standard such that lanes 1–6 contain PCR products amplified from 0.1, 0.05, 0.01, 0.005, 0.001 and 0 pg, respectively, of pCZ21. Lanes 7–26 contain PCR products amplified from 20 samples of DNA extracts from heart tissues as in (A). Lanes marked M contain DNA markers (pUC19 digested with *Hpa*II).

Figure 1 illustrates the detection and quantification of mtDNA^{4977} in 20 heart samples at different ages. The quantity of total mtDNA molecules at a given input of total cellular DNA was first measured (Fig. 1A). The band intensities of the PCR products generated from those heart samples (lanes 5–24) and the reference (pCZ21) inputs (lanes 1–4) were compared. This was followed by a second PCR to measure the mtDNA^{4977} molecules within the same input of total cellular DNA (Fig. 1B). The abundance of mtDNA^{4977} in a given sample was expressed as the percentage of mtDNA^{4977} out of total mtDNA. The same procedures were also carried out to detect and quantify the abundances of mtDNA^{4977} in 22 skeletal muscle and 18 kidney samples at different ages (PCR products in gels not shown).

By this means, it was possible to detect mtDNA^{4977} in most samples from adults, but not from samples of infants and young children (Table 1). The calculated abundances of mtDNA^{4977} in all samples are listed in Table 1. The overall abundance of this particular deletion is generally much higher in skeletal muscle (ranging from 0.015 to almost 0.3% of mtDNA) than in heart (range, 0.0003–0.004%) and kidney (range, 0.0001–0.001%) (Table 1).

Table 1. Detection and quantification of mtDNA^{4977} in different human tissues[a]

Sample number	Age (years)[b]	Tissue		
		Muscle	Heart	Kidney
1	1 h	ND	ND	–
2	5 weeks	ND	–	ND
3	3 months	–	ND	ND
4	5	ND	–	ND
5	6	–	ND	–
6	23	ND	–	–
7	24	0.03	–	–
8	27	ND	–	ND
9	37	0.015	–	0.0005
10	40	0.0355	0.0005	0.00012
11	44	0.044	–	0.00012
12	45	0.293	–	–
13	46	–	0.0008	0.00019
14	50	0.0355	–	0.0001
15	53	0.189	0.0009	–
16	55	0.046	–	0.00022
17	60	–	0.00036	–
18	60	0.116	–	0.00041
19	62	0.03	–	0.00041
20	63	0.151	–	0.0001
21	68	–	0.0019	–
22	69	–	0.002	–
23	69	0.042	0.004	0.0002
24	72	–	0.0017	–
25	72	0.03	0.0008	0.00023
26	75	–	0.00092	–
27	75	ND	–	0.00012
28	76	–	–	0.001
29	76	0.086	0.0012	–
30	78	–	0.00026	–
31	78	–	0.00047	–
32	80	–	0.00034	–
33	81	–	0.00044	–
34	83	–	0.00044	–
35	84	0.08	–	–
36	87	–	0.00088	–
37	90	0.257	–	0.00021

[a]Results are expressed as percentage of mutant mtDNA out of total mtDNA.
[b]Unless indicated explicitly otherwise.
ND indicates not detectable; – indicates tissue not available.

In order to test the relationship of the abundance of mtDNA^{4977} with age, the natural logarithm of abundance of the 4977 bp deletion was plotted graphically against the ages of the samples in each tissue type (Fig. 2A–C). The correlation coefficients (r) and probability (P) values of the regression analysis indicated that mtDNA^{4977} was accumulated in skeletal muscle, heart and kidney tissues in an age-related manner. The regression analyses of ln(abundance) against age of all three tissues show a clear positive correlation ($P < 0.005$) indicating an exponential accumulation of mtDNA^{4977} with age in skeletal muscle, heart and kidney tissues.

It is recognised that in our sample set of subjects, there are relatively few intermediate ages between those of the first decades of life corresponding to non-detection of mtDNA^{4977},

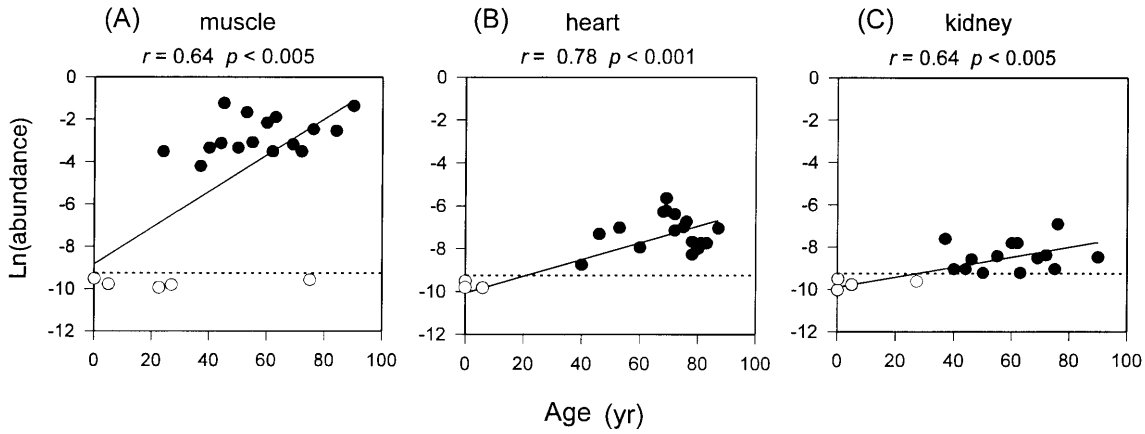

Figure 2. Age-associated accumulation of mtDNA^{4977} in skeletal muscle, heart and kidney tissues. The natural logarithm of abundance of the mutation in skeletal muscle, heart and kidney tissues (as indicated for each panel) was plotted as a function of the age of the subjects (data taken from Table 1). Closed circles represent quantified abundance for the mutation above the limit of detection; open circles indicate samples showing no detectable levels of that mutation. To permit calculation of regression lines (solid lines), data represented by open circles have been denoted a value (between zero and the detection limit) generated randomly (see Materials and Methods). One such set of the 10 used for computation is represented here. Computations yielded the correlation coefficient *r* and the probability *P* value (which are indicated above each panel). The dashed lines represent the threshold of detection of the mutation.

and those of individuals in their fifth decade and above corresponding to the higher levels of that deletion. It would have been desirable for a greater number of samples to be representative of this intermediate interval but human tissue samples in these age groups are relatively difficult to obtain. Nevertheless it is useful to compare the slopes of the regression lines that we were able to obtain for each tissue.

The slopes of the regression lines are different from one another in different tissues. This suggests differences in the rate of accumulation of mtDNA^{4977} in different tissues leading to relative differences in abundance of mtDNA^{4977} in such tissues. On this basis it appears that in skeletal muscle mtDNA^{4977} accumulated at a markedly faster rate than for heart and kidney. A more substantive statistical analysis of the abundance data was undertaken by comparing the mean abundance of mtDNA^{4977} in each of these three tissues, using a subset of adult samples with optimal age matching (to minimise any effects of age, as such, on the mean abundance). The chosen age range for this comparative analysis encompassed subjects from ages 40 to 76 inclusive (n = 13, 11 and 12 for skeletal muscle, heart and kidney, respectively). Using the Student's *t*-test to compare this selected age range between any particular pair of tissues, it was found that there are no significant age differences from one another ($P > 0.3$) in the three subsets of samples used in this analysis. However, by the Student's *t*-test, the mean abundance of mtDNA^{4977} was found to be significantly higher in skeletal muscle than in heart ($P < 0.005$) and in kidney ($P < 0.002$). Moreover, the overall abundance of mtDNA^{4977} in heart is also significantly higher than in kidney ($P < 0.002$). This indicates that the relative extent of accumulation of mtDNA^{4977} is highest in skeletal muscle, intermediate in heart and lowest in kidney. These results clearly indicate the differential accumulation of mtDNA^{4977} in human skeletal muscle, heart and kidney tissues.

Semi-quantitative analysis of multiple deletions in mtDNA in the three tissues

In addition to mtDNA^{4977}, the occurrence of multiple mtDNA deletions in the three different tissue types was also studied (10,19). Semi-quantitative analysis of the relative incidence and abundance of multiple deletions in the three tissue types was possible because a standardised input of total mtDNA molecules from each tissue sample was used for PCR.

Using the widely spaced primer pair encompassing 8.64 kb in the long arc between the two origins of replication, multiple PCR bands, each inferred to represent a specific mtDNA deletion, were readily seen in most of the adult skeletal muscle aged over 40 after 30 cycles of PCR (Fig. 3A, lanes 8–22). No PCR products could be seen in skeletal muscle samples aged below 40 (Fig. 3A, lanes 1–7). Each individual sample gave rise to a unique band pattern, indicating the randomness of generation of mtDNA deletions during ageing. However, no PCR products could be seen in any heart (Fig. 3B, lanes 1–20) or kidney (Fig. 3C, lanes 1–18) tissues, irrespective of age, after PCR under the same conditions (30 cycles) as used for skeletal muscle tissues. This suggests that the abundance of multiple mtDNA deletions is considerably lower in heart and kidney than in skeletal muscle. Nevertheless, at an increased PCR cycle number of 35, using the widely spaced primer pair, multiple PCR bands could be seen in most adult tissues of heart (Fig. 3D) and kidney (Fig. 3E), but again not in those of infants and young children. Unique patterns of products of multiple deletions were observed in adult heart and kidney, much like that of skeletal muscle but at lower abundance. Moreover, different tissue samples obtained from the same subject also gave rise to distinct band patterns (described in more detail below).

Incidence and abundance of the 3243 A→G mutation in mtDNA of human tissues

The incidence and abundance of the 3243 A→G mutation in the same sets of human tissues were determined by quantitative AS-PCR (17). Figure 4 illustrates the detection and quantification of mtDNA carrying the 3243 A→G mutation in the 20 heart samples at different ages. Each sample was first standardised, by PCR amplification using normal-specific primer, to contain mtDNA in copy number equivalent to 0.1 ng of pCZ1 (Fig. 4A). This was followed by AS-PCR to amplify selectively, using mutant-specific primer, the 3243 A→G mutant mtDNA within the same DNA input. By comparison of the intensities of the 459 bp

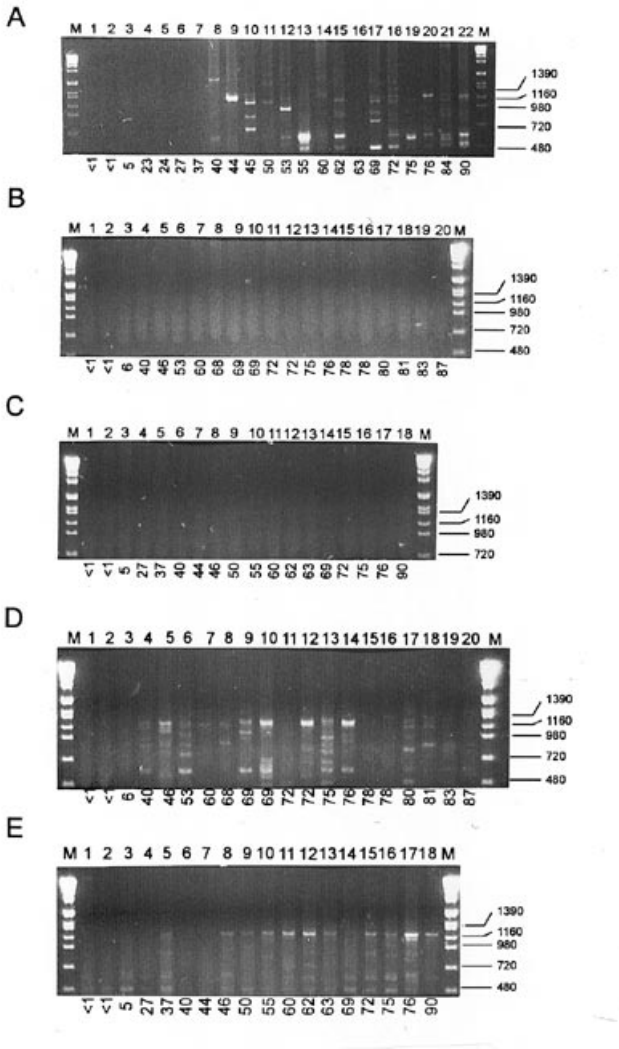

Figure 3. Detection of age-related multiple mtDNA deletions in various human tissues. Detection of multiple mtDNA deletions was carried out using primers L7901[20] and H16540[27] on samples standardised to same input of total mtDNA (cf. Fig. 1A). (**A**) Lanes 1–22 contain PCR products amplified for 30 PCR cycles from skeletal muscle DNA samples. Similar PCR was also carried out on the heart (**B**) and kidney (**C**) tissues under the same conditions (30 cycles of PCR) as for skeletal muscle. PCR was carried out on the same sets of heart (**D**) and kidney (**E**) tissue extracts but for 35 cycles of PCR. Lanes marked M contain DNA markers (SPP1 digested with *Eco*RI). The sizes of some marker bands are indicated (bp). The ages of subjects (year) from whom samples were taken are indicated underneath each gel.

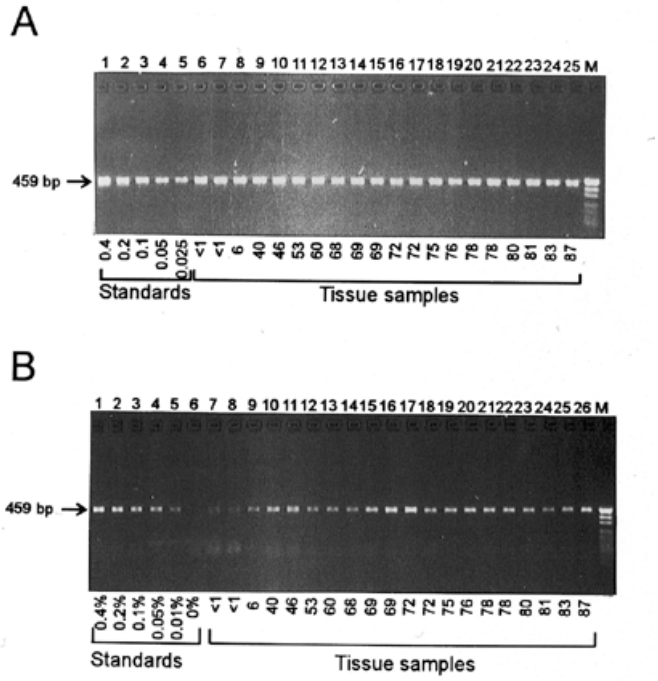

Figure 4. Detection and quantification of the 3243 A→G mutation in mtDNA of heart samples by allele-specific PCR. (**A**) Standardisation of mtDNA input by conventional PCR. PCR was carried out using L2826[24] and H3284[42]T (normal-specific primer) at an annealing temperature of 56°C for 20 cycles. Lanes 1–5 contain PCR products amplified from 0.4, 0.2, 0.1, 0.05 and 0.025 ng of pCZ1, respectively. Lanes 6–25 contain PCR products amplified from 20 samples of DNA in extracts of heart tissues from subjects aged under 1–87 years (indicated underneath the gel), respectively. The mtDNA input of each sample had been standardised to be equivalent in copy number to 0.1 ng of pCZ1. (**B**) Quantification of the 3243 A→G mutation in mtDNA of heart samples by allele-specific PCR. PCR was carried out using L2826[24] and H3284[42]C (mutant-specific primer) at a discrimination temperature of 72.5°C for 35 cycles. Lanes 1–6 contain reference mixtures of normal (pCZ1) and mutant (pCZ2) plasmids (at a total 0.1 ng DNA) at the following percentages of mutant plasmid: 0.4, 0.2, 0.1, 0.05, 0.01 and 0, respectively. Lanes 7–26 contain PCR products amplified from the 20 samples of DNA extracts from heart tissues as in (A). Lanes marked M contain DNA markers (pUC19 digested with *Hpa*II).

PCR products raised from tissue samples (Fig. 4B, lanes 7–26) with those of the products from a series of mixtures of the mutant (pCZ2) and normal (pCZ1) plasmids containing different amounts of mutant plasmid (Fig. 4B, lanes 1–6), the proportions of the 3243 A→G mutation in heart samples were determined. The same procedures were also carried out to detect and quantify the abundances of the 3243 A→G mutation in 22 skeletal muscle and 18 kidney samples at different ages (PCR products in gels not shown).

In all the samples tested, it was possible to detect and quantify the 3243 A→G mutation, above the detection limit of 0.01% of total mtDNA (Table 2). Such global incidence at any ages within these tissue samples should be contrasted with the lower incidence of mtDNA^{4977}, which is mostly not detectable in tissues from infants and young children (Table 1). The quantified abundances of 3243 A→G mutation (Table 2) found in infants or young children (ranging from 0.01 to 0.025% of total mtDNA) are in general lower than those in adults (ranging from 0.05 to 0.75%). In order to test the relationship between abundance and age, the natural logarithm of abundance of the 3243 A→G mutation was plotted graphically against the ages of the samples in each tissue type (Fig. 5A–C). The regression analysis of ln(abundance) against age of all three tissues show a clear positive correlation ($P < 0.01$) in each case, indicating an exponential accumulation with age of mtDNA molecules carrying the 3243 A→G mutation in human skeletal muscle, heart and kidney tissues.

Bearing in mind the same caveat as considered above for the regression lines for mtDNA^{4977} accumulation, in terms of the relative paucity of samples in the second to fourth decades of life, the regression plots for the 3243 A→G mutation gave different slopes for samples of different tissues. This indicates that, as in the case of mtDNA^{4977}, there is a possibility of differential accumulation of this base substitution mutation in different tissues. Applying statistical analysis of the mean abundance of the

3243 A→G mutation on an age-matched subset of samples (aged 40–76 years, inclusive) as for mtDNA^{4977}, the following results were obtained. By Student's *t*-test, the mean abundance of the 3243 A→G mutation in adult skeletal muscle is just marginally lower than in heart ($P < 0.1$), but significantly lower than in kidney ($P < 0.02$). Kidney and heart are indistinguishable by this test ($P > 0.5$). Note that in skeletal muscle, however, mtDNA^{4977} is far more abundant than in heart or kidney (see above).

Table 2. Detection and quantification of the 3243 A→G mutation in mtDNA of different human tissues[a]

Sample number	Age (years)[b]	Tissue		
		Muscle	Heart	Kidney
1	1 h	0.01	0.01	–
2	5 weeks	0.025	–	0.01
3	3 months	–	0.01	0.01
4	5	0.025	–	0.01
5	6	–	0.025	–
6	23	0.01	–	–
7	24	0.07	–	–
8	27	0.06	–	0.025
9	37	0.4	–	0.05
10	40	0.1	0.05	0.1
11	44	0.03	–	0.025
12	45	0.06	–	–
13	46	–	0.065	0.1
14	50	0.06	–	0.6
15	53	0.06	0.025	–
16	55	0.05	–	0.07
17	60	–	0.037	–
18	60	0.07	–	0.06
19	62	0.065	–	0.06
20	63	0.08	–	0.1
21	68	–	0.037	–
22	69	–	0.1	–
23	69	0.05	0.59	0.75
24	72	–	0.365	–
25	72	0.05	0.75	0.65
26	75	–	0.062	–
27	75	0.05	–	0.25
28	76	–	–	0.5
29	76	0.1	0.196	–
30	78	–	0.103	–
31	78	–	0.35	–
32	80	–	0.135	–
33	81	–	0.053	–
34	83	–	0.038	–
35	84	0.09	–	–
36	87	–	0.085	–
37	90	0.25	–	0.1

[a]Results are expressed as percentage of mutant mtDNA out of total mtDNA.
[b]Unless indicated explicitly otherwise.
– indicates tissue not available.

Comparison of the relative levels of mtDNA mutations in various tissues of same subjects

To illustrate further the differential occurrence and accumulation of mtDNA mutations in human tissues, direct comparisons were made of the band intensities of the PCR products obtained from DNA samples of the three sets of skeletal muscle, heart and kidney tissues available from three individual subjects (those aged 40, 69 and 72 years).

These results first serve to indicate the reproducibility of the results already presented above. Second, this comparison makes the point that the generalised relative abundance of particular mtDNA mutations in specific tissues are often reflected in tissue characteristics of individual subjects.

Figure 6 shows the relative intensities of the PCR products generated from each sample when detecting the mtDNA^{4977}, multiple deletions and 3243 A→G mutation. In Figure 6A, for mtDNA^{4977}, the abundance of amplified products from skeletal muscle (lanes 1, 4 and 7) is clearly greater than that from heart (lanes 2, 5 and 8) and kidney (lanes 3, 6 and 9). Multiple band patterns (Fig. 6B) could only be seen after 30 cycles of PCR in skeletal muscle (lanes 1, 6 and 11) but not in heart (lanes 2, 7 and 12) or kidney (lanes 4, 9 and 14). Multiple bands were, however, observable after 35 cycles of PCR in samples from heart (lanes 8 and 13) and kidney (lanes 10 and 15). Moreover, unique band patterns were observed in the three different tissues of each individual subject. These results are indicative of a gross mosaic pattern (30) of mtDNA deletions in each individual. Concerning the 3243 A→G mutation (Fig. 6C), the abundance of amplified products from skeletal muscle (lanes 4 and 7 at ages 69 and 72, respectively) is clearly much less than that from heart (lanes 5 and 8) and kidney (lanes 6 and 9). However, the levels of this mutation in the tissue samples of the subject aged 40 are close to each other (cf. also Table 2). This is consistent with the marginal differences in abundance of the 3243 A→G mutation measured at the population level between skeletal muscle and heart. Note that there can be wide variation in abundance of mtDNA mutations (both mtDNA^{4977} and 3243 A→G base substitution) which may exceed 10-fold for a specific mutation in a particular tissue (even within the subset of samples aged 40–76 years) as shown in Tables 1 and 2. Therefore, it would not be surprising that within some individuals the relative abundance of mutations is not in accord with the relative means determined at the population level.

DISCUSSION

In this study, we have systematically studied the occurrence of mtDNA^{4977}, multiple mtDNA deletions and the 3243 A→G mutation in tissues obtained from human skeletal muscle, heart and kidney. All of the above mtDNA mutations have been found to occur and accumulate in each of these tissues during human ageing. Significantly, different tissues have accumulated different levels of mtDNA^{4977}, multiple mtDNA deletions and the 3243 A→G mutation during ageing.

Occurrence and accumulation of mtDNA mutations during ageing

It has been generally accepted that mtDNA^{4977} and many other large-scale mtDNA deletions occur and accumulate with age in various human tissues (7,8,16,19–27). Our results in this study are consistent with these observations and extend further to

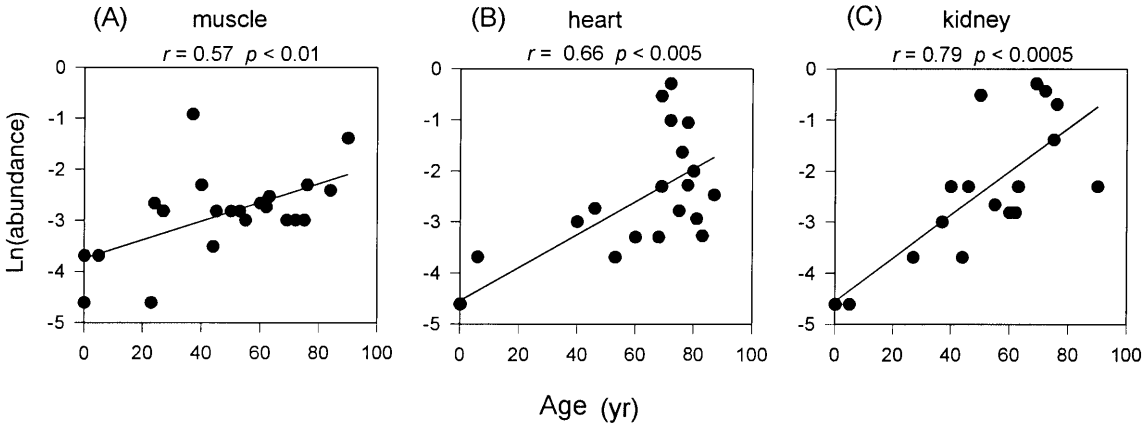

Figure 5. Age-associated accumulation of 3243 A→G mutation in skeletal muscle, heart and kidney tissues. The natural logarithm of abundance of the mutation in skeletal muscle, heart and kidney tissues (as indicated for each panel) was plotted as a function of age of the subjects. Other indications are as for Figure 2.

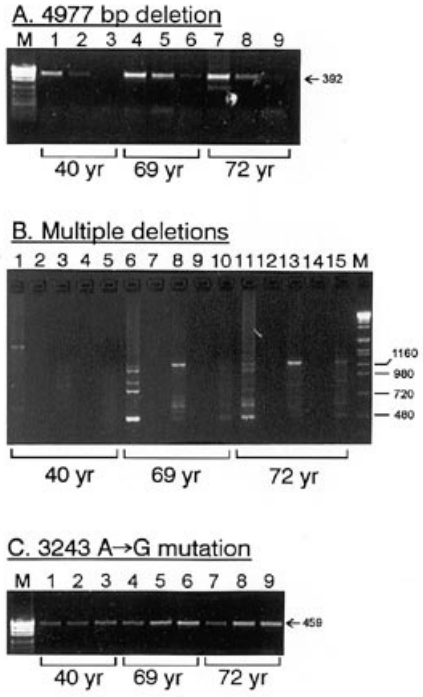

Figure 6. PCR products of deletions and base substitution in mtDNA of different tissues of the same human subjects. PCR products are shown having been generated from DNA samples isolated from the three types of tissues (skeletal muscle, heart and kidney) obtained from three individual subjects aged 40, 69 and 72 years (as indicated below relevant lanes in each panel). (**A**) Detection of the 4977 bp deletion. Lanes 1, 4 and 7, from skeletal muscle; lanes 2, 5 and 8, heart; lanes 3, 6 and 9, kidney. (**B**) Detection of multiple deletions. Lanes 1, 6 and 11, skeletal muscle, 30 cycles of PCR; lanes 2, 7 and 12, heart, 30 cycles of PCR; lanes 4, 9 and 14, kidney, 30 cycles of PCR; lanes 3, 8 and 13, heart, 35 cycles of PCR; lanes 5, 10 and 15, kidney, 35 cycles of PCR. (**C**) Detection of the 3243 A→G mutation. The indications of lanes are as for (A), above. Lanes marked M in (A) and (**C**) contain DNA markers (pUC19 digested with *Hpa*II); the positions of major amplification products are shown. Lane marked M in (**B**) contains DNA markers (SPP1 digested with *Eco*RI); the sizes of some marker bands are indicated (bp).

multiple mtDNA deletions that were also shown to occur in an age-related manner in skeletal muscle, heart and kidney. Strikingly, different tissues obtained from the same subject were found to carry a unique set of multiple mtDNA deletions. This emphasises the independent occurrence of age-related multiple mtDNA deletions in skeletal muscle, heart and kidney, and suggests a similar mechanism of induction of such mutations, although the absolute extent of accumulation differs between tissues.

Our results presented here also clearly indicate that the 3243 A→G mutation occurs and accumulates with age in skeletal muscle, heart and kidney tissues. Previously, contradictory conclusions were drawn about the age-relatedness of the base substitution mutation at the nt 3243 position in skeletal muscle (see Introduction). This discrepancy may be attributed to the fact that only a limited number of samples (13 DNA samples of skeletal muscle) have been analysed in study carried out by Pallotti *et al.* (16). Zhang *et al.* (19) demonstrated a correlation ($P < 0.05$) between the 3243 A→G mutation and age in 18 skeletal muscle samples. The abundance values of the 22 skeletal muscle samples correlate significantly ($P < 0.01$) with age in this study, which further supports the association of this mutation with age in skeletal muscle. In heart and kidney tissues, the abundance of the 3243 A→G mutation strongly correlates with age (heart, $P < 0.005$) (kidney, $P < 0.0005$).

However, in a further human tissue namely skin, the occurrence of the 3243 A→G mutation was found to be sporadic with only 50% (22/44) incidence and without age-association. In contrast, in the same set of skin samples, the occurrence of mtDNA^{4977} was found to be age-related ($P < 0.05$) although the overall incidence is barely above 50% (21/44) (18). The incidence of mtDNA^{4977} is much higher in samples from elderly subjects (18). Moreover, recently, we have studied the occurrence of the 3243 A→G mutation in >100 samples of human lung tissues. Like the case in skin, this mutation occurs sporadically and again without age association (Lim,M., Liu,V.W.S., Zhang,C. and Nagley,P., unpublished data), but age related accumulation of mtDNA^{4977} in a similar set of human lung samples has been reported (25).

In summary, therefore, there is a general phenomenon of age related occurrence and accumulation of mtDNA^{4977} and probably including multiple mtDNA deletions in most human tissues. But this is not the case for the 3243 A→G mutation. The occurrence of this particular mutation may be common in most human tissues, but this mutation does not necessarily accumulate with age in some human tissues during ageing.

Differential accumulation of mtDNA mutations in human tissues during ageing

An important phenomenon observed in this study is the differential accumulation of mtDNA deletion and base substitution mutations in skeletal muscle, heart and kidney. Different tissues do accumulate different levels of deletions and 3243 A→G mutation.

In this study, plus several independent investigations by other groups (20,23,24,26,27), the collective data have given us a clear picture on the differential accumulation of mtDNA^{4977} in various human tissues. The mtDNA^{4977} deletion has occurred much more abundantly in skeletal muscle (this study and reference 20) than many other tissues such as heart (this study), kidney (this study), liver (20), testis (20) and skin (26,27). Both the incidence and abundance of mtDNA^{4977} tend to be higher in those tissues with low mitotic activity and high metabolic rate. However, this general observation has been challenged by a recent study on lung tissues. The relative abundance of mtDNA^{4977} in the lung was found to be ~10 times higher than in skeletal muscle (25). The very specific metabolic function of the lung cells may be the explanation why the abundance of mtDNA^{4977} is higher than those of other energy-demanding tissues such as skeletal muscle, heart and kidney.

Some evidence supports the proposal that the specific function of a particular tissue may induce the occurrence of mtDNA deletions. Two independent studies have found that mtDNA^{4977} increases with age in human brain (23,24). Strikingly, variation of the abundance of mtDNA^{4977} among different anatomical regions of the brain were observed. The abundance of mtDNA^{4977} in putamen (12%) is 2000 times higher than in cerebellum (0.006%) (23). These regional differences in the accumulation of mtDNA^{4977} may be ascribed to different metabolic profiles and functions of the various tissues of the brain, perhaps related to oxidative stress elicited by the activity of monoamine oxidase B, involved in metabolism of certain neurotransmitters (23).

In contrast to mtDNA deletions, the relative abundances of the 3243 A→G mutation are higher in heart and kidney than in skeletal muscle. Furthermore, the abundance of the 3243 A→G mutation in lung is significantly lower than in skeletal muscle, heart and kidney tissues (Lim,M., Liu,V.W.S., Zhang,C. and Nagley,P., unpublished data). Therefore, tissues such as lung and skeletal muscle with relatively higher abundances of mtDNA^{4977} have accumulated lower abundances of 3243 A→G mutation. On the other hand, tissues such as heart and kidney with relatively lower abundances of mtDNA^{4977} have accumulated higher abundances of 3243 A→G mutation. The explanation for this phenomenon is not clear. The different metabolic functions and/or the presence of tissue-specific factors in different tissues may explain the differential accumulation of mtDNA mutations during ageing. Moreover, the different mechanisms of generation of mtDNA mutations may also be a contributing factor. It has been suggested that the deletion mutations are generated through illegitimate recombination (11,31). In contrast, the base substitutions are most probably generated through base modification when damaged in an environment depleted of efficient DNA repair mechanisms. Further investigations on other base substitutions in mtDNA are needed to test the generality of the differential accumulation of mtDNA mutations in tissues during ageing. It will also be necessary to determine the mechanism of generation of deletions and base substitutions in terms of the role of oxidative stress, and to evaluate relevant factors involved in the replication and segregation of mtDNA molecules carrying these mutations in proliferating and post-mitotic tissues.

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REFERENCES

1 Trounce, I., Byrne, E. and Marzuki, S. (1989) *Lancet*, **I**, 637–639.
2 Yen, T.C., Chen, Y.S., King, K.L., Yeh, S.H. and Wei, Y.H. (1989) *Biochem. Biophys. Res. Commun.*, **165**, 994–1003.
3 Linnane, A.W., Zhang, C., Baumer, A. and Nagley, P. (1992) *Mutation Res.*, **275**, 195–208.
4 Wallace, D.C. (1992) *Science*, **256**, 628–632.
5 Wei Y.H. (1992) *Mutation Res.*, **275**, 145–155.
6 Papa, S. (1996) *Biochim. Biophys. Acta*, **1276**, 87–105.
7 Cortopassi, G.A. and Arnheim, N. (1990) *Nucleic Acids Res.*, **18**, 6927–6933.
8 Linnane, A.W., Baumer, A., Maxwell, R.J., Preston, H., Zhang, C. and Marzuki, S. (1990) *Biochem. Int.*, **22**, 1067–1076.
9 Simonetti, S., Chen, X., DiMauro, S. and Schon, E.A. (1992) *Biochim. Biophys. Acta*, **1180**, 113–122.
10 Zhang, C., Baumer, A., Maxwell, R.J., Linnane, A.W. and Nagley, P. (1992) *FEBS Lett.*, **297**, 34–38.
11 Baumer, A., Zhang, C., Linnane, A.W. and Nagley, P. (1994) *Am. J. Hum. Genet.*, **54**, 618–630.
12 Brockington, M., Sweeney, M.G., Hammans, S.R., Morgan-Hughes, J.A. and Harding, A.E. (1993) *Nature Genet.*, **4**, 67–71.
13 Lee, H.C., Pang, C.Y., Hsu, H.S. and Wei, Y.H. (1994) *FEBS Lett.*, **354**, 79–83.
14 Münscher, C., Müller-Höcker, J. and Kadenbach, B. (1993) *Biol. Chem. Hoppe-Seyler*, **374**, 1099–1104.
15 Zhang, C., Linnane, A.W. and Nagley, P. (1993) *Biochem. Biophys. Res. Commun.*, **195**, 1104–1110.
16 Pallotti, F., Chen, X., Bonilla, E. and Schon, E.A. (1996) *Am. J. Hum. Genet.*, **59**, 591–602.
17 Liu, V.W.S., Zhang, C., Linnane, A.W. and Nagley, P. (1997) *Hum. Mutation*, **9**, 265–271.
18 Liu, V.W.S., Zhang, C., Pang, C.Y., Lee, H.C., Lu, C.Y., Wei, Y.H. and Nagley, P. (1998) *Hum. Mutation*, **11**, 191–196.
19 Zhang, C., Liu, V.W.S., Addessi, C.L., Sheffield, D.A., Linnane, A.W. and Nagley, P. (1998) *Hum. Mutation*, (in press).
20 Lee, H.C., Pang, C.Y., Hsu, H.S. and Wei, Y.H. (1994) *Biochim. Biophys. Acta*, **1226**, 37–43.
21 Corral-Debrinski, M., Stepien, G., Shoffner, J.M., Lott, M.T., Kanter, K. and Wallace, D.C. (1991) *J. Am. Med. Assoc.*, **266**, 1812–1816.
22 Hattori, K., Tanaka, M., Sugiyama, S., Obayashi, T., Ito, T., Satake, T., Hanaki, Y., Asai, J., Nagano, M. and Ozawa, T. (1991) *Am. Heart J.*, **121**, 1735–1742.
23 Corral-Debrinski, M., Horton, T., Lott, M.T., Shoffner, J.M., Flint Beal, M. and Wallace, D.C. (1992) *Nature Genet.*, **2**, 324–329.
24 Soong, N.W., Hinton, D.R., Cortopassi, G. and Arnheim, N. (1992) *Nature Genet.*, **2**, 318–323.
25 Fahn, H.J., Wang, L.S., Hsieh, R.H., Chang, S.C., Kao, S.H., Huang, M.H. and Wei, Y.H. (1996) *Am. J. Respir. Crit. Care Med.*, **154**, 1141–1145.
26 Yang, J.H., Lee, H.C., Lin, K.J. and Wei, Y.H. (1994) *Arch. Dermatol. Res.*, **286**, 386–390.
27 Yang, J.H., Lee, H.C. and Wei, Y.H. (1995) *Arch. Dermatol. Res.*, **287**, 641–648.
28 Kadenbach, B., Münscher, C., Frank, V., Müller-Höcker, J. and Napiwotzki, J. (1995) *Mutation Res.*, **338**, 161–172.
29 Vaillant, F. and Nagley, P. (1995) *Hum. Mol. Genet.*, **4**, 903–914.
30 Zhang, C., Liu, V.W.S. and Nagley, P. (1997) *Biochem. Biophys. Res. Commun.*, **233**, 56–60.
31 Shoffner, J.M., Lott, M.T., Voliavec, A.S., Soueidan, S.A., Costigan, D.A. and Wallace, D.C. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7952–7956.