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TECHNICAL NOTE

Detection of age-related duplications in mtDNA from human muscles and bones

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Abstract Several studies have demonstrated the age-related accumulation of duplications in the D-loop of mitochondrial DNA (mtDNA) extracted from skeletal muscle. This kind of mutation had not yet been studied in bone. The detection of age-related mutations in bone tissue could help to estimate age at death within the context of legal medicine or/and anthropological identification procedures, when traditional osteological markers studied are absent or inefficient. As we detected an accumulation of a point mutation in mtDNA from an older individual's bones in a previous study, we tried here to identify if three reported duplications (150, 190, 260 bp) accumulate in this type of tissue. We developed a sensitive method which consists in the use of back-to-back primers during amplification followed by an electrophoresis capillary analysis. The aim of this study was to confirm that at least one duplication appears systematically in muscle tissue after the age of 20 and to evaluate the duplication age appearance in bones extracted from the same individuals. We found that the number of duplications increase from 38 years and that at least one duplicated fragment is present in 50% of cases after 70 years in this tissue. These results confirm that several age-related mutations can be detected in the D-loop of mtDNA and open the way for the use of molecular markers for age estimation in forensic and/or anthropological identification.

Keywords Mitochondrial DNA · Tandem duplications · Heteroplasmic mutation · Aging · Skeletal muscle · Bone

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Introduction

In recent years, various mitochondrial DNA (mtDNA) mutations usually found in patients with neuromuscular diseases or in cancerous tissues have also been found in tissues of healthy elderly individuals [1–6]. According to the mitochondrial theory of senescence, the natural aging process coincides with the appearance of this type of heteroplasmic mutations: An augmentation of the oxidative damages of the mtDNA occurs with the decline in mitochondrial respiratory function and induces an accumulation of nonrepaired lesions on mtDNA on various post mitotic tissues [7–9]. A wide spectrum of these somatic rearrangements including point mutations [1, 10, 11], large scale deletions [10, 12], and tandem duplications [2, 13, 14] have effectively been observed in various tissues like skin fibroblasts, brain, heart, testis, and in the skeletal muscle [6, 15]. The study of these types of somatic mutations is potentially interesting in forensic and/or anthropological identification since they usually occur and accumulate with age in the human subjects. Their detection could thus help to improve the estimation of age at death when the study of classical osteological markers is not conclusive. We undertook to establish whether these mutations are really age-related in bone tissue, by far the most abundant type of biological remains available in anthropological and forensic cases.

In a previous study, we found that one of the most studied point mutations associated with age on muscle tissue (A189G) also accumulates in bone samples of aged individuals [16]. The aim of the present study was to show that the presence of another type of heteroplasmic mutation, the tandem duplications, is also linked with age in human bones. Various types of duplications have been described in the D-loop of the mtDNA, but we chose to use one pair of primers (L336/H335) to detect three main types of mini-duplications frequently described on muscle of aged individuals [14]. The main interest of this pair of primers is that it amplifies the shortest duplications (150, 190, 260 bp). Indeed, we hypothesized that DNA extracted from bones can be partially fragmented, and so we chose to amplify the smallest possible fragments.

The main difficulty of this study was to correctly detect a probably very low rate of heteroplasmy: Due to the multiple copies of mtDNA within cells, when mutated molecules appear, they coexist with wild-type ones and are present in a very low percentage. So in order to easily visualize duplications, we adopted a sensitive "back-to-back" polymerase chain reaction method to screen for the 150-, 190-, and 260-bp duplications [13, 14, 17, 18] in muscle and bone tissues samples. The forward and reverse PCR primers were designed to be located in the same mtDNA region, with their 5′ends, respectively, at nucleotide 336 and nucleotide 335. In this position, the primers did not allow amplification of the wild-type mtDNA but could generate a PCR fragment solely from mitochondrial genomes harboring duplications (Fig. 1).

As only a small percentage of molecules are mutated within cells, the detection of the PCR fragment after separation by electrophoresis on a 2% agarose gel and a UV detection was difficult (very tiny bands could be detected). Thus, in order to more easily visualize duplications, the L336 primer used was 5′-labeled with a flurochrome (FAM). The duplications were then detected after capillary electrophoresis.

Concretely, the main objectives of the present study were (a) to confirm that duplications accumulate in muscle of aged individuals by developing a sensitive and effective technique and (b) to prove that this accumulation is also found in bone tissue.

Materials and methods

Samples and extraction

Bone samples (fragments of the fourth rib) and muscle samples (intercostal associated muscle) were extracted from 82 individuals (28 females and 54 males) ranging from 2 to 87 years at death, exempt from any macroscopic disease and without familial relationship. They were collected during medico-legal autopsies from accidental death victims, secondarily identified. Among these, 29 double bone/muscle samples were extracted and screened for the detection of the A189G heteroplasmic mutation [16]. For the 53 remaining individuals, DNA was extracted from each sample (bone and muscle) with the PrepFiler™ Forensic DNA Extraction Kit (Applied Biosystems, AB, Foster City, CA) according to the manufacturer's instructions and then purified and concentrated with Microcon YM30 filters (Millipore, Billerica, MA, USA). In order to extract DNA from bone cells, rib fragments were boiled and carefully cleaned from flesh tissues and bone marrow remains with distilled water [16, 19].

Quantification

A human DNA quantification was performed with the Quantifiler Human DNA Quantification kit (Applied Biosystems, AB, Foster City, CA). It allows us to verify the good unfolding of extractions and to estimate the DNA concentration in each extract in order to use exactly the same DNA quantity during amplification.

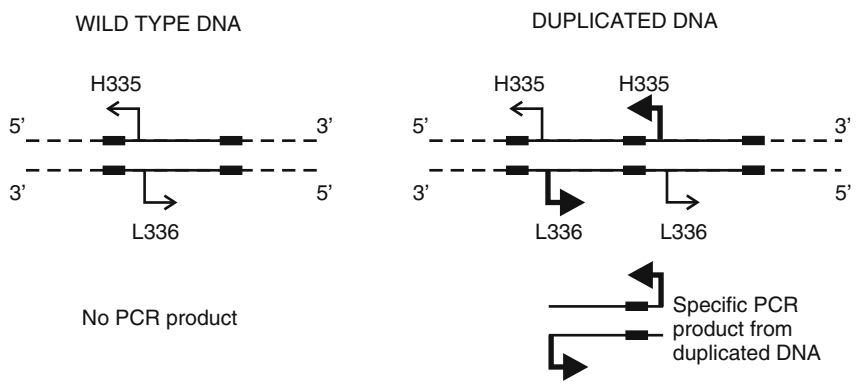

Fig. 1 PCR amplification with L336/H335 "back to back" primers. Under identical conditions, only mtDNA molecules containing the putative tandem duplications were amplified and gave rise to a PCR product of distinct size. The *thicker portions* on templates represent repeated patterns likely to cause the appearance of duplication

Evaluation of DNA degradation

In order to test the quality of the DNA extracts, hypervariable region 1 (HVI) of the mtDNA (422 bp) was amplified and sequenced for 25 samples randomly taken (bone and muscle from individuals of different ages). PCRs were carried out in a T3 Thermocycler (Biometra, Biolabo Scientific Instruments, Archamps, France) and were performed with AmpliTaq Gold polymerase (Applied Biosystems, AB, Foster City, CA) as follows: predenaturation at 94°C for 10 min; 38 annealing cycles at 94°C for 30 s, 48°C for 30 s, and 72°C for 45 s; and final extension at 72°C for 10 min. The sequence reactions were performed on each strand with the BigDye™ Terminator v1.1 kit (Applied Biosystems, AB, Foster City, CA). The products were purified using the protocol recommended by the manufacturer and submitted to capillary electrophoresis on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, AB, Foster City, CA).The results were analyzed using the Sequencing Analysis 3.7 (Applied Biosystems, AB, Foster City, CA) and Sequencher (Gene Codes Corporation) softwares. More details about the procedure are available in Keyser-Tracqui et al. 2003 [20].

Duplication amplification and analyses

Duplication amplifications were performed in a final volume of 25 µl containing, 10× Buffer, MgCl2 (1.5 mM), 0.2 mM of dNTPs, 0.05 µM of each primers L336 (5′-AACACATCTCTGCCAAACCC-3′) and H335 (5′-TAAGTGCTGTGGCCAGAAGC-3′) [14], and 1 U of Taq HotGoldstar DNA polymerase (Eurogentec, Seraing, Belgium). A dilution range from 10 to 0.1 ng DNA was performed in order to test the sensibility of the method, but all the amplification reactions were realized with 1 ng of DNA. After 4 min of predenaturation at 94°C, 34 cycles were realized using the following conditions: 94°C during 1 min, 60°C during 30 s, 72°C during 1 min. Seven minutes of final extension were performed at 72°C. PCR products were thus analyzed by electrophoresis in a 2% agarose gel. Because of the low intensity of the bands detected on the gel, a second amplification was performed in the same conditions with L336 primer 5′FAM-labeled. The products were analyzed after a capillary electrophoresis: 1 µl of PCR product was added to 9.5 µl of Hi-Di Formamide and 0.5 µl of Genescan GS 500 LIZ (Applied Biosystems, Foster City, CA). Electrophoresis were also carried out on an ABI Prism® 3100 Genetic Analyzer and electropherograms were studied using the GeneMapper® Software. Each amplification with the labeled primer was performed at least twice.

To verify that the amplicons visualized are really the duplicated fragments previously described in tissues of healthy elderly individuals, products obtained were also excised as soon as they were clearly visible on the gel. Recovered DNA was then purified with the NucleoSpin® Extract II kit (Macherey-Nagel) and sequenced. Sequencing reactions were performed using the same protocol than for HVI.

Results

The quantification result confirmed that for or all the samples (muscles and bones), DNA can be correctly

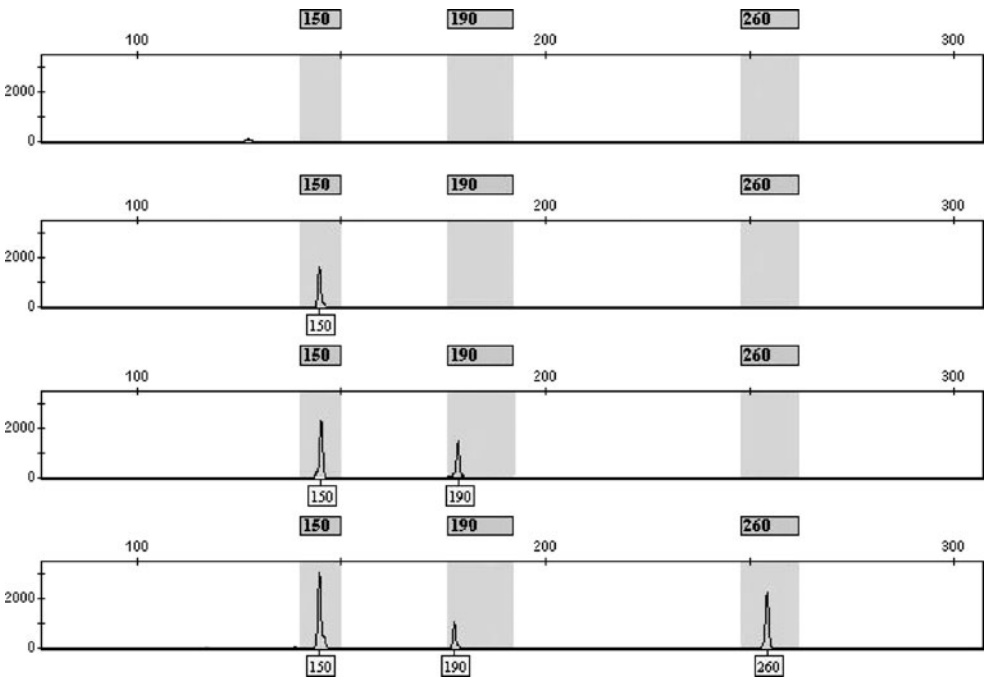

Fig. 2 Examples of electropherograms obtained for muscle samples from 2-, 42-, 71-, and 78-year-old individuals (from *top* to *bottom*)

Table 1 Details about the individuals studied (age and sex) and the presence of the three types of duplications (150, 190, and 260 bp) according to tissue type (muscle and bone)

Age (years)	Sex	Duplications in muscle		Duplications in bone	
2	F	–		–	
2	M	–		–	
15	F	–		–	
16	F	–		–	
16	***M***	–		–	
21	***F***	–		–	
22	***M***	–		–	
22	M	190		–	
23	F	–		–	
24	F	–		–	
26	***F***	***150***	***190***	–	
26	M	150	190	–	
28	M	190		–	
30	F	–		–	
30	***M***	***150***	***190***	–	
31	M	150	190	–	
33	M	150	190	–	
34	M	–		–	
35	M	150	190	–	
35	M	150		–	
36	***M***	***190***		–	
37	M	–		–	
38	***F***	***150***		–	
38	F	150		–	
38	***M***	***150***	***190***	***190***	
38	M	–		–	
38[a]	***M***	***150***	***190***	***150***	
38	***M***	***150***	***190***	–	
39	***F***	***190***		–	
39	***F***	***150***	***190***	–	
39[a]	***M***	***150***	***190***	***150***	***190***
39[a]	***M***	***150***		***150***[a]	
39	M	–		–	
42	M	260		150	260
42	F	–		–	
43	M	–		–	
43	***M***	***150***	***190***	***150***	
44	M	–		–	
44	F	150	190	150	
47	M	–		–	
47	M	150		–	
48[a]	***F***	***150***	***190***	***150***	
48	***F***	***150***	***190***	–	
48[a]	***M***	***150***		–	
49	M	150	190	–	
49	M	—		–	
50	***F***	***150***	***190***	–	
51	M	150		–	

Table 1 (continued)

Age (years)	Sex	Duplications in muscle			Duplications in bone	
51	F	150			–	
54	M	–			–	
54	***M***	***150***	***190***		–	
55[a]	***F***	***150***	***190***		***150***	***190***
55	M	–			–	
56	F	150	190		–	
56	F	150				
58	***M***	***150***	***190***		***150***	
58	***M***	***150***	***190***		–	
59	M	–			–	
60	M	–			–	
61	F	150			–	
63	M				–	
65[a]	***M***	***150***	***190***		***150***	
66	M	150	190		150	
66	M	190			–	
67	F	–			–	
71	M	150			–	
71	***M***	***150***	***190***		–	
72	M	150	190		150	
73[a]	***F***	***150***	***190***		***150***	
75	M	150	190		–	
77	M	150	190		150	
77	***M***	***150***			–	
78	M	150	190	260	150	
78	M	150	190		–	
79	M	150	190		–	
84	M	150			190	
85	M	150			–	
85	F	150	190		150	
86	F	150	190		150	190
86	M	150	190		–	
87[a]	***F***	***150***	***190***		***150***	
87	***F***	***150***	***190***		–	

The 29 individuals (rows in italics and in bold) were previously studied for the A189G point mutation [16]

[a] Indicates the nine bone tissue samples which were also found to carry the A189G heteroplasmic mutation

extracted and that the quantities obtained were sufficient for all the analyses realized. The HVI amplification and sequencing has yielded sequences of 422 bp of good quality for all samples tested (data not shown). These results confirmed that a good DNA quantity and quality was available for the duplication amplification.

The three types of expected duplications (150, 190, and 260 bp) were successfully detected on both bone and muscle tissues. An example of electropherograms obtained for DNAs from muscle tissue of 2-, 42-, 71-, and 78-year-old individuals is given in Fig. 2. Duplications were detected from as low as 0.1 ng of genomic DNA with the Quantifier Human DNA Quantification kit (Applied Biosystems, AB, Foster City, CA).

In the two tissue types, only DNA from the older individuals carried several duplications. The most frequent duplications were at 150 and 190 bp. Interestingly, the 260-bp duplication, the first described in the literature, was the less frequently observed in our study. Only two of the 164 electropherograms obtained (82 individuals; bone

and muscle samples) contained the 260-bp duplication, more precisely those obtained from muscle tissue of a 78-year-old individual and from bone tissue of a 42 year old individual (see Table 1).

In muscle tissue, the number of duplications detected in a DNA sample can vary from 0 to 3. Single duplications were detected in individuals over 22 years old and double duplications were observed in individuals over 26 years old, whereas triple duplications were only found once in a 78-year-old individual (Fig. 3a). Seventy percent of subjects over 22 years old, 85% of individuals over 50, and 100% individuals over 70 exhibited at least one duplication (Fig. 3a).

On bone tissue, only zero to two duplications can be detected in each individual's DNA. At least one duplication can be detected in individuals over 38 years old. We never observed three duplications even in oldest individuals. Thirty-five percent of the individuals over 50 years old and 50% above 70 presented at least one duplication (see Fig. 3b).

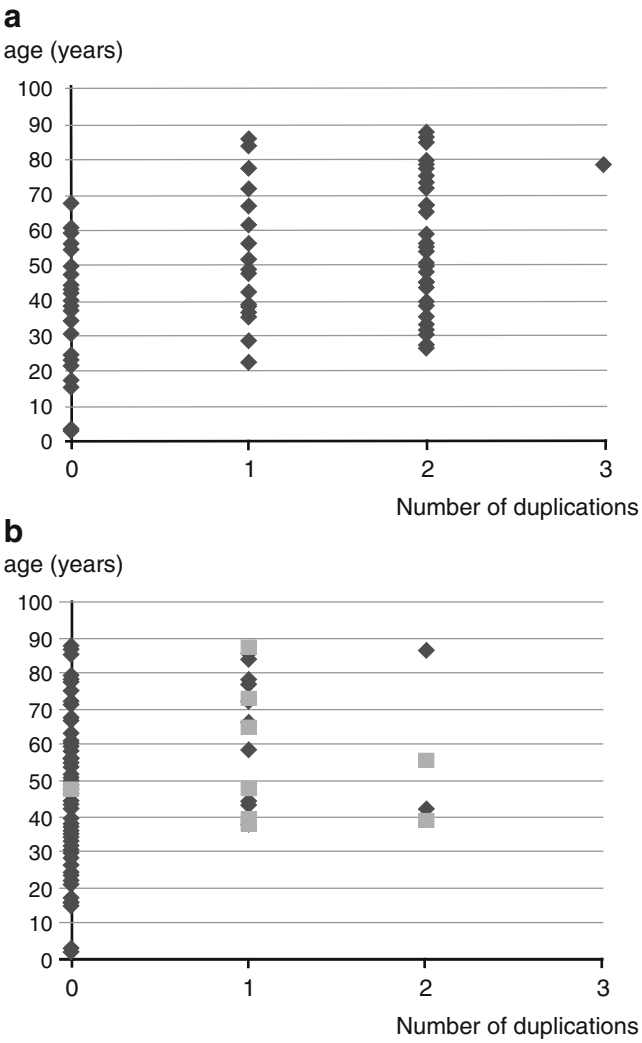

Fig. 3 Number of duplications detected in muscle samples (**a**) and in bone samples (**b**) from the same individuals according to their age. The *squares* on the *bottom graph* indicate DNAs from bone which also carried the A189G mutation

Discussion

The results of the quantification and of the HVI sequencing confirmed that extractions were successful and that the DNA extracts were of good quality and allowed amplification of fragments of at least 422 bp in length.

We so can observe clearly that the three types of duplications tested were found in both muscle and bone tissues and that, furthermore, the duplications could be detected from very low DNA quantities. This result confirmed that the use of "back-to-back" primers during amplification followed by a capillary electrophoresis is a very sensitive technique able to amplify a heteroplasmic mutation probably present in low levels and from small amount of DNA. Thus, this technique seems to be adapted for the identification of duplications in bone tissue samples from which only minute amounts of DNA can sometimes be obtained.

As described in Wei et al. 1996 [14], the presence of a duplication was taken into account if an amplicon was detectable at the three sizes, taking into account residues due to a poly-C variation at each side of the duplicated fragment. For each duplication size, the sequencing confirmed that the detected products really consisted in the rearrangement described above. The 260-bp duplication could be detected between the repeated motif 5′-ACCCC C-3′ at positions 302/567 [21, 22] and at positions 302/493 for the 190 bp [13, 14]. For the 150-bp duplication, the repetition was found between the two motifs 5′-CCCCTCCC-3′ at positions 306/456 of the control region of the mtDNA [14]. The end of the 260-bp duplications sequences could, however, never be obtained because of poly-C stretch length variations present around nucleotide 568 that made the end of the sequence analysis unreadable [17].

In muscle tissues, as previously described in Lee et al. 1994 [2], the duplicated fragments started to be observed on individuals from around 20 years old and accumulated in the most aged individuals. Our results also show that beyond 70 years old, all muscle DNAs were duplicated whereas on bone tissue, only 50% of the same individuals carry at least one duplication. As expected for this mutation type, it confirms that a strong tissue specificity exists for this type of rearrangement. It is interesting to note that quite similar results were obtained for the A189G mutation detection for which only 41% of the DNA from individuals over 50 years old carry the mutated position at a rate up to

30%, while 100% of the DNA from muscle were mutated in individuals over 60 years old at a rate up to 30% in bone [16]. Moreover, eight individuals of the nine who carry the A189G mutation on bone also show duplications (see Table 1). The number of individuals which carry both mutations is of course too small to conclude about the origin of the two mutations, but it can suggest that a link between both could be suggested.

Anyway, if duplications clearly seem to have a somatic origin, their mechanism of appearance stays still unclear. Some models propose that an insertion of C due to oxidative stress in the motifs flanking the duplicated DNA will provoke a misalignment in the repeated region prior to elongation combined with single-strand breaks which might explain the duplication. Originally proposed to explain frame shift mutations in sequence repeats, this model has been since invoked for larger repeat rearrangements [23–26].

Conclusion

Adult age-at-death assessment is one of the most difficult problems encountered in paleoanthropology as well as in legal medicine. Many procedures using either skeletal remains or dental records are sometimes inaccurate or insufficient. We proposed to examine whether it is possible to find molecular markers which can help the determination of age at death. If the number of duplications does not correlate totally with age in DNA from bone samples, the presence of duplicated fragments can give a first indication of the age of the individual. Indeed in bone, one duplication is systematically detected in over 38-year-old individuals. This result is of course not sufficient to determine the age at death, but with the A189G detection, it confirms that it occurs on the D-loop of mtDNA, a phenomenon which induces a heteroplasmic mutation accumulation in a bone tissue of the oldest individuals. We can make the hypothesis that the appearance of various other heteroplasmic rearrangements could be found linked with age in the same region of mtDNA extracted from bone tissue.

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