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TECHNICAL NOTE

Detection of the A189G mtDNA heteroplasmic mutation in relation to age in modern and ancient bones

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Abstract The aim of this study was to demonstrate the presence of the A189G age-related point mutation on DNA extracted from bone. For this, a peptide nucleic acid (PNA)/DNA sequencing method which can determine an age threshold for the appearance of the mutation was used. Initially, work was done in muscle tissue in order to evaluate the sensitivity of the technique and afterwards in bone samples from the same individuals. This method was also applied to ancient bones from six well-preserved skeletal remains. The mutation was invariably found in muscle, and at a rate of up to 20% in individuals over 60 years old. In modern bones, the mutation was detected in individuals aged 38 years old or more, at a rate of up to 1%, but its occurrence was not systematic (only four out of ten of the individuals over 50 years old carried the heteroplasmy). For ancient bones, the mutation was also found in the oldest individuals according to osteologic markers. The study of this type of age-related mutation and a more complete understanding of its manifestation has potentially useful applications. Combined with traditional age markers, it could improve identification accuracy in forensic cases or in anthropological studies of ancient populations.

Keywords Mitochondrial DNA · Heteroplasmic point mutation · Ageing · Modern and ancient bones

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Introduction

The ageing theory presented by Harman [1] and supported by Beckman and Ames [2] postulates that the production of free radicals rises with age, and plays a key role in the degenerative processes of senescence. This oxidative stress increase could thus be at the origin of all cellular molecule damage and in particular, could induce the accumulation of lesions on mitochondrial DNA (mtDNA). These lesions were found in numerous tissues with high metabolism, especially in post-mitotic tissues [3–5]. They took several forms—mostly insertions, deletions, and heteroplasmic point mutations. The presence of this latter mutation type has never been closely studied in bone, for which only deletions have been highlighted [6]. However, observing the presence or absence of heteroplasmic mutations in bones could be useful in skeleton identification or in mitochondrial analysis of archaeological bones. Indeed, when heteroplasmic mutations are found, they could have been inherited from the mother (because of mtDNA maternal inheritance) or induced by oxidative stress and the lack of lesion repair. In this latter case, the mutations are somatic and accumulate during the natural ageing process. Consequently, their study might be helpful in age evaluation (e.g. forensic sciences), where burnt or damaged bones prevent the use of traditional osteology markers. In the same way, it could be an important tool in anthropological studies where the detection of these possible age-related

mutations could be used along with osteology markers and provide an interesting approach to determining the state of health of past populations.

The general aim of this study was to show that mtDNA heteroplasmic point mutation can be linked with ageing in DNA extracted from bone. The mutation chosen for this was an adenine to guanine transition at position 189 (A189G). This transition presents high, easily detectable heteroplasmic mutation rates in muscle tissue. Its level been detected at an increasing rate in muscle tissue from older individuals [4, 5, 7–9]. In the present study, this mutation was investigated in both muscle and bone from the same individuals, thus providing a comparison of heteroplasmic variability between the two tissues. Heteroplasmy detection requires a very sensitive method able to detect mutations at low levels, hence the selection of polymerase chain reaction (PCR) with peptide nucleic acid (PNA)/DNA sequencing method which provides an age threshold for the appearance of the mutation [10, 11]. The peptide nucleic acid molecule is a DNA mimic, in which the negatively charged sugar–phosphate DNA backbone is replaced by an achiral, neutral polyamide backbone formed by repetitive units of *N*-(2-aminoethyl) glycine. The PNA probe perfectly hybridizes to a target DNA region and prevents PCR primer binding, whereas single base-pair mismatches containing PNA exhibit minimal primer binding inhibition [9, 11]. Thus, incorporating the PNA probe in the PCR reaction mixture allows preferential amplification of the mutated fragments and is more sensitive in detecting the mutation during sequencing.

Thus the three specific objectives of this study were:

1. To detect the mutation in the mtDNA extracted from bone tissues
2. To confirm that it is a somatic mutation, to determine the age at appearance and its possible variations depending on the tissues under consideration, and then
3. To show that these somatic heteroplasmy types can also be evaluated in bone from ancient skeletal remains

To this end, bone and muscle from the same individuals (called "modern samples") as well as bones from archeological specimens (called "ancient bones") were analyzed. In addition, individuals were described as young or old at death to distinguish between the modern and ancient bones.

Materials and methods

Samples

Modern samples were collected during medico-legal autopsies from accidental death victims, secondarily identified and exempt from any specific macroscopically detectable diseases. The sample from each individual consisted of a sternal fragment extremity of the fourth rib, and a part of the associated intercostal muscle. This double sampling had a dual purpose: firstly, to check the exact correlation between sequences of different tissues from the same individual and secondly, to specifically evaluate the level of the 189 heteroplasmy in the bones. In a laboratory dedicated to modern samples, 34 unrelated individuals ranging in age from 1–87 years at death were thus analyzed, including seven individuals from whom only muscle was collected. First, the muscle was carefully removed from the bone with a scalpel. Then, the bones were placed in a microwave oven (5×1 min) to eliminate soft tissues [12]. The marrow remaining in the bone matrix was cleaned with distilled water and the bone was then crushed with a mortar.

The six ancient samples dating from the seventeenth to the early nineteenth century were collected during excavations performed by the MAFSO (French Archaeological Mission in Oriental Siberia) team in 2003 and 2004 in Central Yakutia (north-eastern Siberia). The extremely cold, dry climate of this region generally provided a well-preserved nucleic acid chain [13]. Of the six specimens studied, two were identified as newborns using the method described by Scheuer and Black [14]. Two adults and two old adults were identified by the ageing process of the human skeleton as presented elsewhere [15, 16]. Ancient DNA samples were collected from femur, tibia, humerus, and patella bones. The protocol to eliminate surface contamination and to generate the powdered bone from bone fragments is described elsewhere [17]. The ancient bones were treated with extreme precautions during each step of sample preparation. DNA extraction and PCR amplification were carried out in a DNA laboratory specifically for ancient bones as described elsewhere [13, 17]. In both laboratories, the technical personnel handling bone material were genotyped for the neighboring 189 mtDNA region. Extraction and amplification blanks were used as negative controls for both modern and ancient samples

Digestion and extraction protocol

DNA from approximately 1 cm^3 of muscle and bone samples were extracted with buffer as described in Thèves et al. [9]. The aqueous phase was purified with the Cleanmix kit (Talent, Trieste, Italy) and eluted in 100 μl of sterile water. DNA extracted from the bones was then concentrated in Microcon YM30 filters (Millipore, Billerica, MA, USA) in 40 μl for the modern samples and 30 μl for the ancient ones.

Amplification, purification, and sequencing

For each sample, two conditions of PCR were carried out with and without a PNA probe using the same reaction

volume as applied elsewhere [9], with additional PNA of 8 μM final when amplifications were performed with this probe (CAGGCGA*A*CATACTT). Amplification reactions were carried out in a thermocycler T3 (Biometra, Archamps, France) using the following steps: initial denaturation (95°C, 10 min), 35 cycles of denaturation (95°C, 30 s), primer hybridization (60°C, 30 s), elongation (72°C, 1 min), and a final elongation (72°C, 7 min). PCR with PNA was carried out with the additional step of probe hybridization (69°C, 30 s) between the denaturation and primer hybridization steps. For DNA extracted from the ancient bones, the number of cycles was raised to 40, and probe concentrations of 2 and 8 μM were tested, in order to obtain a sufficient quantity of PCR products before sequencing. All PCR products were purified with QIAquick® kit (Qiagen GmbH, Hilden, Germany). The sequencing reactions were performed on both forward and reverse DNA strands for each sample and for both conditions using the BigDye™ Terminator v1.1 kit (PE Applied Biosystems, Courtabeuf, France). The products were purified as recommended by the manufacturer and submitted to an ABI Prism 310 Genetic Analyser (PE Applied Biosystems). The electropherograms were analyzed using the Sequencing Analysis 5.2 Software (PE Applied Biosystems).

Southern blotting

To confirm the results obtained after sequencing, mtDNA from ancient bones was also tested by Southern blotting. The amplification of PCR products from the samples and the chemiluminescent technique were performed under the same conditions as described in Thèves et al. [9]. However, the quantity of ancient DNA was increased: 40 cycles of PCR were carried out and a total of 25 μl PCR products were blotted onto membranes.

Results

The sequences obtained are located between positions 133 and 252 in the HVR-II region of mtDNA. Figure 1 shows the results obtained at position 189 with and without PNA on three bone samples, representing a young individual (Fig. 1a), an old individual from modern bones (Fig. 1b), and an old subject from ancient bones (Fig. 1c). As described in the guidelines of Brandstätter et al [18], at position 189, the G nucleotide was considered heteroplasmic if a secondary peak of more than about 10% of the height of the primary peak was present. This was confirmed for both the forward and reverse sequencing reactions.

In muscle tissues A total of 34 DNA sequences were studied from muscles (Table 1). Of these sequences, ten

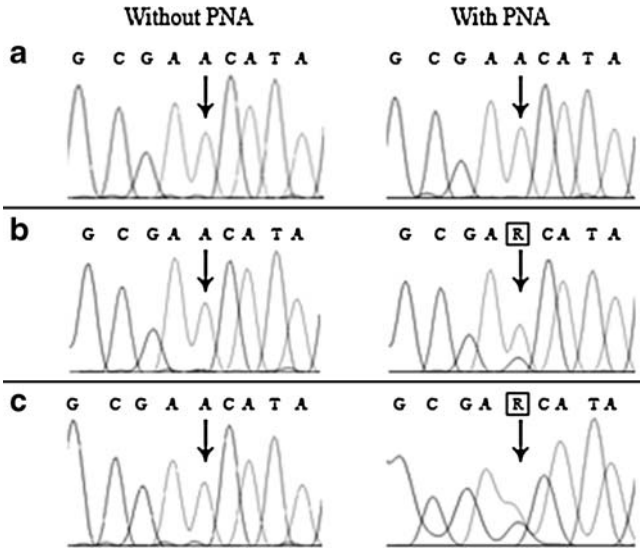

Fig. 1 Electrophoregrams visualized after sequencing without and with PNA probe in bone tissues. The *arrow* indicates the 189 position. **a** for 26-year-old individual, in both cases (without and with PNA), any heteroplasmy to 189 position is visible in bone. **b** In 55-year-old subject, sequencing without PNA presents an alone 189A peak. However, with 8 μM of PNA probe, the 189R heteroplasmy is detected in bone. **c** Once again, for the archaeological specimen YAK32 (old adult), the 189R heteroplasmy is visible with PNA (8 μM of probe) in ancient bone

taken from people over 39 years of age presented a heteroplasmy at position 189 with conventional sequencing. After the addition of the probe in the PCR mixture before sequencing, only three of the samples did not carry the heteroplasmic mutation (1, 14 and 16 years old). All remaining muscle samples showed the coexistence of the two nucleotides (A/G noticed R) at position 189 or only the presence of the G peak in the electropherogram.

In modern bones Without using the probe, no heteroplasmy could be highlighted (Table 1), which could mean that the mutation never occurs in this tissue at a rate higher than 20–30%. In fact, the sequencing technique alone detected no secondary peak below this threshold according to the neighboring sequence [9, 11, 19]. When a PNA probe was added during amplification it showed that nine out of the 27 individuals tested carried the heteroplasmic mutation in bones, all of whom were over 38 years of age (Table 1). For bone from individuals over 50 years of age, the mutation was only present in 40% of cases, whereas for muscle tissue the mutation was always found.

In ancient bones No A189G heteroplasmy was detected in any of the six ancient samples analyzed using the conventional sequencing technique (Table 1). On the other hand, the sequencing of PCR products with PNA revealed that three of them (YAK20, YAK31, and YAK32) showed a second fluorescence peak at position 189, which is clearly

Table 1 Detection of the A189G heteroplasmy for each individual according to different techniques used

Modern samples (n=34)		Muscle 189 position		Bone 189 position	
Age (years)	Sex	Simple sequencing	Sequencing with PNA probe	Simple sequencing	Sequencing with PNA probe
1	Y	189 A	189 A	ns	ns
13	Y	189 A	189 R	ns	ns
14	Y	189 A	189 A	ns	ns
16	X	189 A	189 A	ns	ns
19	Y	189 A	189 R	ns	ns
21	X	189 A	189 G	189 A	189 A
22	X	189 A	189 R	189 A	189 A
26	X	189 A	189 R	189 A	189 A
30	X	189 A	189 G	ns	ns
30	Y	189 A	189 R	189 A	189 A
36[a]	Y	189 A	189 G	189 A	189 A
38	X	189 A	189 G	189 A	189 R
38	Y.1	189 A	189 G	189 A	189 A
38	Y.2	189 A	189 G	189 A	189 A
39	X.1	189 A	189 G	189 A	189 A
39	X.2	189 R	189 G	189 A	189 A
39	Y.1	189 A	189 G	189 A	189 R
39	Y.2	189 A	189 G	189 A	189 R
43	Y	189 R	189 G	189 A	189 A
48	X	189 A	189 G	189 A	189 R
48	X.2	189 A	189 G	189 A	189 A
48	Y	189 R	189 G	189 A	189 R
50	X	189 A	189 G	189 A	189 A
54	Y	189 A	189 G	189 A	189 A
55	X	189 A	189 G	189 A	189 R
58	Y.1	189 R	189 G	189 A	189 A
58	Y.2	189 R	189 G	189 A	189 A
65	Y	189 A	189 G	189 A	189 R
66	X	189 R	189 G	ns	ns
71	Y	189 R	189 G	189 A	189 A
73	X	189 R	189 G	189 A	189 R
77	X	189 R	189 G	189 A	189 A
87	X.1	189 R	189 G	189 A	189 R
87	X.2	189 A	189 G	189 A	189 A
Ancient samples (n=6)					
YAK 30: nb	Nd	ns	ns	189 A	189 A
YAK 29: nb	Nd	ns	ns	189 A	189 A
YAK 15: a	Nd	ns	ns	189 A	189 A
YAK 31: a	Nd	ns	ns	189 A	189 R
YAK 20: oa	Nd	ns	ns	189 A	189 R
YAK 32: oa	X	ns	ns	189 A	189 R

In modern bones, 9/23 individuals of 30 years old and over carried the mutation and 4/10 individuals over 50 years old. In ancient bones, the heteroplasmy was found in 3/4 adult subjects by sequencing with PNA probe. Age and sex were known in muscles and bones from modern samples. Class of age was assigned to ancient bones

nb Newborn, *a* adult, *oa* old adult, *Nd* sex not determined, *X* female subject, *Y* male subject, *ns* no bone or muscle sample, *R* indicating the heteroplasmy 189 A/G

[a] heteroplasmy to 185 position

visible in Fig. 1c for YAK32. In the two newborn individuals and in one of the two "adult" individuals, the 189R heteroplasmy was not found. These results were obtained with both probe concentrations (2 and 8 μM), the G peak being higher and more clearly identifiable for the latter PNA concentration. Since all the ancient bone samples were identical to CRS [20] next to the probe's hybridization region, they could be analyzed by Southern blotting. The 189G mutant molecules were only detected on two old individuals (the detection limit being 10% with the

protocol applied [9]). The 189G mutation was not detected in subjects belonging to "newborn" and "adult" age classes by the Southern blot technique.

Discussion

The study of adjacent polymorphisms—flanking position 189 in the electropherograms—confirmed that all samples were identical to the CRS sequence [20] next to the probe hybridization region, except for one sample from a 36-year-old individual (Table 1, subject identified by superscript letter a). This subject presented one heteroplasmy (G→A) at position 185, visible by sequencing without probe in both tissues. Present in a young individual, this mutation was probably not somatic and was of no interest in the context of our study. Furthermore, this mutation did not interfere with the probe hybridization since the 185A mutated peak is preferentially amplified with the 189G peak after the addition of the PNA probe.

In Table 1, the observation of only a G peak after additional PNA may generate discussion. For example, in the case of a female subject of 21 years old, the identification of only one G peak could be explained in two ways. Firstly: the level of heteroplasmy present in this subject is not known. As described in our previous study [9], for position 189, heteroplasmy is not detectable at level ≤30% for conventional sequencing, and the possible level of heteroplasmy could be between 0–15% in muscle for subjects of 20–30 years old (see Fig. 5 in [9]). Secondly, in Hancock et al. [21], their Fig. 4 shows the sequence analysis of a representative A3243G sample amplified with no PNA or with various concentrations of PNA. They found that a PNA concentration of 2 μM was optimal for the greatest suppression of the wild-type 3243 mtDNA. Moreover the mutant 3243 is preferentially amplified and is the dominant species with all PNA concentrations. For 8 μM of PNA, the G mutant peak is heavily predominant, whereas the A wild-type peak is at a low level, and it may be identified as baseline noise. In the majority of the electropherograms in our study, the 189A peak is absent or at a low level for 8 μM of 189A PNA probe.

To evaluate the detection level of the PNA/DNA sequencing method, five DNA samples from individuals aged 1, 13, 14, 16, and 19 years old whose mutation level had been estimated by real-time PCR (qPCR) in a previous muscle study [9], were analyzed. For the 1, 14, and 16-year-old individuals—whose rate was less than 0.3%—no heteroplasmy was detected with the PNA/DNA sequencing method (Table 1). Above this percentage, heteroplasmy was always seen in the electropherograms. Considering a possible range of error inherent in the qPCR technology, the minimum detection threshold of heteroplasmy may be estimated at 1% in the conditions applied using this PNA/DNA method. This minimum was also found in other studies which used the same technology [21].

An observation can be made about the quality of the sequences generated in this study. For modern samples, the quantity of DNA was always sufficient to obtain high quality electropherograms. As in the work of Brandstätter and Parson [22], for low quality sequences or in the case of sequences with very high background noise, detecting a low level of heteroplasmy is almost impossible. This could also be a problem for ancient bones. PCR and sequencing with or without PNA were repeated two or three times to obtain a good quality sequences. It was observed that with PNA, the 189G peak is better individualized and there is no uncertainty in the detection of heteroplasmy (see Fig. 1c). However, while this PNA/DNA technique is certainly applicable to ancient DNA, a very large quantity is required.

The principal findings are that somatic 189 heteroplasmy can be visualized from bones and that its appearance occurs later when compared to muscle tissue. These facts lead to several discussion points:

Firstly, the difference in (a) the appearance age and (b) the heteroplasmy rate variability between muscle tissue and bone for each individual demonstrate that the independent extraction of each tissue was properly carried out: consequently, the heteroplasmy found in bone DNA can only be from bone cells.

Secondly, this study emphasizes that tissue specificity is present within the same individuals for the 189 mutation. Similar cases had already been found in other tissues [3, 4] and for other somatic mutations [23]. In the present case, the A189G tissue variability (that is, the difference in both appearance age and heteroplasmy rate variability), could be explained by a difference in the metabolism of muscle and bone [24].

Finally, this study demonstrates for the first time, that this type of somatic point mutation exists in bone tissue and is linked to age. The age threshold for the appearance of the mutation A189G in muscle is about 20 years [9]. In addition, although the sample is reduced for this tissue, a later age for the appearance threshold in modern bone is suggested: 38 years. However, while the presence of the 189 mutation seems fairly well correlated with age in the muscle, it is more difficult to demonstrate a direct correlation in the bone. In fact, only nine modern individuals out of 22 over 38 years old (or approximately 41%) carried the heteroplasmic mutation in bone at a rate higher than 1%.

In Table 1, bone tissue was not collected from six of the youngest individuals. These samples came from a previous study in which the heteroplasmy level was quantified using PNA/qPCR [9]. The autopsies on these individuals were

performed before the beginning of this current work, and the extraction of bone was not part of the autopsy protocol [9]. Consequently, no bone tissue samples from young subjects (less than 20 years old) were available for testing the occurrence of bone heteroplasmy. However, the 189 muscle heteroplasmy was only detected in two subjects (13 and 19 years old) at a low level of 0.3%. So, in bones, where heteroplasmy occurs later than in muscle, bone heteroplasmy for these youngest subjects should, in principle, be absent.

These results obtained using PNA/DNA sequencing appear consistent with those obtained from muscle using qPCR [9]. Indeed in Thèves et al. [9], where the heteroplasmy rate was evaluated in 50 individuals, the mutation was present at a rate of 5% from 20 years old and increased above this age. So in both cases (with the two methods), heteroplasmy was detected in individuals over 20 years of age. Therefore, the technique described in the present study appears to be a fast and reliable method to determine the presence of these somatic mutations. A multifactorial approach combining this mutation detection method with other markers currently employed to study skeletal remains (such as bone remodeling, cranial suture synostosis, degenerative diseases, dental indicators) is likely to increase the reliability of age evaluation.

For samples taken from ancient bone remains, cold conservation in permafrost provided a sufficient quantity of good quality DNA to analyze the position 189 in the ancient Yakut bones. Our study revealed that only one adult and the two oldest individuals carried the mutation detected using the PNA/DNA sequencing method. Furthermore, the fact that the 189 heteroplasmy was only detected in the oldest individuals, and not in the youngest, demonstrates that this mutation is not due to oxidation reactions which could start after the individual's death. Actually, modifications of transition types could occur at death in certain sites known to have high mutation rates [25]. In addition, by providing new indicators for age evaluation, this mutation study could also be very helpful during anthropological studies. In such studies, the state of health of past populations could be analyzed by comparing the mutation appearance age with modern populations (still living in a traditional way).

Outlook The presence of the 189 mutation in bone is not systematic even in old individuals. The study of a single mutation is obviously not sufficient. It would be interesting to relate the presence of this mutation in bone tissue to other mutations known to be highly polymorphic. Specifically, the mutations T408A, C150T, or others in a control region whose presence seems to be clearly correlated with ageing [23] could be studied together with the SNaPshot technology [26]. These mutation studies can also be applied to ancient DNA [27]. If the combination of these point mutations is not sufficient to obtain accurate ageing indicators, other mutations in the coding region, whose rate also seems to increase with age in muscle tissues, may possibly complete the study. More specifically, the 8344, 3243, and 10006 [23, 28] positions as well as modification types like mini-duplications could be also tested [29].

Conclusion

This study demonstrates for the first time that the 189 heteroplasmy correlated with age in muscle, is also found in bone, despite strong tissue specificity for the appearance of this mutation. Furthermore, it is shown that this mutation is found in archaeological specimens and therefore probably in old and degraded bones studied in forensic cases.

This first result should open up many research lines in the biological field, in the study of ageing mitochondrial DNA, and more specifically in musculo-skeletal tissue. Pending the discovery of new age-related mtDNA positions, determining the 189 heteroplasmy point could be added to the current osteology markers. Since the presence of this heteroplasmic mutation in bone gives a first indication of the age at death (38 years old or more), the position 189 on the mtDNA can be considered as an interesting potential marker for age estimation in forensic identification. New information about its presence, gathered in a common database for example, could permit researchers to improve its use in identification.

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