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**STERNAL PERFORATION AND BIFID RIBS
A POSSIBLE FAMILIAL CASE 5400 YEARS OLD. AN EXAMPLE
OF EPIGENETIC CONTROL OF DEVELOPMENT?**

M.L. FILY ^{1, 2}, E. CRUBÉZY ¹, B. LUDÉ ^{1, 2}, D. ROUGÉ ¹, B. MIDANT-REYNES ¹

ABSTRACT

Two men buried in the same grave in the predynastic burial ground of Adaima (Upper Egypt, around 3400 B.C. evince thoracic anomalies. One man had a bifid rib and the other a sternal perforation. Amplification and sequencing of mitochondrial DNA suggested they were maternally related. In the light of these findings, we therefore sought the genetic determinism of these characteristics; they may be minor developmental anomalies arising from a similar genetic determinism with epigenetic control of development. This hypothesis raises a number of implications for anthropobiological study.

Keywords: ancien DNA, predynastic egypt, discret trait, epigenetic trait.

RÉSUMÉ

Deux hommes enterrés dans la même tombe du cimetière prédynastique d'Adaima (Haute Egypte, environ 3400 av. J.-C.) présentent des variations thoraciques. L'un a une côte bifi - de et l'autre une perforation sternale. L'amplification et le séquençage de l'ADN mitochon - drial suggèrent que ces deux sujets étaient apparentés du côté maternel. À la lueur de ces données nous avons tenté d'appréhender le déterminisme génétique de ces variations: elles pourraient correspondre à des anomalies mineures du développement liées à un déterminis - me génétique semblable avec des modalités de contrôle épigénétiques. Cette hypothèse pour - rait avoir de nombreuses implications dans les études anthropologiques à venir.

INTRODUCTION

Since 1967 (Berry and Berry, 1967) an increasing number of anthropological studies based on the epigenetic characteristics of the skeleton or discrete characteristics have been carried out in man (review in Hauser and De Stephano, 1989; Saunders, 1989; Crubézy *et al.*, 1999; Zimmer, 1939). These studies often revealed correlations between various characteristics which led certain authors (Molto, 1983; Saunders, 1989; Crubézy, 1992a)

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to speculate on their underlying determinism. At the same time, there have been relatively few familial studies of these characteristics (Lasker, 1947; Torgersen, 1951; Hertel, 1959; Sjøvold, 1984; Crubézy, 1992b). Today, thanks to the possibilities of amplification of the remains of the DNA molecule in ancient bone, we can look for parental relationships in past populations (Hummel and Herrmann, 1997; Fily *et al.*, 1998) as well as the discrete characteristics these individuals have in common. In the near future this should result in improved knowledge of the genes involved in human morphology as well as of developmentally associated epigenetic modifications.

With this in mind, we used mitochondrial DNA (mtDNA) to look for a familial relationship on the maternal side between two male individuals in the predynastic burial ground of Adaima, Upper Egypt, dating from about 3400 B.C. (Midant-Reynes *et al.*, 1996), who were buried in the same grave and who both had morphological anomalies of the thorax.

MATERIAL AND METHODS

The predynastic burial ground of Adaima is characterized by an exceptional preservation of organic remains (Ludes *et al.*, 1999). Over 550 tombs have now been excavated (Crubézy *et al.*, *in press*). The two individuals studied here come from the same grave (S34) which has been dated to the Nagada IIA period (3400 B.C.) on the basis of its location in the burial ground and the funeral practices. The two individuals, who laid in matting separated by two to three centimeters of sand, were slightly out of alignment in relation to each other and from a purely archeological point of view it was not possible to determine whether they were buried at the same time. However, the first individual was in an unusual position and this may possibly have been planned with the second burial in mind. The second burial did not disturb the first. Two simultaneous burials from the same chronological period (Nagada IIA) have been excavated in the same area, and in the case of a woman and a child, they have so many rare vertebral anatomical variation in common, that they could be relatives (Crubézy *et al.*, *in press*). In the grave S 34, sex was determined as male using the visual characteristics of the pelvic bones (Bruzek, 1992). All the epiphyses of the upper individual were closed except for the medial clavicle; so his age was estimated between 25 and 30 years. All the epiphyses of the lower individual were closed but neither the pubic symphysis nor the auricular surface were at an advanced phase and there were no signs of degeneration. He was thus probably aged between 30 and 40.

Morphological analysis: Conventional osteometric analysis was carried out in the first 200 adults exhumed and several discrete characteristics were scanned (Crubézy *et al.*, *in press*). In this paper we will deal only with those characteristics whose frequency in the population is less than 1% and which are thus rare.

Genetic analyses: Genetic analyses were performed on the two left calcanei. The samples were numbered 12AB (upper skeleton) and 13AB (lower skeleton).

DNA extraction: After removal of the outer surfaces of the bones by scraping, the samples were mechanically crushed into powder, avoiding heating of the tissues. Extractions were then performed on 2 g of bone powder as described by Fily *et al.*, 1998.

DNA amplification and sequencing: To determine the possible relationships between these two men, we decided to work on mitochondrial DNA (mtDNA). MtDNA is present in considerable quantity per cell and is therefore more likely to be preserved than nuclear DNA. Moreover, mtDNA is haploid, inherited only through the maternal lineage and does not recombine. In mtDNA, the most informative region for genetic relationships is the D-loop, which contains a hypervariable region divided into two parts (HVI and HVII) of about 400 bp each. Comparison of the sequences obtained excludes kinship through maternal lineages if at least three differences are found between two persons in the 700 bp or so compared (Pascal, 1998). Working on DNA extracted from ancient tissues and probably highly degraded (Pääbo, 1989), we tried to sequence the second hypervariable region (HVII) of the mitochondrial DNA (about 400 bp long) and therefore we chose to amplify two short overlapping fragments each 250 bp long. The primers used were those described by Vigilant *et al.* (1989) and Sullivan *et al.* (1995). PCR conditions were as follows: 5 min of denaturation at 93°C and then 40 cycles of denaturation at 93°C for 45 s, 53°C annealing for 1 min and 72°C extension for 2 min. Final extension was carried out for 10 min. PCR reactions were performed in a final volume of 50 µl containing 2 units of Taq polymerase (MBI Fermentas) and up to 5% of the sample extract. The PCR products were run on a 1% agarose gel stained with ethidium bromide and visualized on a transilluminator. The expected bands were excised from the gel and DNA was extracted using the Clean-Mix kit (Talent®, France). Sequencing was then performed on an ABI 373 (Applied Biosystems) automatic sequencer using a Dye Deoxy Terminator kit (Applied Biosystems).

Precautions against contamination: To avoid contamination with modern DNA, many precautions were taken. The experiments were performed in a dedicated room with products and material only used for study on DNA from ancient tissues. Pre-PCR operations were undertaken under a laminar-flow hood regularly washed with ethanol and exposed to ultraviolets. Many controls were performed, such as blanks during extractions, which were submitted together with the samples to the PCR reactions in order to detect any possible contamination.

RESULTS

Morphological data: Both individuals had unique thoracic anomalies which were present in less than 1% of the population studied in Adaima. The individual aged 25-30 had (*fig. 1B*) a bifid right sixth thoracic rib (the only case in 200 skeletons) and the older individual had (*fig. 1A*) a sternal perforation (two cases in 200 skeletons).

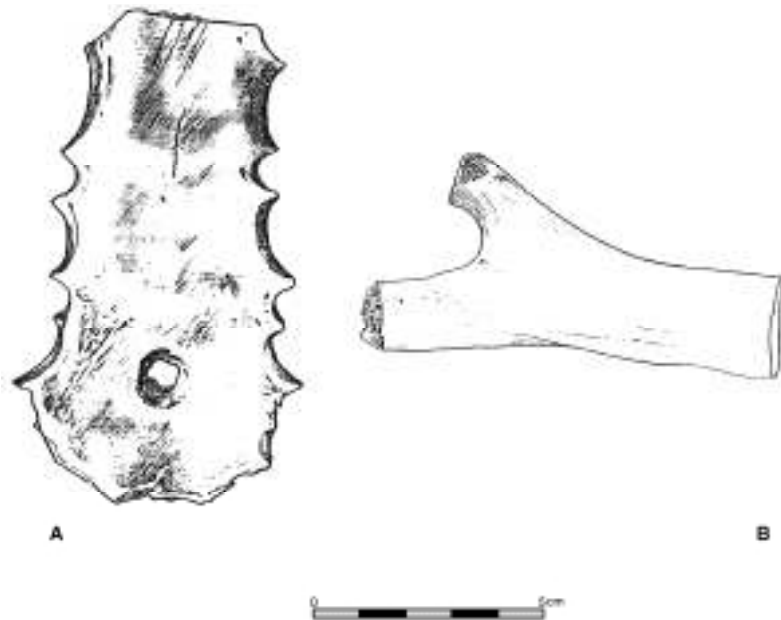
Genetic data: Three extractions of sample 13AB and two extractions of sample 12AB, which was smaller, have been performed. 71 PCR were made, among them 11.3% were contaminated by modern DNA (a band appeared in the negative control). The main source of contamination was the primers which were regularly replaced. From the non contaminated amplifications, 17.5% gave positive results with specific products of amplification. We tried to sequence in parallel HVI and HVII regions. One sequence had been obtained for HVI from sample 13 AB but it was not usefull to understand relationships between the two skeleton remains as it could not be compared to a 12AB sequence (there were not enough extracts to repeat the experience). Sequences of the HVII region were obtained from 2 independant extractions which allowed us to resolve some ambiguities in the sequences. The results are summarized in tables 1 and 2. Compared to the reference sequence (Anderson *et al.*, 1981), only two transitions were observed at positions 73 and 263 as well as a length polymorphism in the cytosine stretches. Both sequences were identical for the 286 base pairs compared except for an ambiguity in the length of the first C stretch observed in sample 13AB.

	Extraction	from	to	73	109	242	263	302.1	310.1	310.2
Anderson		29	408	A	G	C	A	0	0	0
12AB	1	56	244	G	N					
12AB	2	35	254	G						
12AB	2	196	320			C/G	G	C	C	C
consensus		35	320	G	-	-	G	C	C	C

Table 1 - Sequences obtained for the sample 12AB.

	Extraction	from	to	73	85	263	302.1	302.2	302.3	310.2
Anderson		29	408	A	G	A	0	0	0	0
13AB	2	33	254	G						
13AB	3	36	254	G						
13AB	3	39	288	A/G	G/C					
13AB	2	169	407			G	C	C	C?	C
consensus		33	407	G	-	G	C	C	C?	C

Table 2 - Sequences obtained for the sample 13AB.



*Fig. 1 - A: Sternum with a sternal perforation from the older individual. Anterior view.
B: Fragment of the medial part of the bifid right sixth thoracic rib from the younger male. Anterior view.*

DISCUSSION

Authenticity of the sequences is one of the main difficulties when dealing with ancient human DNA. We have detected number of modern DNA contamination, essentially coming from the primers solutions. Many precautions were taken to avoid contaminations in the lab. The first authenticity criterion is the good reproducibility of the sequences. We have been able to obtain this reproducibility for 50% of the HVII region, and the lack of bone material prevent us to obtain many sequences of the other part of the HVII region. Nevertheless, we can notice that when a sequence was obtained from two independant extractions of the same sample, the reproducibility was good.

A second criterion which has been developed by Pääbo (Pääbo, 1989) consists in verifying if a negative correlation is observed between the fragment length and the amplification yield. During our work, the yield of positive results was 7.7% for the longer fragments (HVI and HVII) and was 33.3% for the smaller fragments (HVIIA, HVIIIB, HVIIAand HVID). This second criterion was verified. And last, we looked at the

73 site of the HVII region: for both sequences, the nucleotide is a guanin, as expected in most non-european populations excepted a few pygmys and a few Japanese (Wilkinson-Herbots *et al.*, 1996).

Few DNA results have been obtained, mainly because of the small size of our samples. Nevertheless, the little informations obtained are quite informative on a relationship point of view. From the two calcanei of the two putative related males found in grave 34, it was possible to extract, amplify and sequence DNA. The sequences of the HVII region of the mitochondrial D-loop were compared with each other: they were identical except for a single site where an ambiguity was released for sample 13AB. Three main hypotheses could account for the sequence ambiguity: i) a Taq polymerase error during the early cycles, as slippages of the polymerase are very easy on such a stretch; ii) a damaged DNA template randomly “repaired” by the polymerase; iii) the presence of a heteroplasmy in this sample, which could only be proved through cloning (Ivanov *et al.*, 1994) and which was not performed in this study. These genetic results do not exclude the possibility that these two men were relatives. They are in fact in support of a genetic relationship between these two individuals, at least through the maternal lineages. It has been observed that in present-day Caucasians the probability of identity in the HVII region between two non-related persons is about 4,7% (Piercy *et al.*, 1994). So, even if we do not have a reference population for the predynastic Egyptian population, we could consider that these two men were maternally related. As they were buried at the same time and there may well have been little difference in age, they could be brothers, half brothers or cousins through maternal lineages.

Sternal perforation is an anatomic variation whose frequency varies from 1 to 10% in different populations (Crubézy, 1992b) excepted in the sample of East African remains —not so far away from the Nile Valley— studied by Ashley (1956) where it was noted in 13% of the total cases. It's frequency in Adaïma is less than 1%, and for the Nagada IIa period, the present case is the only one which has been found. Considered as an epigenetic characteristic, it has been used in anthropological studies to compare populations (Saunders, 1978) or to look for family groups in ossuaries (Crubézy, 1992a). Detailed studies (Zimmer, 1939; Crubézy, 1992b) have shown that this anomaly is related to highly specific patterns of ossification of the sternum. Bifid ribs, which have also long been known seem to have been little used in anthropological study of past populations as the ribs are often poorly preserved. However, they do not seem uncommon (Barnes, 1994) in living populations (specially in ribs 3-5), but in Adaïma the frequency is under 1% and like for the sternum aperture, the present case is the only one which has been found for the Nagada IIa period. Embryologically, they correspond to an anomaly of the process of segmentation that cleaves skeletal precursors into separate elements.

These two individuals, who were buried in the same tomb and who may well be related according to genetic data, raise the question of the underlying genetic variable or variables which could be responsible either for particular patterns of ossification of the sternum or for anomalies of the process of segmentation that cleaves skeletal precursors into separate elements. Studies on the morphogenesis of the mouse sternum suggest the ribs do not give rise to the sternal tissue, it seems likely that they may be concerned with the segmentation of the sternum (Chen, 1986). At the present time, we could put forward the hypothesis of minor variations in the expression or regulation of at least two gene families: the Hoxc-9 gene involved in segmental determination and the family of the gene of growth differentiation factor 5 (GDF5). The modifications of these genes are mainly known in mice. In order to clarify the function of the Hoxc-9 gene, Suemori *et al.* (1995) introduced a mutation into the Hoxc-9 locus by gene targeting. Skeletal analysis of homozygous mice revealed bending and fusion of the ribs and an abnormal pattern of ossification of the sternum. According to these authors (Suemori *et al.*, 1995), the data suggested that a functional interaction between the Hoxc-8 and Hoxc-9 proteins is involved in segmental determination. Moreover, changes in the Hoxc-8 gene expression pattern suggested that the Hoxc-9 gene regulates expression of the Hoxc-8 gene. In the same way, Storm and Kingsley (1996) have shown that particular bone morphogenetic protein family members may also play an essential role in the segmentation process that cleaves skeletal precursors into separate elements. Mice carrying null mutations in both GDF5 and another BMP family member, BMP5, show pathological abnormalities including disruption of the sternbrae within the sternum and abnormal formation of the sternocostal articulations. Our examples suggest that minor variations in these gene families or in their epigenetic control of gene expression (Wolffe and Matzke, 1999) could result in characteristics which differ in their expression or localization. If so, in order to study parental links or group relationships using these characteristics, it would be more pertinent to determine those characteristics which are associated with the same underlying genetic variant rather than to treat them as distinct entities, since the difference between them probably only arises from epigenetic control of development.

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