Int J Legal Med (2001) 114 : 186–190
© Springer-Verlag 2001

CASE REPORT

A. Salas · M. V. Lareu · A. Carracedo

Heteroplasmy in mtDNA and the weight of evidence in forensic mtDNA analysis: a case report

Received: 26 January 2000 / Accepted: 19 April 2000

Abstract Mitochondrial DNA (mtDNA) sequencing has been validated as a useful tool for forensic analysis. However, there are several aspects of the analysis which need to be considered in order to evaluate the value of the evidence. One of these aspects is related to heteroplasmy which is the state when two or more mtDNA populations occur in a single individual, cell or mitochondrion. In this report a case is described where the mtDNA profile of the blood sample of a raped woman was compared with the mtDNA profile of a single hair found in the suspect's car. The results obtained show differences in sequence between different portions of the hair and the victim's sequence. These differences are related to various heteroplasmy events. The concordance between the hair sample and the potential source (victim) of this sample is questionable and the strength of the evidence depends on how the sequence information is interpreted by the expert. The discussion of the results emphasises the necessity to evaluate heteroplasmic events in routine forensic work.

Key words MtDNA · Heteroplasmy · Hair shafts · Control region · Hypervariable regions

Introduction

Sequencing of the mitochondrial DNA (mtDNA) control region has become an essential tool in forensic genetics for the analysis of degraded material or samples containing little or no genomic DNA, notably hair shafts (Holland et al. 1993; Wilson et al. 1995). The results of mitochondrial DNA analysis have been accepted as evidence in courts of law in Europe and North America (e.g. Sumario 1/93 against M. Ricart 1996, Tennessee vs Ware 1996, respectively). Despite a diversity of methodologies, the ability to obtain consistent results and to compare results between laboratories has been demonstrated (Carracedo et al. 1998). However, there are still some questions that need to be addressed by the scientific community so that common criteria are achieved, such as heteroplasmy, analysis, interpretation, etc.

Heteroplasmy is the co-existence of two or more populations of mtDNA in a single mitochondrion, cell or individual. Mutations accumulate during the lifetime of an individual, therefore heteroplasmy probably exists in all individuals, although it is often at such a low level that it cannot be detected. In order for a mutation to be detected by sequencing, it must be present at a level approaching 20% to be distinguished from the background. Jazin et al. (1996) showed that non-coding regions have high levels of heteroplasmy, while they did not detect variability in the heteroplasmy of the coding regions. Several authors (Gocke et al. 1998; Bendall et al. 1996; Parsons et al. 1998) showed that heteroplasmy levels can vary among different family members. In addition, several authors (Jazin et al. 1996; Huhne et al. 1999) analysed the level of intra-individual sequence variability of mtDNA. Jazin et al. (1996) reported high levels and different proportions of heteroplasmy in different brain regions of the same individual. Sullivan et al (1997) reported heteroplasmic point mutations in hair shafts from a single individual with a homoplasmic blood sample. Some collaborative exercises (EDNAP exercises; details available on http://www.uni-mainz.de/FB/Medizin/Rechtsmedizin/ednap/ednap.htm) are currently being carried out to analyse the real importance of heteroplasmy when sequencing is used for analysing mtDNA variation.

In this report we consider a forensic case where mtDNA analysis was performed to compare a blood sample obtained from a raped woman with a single hair shaft found in the suspect's car. Two different portions of a single hair shaft were extracted and sequenced for the two non-coding hypervariable segments (HVI and HVII) of the control region. Surprisingly, different levels of heteroplasmy were found in each of these two portions. Also, with respect to

A. Salas · M. V. Lareu · A. Carracedo (✉)
Institute of Legal Medicine, Faculty of Medicine,
University of Santiago de Compostela, C/ San Francisco s/n,
15705 Santiago de Compostela, Spain
e-mail: apimlang@uscmail.usc.es,
Tel.: +34-981-582327, Fax: +34-981-580336

the victim, there were differences in the magnitude of the heteroplasmy. This case allowed us to discuss the interpretation and value of the evidence of these types of findings in forensic casework.

Material and methods

The single hair shaft was rinsed in ethanol (80%) in a small sterile test tube followed by sterile distilled H_2O, two portions of 3 cm of the hair shaft were cut into small pieces with scissors, a proximal (portion 1) and a distal portion (portion 2), and treated separately with 200 µl of chelex (5%), 2 µl proteinase K (10 ng/µl) and 7 µl of DTT (1 M dithiothreitol). Samples were mixed and the extraction mix was incubated for 2 h at 56 °C. Samples were boiled for 8 min, centrifuged for 3 min at 14000 rpm and the supernatants were transferred to separate sterile tubes. The blood sample of the victim was extracted using a phenol-chloroform procedure and DNA was quantified using a Hoefer DyNA Quant 200 Fluorometer (APB, Uppsala, Sweden). The amplifications of HVI and HVII regions were carried out in a Perkin Elmer (PE) 480-A thermocycler using 10 ng of DNA in a 25 µl reaction volume. The temperature profile for 32 cycles of amplification was 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. Two sets of primers were used to amplify 403 bp of the HVI region (L15997 and H16401) and 360 bp of the HVII region (L47 and H408). Before cycle sequencing, PCR products were purified with Microspin HR S-300 columns (APB, Uppsala, Sweden). Sequencing reactions were carried out using the DNA sequencing kit with dRhodamine terminators (PE–Applied Biosystems) with 100 ng of template DNA and 0.5 µM of sequencing primers. The sequencing profile for 10 cycles was 95 °C for 30 s, 55 °C for 30 s and 70 °C for 90 s, followed by a final extension at 72 °C for 5 min. The sequenced products were then purified again by chemical precipitation to remove unincorporated PCR primers and ddNTPs. Samples were resuspended in 4 µl of loading solution (95% deionizide formamide, 20 mM EDTA, and 0.05% bromophenol blue) and were run in a 6% PAGE in an ABI 377 automatic sequencer (PE-Applied Biosystems). Each template was sequenced in both forward and reverse directions in order to rule out artefacts, and several amplifications and sequence reactions were carried out in order to confirm the sequencing results. Negative controls (blanks during extractions and amplifications) and positive controls were included for extraction and PCR stages in order to detect possible sample contamination. To prevent carry-over contamination, crime and reference samples were analysed separately with the analysis of crime samples carried out prior to analysis of reference samples. All equipment used during all steps of the protocol were previously autoclaved (e.g. distilled water, pipette tips and plastic tubes) and all technical precautions were taken in order to minimise the possibility of contamination. All additional recommendations of the DNA Commission of the International Society for Forensic Genetics (ISFG) to monitor contamination were strictly followed (Carracedo et al.2000).

Results

The complete sequences of the 403 bp HVI region and the 360 bp HVII region were determined from the victim's DNA as well as from mtDNA obtained from the hair. The results of the HVI and HVII sequence of the victim and hair samples (portion 1 and portion 2) are summarised in Table 1. Some differences in the sequences were observed. The victim and the hair sample portion 1 had the same HVI sequence, which coincides with the Cambridge reference sequence (CRS, Anderson et al. 1981), but portion 2 of the hair showed three differences with respect to the other samples. At position 16093, the HV1 sequence of portion 2 showed a clear point heteroplasmy C/T (ratio close to 50:50), while the other samples clearly showed only one T peak (Fig. 1 a1, a2 and a3). A second heteroplasmy could be observed in the electropherogram of Fig. 1 b3 and consisted of two clearly defined peaks (C and T) at position 16189 (a well known hot-spot) in the hair portion 2 sample. A third heteroplasmy was probably a consequence of that at position 16189 and was present in the homopolymeric tract after position 16189 (C-stretch, positions 16 190–16 193). These cytosine stretches are often heteroplasmic (length heteroplasmy) and the mixture of the different length variants is often problematic to interpret (Parson et al. 1998). Nevertheless, the readings of the electropherograms in both directions (forward and reverse) seemed to confirm that molecules with a single insertion of a C and with an insertion of a CC co-exist in the same track in approximately the same proportion. This length heteroplasmy associated with a substitution at position 16189 has previously been reported by several authors (Bendall and Sykes 1995; Parson et al. 1998). This position shows a C in approximately 20% of the general population creating an unbroken run of Cs that is apparently replicated with low fidelity by the mitochondrial

Table 1 Variable positions in HVI and HVII region. Victim and portion 1 match with the Cambridge reference sequence at HVI, while portion 2 shows some difference. Apparent heteroplasmy at position 16189 in portion 2 (HVI) is an artefact produced by the length heteroplasmy of the homopolymeric tract beside these positions. This is corroborated by the sequence of the H-strands in each case. It is important to note that all sequences showed minor traces of background peaks underneath the principal peaks, the reading of the electropherogram being unambiguous. *Ins.* Insertion, *CRS* Cambridge reference sequence (Anderson et al. 1981), *Het.* heteroplasmy, *[1]homopolymeric tract

Sample	HVI			HVII			
	16093	16189	*[1]16184/16188	263	*[1]303/309	310	*[1]311/315
CRS	T	T	–	A	–	T	–
Victim	–	–	–	G	Ins. CC/CCC (length het)	Apparent het. (50%) C/T	Ins. C
Portion 1	–	–	–	G	Ins. C/CC (length het)	Apparent het. (50%) C/T	Ins. C
Portion 2	C/T Het (50%)	C/T Het (50%)	Ins C/CC (length het.)	G	Ins. C	–	Ins. C

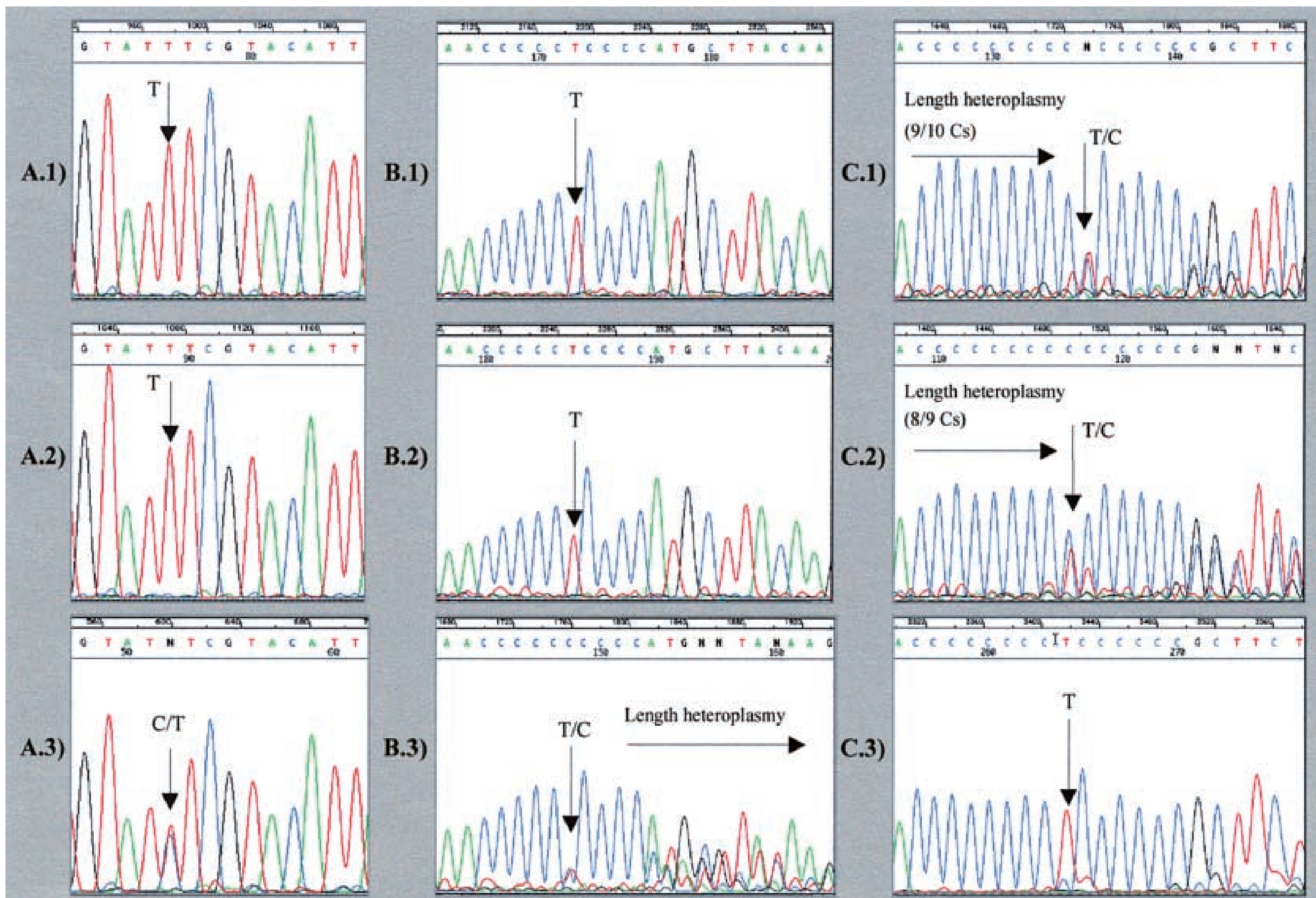

Fig. 1 a–c Partial electropherograms showing sequence results of the samples analysed in this report. **a1, b1, c1** Blood sample of the victim; **a2, b2, c2** portion 1 of the hair; **a3, b3, c3** portion 2 of the hair (*Arrows* indicate sites of heteroplasmy at different positions. Length variation in the homopolymeric tract around position 16189 (HVI) (HVII) has been reported by several authors (Bendall and Sykes 1995). These length heteroplasmies produce a characteristic unclear sequence in the nucleotides following this tract as seen in **b3, c1 and c3** when the L-strand is analysed)

DNA polymerase system (Holland and Parsons 1999). Replication slippage is thought to create length polymorphisms in these and another homopolymeric cytosine tracts in the control region.

With respect to the second hypervariable region, a similar situation could be observed (Table 1). The three samples show the most frequent variant for the HVII region in Caucasians (Salas et al. 2000), 263G and 315.1C. However, there were some differences among the samples. The victim's blood sample showed a length heteroplasmy in the tract 303–309 where molecules with a CC insertion coexist with those with a CCC insertion. Portion 1 showed similar heteroplasmy but with molecules with a single C insertion co-existing with those with a double CC insertion. As occurs in the HVI region, length heteroplasmy is also frequently observed in HVII. Portion 2 shows a single insertion 303.1C. When the L-strand of the HVII region was sequenced for the victim and hair portion 2 samples, the electropherograms seemed to show a heteroplasmy C/T (ratio 50%) at position 310. This is a common artefact produced by the existence of length heteroplasmy in the adjacent homopolymeric tract (positions 303–309). Sequencing of the H-strand resolves the problem of all possible artefacts.

Discussion

The evaluation of mtDNA evidence in cases of matching sequences is currently under debate. Due to the small size of the databases, different methods have been proposed to correct for sampling errors and to obtain the gene frequencies. Although the method described by Holland and Parsons (1999) and especially bootstrapping could alternatively be used, using the method of Balding and Nichols (1994) a frequency estimate of 0.0462 was obtained in a population database for the haplotype 263G/315.1C (the nomenclature gives changes from the CRS), the most common in Caucasians. Taking into account this value and correcting for population substructure (Salas et al. 2000), a likelihood ratio could be derived. Nevertheless, the fact that heteroplasmy was found must be taken into consideration. It is a question of debate whether the heteroplasmy should not be considered at all or if the heteroplasmic findings should be treated as inconclusive.

Nevertheless, it is obvious that in some cases, the co-occurrence of heteroplasmy in two otherwise identical sequences increases the significance of a match. In a typical heteroplasmic case where a heteroplasmy of 50:50 (or close to this ratio) is found in both reference and crime samples, this adds to the value of the evidence and increases the evidential value depending on factors such as the position where the heteroplasmy was found. However in some cases weighting the value of the mtDNA evidence is not an easy task. An example is the case considered here. To analyse this particular case we can start by analysing the value of the evidence using portion 1 and portion 2 of the hair in isolation. The sequence of the victims blood and hair portion 1 showed only one difference in the homopolymeric tract of the HVII region in the number of Cs inserted, and consequently in the state of heteroplasmy. This region has been classified as extremely mutable (1 out of 10 individuals) and is often associated with length heteroplasmy. In fact, as some authors (Marchington et al. 1997) have described, the existence of heteroplasmic length variants at position 310 in the same individual, then for our purposes, we can consider that these two sequences share exactly the same heteroplasmy state, i.e. length heteroplasmy in the tract 303–315 (therefore, the two sequences are identical). Although it is difficult to give a statistical value for this match (and this is not the aim of this argument) it is evident that the value of the evidence would be significantly increased. However, if we used portion 2 of the hair, the concordance with the victims sequence is more questionable and the results of much of the forensic research would indicate that the sources of the two samples were different, if only this portion had been analysed. In this case, the existence of clear differences in some positions decreases the match probability, but it would be difficult to establish to what extent.

Since all the findings for the evaluation must be considered as a whole, it would be even difficult to give a verbal opinion in this case. We could of course conclude that the results were inconclusive, or that it was not possible to exclude the hair and the blood sample as potentially originating from the same source or from the same matrilineage. However, it would be much more desirable to precisely weight the value of the evidence, but insufficient data is available at present for an exact evaluation. The differences found between both parts of the hair also provoked the question of criteria for matching when heteroplasmy or single base changes are found.

Our results confirm the possibility of finding different levels of heteroplasmy in a single hair and suggest the existence of narrow bottlenecks in mitochondrial transmission due to the stochastic segregation of mitochondrial DNA types during the growth of the hair. As a general conclusion, the expert should take into account the possibility of using estimates of empirical mutation rates and other parameters concerning mtDNA to evaluate the probability of a match among very closely matching sequences. For instance, it is well known that hair DNA is prone to somatic mutation and to strong genetic bottlenecks. As a consequence, it is important to estimate the frequency of these events to be included in the calculations. In this way, the expert could evaluate complex cases like this more accurately. These factors are becoming more important in the forensic routine as the analysis of mtDNA variation is sometimes the only possibility for analysing evidence in criminal cases. MtDNA analysis can undoubtedly be used in casework and in many cases (clear exclusions of crimes and evidentiary samples or matches of two samples for common mtDNA sequences) the calculation of the value of the evidence is not complicated. The case reported here is probably not common but it illustrates the difficulty of the evaluation of mtDNA evidence when heteroplasmy is involved. Probably only with more data on mutation and population demography, would it be possible to assign numerical values to these types of findings.

Acknowledgements This work was supported in part by grants from the Xunta de Galicia (XUGA 20806B97 and PGIDT 99PXI20807B). A.S. was the recipient of a grant from the Xunta de Galicia.

References

Anderson S, Bankier AT, Barrell BG, De Bruijn MH, Coulson AR, Sanger F, Schreier PH, Smith AJH, Staden R, Young G (1981) Sequence and organisation of the human mitochondrial genome. Nature 290: 457–465

Balding DJ, Nichols RA (1994) DNA profile match probability calculations: how to allow for population stratification, relatedness, database selection and single bands. Forensic Sci Int 64: 125–140

Bendall KE, Sykes BC (1995) Length heteroplasmy in the first hypervariable segment of the human mtDNA control region. Am J Hum Genet 57: 248–256

Bendall KE, Macaulay VA, Baker JR, Sykes B (1996) Heteroplasmic point mutations in the human mtDNA control region. Am J Hum Genet 59: 1276–1287

Carracedo A, D'Aloja E, Dupuy B, Jangblad A, Karjalainen M, Lambert C, Parson W, Pfeiffer H, Pfitzinger H, Sabatier M, Syndercombe Court D, Vide C (1998) Reproducibility of mtDNA analysis between laboratories: a report of the European DNA Profiling Group (EDNAP). Forensic Sci Int 97:165–170

Carracedo A, Bär W, Lincoln P, Mayr W, Morling N, Olaisen B, Schneider P, Budowle B, Brinkmann B, Gill P, Holland M, Tully G, Wilson M (2000)DNA commission of the International Society for Forensic Genetics. Forensic Sci Int 110: 79–85

Gocke CD, Benko FA, Rogan PK (1998) Transmission of mitochondrial DNA heteroplasmy in normal pedigrees. Hum Genet 102: 182–186

Holland MM, Parsons TJ (1999) Mitochondrial DNA sequence analysis – Validation and use for forensic casework. Forensic Sci Rev 11: 21–50

Holland MM, Fisher DL, Mitchell LG, Rodriguez WC, Canik JJ, Merril CR, Weedn VW (1993) Mitochondrial DNA sequence analysis of human skeletal remains: identification of remains from the Vietnam War. J Forensic Sci 38: 542–553

Huhne J, Pfeiffer H, Waterkamp K, Brinkmann B (1999) Mitochondrial DNA in human hair shafts – existence of intra-individual differences? Int J Legal Med 112: 172–175

Jazin EE, Cavelier L, Eriksson Y, Oreland L, Gyllensten U (1996) Human brain contains high levels of heteroplasmy in the noncoding regions of mitochondrial DNA. Proc Natl Acad Sci USA 93: 12382–12387

Marchington DR, Hartshorne GM, Barlow D, Poulton J (1997) Homopolymeric tract heteroplasmy in mtDNA from tissues and single oocytes: support for a genetic bottleneck. Am J Hum Genet 60: 408–416

Parson W, Parsons TJ, Scheithauer R, Holland MM (1998) Population data for 101 Austrian Caucasian mitochondrial DNA d-loop sequences: application of mtDNA sequence analysis to a forensic case. Int J Legal Med 111: 124–132

Parsons JT, Muniec DS, Sullivan K, Woodyatt N, Alliston-Greiner R, Wilson MR, Berry DL, Holland KA, Weedn VW, Gill P, Holland M (1997) A high observed substitution rate in the human mitochondrial DNA control region. Nat Genet 15: 363–368

Salas A, Lareu MV, Calafell F, Bertranpetit J, Carracedo A (2000) mtDNA hypervariable region I (HVII)sequences in human evolution studies. Eur J Hum Genet (in press)

Sullivan KM, Alliston-Greiner R, Archampong FIA, Piercy R, Tully G, Gill P, Lloyd-Davies C (1997) A single difference in mtDNA control region sequence observed between hair shaft and reference samples from a single donor. Proceedings from the 17th International Symposium on Human Identification. Promega Corporation, Madison, Wis, pp 126–129

Wilson MR, Polanskey D, Butler J, DiZinno JA, Replogle J, Budowle B (1995) Extraction, PCR amplification and sequencing of mitochondrial DNA from human hair shafts. Biotechniques 18: 662–669