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ORIGINAL ARTICLE

Heidi Pfeiffer · Sabine Lutz-Bonengel · Stefan Pollak
Rolf Fimmers · Max P. Baur · Bernd Brinkmann

Mitochondrial DNA control region diversity in hairs and body fluids of monozygotic triplets

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Abstract Length heteroplasmy of the homopolymeric cytosine stretch in the hypervariable region II of the mitochondrial D-loop was investigated in blood, buccal cells and hair shafts of monozygotic triplets. The proportions of length heteroplasmy were determined by cloning and sequencing of multiple independent clones. Blood and buccal cells showed an accumulation of molecules with one and two insertions of cytosine residues in relation to the Cambridge Reference Sequence (CRS). The results did not show statistically significant differences between blood and buccal cells of one and the same individual and also not between the three monozygotic brothers. In the hair samples a loss of cytosine residues was established in all three monozygotic individuals compared to blood and buccal cells, suggesting that this must be a regular process. Furthermore, the hair shaft samples showed significant differences between the frequencies of 7, 8 or 9 Cs in the poly C region comparing the three individuals ($p<0.008$) and in addition there were highly significant differences ($p<0.0001$) when comparing the results for six different hairs of each individual separately. From these results it can be assumed that besides a common genetic bottleneck during embryonic development, a post-embryonic bottleneck seems to exist in each hair follicle.

Keywords Mitochondrial DNA · Length heteroplasmy · Monozygotic triplets · Body tissues

H. Pfeiffer (✉) · B. Brinkmann
Institute of Legal Medicine, University of Münster,
Röntgenstrasse 23, 48149 Münster, Germany
Tel.: +49-251-8352782, Fax: +49-251-8355158,
e-mail: pfeiffh@uni-muenster.de

S. Lutz-Bonengel · S. Pollak
Institute of Legal Medicine, University of Freiburg, Germany

R. Fimmers · M. P. Baur
Institute for Medical Biometry Informatics and Epidemiology,
University of Bonn, Germany

Introduction

Mitochondrial heteroplasmy is defined as the occurrence of two or more populations of mitochondrial DNA (mtDNA) in the same individual, the same tissue, the same cell or the same mitochondrion (Holland and Parsons 1999).

The occurrence of heteroplasmy improves the value of mtDNA analyses for forensic purposes. If a suspect and a sample match at all mtDNA positions and share a heteroplasmic constellation at the same position, the mtDNA evidence is strengthened (Ivanov et al. 1996; Holland and Parsons 1999; Carracedo et al. 2000).

Length heteroplasmy was found to occur in the first and the second hypervariable segments of the mtDNA D-loop (Bendall and Sykes 1995; Bendall et al. 1996; Marchington et al. 1997; Parson et al. 1998; Lutz et al. 2000). The homopolymeric cytosine tracts (poly C tracts) in both hypervariable regions (HVI and HVII) represent hot spots for mutations and replication slippage is suspected to be the cause of this length polymorphism (Hauswirth et al. 1984; Hauswirth and Clayton 1985). In the second hypervariable region (HVII) length heteroplasmy is due to cytosine insertions between the positions 303 and 309 of the reference sequence (Anderson et al. 1981). These heteroplasmic mixtures in the HVII cytosine stretch usually have an identifiable prominent type (Parson et al. 1998).

Recently, heteroplasmy has been shown to occur with different proportions in individual hairs from a single person. The interpretation of this fact in forensic routine is still a matter of debate (Sullivan et al. 1996; Bendall et al. 1997; Wilson et al. 1997; Stewart et al. 2001; Alonso et al. 2002).

The aim of our study was to investigate the variation of length heteroplasmy in HV II in hair shafts, blood and saliva of monozygotic triplets. For this purpose an extensive study was carried out by cloning of various PCR amplicons in bacteria and determination of the proportion of cloned length variants.

Materials and methods

Samples

Samples of peripheral blood, saliva (buccal cells) and hair shafts were taken from 30-year-old monozygotic triplets. Monozygocity was confirmed by a set of nine short tandem repeat systems (AmpF/STR Profiler PCR Amplification Kit, PE Applied Biosystems, users guide 1997). The study was approved by the institutional review board and informed consent was obtained from human subjects.

DNA extraction and amplification

DNA was extracted from EDTA blood samples using the QIAamp Blood kit, from the saliva samples with Chelex-100 (Walsh et al. 1991) and from hair shafts as described by Hellmann et al. (2001).

Amplification of the HVII region was performed using the primer set F15/R484 (Holland et al. 1995) as described before (Pfeiffer et al. 1999).

Cloning and subsequent cycle sequencing

PCR products were cloned into the pCR2.1 TOPO-vector using the Topo-TA cloning kit (Invitrogen, USA). The plasmids were isolated and purified using the Plasmid Mini kit (Qiagen, Germany). Subsequent sequencing of the purified plasmids was performed as described before (Lutz et al. 2000) with the exception that the analysis was carried out by capillary electrophoresis on an ABI Prism 310 (Applied Biosystems) according to the manufacturer's instructions.

For each sample two independent PCR products were cloned to minimise the effects of a possible error of the Taq DNA polymerase in the first cycles of the amplification. The resulting clones of the different PCR products of one sample always showed similar distributions of the fragment lengths.

Statistical analysis

For statistical comparisons the absolute counts of the different insertions of cytosine residues were analysed with a two-dimensional (insertions by individuals for blood and buccal cells) and a three-dimensional (insertions by individuals by hairs) contingency table analysis. Deviations from the null hypothesis of equal frequencies were tested with the χ^2 statistic or the likelihood ratio test using the SAS statistical analysis package.

Results

Blood and buccal cells

Between the individuals and also between both body fluids of one and the same individual, slight differences in the proportion of the different length variants were found (Table 1). In all samples the molecules with 8 Cs clearly predominated in relation to molecules with 9 Cs and only 1 out of 124 clones showed another different length variant (7 Cs). As expected, the statistical analysis showed no significant deviation from the null hypothesis of equal proportions in the three individuals ($p \approx 0.19$ for blood cells, $p \approx 0.41$ for buccal cells).

Table 1 Percentages of different length variants in the homopolymeric C-tract of HVII in blood, buccal cells and hair shafts of the monozygotic triplets determined by cloning

Individual	Type of tissue	Total number of clones	Percentages of the length variants			
			C7 %	C8 %	C9 %	Others %
1	Blood	30	0	67	33	0
2	Blood	25	0	80	20	0
3	Blood	18	0	89	11	0
1	Buccal cells	14	0	86	14	0
2	Buccal cells	19	0	63	37	0
3	Buccal cells	18	5	67	28	0
1	Hair 1	40	57	30	0	13
	Hair 2	38	16	58	5	21
	Hair 3	39	18	79	3	0
	Hair 4	39	23	64	3	10
	Hair 5	49	12	78	4	6
	Hair 6	49	4	84	2	10
2	Hair 1	38	11	65	11	13
	Hair 2	40	20	58	2	20
	Hair 3	30	0	77	7	16
	Hair 4	41	27	71	2	0
	Hair 5	50	2	92	2	4
	Hair 6	37	0	84	16	0
3	Hair 1	38	11	81	3	5
	Hair 2	40	10	75	3	12
	Hair 3	38	3	94	0	3
	Hair 4	21	0	90	5	5
	Hair 5	15	93	7	0	0
	Hair 6	13	23	62	15	0

Hairs

For 6 hair shafts of each monozygotic individual, between 13 and 50 clones per hair were generated (Table 1). The predominant type was the C8 variant with two exceptions where C7 predominated over C8. The distribution of the length variants in a single individual exhibited gross differences (Table 1). In contrast to the blood/buccal cell pattern in hair shafts C7 was the secondary variant and C9 was only tertiary. In hair shafts there existed a multiplicity of further length variants which were neither present in buccal cells nor in blood. Statistically significant differences between the three brothers could be established, when summing up over all six hairs for each individual ($p<0.008$). Furthermore the analysis revealed that for each of the three monozygotic brothers the frequencies of the different lengths variants differed significantly ($p<0.0001$) within the six different hairs of each individual.

Discussion

The clones generated from blood and buccal cells were relatively homogeneous and the length polymorphism was mainly restricted to one and two insertions (C8 and C9). The clones from the hair shafts revealed different relationships between the main length variants, C7:C8:C9. As the main result of this investigation we found a loss of C-insertions in hair shafts compared to blood and buccal cells in one and the same individual. These findings have not yet been described before. Since this phenomenon is obviously the same in all three monozygotic individuals, the mechanism responsible seems be the same as well.

A genetic bottleneck is thought to be responsible for the regulation of heteroplasmy (Hauswirth and Laipis 1982). Within the maternal germline or during embryogenesis a small number of mtDNA molecules is assumed to be transmitted to the offspring.

The occurrence and the proportions of heteroplasmic HV II length variants have been found to be almost identical among different tissues of healthy individuals (Marchington et al. 1997). This would suggest that mtDNA in each tissue arises from the same founder pool of mtDNA and that small numbers of additional variants as present in selected tissues may be generated by replication slippage during expansion of the mtDNA population (Marchington et al. 1997). In contrast, in a study by Calloway et al. (2000) the proportions of HV II sequence variants were detected at different levels within various tissues and such differences showed an increase with age. This discrepancy between finding heteroplasmy and not finding heteroplasmy in healthy individuals has not yet been resolved.

Mutagenesis is expected to be elevated in cells with high energy demands due to the oxidative environment and mtDNA is permanently exposed to reactive oxygen radicals as by-products of the oxidative metabolism (Harman 1972).

The hair bulb contains a small pool of undifferentiated epithelial cells (matrix cells) which are able to proliferate extremely rapidly with a doubling time of 18–24 h during the growth phase (anagen). They differentiate, move upward and produce the hair shaft (Paus and Cotsarelis 1999). The transformation of the follicle from the growth (anagen) to the regression (catagen) phase is associated with the apoptosis of keratinocytes (Lindner et al. 1997). The high energy demand of the rapidly proliferating matrix cells on the one hand and the mechanisms of apoptosis during hair growth on the other hand, could account for the frequent occurrence of mtDNA mutations in hairs. Furthermore, mtDNA deletions seem to be age-related (Cortopassi and Wong 1999) and a similar mechanism could be discussed for hairs.

In our study, mtDNA deletions due to aging can be suspected as being responsible for the observed loss of cytosines in the C tract of hair shafts in relation to blood and buccal cells. From this it could be assumed that mtDNA in the hair matrix cells seems to be subject to more rapid aging in comparison to mtDNA in blood and buccal cells due to their high energy demand.

Furthermore, the widely differing heteroplasmic length ratios in hairs of one and the same individual lead to the conclusion that there must be a post-zygotic bottleneck for each hair follicle.

Conclusions

The proportions of the major length variants are different between blood/buccal cells on the one hand and hair shafts on the other hand, whereas a loss of C-residues was found in hair shafts. Since this phenomenon is obviously the same in the three identical siblings, the mechanism behind it seems to be the same as well and could be explained by the rapidly proliferating matrix cells causing more rapid aging in hair follicles compared to blood and buccal cells. Furthermore, the statistically significant intra-individual variation on the same head, i.e. between different hairs, implicates the existence of a post-zygotic bottleneck occurring in each hair follicle.

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