Original Article

HVRII of mtDNA in Cord Blood Cells of Newborn Children and in Their Saliva 10 Years Later

(variability of mtDNA / hypervariable region II / comparison of cord blood cells and saliva cells from ten years old children / comparison of two regions)

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Abstract. Comparison of hypervariable region II nucleotide sequences of mitochondrial DNA obtained from cord blood cells and saliva cells of the same individual at birth and after ten years revealed a few differences at the so-called mutation hot spots (three transitions and three indels within the C-tract). The personal identity of samples was proved by short tandem repeat profiling. Comparison of individuals living in two regions that differ by air pollution, however, did not reveal statistically significantly increased number of mutations in the population from the region of poorer environmental conditions, although indicating such tendency.

Introduction

Mitochondrial genome has become an object of studies of physiological and patho-physiological processes, as well as population studies in anthropology, human evolution, etc. Because of its reduced capacity of DNA repair, limited to base excision repair (BER), the variability both relative to inter-individual and intra-individual differences among various tissues and cells is very high. The mitochondrial DNA (mtDNA) differs between young and old individuals and deterioration of its activity was considered to be one of the most often discussed causes of ageing (Iwata et al., 2007; Passarino, 2010). As differences between young and old were based mainly on observations of non-identical individuals, and in fact two different populations were compared, the value of such observations is always only relative. From this point of view our study has brought new aspects by comparing identical individuals during the first ten years of their life.

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Abbreviations: BER – base excision repair, CRS – Cambridge reference sequence, HVRII – second hypervariable region of mtDNA, mtDNA – mitochondrial DNA, STR – short tandem repeats.

Material and Methods

Sample collection

Cord blood and saliva samples used in this study were obtained from 269 deliveries and 269 children from the Prachatice region and Teplice region with informed consent of their mothers. Cord blood samples were collected within the period of 1994–1998, and saliva at the age of 8–12 years of the children was collected during 2006 (see Schmuzerova et al., 2009).

DNA isolation

DNA isolation from cord blood samples was performed according to a method described previously (Miller et al., 1988). DNA isolation from saliva samples (Rogers et al., 2007) was performed using the Oragene DNA kit (DNA Genotek, Ontario, Canada) according to the protocol recommended by the manufacturer.

DNA sequencing

The hypervariable region II (HVRII) of the mitochondrial genome was analysed by bidirectional sequencing of mtDNA isolated from the cord blood and the saliva. The HVRII region was amplified in a total volume of 20 μl (10× Taq buffer 2 μl; 25 mM $MgCl_2$ 1.7 μl; 10 mM

dNTP 0.5 μl; 20 μM primer MIThvr2 long-A 0.5 μl; 20 μM primer MIThvr2 short-C 0.5 μl; Taq platinum polymerase 1.0 μl; 20 ng/ml DNA 1.0 μl; deionized water 12.8 μl) using the pair of primers MIThvr2 long-A 5´-GGTCTATCACCCTATTAACCAC-3´ (8-19) and MIThvr2 short-C 5´-CTGTTAAAAGTGCATACCG-CCA-3´ (429-408).

The temperature profile of the amplification reaction was: 95 °C for 2 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min (annealing), 72 °C for 1 min, 72 °C for 10 min, 15 °C for 30 min. Five μl of the PCR product was separated on 2% agarose gel and a 100 bp ladder was used to identify the 409 bp PCR product. The PCR product was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and QIAquick spin columns, according to the protocol recommended by the manufacturer. The PCR for sequencing was performed using the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA) in a total volume of 20 μl (Big Dye v3.1 sequencing kit 4 μl; dilution buffer 2 μl; oligo A (for forward sequencing) 1 μl; oligo C (for reverse sequencing) 1 μl; purified PCR product 4 μl; deionized water 9 μl). The temperature profile of the PCR reaction was: 95 °C for 5 min, 28 cycles, 95 °C for 30 s, 53 °C for 15 s (annealing), 60 °C for 4 min, 15 °C for 30 min. The product (20 μl) was precipitated for 30 min in the dark by adding 3 μl of 3 M NaAc and 50 μl of 96% ethanol. The precipitate was centrifuged and evaporated until dry, dissolved in 20 μl formamide and denatured for 2 min at 96 °C. The samples were sequenced using an ABI PRISM Analyzer 3100 (Applied Biosystems). The sequenced region covered nucleotides 90–325. The obtained sequences were controlled visually and compared with previously published sequences (http://www.mitomap.org) by the Bioedit software.

Identity verification

In order to eliminate personal nonidentity of sample pairs cord blood/saliva, all pairs with observed mutual differences underwent short tandem repeat (STR) profiling by Powerplex 16 kit (Promega, Madison, WI). All samples with problems – nonidentity, low amount of DNA or degraded DNA – which did not allow repeating the tests were excluded from the study. This caused reduction of the sample pairs to 245.

Statistical analysis

Contingence tables using Fisher's exact test were employed to verify statistical significance of the observed changes at the level of significance 0.05. The differences observed were compared with a null hypothesis suggesting no difference between mtDNA sequences in cord blood and saliva of the same person.

Results

Comparison of observed sequences with the sequence mentioned at http://www.mitomap.org/MITOMAP/PolymorphismsControl (MITOMAP:MtDNA Control Region Sequence Polymorphisms, ed. 2010) proved that all "deviations" from the "standard" were already observed and included in haplogroups identified in the Czech population (Brega et al.,1994; Vanecek et al., 2004; Malayarchuk et al., 2006, 2008) with the exception of nucleotides Nos. 243, 291 and nucleotides Nos. 296 a 299 (Table 1). (Consultation with Prof. A. Torroni from University of Pavia, Italy, who probably possesses the largest collection of data on mtDNA polymorphism, revealed that, although not published, these changes were already observed in other than Czech populations.)

Sample pairs cord blood/saliva analysed for identity and mentioned in this paper were proved to come from the same body. The level of matching probability was 1 in 1.83×10^{17} as indicated by Promega and the power of exclusion was 0.9999998. The observed differences between cord blood and saliva cells are summarized in Tables 2a, 2b and 2c. Fisher's exact test at the 0.05 level of probability revealed significant differences between cord blood and saliva samples' HVRII sequences ($P = 0.0306$). Blood samples were also collected from 20 mother/child pairs selected randomly at aproximately 10 years of child age to compare the pattern of mtDNA with already tested saliva samples. All of them gave pat-

Table 1. Observed differences between standard reference sequence and our samples

position	CRS	changed to	N	type of mutation
243	A	G	1	transition
291	A	T	1	transversion
296	C	A	1	transversion
299	C	A	1	transversion

Table 2a. Observed differences between cord blood and saliva cell mtDNA

transition	transversion	indel	Σ
3	0	3*	6

*with numerical heteroplasmy

Table 2b. Substitutions

Sample identification No.	Cord blood cells	Saliva cells
910	73A*	73G
972	73A*	73G
1025	242C**	242T

* Reference allele
** at position 242 a low level of C/T heteroplasmy already appeared in cord blood (Fig 1)

Table 2c. Insertion 309.nC

Sample	Cord blood cells	Saliva
1025	309.C*	309.1C
1779	309.2C	309.1C
1920	309.2C	309.1C

* Reference allele

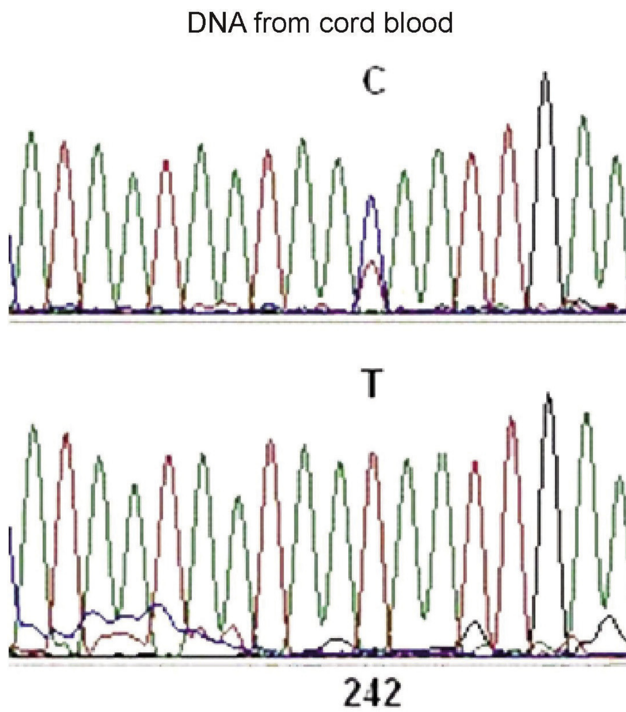

Fig.1 Low-level position heteroplasmy in mtDNA from cord blood at position 242 not visible in mtDNA from saliva cells taken 10 years later

terns identical to the mother or to umbilical cord blood taken at birth, though two of them exhibited different patterns in saliva samples.

Discussion

Our results are in agreement with other reports dealing with the sequence analyses of mtDNA HVRII, which seems to be the most variable region of the molecule, and only few changes observed by us have not yet been published. Most of the observed deviations from the standard sequence could originate from some of the haplogroups known to be present in Slavic populations (Vanecek, 2004; Malyarchuk, 2006, 2008) and some of them were already detected by Brega (1994).

The attempt to find differences between the analysed sequences of mtDNA (cord blood/saliva) between regions that substantially differed in air pollution gave negative results as shown in Table 3a. Nevertheless, comparison between boys and girls has shown higher incidence of differences among boys coming from the Teplice region (Table 3b). The non-significance (Fisher's exact test) is probably due to low numbers of observed different samples. At this place we have to mention observations that suggest that changes observed within the control region of mtDNA need not be completely innocent as was supposed (Coskun et al., 2004).

Our results on differences between cord blood and saliva samples taken from 10 years old children proved to

Table 3a. Sample comparison of two regions (Prachatice and Teplice) ♂ + ♀

1	85	86	**Prachatice r.**
4	155	159	**Teplice r.**
5	240	245	Σ
different	**identical**	Σ	

OR = 2.19 CI
P value (Fisher's exact test) = NS

Table 3b. Comparison of two regions according to gender

1	44	45	**Prachatice r.**	♂
0	41	41		♀
3	65	68	**Teplice r.**	♂
1	90	91		♀
5	240	245	Σ	
different	**identical**	Σ		

OR = 4.81 CI
P value (Fisher's exact test) = NS

be significant (Fisher's exact statistical test) and are also in agreement with observations on step-vise growing structural differences among tissues and their derivates and support the view that epithelial cells exhibit more substantial changes in comparison with blood forming tissue, which seems to be relatively stable (Nekhaeva et al., 2002; Fellous et al., 2009). We have shown that the mtDNA divergence between tissues starts early in our life and as suggested by many other authors (Shanske et al., 2004, Trifunovic and Larson, 2007) is increasing during life. The possibility that the degree of variability between different tissues within one body could get over inter-haplogroup borders could not be excluded (Fig. 2).

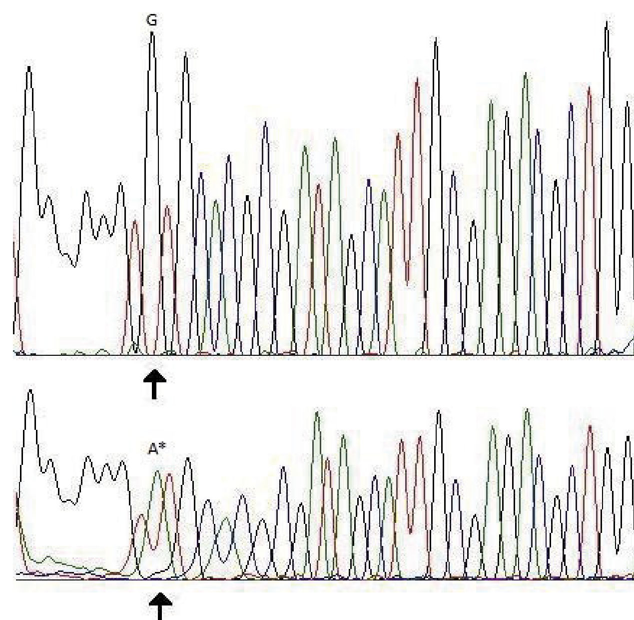

Fig. 2. Difference at position 73 between cord blood and saliva cells

References

Brega, A., Mura, G., Caccio, S., Semino, O., Brdicka, R., Santachiara-Benerecetti, A. S. (1994) MtDNA polymorphisms in a sample of Czechoslovaks and in two groups of Italians. *Gene Geogr.* **8**, 45-54.

Coskun, P. E., Beal, M. F., Wallace, D. C. (2004) Alzheimer's brains harbor somatic mtDNA control-region mutations that suppress mitochondrial transcription and replication. *Proc. Natl. Acad. Sci. USA* **101**, 10726-10731.

Fellous, T. G., McDonald, S. A. C., Burkert, J., Humphries, A., Islam, S., De-Alwis, N. M. W., Gutierrez-Gonzales, L., Tadrous, P. J., Elia, G., Kocher, H. M., Bhattacharya, S., Mears, L., El-Bahrawy, M., Turnbull, D. M., Tailor, R. W., Greaves, L. C., Chinnery, P. F., Day, C. P., Wright, N. A., Alison, M. R. (2009) A methodological approach to tracing cell lineage in human epithelial tissues. *Stem Cells* **27**, 1410-1420.

Iwata, N., Zhang, J., Leanza, S., Cho, J., Chomyn, A., Burk, R. D., Barzilai, N., Attardi, G. (2007) Aging-related occurrence in Ashkenazi Jews of leukocyte heteroplasmic mtDNA mutation adjacent to replication origin frequently remodeled in Italian centenarians. *Mitochondrion* **7**, 267-272.

Malyarchuk, B. A., Vanecek, T., Perkova, M. A., Derenko, M. V., Sip, M. (2006) Mitochondrial DNA variability in the Czech population, with application to the ethnic history of Slavs. *Hum. Biol.* **78**, 681-696.

Malyarchuk, B. A., Grzybowski, T., Derenko, M., Perkova, M., Vanecek, T., Lazur, J., Gomolcak, P., Tsybovsky, I. (2008) Mitochondrial DNA phylogeny in Eastern and Western Slavs. *Mol. Biol. Evol.* **25**, 1651-1658.

Miller, S. A., Dykes, D. D., Polesky, H. F. (1988) A simple salting-out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* **16**, 1215.

Nekhaeva, E., Bodyak, N. D., Kraytsberg, Y., McGrath, S. B., Van Orsouw, N. J., Pluzhnikov, A., Wei, J. Y., Vijg, J., Krapko, K. (2002) Clonally expanded mtDNA point mutations are abundant in individual cells of human tissues. *Proc. Natl. Acad. Sci. USA* **99**, 5521-5526.

Passarino, G., Rose, G., Bellizzi, D. (2010) Mitochondrial function, mitochondrial DNA and ageing: a reappraisal. *Biogerontology* **11**, 575-588.

Rogers, N. L., Cole, S. A., Lan, H., Crossa, A., Demerath, E. W. (2007) New saliva DNA collection method compared to buccal cell collection techniques for epidemiological studies. *Am. J. Hum. Biol.* **19**, 319-326.

Schmuczerova, J., Brdicka, R., Dostal, M., Sram, R. J., Topinka, J. (2009) Genetic variability of HVRII mtDNA in cord blood and respiratory morbidity in children. *Mutat. Res.* **666**, 1-7.

Shanske, S., Pancrudo, J., Kaufmann, P., Engelstad, K., Jhung, S., Lu, J., Naini, A., DiMauro, S., De Vivo, D. C. (2004) Varying loads of the mitochondrial DNA A3243G mutation in different tissues: Implications for diagnosis. *Am. J. Med. Genet. A* **130**, 134-137.

Trifunovic, A., Larson, N. G. (2007) Mitochondrial dysfunction as a cause of ageing. *J. Intern. Med.* **263**, 167-178.

Vanecek, T., Vorel, F., Sip, M. (2004) Mitochondrial DNA D-loop hypervariable regions: Czech population data. *Int. J. Legal Med.* **118**, 14-18.