ORIGINAL ARTICLE

LENGTH HETEROPLASMY IN THE PREDOMINATE MITOCHONDRIAL DNA HAPLOGROUPS IN THE CROATIAN POPULATION

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Abstract: Mitochondrial control region represents the most variable segment of the mitochondrial genome. The frequency and pattern of heteroplasmy has been described in several studies; however, none of the reports documented the Croatian population. In the present study, we screened the control region (1122 bp) of 95 individuals belonging to two predominant mitochondrial phylogenetic branches in the Croatian population, haplogroups H and U. Length heteroplasmy occurred in polycytosine (poly-C) tracts within three hypervariable segments of the control region with the following frequencies: HVSI - 26.3%, HVSII - 52.6% and HVSIII - 7.4%. Furthermore, the association between certain polymorphisms in HVSI and length heteroplasmy was investigated. Our results indicate that only polymorphisms located in the poly-C tract are associated with HVSI length heteroplasmy. The T to C transition at np 16189 is significantly associated with the occurrence of length heteroplasmy ($p<0.0001$). This effect was even stronger if the C insertion was present in the position 16193. The data support the hypothesis that an uninterrupted poly-C tract of more than eight cytosines leads to length heteroplasmy. Length heteroplasmy associated with the T to C substitution in np 16189 was predominantly found in haplogroup U.

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INTRODUCTION

Mitochondrial DNA (mtDNA) has proven to be a useful tool in evolutionary, anthropological and forensic research considering its features such as the high copy number,[1] apparent lack of recombination,[2] high substitution rate,[3] and predominant maternal inheritance.[4] The mutation and substitution rates of mtDNA are orders of magnitude higher than that of nuclear DNA, thus generating considerable population variability.[5-7] Hence, numerous population studies have been conducted in the last few decades that have defined population-specific DNA lineages, which can be traced back to African origin of the mitochondrial gene pool.[8-11] The highest degree of polymorphism in mtDNA is concentrated within three hypervariable segments of the control region: hypervariable segment I (HVSI, np 16024-16383), hypervariable segment II (HVSII, np 73-340) and hypervariable segment III (HVSIII, np 438-574). The control region is a relatively small, non-coding section of mtDNA with an important, mainly regulatory, function in mtDNA replication, as well as in transcription initiation and regulation.[9] Thus, sequence alterations in the control region may affect mitochondrial function via modified replication and/or transcription.[12] mtDNA exhibits the feature of heteroplasmy, i.e. the presence of multiple sequence types within a cell, a tissue, or an individual. Two types of heteroplasmy are present in the human mitochondrial genome: point and length heteroplasmy.[13-15] Point heteroplasmy is defined as the presence of two distinct bases at a single nucleotide position (np), while length heteroplasmy is detected as a co-existence of at least two populations of mtDNA molecules that differ in the number of nucleotides and therefore in their length.[14-16] Length heteroplasmy is usually observed within the cytosine polymeric tracts,

when the number of identical adjacent nucleotides is greater than eight.[17] The biological relevance of length heteroplasmy is not yet fully understood. According to previous reports, heteroplasmy accumulates during aging[18, 19] and has been associated with several multifactorial disorders.[20-22]

The main goals of this study were to determine the frequency of length heteroplasmy in two major mtDNA haplogroups in the Croatian population sample and to investigate the association between certain polymorphisms in HVSI and the occurrence of length heteroplasmy.

MATERIAL AND METHODS

Buccal swabs were collected from randomly selected volunteer donors who had given written informed consent to participate in the Croatian mitochondrial population study. To the best of our knowledge, individuals were healthy and were not close maternal relatives. All samples included in this study (n=95) belong to haplogroups H and U (53.7% and 46.3%, respectively). The characteristics of the population sample are shown in Table 1.

Table 1. Characteristics of the study population

	N (%)	Age
Female	72 (75.8)	29.7±10.5
Male	23 (24.2)	33.0±10.2
Total	95 (100)	30.5±10.5

N- number of individuals; age - mean±SD

Genomic DNA was extracted from buccal cells using the EZ1® DNA Investigator Kit for EZ1® Advanced XL instrument (Qiagen, Germany) following the manufacturer's instructions. The entire control region was amplified and sequenced according to the principle outlined in Lyons et al[23] with the following modifications. Amplification was performed using forward primers (F15971/F15851) and reverse primers (R599/R639) in final volume of 25 µL containing AmpliTaq Gold 360®Master Mix (Applied Biosystems, Thermo Fisher Scientific, USA), 10 µM of each primer, ultrafiltered water and 1 ng DNA. The amplification conditions were: 95ºC for 10 min, followed by 35 cycles at 95 ºC for 30 sec, 56ºC for 30 sec and 72ºC for 1 min, and final elongation at 72ºC for 7 min. In order to provide full reads of the poly-cytosine (poly-C) tracts, multiple primers were used (Table 2). PCR products were purified using ExoSAP-IT (Affimetrix, Thermo Fisher Scientic, USA) and sequenced with the Big Dye Terminator v.3.1 (Applied Biosystems, Thermo Fisher Scientific, USA). Sequencing products were separated on the 3500xL Genetic Analyzer (Life Technologies, Thermo Fisher Scientific, USA). Consensus sequences were compared

Table 2. Amplification and sequencing primers used for mtDNA control region analysis

Primer designation	Sequence (5' → 3')
Amplification Primers	
F15851 / R639	ATC TCC CTA ATT GAA AAC AAA ATA CTC AAA GGG TGA TGT GAG CCC GTC TA
F15971 / R626	TTA ACT CCA CCA TTA GCA CC TTT ATG GGG TGA TGT GAG CC
Sequencing Primers	
F15851	ATC TCC CTA ATT GAA AAC AAA ATA CTC AAA
F15971	TTA ACT CCA CCA TTA GCA CC
F16190	CCC CAT GCT TAC AAG CAA GT
F16450	GCT CCG GGC CCA TAA CAC TTG
F34	GGG AGC TCT CCA TGC ATT TGG TA
F314	CCG CTT CTG GCC ACA GCA CT
F361	ACA AAG AAC CCT AA ACC AGC
R16410	GAG GAT GGT GGT CAA GGG A
R285	GTT ATG ATG TCT GTG TGG AA
R484	TGA GAT TAG TAG TAT GGG AG
R599	TTG AGG AGG TAA GCT ACA TA
R626	TTT ATG GGG TGA TGT GAG CC
R639	GGG TGA TGT GAG CCC GTC TA

to the revised Cambridge Reference Sequence (rCRS) using Sequencher v.5.4.6 (Gene Codes, USA). Haplogroup assignment based on PhyloTree mtDNA tree build 17[24] was previously performed as part of a Croatian mitochondrial population study (unpublished data). Length heteroplasmy was interpreted by calling the dominant variant, following the interpretation guidelines.[25] Sequence variants were checked against MITOMAP.[26] The frequency distribution was analyzed using Fisher's exact test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Buccal swab samples from healthy individuals belonging to the most prevalent haplogroups in the Croatian population (H and U) were analyzed in this study. In the context of length heteroplasmy, 35.8% of samples are fully homoplasmic, while the rest displayed length heteroplasmy distributed as shown in Table 3.

The prevalent form of length heteroplasmy is found to be in the poly-C tract of HVSII and is present in 52.6%

Table 3. Distribution of the length heteroplasmies along the mitochondrial control region

	N	Frequency (%)
Homoplasmy		
LHP HVSI	25	26.3
LHP HVSII	51	52.6
LHP HVSIII	7	7.4

N - number of individuals; LHP – length heteroplasmy; HVSI – hypervariable segment I; HVSII – hypervariable segment II; HVSIII – hypervariable segment III

of the samples, while 26.3% of the total analyzed samples showed length heteroplasmy in the C-stretch of the HVSI. Moreover, 7.4% of all individuals demonstrated length heteroplasmy in the poly-C tract of HVSIII. In total, 83 length heteroplasmies were found in 61 individuals, which shows that some individuals carry more than one length heteroplasmy (Table 4).

Table 4. Frequency (%) of length heteroplasmy in Croatian population sample

	N	%
LHP HVSI		
LHP HVSI & HVSII	14	14.7
LHP HVSI & HVSIII	3	3.2
LHP HVSII	32	33.7
LHP HVSIII	0	0
LHP HVSII & III	4	4.2

N - number of individuals; LHP – length heteroplasmy; HVSI – hypervariable segment I; HVSII – hypervariable segment II; HVSIII – hypervariable segment III

Overall, more than 92% of the samples exhibiting length heteroplasmy in HVSI displayed a mixture of molecules with at least 10 cytosines in the C-strech. Individuals with the transition at np 16189 (resulting in a string of 10 cytosines) and C or CC insertion at np 16193 are more prone to length heteroplasmy (Figure 1). In HVSII, length heteroplasmy was observed when a minimum number of 8 cytosines was present. All heteroplasmic individuals displayed a mixture of molecules with 8 (one C insertion at np 309) or 9 cytosines (two C insertion at np 309). Although the majority of length variants were found in the HVSI (np 16184-16193) and HVSII (np 303-315) C-stretches, heteroplasmy was also observed in the HVSIII C-stretch (np 568-573). In our study, length heteroplasmy was associated with the expansion of the C-stretch beyond 7 cytosines (one C insertion in 573) with a maximum number of 12 cytosines. Besides C insertions, there were also CA dinucleotide insertions detected in the poly-AC tract (np 515-524) of

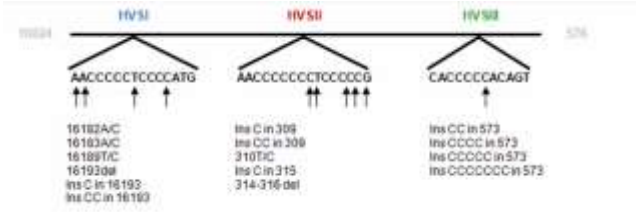

Figure 1. Detected length heteroplasmies in two major mtDNA haplogroups in the Croatian population. Length heteroplasmies occurred at three locations in the control region (np 16024-576). Numbering according to rCRS.

hypervariable segment III. However, the latter insertions did not cause length heteroplasmy in our sample pool.

In order to test the association between HVSI polymorphisms and length heteroplasmy in the Croatian population sample, we focused on 13 of 57 polymorphisms which had >5% minor allele frequency (MAF) (Table 5). Selected polymorphisms were then subjected to further statistical analysis. Five mtDNA polymorphisms (A16051G, G16129C, A16183C, T16189C, 16193C ins) were significantly more frequent in individuals with heteroplasmy than in individuals without heteroplasmy (homoplasmic state) (Table 4). Two out of five detected polymorphisms were located in the poly-C stretch. To analyze the combined effect of HVSI polymorphisms on length heteroplasmy, the haplotype frequencies for significant loci were estimated. Haplotype analysis revealed 16189T-16193C as the most common haplotype in the Croatian population sample, and was thus used as a reference (Table 6). Our results demonstrated that the frequency of length heteroplasmy ($P<0.0001$) is significantly increased when T to C substitution at position 16189 is present. This effect was even stronger if the C or CC insertion was present at np 16193 that generated a polyC-stretch of 11 and 12 cytosines, respectively. Importantly, 96% of samples with T16189C transition and all samples with C or CC insertion at np 16193 exhibited length heteroplasmy. Reversely, length heteroplasmy was absent in one

Table 5. Mitochondrial HVSI polymorphisms with >5% minor allele frequency (MAF) observed in Croatian population sample

Nucleotide position	rCRS	Base change	MAF (%)	HTP (%)	HMP(%)	*P-value*
16051	A	G	8.4	7.4	1.0	0.0003
16129	G	C	8.4	7.4	1.0	0.0003
16183	A	C	10.6	9.5	1.1	<0.0001
16189	T	C	28.4	26.3	2.1	<0.0001
16192	C	T	14.8	1.1	13.7	0.104
16193	C	ins C	17.9	17.9	0	<0.0001
16256	C	T	13.7	4.2	9.5	0.739
16270	C	T	21.1	7.4	13.7	0.393
16304	T	C	16.8	2.1	14.7	0.231
16311	T	C	6.3	1.0	5.3	0.684
16343	A	G	5.3	0	5.3	0.321
16356	T	C	7.4	2.1	5.3	0.595
16362	T	C	10.6	5.3	5.3	0.122

HVSI - hypervariable segment I; rCRS - revised Cambridge reference sequence; HTP - frequency of heteroplasmy; HMP - frequency of homoplasmy

Table 6. Haplotype frequencies of significant polymorphisms in hypervariable segment I

Haplotypes		Haplotype frequency (%)		*P*-value
16189	16193	Heteroplasmic	Homoplasmic	
T	C	0	71.6	reference
C	C	8.4	1.1	<0.0001
T	ins C	0	0	-
C	ins C	17.9	0	<0.0001

sample probably due to C to T transition in np 16192 which interrupted the poly-C stretch. Moreover, the frequency of length heteroplasmy was comparable in haplotypes 16183C-16193C and 16183A-16193C (3.2% and 4.3%, respectively), which indicates that an elevated level of length heteroplasmy is associated with C insertion at the position 16193 ($p<0.0001$), and not to A to C transversion at 16183. Similar effect was observed in case of 16051 transition or 16129 transversion, which did not affect the frequency of length heteroplasmy (5.3% and 3.2%, respectively).

In order to compare the frequencies of length heteroplasmy between haplogroups H and U, additional statistical testing was performed. The frequency of length heteroplasmy in haplogroup U was significantly higher compared to haplogroup H ($p=0.0045$).

DISCUSSION

The mitochondrial control region is the most variable region of the mitochondrial genome. In the present study, we explored the frequency of length heteroplasmy in two predominant Croatian haplogroups, H and U. Moreover, the association between polymorphisms and HVSI length heteroplasmy was investigated.

Overall, 64.2% of individuals showed length heteroplasmy in the mitochondrial control region and the majority of observations were localized in the poly-cytosine stretches of HVSI and HVSII. The frequency of length heteroplasmy obtained in HVSI (26.3%), HVRII (52.6%) and HVSIII (7.3%) was considerably higher than previously reported (15% - HVSI, 45% - HVSII and 4.3% - HVSIII),[27] even though the same methodology was used. It is not surprising, considering that the mentioned study included numerous mtDNA lineages,[27] in contrast to our study with just two analyzed lineages.

In order to explore the association between polymorphisms and HVSI length heteroplasmy, five polymorphisms (A16051G, G16129C, A16183C, T16189C and C16193C ins) were further analyzed. Our results indicate that 16189 and 16193, which are located in the poly-C tract, are crucial. The T to C transition at np 16189 is significantly associated with the occurrence of length heteroplasmy ($P<0.0001$). In addition, length heteroplasmy was even two times more frequent if the C insertion was present at np 16193. As a result, an uninterrupted stretch of 10 or 12 cytosines was generated that supports the hypothesis that an uninterrupted poly-C tract of more than eight cytosines leads to length heteroplasmy.[14, 16, 27-29] The T16189C mutation was suggested to interfere with the replication process of mtDNA generating instable C-stretch, which in turn decreases mtDNA copy number and causes mitochondrial disfunction.[30] This substitution is described as a strong mutational hotspot and has been associated with aging,[18, 19] coronary artery disease,[20] metabolic syndrome,[31] type 2 diabetes,[32] obesity[33] and a variety of tumors.[13, 34-36]

In the context of phylogeny, the position 16189 is often observed in different mtDNA lineages.[37] This locus is among the top 50 fastest evolving sites.[27] Length heteroplasmy associated with the T to C substitution at np 16189 was predominantly found in two mitochondrial lineages, haplogroups H and U[17, 19] which encompass more than 60% of all mitochondrial genomes in the Croatian population.[38] In the present study, of all individuals with observed HVSI length heteroplasmy, more than 70% belong to haplogroup U. Importantly, the findings from this study indicate that every fourth individual belonging to H and U lineages carries heteroplasmic length variation.

In conclusion, this is the first report on length heteroplasmy in mitochondrial control region in two major mtDNA haplogroups in the Croatian population sample. With regards to obtained data, comparisons with various patient groups affected by mitochondrial-associated diseases might be used to explore the association between T16189C and certain diseases in Croatian population. In future studies, heteroplasmy screening should be expanded to the entire control region, as it would be valuable information for medical, evolutionary, and forensic purposes.

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