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Title: The link between mitochondrial DNA hypervariable segment I heteroplasmy and ageing among genetically unrelated Latvians

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Abstract

Various studies have demonstrated that mitochondrial DNA (mtDNA) heteroplasmy tends to increase with age and that the observed frequency of heteroplasmy among populations mostly depends on the way it is measured. Therefore, we investigated age-related association on the presence of mtDNA heteroplasmy within the hypervariable segment 1 (HVS-I) in a selected study group. The study group consisted of 300 maternally unrelated Latvians ranging in age from 18 to over 90 years. To determine the optimal method for mtDNA heteroplasmy detection, three approaches were used: (i) SURVEYORTM Mutation Detection Kit, (ii) sequencing and (iii) denaturing gradient-gel electrophoresis (DGGE). Among the studied individuals, 30.3% were found to be heteroplasmic. The distribution of heteroplasmy statistically significantly increased with individuals' age (17%; 95% confidence interval [CI] 0.095-0.244 in the 18-40 year age group vs. 39%; [CI] 0.294-0.487 in the > 90 year age group). Heteroplasmy occurred in a total of 21 different positions within HVS-I, and was the most frequent at fast-mutated positions 16189, 16304 and 16311. The results indicate that heteroplasmy in HVS-I is relatively common and occurs in a broad spectrum of sites. The above is supported by evidence to eventual increase of the probability of heteroplasmy with age due to specific mitochondrial haplogroup background.

Research Highlights

- The results indicate that heteroplasmy in HVS-I is relatively common and occurs in a broad spectrum of sites. Consequently, a strong association is found to exist between the mtDNA heteroplasmy and ageing in the studied population.
- We found that the Denaturing Gradient Gel Electrophoresis (DGGE) and the SURVEYOR™ Mutation Detection Kit assay exhibited a lower limit of detection (LOD) of mtDNA heteroplasmy and revealed the presence of heteroplasmy at the already existing 1% and 5% occurrence in the sample.

Introduction

The non-coding control region (CR) of mitochondrial DNA (mtDNA) contains a high degree of sequence variability between individuals and has mainly regulatory functions (Brown, 1980). The highest degree of polymorphisms is concentrated within two hypervariable segments of CR: hypervariable segment I (HVS-I, 16024-16365 nt) and hypervariable segment II (HVS-II, 73-340 nt) (Wilkinson-Herbots et al., 1996). The high level of polymorphism is thought to reflect an extremely high and variable rate of mutation in the CR (Parsons et al., 1997; Jazin et al., 1998; Excoffier and Yang, 1999). When comparing features of nuclear DNA with mtDNA three main differences arise: 1) mtDNA is characterized by a high rate of mutation due to the elevated concentration of mutagenic oxygen free radicals generated during oxidative phosphorylation (OXPHOS); 2) mtDNA has a higher turnover rate than nuclear DNA, requiring more replications per unit time; 3) the lack of protecting histone-like proteins and less effective repair systems (Shadel and Clayton, 1997; Bogenhagen, 1999). The presence of many hundreds of copies of mtDNA per cell, together with a high mutation rate, creates the potential for widespread heteroplasmy, that is, the presence of multiple sequence types within a cell, a tissue, or an individual. Heteroplasmic mtDNA variants might be inherited passing through a bottleneck at some point in oogenesis between the primordial germ cells and the primary oocyte, though inheritance of heteroplasmy is limited by replicative segregation (Jenuth et al., 1996; Lightowlers et al., 1997). In contrast, acquired heteroplasmy is quite commonly observed and tends to increase with age (Michikawa et al., 1999). Usually, a low level of heteroplasmy does not impair mitochondrial function, but once the level of mutant mtDNA exceeds a certain threshold, OXPHOS dysfunction may arise (Rossignol et al., 2003). As a rule, the age-related

mtDNA somatic mutations lead to a decline in mitochondrial function, which contributes to ageing and degenerative diseases (Linnane et al., 1989; Wallace, 2005). The fate of heteroplasmic mutations depends on several factors, including the replication rate of the cell, type and location of the variation (DiMauro and Davidzon, 2005; Lacan et al., 2008). Several mutant mtDNA varieties may be present at undetectable low concentrations, and the degree of heteroplasmy may also be tissue-specific. Recently, He at al. (2010) showed that the distribution of mtDNA heteroplasmy pronouncing varied across different normal tissues from the same individual and was widespread through the entire mitochondrial genome. Two types of heteroplasmy are recognized in mitochondrial genome: point and length heteroplasmy (Bendall and Sykes, 1995; Bendall et al., 1996). Point (sequence or site) heteroplasmy is defined by sequences presenting different bases at the same nucleotide position, which are usually observed in a sequence electropherogram as a superimposition of two different bases at this nucleotide position (Lutz-Bonengel et al., 2008). Length heteroplasmy is identified by repetitive tracts of consecutive cytosines, and has been found in four regions within the mtDNA control region (Bendall and Sykes, 1995; Lee HY et al., 2004; Forster et al., 2010). However, the observation of heteroplasmy in routine sequencing studies is the exception rather than the rule. From this point of view, different methodological strategies (e.g. denaturing high performance liquid chromatography, pyrosequencing, sequencing-by-synthesis) were applied to reveal mtDNA heteroplasmy at very low levels (Rose et al., 2007; Ballana et al., 2008; He et al., 2010). The main goals of the present study were to determine by means of different approaches, the distribution of mtDNA heteroplasmy in the HVS-I among maternally unrelated Latvians subdivided into three age groups, and to establish relationship between polymorphisms in the mtDNA coding region (mtDNA haplogroups) and HVS-I heteroplasmy.

Materials and methods

Samples

Whole blood samples were collected from 300 randomly chosen healthy Latvians, none of whom were known to be maternally related. The samples were divided into three groups according to individual age: 18-40 years (control group, n=100), 70-80 years (n=100) and over 90 years (n=100). The gender ratios (female/male, %) in the groups were 57/43, 49/51, and 68/32, respectively. Studied individuals represented four different regions of Latvia, and maternal ancestry over the last three generations was established from interviews. All participants provided appropriate written informed consents to use their phenotypic and genetic data from detailed health and heredity questionnaires. The Latvian Central Medical Ethics Committee approved the research protocol. Based on the information provided, individuals included in the current study were characterized by relative healthy status without tumors, pronounced cardiovascular diseases, dysfunctions of endocrine and musculoskeletal systems. The leukocyte content of blood among studied individuals was indicated in each individual questionnaire, and leukocyte subsets were presented in appropriate proportion without abnormalities (neutrophils ~54-62%; lymphocytes ~ 25-33%; monocytes ~ 2-10%; eosinophils ~ 1-6%; basophils <1%).

All DNA samples were taken anonymously. DNA was extracted from 5 ml venous blood using the standard phenol–chloroform method as described in Sambrook et al. (1989). The obtained DNA concentration of samples was in a range from 450 ng/µl to 550 ng/µl. Subsequently, DNA samples were diluted with sterile distilled water to the working concentration, 10 – 50 ng/µl. Artificial heteroplasmy was produced by mixing DNA samples from two different individuals with known mtDNA HVS-I sequences. Samples for artificial heteroplasmy contained the same

initial DNA concentration (10ng/µl). The first sample was defined as the rCRS (revised Cambridge Reference Sequence; Andrews et al., 1999) mtDNA haplotype and the second sample as having one substitution at position 16304. The two DNAs were combined to generate samples having heteroplasmy levels of 1%, 2%, 5%, 10%, 30 and 50%.

Detection of mtDNA heteroplasmy

In order to quantify the levels of heteroplasmy in the studied DNA samples, we applied and compared three different methods: (i) SURVEYORTM Mutation Detection Kit, (ii) DGGE assay and (iii) sequencing analysis, to the artificially produced heteroplasmic samples. Further, a combination of three methods were used to analyze the HVS-I mtDNA heteroplasmic variation in three age groups of unrelated Latvians and to characterize the spectrum of sites at which heteroplasmy occurred.

1. Mismatch-specific Surveyor nuclease

The presence of mtDNA heteroplasmy was determined using the SURVEYOR™ Mutation Detection Kit, which is based on the use of a mismatch-specific endonuclease, SURVEYOR Nuclease (Transgenomic, USA) (Bannwarth et al., 2006). The DNA fragment encompassing the mtDNA HVS-I between nucleotide positions (nps) 16024-16390 was amplified and sequenced in all samples using forward (L15996) and reverse (H16401) primers (0.2 M each) (Vigilant et al., 1989) in a final volume of 25.5 µl containing 10-50 ng DNA, 1.5 mM $MgSO_4$, 1x Optimase reaction buffer, 10 mM each dNTP, 1.25 U/µl Optimase polymerase and sufficient sterile distilled water (Transgenomic, USA). Amplification was performed in a Mastercycler® ep PCR gradient thermal cycler (Eppendorf, Germany). The amplification conditions were as follows: initial denaturation at 94^0C for 2 min, followed by 30 cycles at 94^0C for 30 sec, 56^0C for 30 sec and 72^0C for 90 sec, and final elongation at 72^0C for 5 min. Negative controls were prepared for both the DNA extraction and the amplification processes. PCR products were checked by 2% agarose gel electrophoresis in 1x Tris-Borate-EDTA (1xTBE, pH 8.3) buffer with ethidium bromide (0.2 mg/ml) staining and visualization using an ultraviolet-wavelength transilluminator.

The PCR products were heated to denature the DNA and were then slowly cooled to room temperature to allow for reannealing of the DNA strands using the following protocol: initial denaturation at 95^0C for 2 min, followed by a 95^0C to 85^0C (-2^0C/sec) decrease, and then a 85^0C to 25^0C (-0.10^0C/sec) decrease. This heating-and-cooling process resulted in the formation of a single homoduplex fragment in homoplasmic individuals and a series of homoduplex and heteroduplex fragments in heteroplasmic individuals. Afterwards, products were incubated with Surveyor enhancer S 0.5 µl and Surveyor nuclease S 0.5 µl at 42^0C for 20 min; 1 µl of stop solution was added at the end of reaction. The samples were analyzed on a 6% polyacrylamide gel electrophoresis with ethidium bromide staining and visualized using an ultraviolet wavelength transilluminator.

2. Denaturing Gradient Gel Electrophoresis (DGGE)

HVS-I was analyzed as two overlapping fragments using PCR primer pairs F15989/R16258GC and F16144GC/R16410 (Anderson et al., 1981; Tully et al., 2000). A GC clamp added to the 5′ end of one primer in each pair (GC) made the resulting PCR products suitable for DGGE analysis (Myers et al. 1985; Tully et al. 2000). PCR amplification was performed by combining 10-50 ng DNA templates, 10xPfu buffer with $MgSO_4$, 10 mM primers, 10 mM dNTPs, deionized water, and 2,5 U/µl Pfu DNA polymerase (Fermentas, Lithuania). In order to decrease the artificial generation of amplification and sequencing errors by Taq DNA polymerase during PCR, Pfu DNA polymerase was used for the amplification of samples for DGGE and prior to sequencing analysis. PCR cycling consisted of initial denaturation at 94^0C for 2 min, followed by 35 cycles at 94^0C for 30 sec, 50^0C for 30 sec and 72^0C for 2 min, and final elongation at 72^0C for 5 min. PCR products were checked by 2% agarose gel electrophoresis in 1xTBE buffer with ethidium bromide staining and visualized using an ultraviolet-wavelength transilluminator. Prior to DGGE analyses we used the same protocol for homo-and hetero-duplexes produced as described above. DGGE was performed using a 5% to 10% polyacrylamide gel gradient and 40% -50% chemical (i.e., urea/formamide) denaturing gradient for 15 h at 100 volts, 45 mA,

60^0C. The gel was stained with ethidium bromide and 1x Vistra green (Molecular Probes, USA) for 20 min and visualized using an UV transilluminator (Figure 1). DGGE separation of homoduplex and heteroduplex fragments, followed by elution reamplification and sequencing of the DNA heteroplasmic bands, allowed to identify the heteroplasmic positions.

3. Sequencing

Prior to sequencing, the PCR products of the HVS-I, which were amplified using the primer set from Vigilant et al., 1989, were purified with shrimp alkaline phosphatase (SAP) and exonuclease I (ExoI) (Werle et al., 1994). PCR products were sequenced directly from both strands using the DYEnamic™ ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Sweden) according to the manufacturer's protocol. The cycle-sequencing profile was 25 cycles of 96^0C for 10 sec, 50^0C for 5 sec, and 60^0C for 4 min. Sequencing reactions were run on an ABI Prism 3100 DNA automated sequencer (Applied Biosystems, USA). The sequences were compared with the rCRS using Contig Express software (Invitrogen, USA). All mutations in this study are reported as differences from rCRS; only transversions are further specified.

mtDNA genotyping

To confirm the haplogroup affiliation of mtDNA sequences, hierarchical PCR – RFLP analysis was performed using 17 restriction endonucleases: AluI, AvaII, DdeI, Bsh1236I, HaeIII, HhaI, HinfI, MboI, RsaI, NlaIII, AccI, BstOI, MseI, Alw44I, SspI, Eco47I, BsuRI. Classification of haplogroups was based on Torroni et al., (1996), Richards et al., (1998), Macaulay et al., (1999), Finnila et al., (2001), Kivisild et al., (2002).

Statistical analysis

Confidence intervals for the observed population frequency of mtDNA heteroplasmy were calculated using the standard equation for true proportions (Colton 1974). Statistical significance of the observed mtDNA haplogroup differences in heteroplasmic samples taken from three age groups was evaluated with the G-test, and the significance level was adjusted (Bonferroni) for the multiple pairwise comparisons. The combined influence of the age group and mtDNA

haplogroup on the probability of heteroplasmy was tested by binary logistic regression analysis using SPSS software, version 17.0 (Anonymous, 2007). The response variable (heteroplasmy) was binary (presence=1; absence=0). Two predictor variables presenting interest included: age class (three age groups), and mtDNA haplogroup (ten haplogroups, namely, H, HV, V, J, T, U, I, W, X and M). Haplogroup U encompassed subhaplogroups U*, U2, U3, U4, U5, and U8. Actual levels for age and haplogroup were changed to "dummy variables" (the first levels – haplogroup U and age group of 18-40 years – were used as a reference). Differences with p values equal to or under 0.05 were considered significant. In Table 3 "dummy variable" is changed back to the initial levels.

Results

1. Distribution of mtDNA heteroplasmy within HVS-I region in three age groups

Heteroplasmy was reproducibly found in 91/300 individuals in the Latvian population, resulting in an overall incidence of 30.3%. Among the 91 heteroplasmic individuals, mtDNA heteroplasmy was found at 21 different positions; it was observed more frequently in nucleotide substitutions of pyrimidines than purines (17 nps vs. 4 nps). This can be explained by the earlier observed fact that overall transitions between pyrimidines (C/T) are nearly three times as abundant as transitions between purines (A/G) in HVS-I (Wakeley 1993). The wide range of nucleotide positions, in which heteroplasmy appeared, are characterized by a diverse substitution rate, from fast-to slowly-mutated nucleotide positions. Transitions at nps 16093, 16129, 16189, 16311 and 16362 in HVS-I are considered as mutational "hotspots" and are often observed in different phylogenetic branches of mtDNA (Hasegawa et al., 1993; Finnila et al., 2001). Among Latvians, heteroplasmy was found at six positions with marked frequency of its occurrence (more than 5%): 16093, 16126, 16189, 16192, 16304, and 16311 (Table 1a). Furthermore the relative rate of evolutionary substitutions of these sites is also quite high (Hasegawa et al., 1993).

Therefore, our results and those of other reports on mtDNA heteroplasmy in the HVS-I indicated that fast-mutated positions are predisposed to form heteroplasmy (e.g. Bendall et al., 1996; Parsons et al., 1997; Howell and Smejkal, 2000; Sigurdardottir et al., 2000; Tully et al., 2000; Santos et al., 2008). Despite the fact that heteroplasmy was more frequently found at position 16189, the proportion of non-heteroplasmic and heteroplasmic individuals at this particular position was almost equal (9.0% vs. 10.3%) (Table 1b). Similarly to our findings, Chen et al. (2009) identified C-stretch length heteroplasmy at position 16189 in ~9% of 919 analyzed samples from various Chinese ethnic groups. Among the studied age groups the 16189C variant was found in two predominate mtDNA phylogenetic branches, haplogroup H and subhaplogroups U (U4, U5 and some minor subgroups of U), which encompass almost 70% of all mitochondrial genomes in the Latvian population (Pliss et al., 2006). Similarly, Forster et al., (2010) showed that a high proportion of 16189C alleles among 1172 mtDNA analyzed sequences were associated with the mtDNA type U1a. The T to C substitution at nucleotide position 16189 leads to C-stretch length heteroplasmy approximately ranging from nps 16184 to 16193 (Bendal and Sykes, 1995; Forster et al., 2010). In addition, length heteroplasmy found in this region of the HVS-I reproduced a complex DGGE pattern. In our study, individuals with HVS-I motif 126-163-186-189-294 (haplogroup T1) resulting in a string of seven cytosines did not exhibit length heteroplasmy, thereby supporting the hypothesis that eight cytosines is likely the critical number (Melton 2004; Chen et al., 2009; Irwin et al., 2009; Forster et al., 2010). Interestingly, at some positions with a relatively pronounced substitution rate, heteroplasmy was occasionally found, e.g. positions 16129 (4.4%) and 16294 (1.1%) (Table 1a). The distribution of heteroplasmy in three age groups of the Latvian population was non-uniform: it was more frequently observed in elderly individuals at various mtDNA HVS-I positions in comparison with the control group, and the observed age-related pattern of mtDNA heteroplasmy was statistically significant. In the 18-40 year age group mtDNA heteroplasmy was reproducibly detected in 17 cases of 100 individuals (17%; CI 0.095-0.244) at eight positions compared to 35

cases of 100 individuals (35%; CI 0.256-0.444) at 13 different positions in the 70-80 year group, and 39 cases of 100 individuals (39%; CI 0.294-0.487) at 14 different positions in the nonagerian group (Table 1a). Comparing the age groups of 70-80 years and over 90 years, respectively, frequency of heteroplasmy was higher in the latter, while the observed differences between the two age groups were not significant (Table 1a). Lack of further increase in heteroplasmy with age may be explained by slight difference in the age range between the age groups of 70-80 years and over 90 years, and the relatively small portion of centenarians (32% of the samples in the nonagerian group).

2. Age-related association of heteroplasmy with mtDNA haplogroup-defining polymorphisms

The results obtained on the pattern of heteroplasmy in three age groups of the Latvian population provoked us to search for a possible association between non-coding and coding region mtDNA variability. Thus, we screened mtDNA haplogroup variability in the same individuals, and determined the frequency distribution of heteroplasmic subjects within each haplogroup category. In addition to the aforesaid, our analysis of mtDNA haplogroups' variation in three age groups revealed that randomly chosen individuals represent the gene pool of mtDNA variants, which is characteristic of the Latvian population, and the composition of observed mtDNA haplogroups was similar in all studied age groups (Table 2).

Heteroplasmy was found in three mtDNA haplogroups (H, U, and I) in the control group. In the meanwhile, in elderly individuals it was distributed among a wider set of haplogroups (Table 2). Examination of the data included investigation of whether or not the distribution of heteroplasmy was different between haplogroups in the three age groups. G-test was applied to verify this relationship. Notably higher abundance of haplogroup H was found in the age group of 18-40 years (82.4%), compared to the mid and nonagerian groups (31.4% and 41.0%; $p \leq 0.05$, respectively). The opposite gradient of frequency distribution was observed for haplogroup U, as

the frequency increased with age: 5.9%, 37.1% and 35.9% in the young, mid and older age groups, respectively (Table 2). However, the differences between the three analyzed age groups with respect to the frequency of haplogroup U were not significant.

In the binary logistic regression analysis, we have tested the role of age and mtDNA haplogroups on the presence of mtDNA heteroplasmy. We have not only included in our analysis the interaction between haplogroups and age groups but also separately checked the relation of the two predictor variables (haplogroups and age groups) to heteroplasmy (Table 3). Haplogroup U was used as the reference haplogroup in the binary logistic analysis, because it corresponds to overall pattern of age-based gradual increase of heteroplasmy. Difference between the most frequent haplogroups H and U was significant in the studied population. As regards the other mtDNA haplogroups, only haplogroup J was showed significant relation to heteroplasmy, compared to haplogroup U. The number of heteroplasmic individuals in haplogroup J was low, however, in all age groups (Table 2); therefore this interaction term should be taken with caution. We have also evaluated age-based difference progress in these haplogroups with the interaction term in logistic regression. Our results show that the age-based change in heteroplasmy depends on phylogenetic background: heteroplasmic samples belonging to haplogroup H were found to be significantly less frequent in individuals over 90 years than in the controls (odds ratio = 0.051, 95% CI =0.005 – 0.511, p = 0.011 (Table 3), compared to haplogroup U.

Besides, we have observed significant relation of heteroplasmy distribution to the age of the studied individuals in the analysis of logistic regression where only the influence of age on the occurrence of mtDNA heteroplasmy was considered, and this matches the calculated confidence intervals for heteroplasmy distribution in three age groups (Tables 1a, 3).

Cox and Snell R^2 coefficient in analysis of the logistic regression indicated that 19.4% of the observed variability of the heteroplasmy could be captured by the mtDNA haplogroup background and age. However, there is still a vast portion of undiscovered genetic variability that may influence the presence of mtDNA heteroplasmy in the analyzed samples. We can

propose that the remainder of heteroplasmy distribution is due to possible interactions between nuclear genetic factors, mitochondrial genes, and environmental components.

3. Comparison of mtDNA heteroplasmy detection methods and their limit of detection

We found that the DGGE and the SURVEYORTM Mutation Detection Kit assay exhibited a lower limit of detection (LOD) of mtDNA heteroplasmy and revealed the presence of heteroplasmy at the already existing 1% and 5% occurrence in the sample, respectively, while the LOD of heteroplasmy detectable by sequence analysis was about 20–25%. Sequencing analysis showed pronounced differences in the intensity of the fluorescent sequence signal for different variants at the position of interest, indicating sequence-specific-strength artefacts. Advantages and drawbacks of the exploited methods for detection of mtDNA heteroplasmy were evaluated by three parameters (time and labor, LOD and costs) and are summarized in table 4.

The assays used for detection of mtDNA heteroplasmy together with technical supplements required for these methods were combined in an efficient methodological protocol. First, all samples were screened with the most rapid, precise and non-labor intensive method: the SURVEYOR™ Mutation Detection Kit. Second, to confirm mtDNA heteroplasmy, DNA samples were analyzed by DGGE. To identify heteroplasmic positions in the HVS-I, both homoduplex bands were excised from the DGGE gel, eluted, reamplified and sequenced (Figure 2). Our results showed that heteroplasmy within this detection range was quite common, occurring in 30.3% of the studied age groups at a broad spectrum of sites. The percentage of heteroplasmy revealed among individuals in our study groups reflected the minimum LOD of heteroplasmy (~1.0%) by the methods used. However, within these age groups, we can not exclude the existence of additional heteroplasmies whose level of occurrence was below our ability to resolve them.

Discussion

Most age-related studies on mtDNA heteroplasmy have focused on the common deletions and point mutations in the coding region of mtDNA (e.g. Hattori et al., 1991; Cortopassi et al., 1992; Melov et al., 1995). However, over the course of the last decade the non-coding region of mtDNA has been preferred for studies on heteroplasmy distribution (e.g. Tully et al., 2000; Calloway et al., 2002; Malik et al., 2002; Rose et al., 2007; Santos et al., 2008). Having selected the mtDNA HVS-I region for an age-related association study, we estimated heteroplasmy frequency to be 30.3%, and it was observed across a broad spectrum of sites with different mutation rates. The incidence of heteroplasmy found in the present study was higher than the level of 14% previously reported for

HVS-I heteroplasmy determined using DGGE and sequencing (Tully et al., 2000), but was considerably higher than those reported in studies where sequencing analysis (Bendall et al., 1996) or single – strand conformation polymorphism (SSCP) analysis (Gocke et al., 1998) was used alone: respectively, frequencies of 1.48% and 2.53%. Technical approaches used for detection of mtDNA admixture were usually unable to reveal the heteroplasmy at <10% occurrence in the clinical samples due to an insufficient level of LOD. Recently, a vast spectrum of modern technologies for genetic analyses has appeared and has greatly increased the chance of identifying the presence of the mtDNA heteroplasmy. Among these technologies, mostly a combination of DHPLC and pyrosequencing has been used (Rose et al., 2007; Ballana et al., 2008). DHPLC as well as DGGE are characterized by a low level of LOD allowing to heteroplasmy at 1% occurrence in the sample to be distinguished. However, both techniques do not specify the position of the mutated nucleotide. In contrast, pyrosequencing is able to identify the level of heteroplasmy up to 5% and the mutated nucleotide position (Biggin et al., 2005; Rose et al., 2007).

He et al., (2010) used the parallel sequencing-by-synthesis approach for the analysis of the whole mitochondrial genome from normal and colorectal tumor cells. By this very precise assay heteroplasmy was detectable at a 1.6% level in the sample, and more importantly, it allowed the mutated nucleotide to be simultaneously revealed. In our study we have evaluated a protocol consisting of three detection strategies (Table 4), each of which, by itself was not able to detect heteroplasmy and the position at which it occurred in the sample. The most promising are the so-called next generation sequencing (NGS) methods exemplified by the 454 (Roche), Gene Analyzer II (Solexa/Illumina), ABI and Helicos Systems. Resolution of mtDNA heteroplasmy at a high level of detail would appear within reach of NGS methods. As DNA sequencing methods move toward lower costs coupled with high throughput, increased resolution and improved limits of quantification of mtDNA heteroplasmy, may change and evolve with improvements in data (Barker and Murthy, 2009). Therefore, the development of high-throughput, rapid and with low LOD advanced technologies for detection of heteroplasmy is still an important goal.

The clinical relevance of mtDNA inherited polymorphisms has been shown by correlation of mtDNA haplogroups with longevity (e.g. De Benedictis et al., 1999; Niemi et al., 2003; Tanaka et al., 2000) and degenerative disease (Wallace, 2005). Hence, certain mtDNA lineages from Europe and Asia are protective against the ravages of aging but are population specific. mtDNA variability depends on both the genetic and environmental background (Wallace, 2005). However, there are few studies on HVS-I heteroplasmy affiliation to specific mtDNA phylogenetic branches, i.e. haplogroups. In particular, persistent heteroplasmy at nucleotide location 16192 among some members of the mtDNA haplogroup U has been detected, and it is suspected to be related to polymorphism at 16189 (Howell and Smejkal 2000). A similar mechanism was observed among the members of haplogroup T, which harbour the polymorphism at 16294 helping to produce or sustain heteroplasmy at 16296 (Pike 2006).

Observations made during our study show that individuals carrying haplogroup U would have higher risk of developing age-based heteroplasmy. The opposite association was found in respect

of the haplogroup H: the frequency of heteroplasmy within this mtDNA lineage trends to decrease with the age. We have found no association of the presence of heteroplasmy and mtDNA haplogroups between the age groups of 70-80 years and over 90 years, respectively. Similarly, Rose et al., (2007) have found that by comparing the distribution of heteroplasmic subjects within haplogroups/haplotypes the differences between centenarians and the controls (median age 71 years) were not significant.

There could be several explanations for established genetic association in our study. Firstly, the observed relationship might be related to the genetic composition of the studied age groups, mtDNA genotypes which mainly belong to two main haplogroups H (mean frequency 44.0%) and U (mean frequency 30.7%) (Table 2). A further possibility may be related to the fast-mutating position affiliation to specific mtDNA haplogroups. For example, samples that bore heteroplasmy at nps 16304 and 16311 were characterized by relative high substitution rates (Hasegawa et al., 1993) were linked to haplogroup H. Another similar observation was found among heteroplasmic samples at nps 16189 and 16192 affiliated to haplogroup U.

In this study, we provide evidence to the allegation that specific mitochondrial haplogroup background may affect the probability of heteroplasmy with the age. mtDNA haplogroup defining polymorphisms – the 12308A>G (haplogroup, hg U) and the 7028C>T (hg H) – have been previously found to be associated with such diseases as neurodegenerative disorders, coronary artery disease, and mitochondrial disorders (e.g. Majamaa et al., 1998; Hudson et al., 2007). These mtDNA coding region polymorphisms may cause alterations in OXPHOS performance and ROS production, and consequently influence the risk of heteroplasmy occurrence. However, further investigations need to be performed to understand better the functional significance of haplogroup defining polymorphisms on the occurrence of heteroplasmy in different age groups. Since a number of haplogroup associations are population specific, additional populations should be examined to validate these results. Furthermore, the genomewide association studies revealed that disease prevalence, severity, and resistance vary

considerably among ethnic groups as a consequence of inherited factors and non-inherited causes (Rotimi and Jorde, 2010).

In conclusion, a thorough analysis of mtDNA point and length heteroplasmy was performed during the course of this study. The developed methodological protocol can provide not only satisfactory solutions for previously unsolved problems concerning mtDNA heteroplasmy detection, but can also be used as a diagnostic tool for understanding pathogenic mechanisms of mtDNA-associated pathologies.

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Table 1. Frequency of mtDNA heteroplasmy within HVS-I region in 300 maternally unrelated individuals from three age groups.

A. Distribution of heteroplasmic positions in three age groups.

Position (type)[1] (between 16021-16399)	18-40 years		70-80 years		over 90 years		Total number of observations in three age groups		Relative rates of evolutionary substitutions per site (according to Hasegawa et al., 1993)
	Number of observations	Relative frequency (%)	Number of observations	Relative frequency (%)	Number of observations	Relative frequency (%)	Number of observations	Relative frequency (%)	
16092 (C/t)	-	-	-	-	1	2,6	1	1,1	3
16093 (C/t)	2	11,8	1	2,9	2	5,1	5	5,5	8
16126 (T/c)	-	-	3	8,5	5	12,8	8	8,8	6
16129 (A/g)	2	11,8	-	-	2	5,1	4	4,4	15
16168 (C/t)	1	5,9	-	-	-	-	1	1,1	2
16172 (T/c)	-	-	-	-	1	2,6	1	1,1	8
16189 (T/c)	7	41,0	11	31,4	13	33,3	31	34,0	15
16192 (C/t)	-	-	2	5,7	4	10,3	6	6,6	4
16237 (A/g)	-	-	-	-	1	2,6	1	1,1	1
16256 (C/t)	-	-	1	2,9	-	-	1	1,1	5
16263 (T/c)	-	-	1	2,9	-	-	1	1,1	2
16278 (C/t)	1	5,9	-	-	1	2,6	2	2,2	8
16294 (C/t)	-	-	1	2,9	-		1	1,1	9
16298 (T/c)	-	-	1	2,9	1	2,6	2	2,2	6
16304 (C/t)	1	5,9	6	17,0	3	7,6	10	11,0	6
16311 (C/t)	2	11,8	5	14,2	3	7,6	10	11,0	14
16319 (G/a)	-	-	1	2,9	-	-	1	1,1	5
16343 (A/g)	-	-	-	-	1	2,6	1	1,1	1
16354 (C/t)	1	5,9	-	-	-	-	1	1,1	2
16356 (T/c)	-	-	1	2,9	-	-	1	1,1	2
16362 (T/c)	-	-	1	2,9	1	2,6	2	2,2	8
Total number of observations	17	100,0	35	100,0	39	100,0	91	100,0	
Number of tested individuals	100 (100,0%)		100 (100,0%)		100 (100,0%)		300 (100,0%)		
Frequency of heteroplasmic individuals (95% CI)	17% (0.095-0.244)		35% (0.256-0.444)		39% (0.294-0.487)		30,3%		

B. Distribution of heteroplasmy at position 16189 among studied individuals.

Heteroplasmy at position 16189 among studied individuals	18-40 years		70-80 years		over 90 years		Total number of observations in three age groups	
	Number of observations	Relative frequency (%)	Number of observations	Relative frequency (%)	Number of observations	Relative frequency (%)	Number of observations	Relative frequency (%)
16189 (non-heteroplasmic individuals)	9	9.0	8	8.0	10	10.0	27	9.0
16189 (heteroplasmic individuals)	7	7.0	11	11.0	13	13.0	31	10.3
Number of tested individuals	100	16.0	100	19.0	100	23.0	300	19.3

Notes: [1]Nucleotide positions where heteroplasmy was observed. Numbering and sequence are according to Anderson et al. (1981) and Andrews et al. (1999). CI – confidence intervals.

Table 2. Distribution of heteroplasmic individuals among mtDNA haplogroups in three age groups.

mtDNA haplogroups	18-40 years		70-80 years		over 90 years		Total number of observations in three age groups	
	Heteroplasmic samples (relative frequency, %)	Determined haplogroups in all studied individuals (relative frequency, %)	Heteroplasmic samples (relative frequency, %)	Determined haplogroups in all studied individuals (relative frequency, %)	Heteroplasmic samples (relative frequency, %)	Determined haplogroups in all studied individuals (relative frequency, %)	Heteroplasmic samples (relative frequency, %)	Determined haplogroups in all studied individuals (relative frequency, %)
H	14 (82,4)	55 (55,0)	11 (31,4)	34 (34,0)	16 (41,0)	43 (43,0)	41 (45,1)	132 (44,0)
HV	-	2 (2,0)	2 (5,7)	3 (3,0)	-	2 (2,0)	2 (2,2)	7 (2,3)
V	-	1 (1,0)	-	2 (2,0)	-	6 (6,0)	-	9 (3,0)
J	-	7 (7,0)	1 (2,9)	5 (5,0)	4 (10,3)	6 (6,0)	5 (5,5)	18 (6,0)
T	-	3 (3,0)	6 (17,2)	9 (9,0)	2 (5,1)	11 (11,0)	8 (8,8)	23 (7,7)
U	1 (5,9)	28 (28,0)	13 (37,1)	39 (39,0)	14 (35,9)	25 (25,0)	28 (30,8)	92 (30,7)
I	2 (11,7)	3 (3,0)	2 (5,7)	2 (2,0)	1 (2,6)	1 (1,0)	5 (5,4)	6 (2,0)
W	-	1 (1,0)	-	5 (5,0)	2 (5,1)	5 (5,0)	2 (2,2)	11 (3,7)
X	-	-	-	-	-	1 (1,0)	-	1 (0,3)
M	-	-	-	1 (1,0)	-	-	-	1 (0,3)
Total number of observations	17 (100,0)	100 (100,0)	35 (100,0)	100 (100,0)	39 (100,0)	100 (100,0)	91 (100,0)	300 (100,0)

Table 3. Logistic regression of mtDNA.

	Parameter	Standard error	Wald z-statistic	df	p-values	Odds Ratio	Lower 95% CI for Odds Ratio	Upper 95% CI for Odds Ratio
Age			**11,242**	**2**	**,004**			
70-80 years	**2,603**	**1,074**	**5,878**	**1**	**,015**	**13,500**	**1,646**	**110,689**
over 90 years	**3,537**	**1,095**	**10,431**	**1**	**,001**	**34,364**	**4,017**	**293,968**
Haplogroup			6,764	9	,662			
H	**2,221**	**1,064**	**4,356**	**1**	**,037**	**9,220**	**1,145**	**74,249**
M	-20,510	40192,970	,000	1	1,000	,000	,000	.
HV	-17,907	28420,723	,000	1	,999	,000	,000	.
V	-17,907	40192,970	,000	1	1,000	,000	,000	.
J	-17,907	15191,515	,000	1	,999	,000	,000	.
T	-17,907	23205,420	,000	1	,999	,000	,000	.
J	**3,989**	**1,593**	**6,272**	**1**	**,012**	**54,000**	**2,380**	**1225,164**
W	-17,907	40192,973	,000	1	1,000	,000	,000	.
X	-21,444	40192,970	,000	1	1,000	,000	,000	.
Age * Haplogroup			13,957	14	,453			
70-80 years by H	-2,266	1,176	3,713	1	,054	,104	,010	1,040
70-80 years by HV	19,293	28420,723	,000	1	,999	,000	,000	.
70-80 years by V	-2,603	49226,134	,000	1	1,000	,074	,000	.
70-80 years by J	17,214	15191,515	,000	1	,999	,000	,000	.
70-80 years by T	19,293	23205,420	,000	1	,999	,000	,000	.
70-80 years by I	17,907	28420,722	,000	1	,999	,000	,000	.
70-80 years by W	-2,603	44029,196	,000	1	1,000	,074	,000	.
over 90 years by H	**-2,986**	**1,181**	**6,392**	**1**	**,011**	**,051**	**,005**	**,511**
over 90 years by HV	-3,537	40192,971	,000	1	1,000	,029	,000	.
over 90 years by V	-3,537	43413,370	,000	1	1,000	,029	,000	.
over 90 years by J	18,359	15191,515	,000	1	,999	,000	,000	.
over 90 years by T	16,162	23205,420	,000	1	,999	,000	,000	.
over 90 years by I	16,973	40192,970	,000	1	1,000	,000	,000	.
over 90 years by W	17,260	40192,973	,000	1	1,000	,000	,000	.
Constant	-3,296	1,018	10,475	1	,001	,037		

Table 4. Opportunities and drawbacks of three methods used for detection of mtDNA heteroplasmy.

Parameters	Denaturing Gradient Gel Electrophoresis (DGGE)	Sequencing	Mismatch-specific Surveyor nuclease
Time and Labor	Time consuming (~ 14 hours); Intensive: consists of a lot of pipetting steps, procedures in thermal cycler, accurate preparation of lagger sized (16 cm x 18 cm) denaturing gradient polyacrilamide gel for discrimination of homo- and heteroduplexes.	Intermediate time consuming (~ 9 hours); Intermediate intensive: consists of a lot of pipetting steps, procedures in thermal cycler, additional treatment of samples after PCR product labeling reaction, capillary electrophoresis.	Rapid (~ 5 hours); Not intensive: consists of few pipetting steps and procedures in a thermal cycler.
Load of detection (LOD)	High: allow to detect the presence of heteroplasmy at 1% occurrence in the samples achieved by use of denaturing agents and polyacrylamide gel gradient. Due to limit of analyzed fragment size (max. 400 bp) two overlapping fragments were used.	Low: direct sequencing can provide a poor means for detection of heteroplasmy (20-25%), unless extensive confirmatory experiments are performed. Limit of analyzed fragment size is ~ 2000 bp.	Intermediate: allow to detect the presence of heteroplasmy at 5% occurrence in the samples. Limit of analyzed fragment size is ~ 3000 bp.
Costs	Low: the stock reagents (e.g. urea, formamide, acrylamide, bis-acrylamide) once have been purchased and afterwards are used for a long time (approximate costs per sample is 1 euro).	High: dye - labeled dideoxynucleotides (ddNTPs or terminators) and capillary electrophoresis are costs - consuming (approximate costs per sample is 20 euro).	Intermediate: approximate costs of mismatch - specific Surveyor nuclease per sample is 3 euro.

Figures legends:

Figure 1. Detection of mtDNA HVS-I heteroplasmy by DGGE system. Size markers: pUC19 (lane 1) and 100bp ladder plus (lane 2) (Fermentas, Lithuania); PCR products with rCRS mtDNA haplotype (lanes 3 – 5); PCR products with 16129 A/g heteroplasmy (lanes 6 – 8).

Figure 2. Sequencing results of heteroplasmic sample. The original PCR product (A) appears to be heteroplasmic (C/t) at position 16093. Sequence data of excised homoduplexes from DGGE gel (B, C) confirmed the heteroplasmic position.

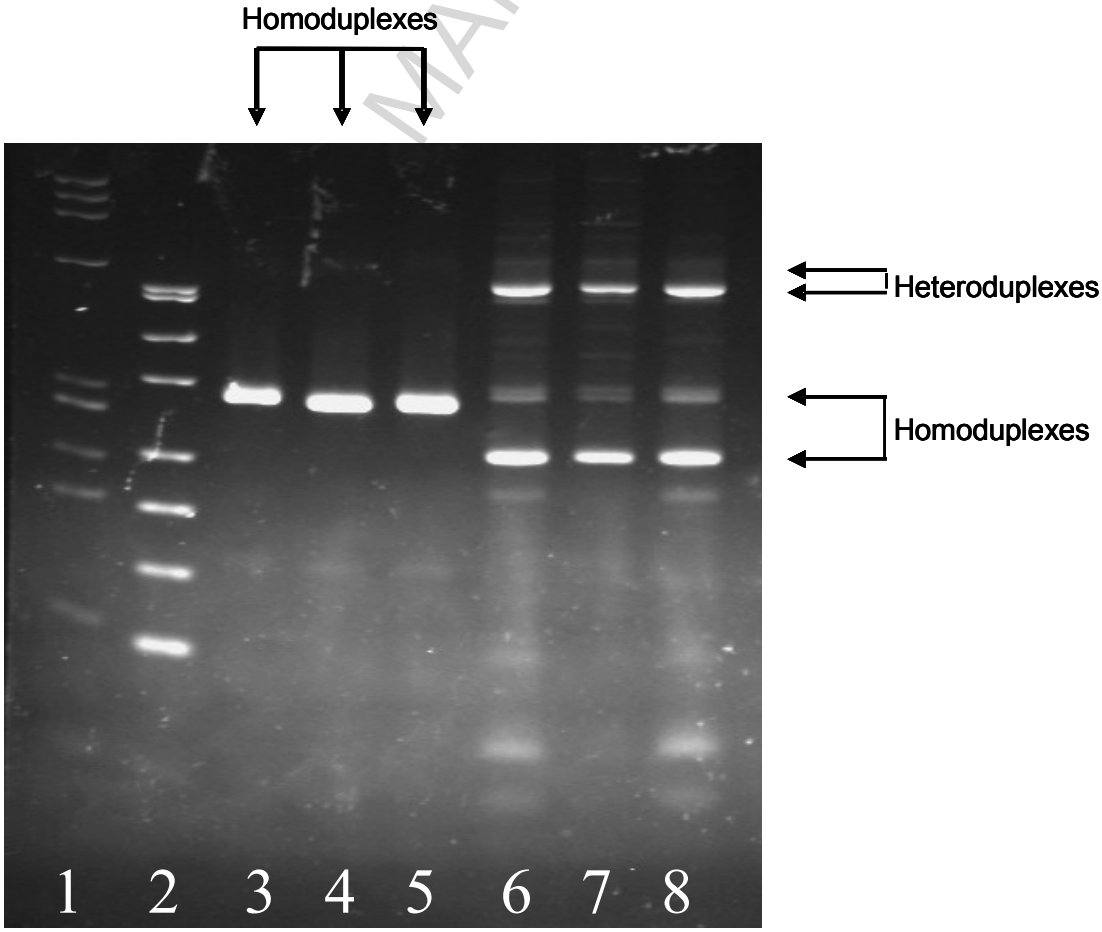

Fig. 1

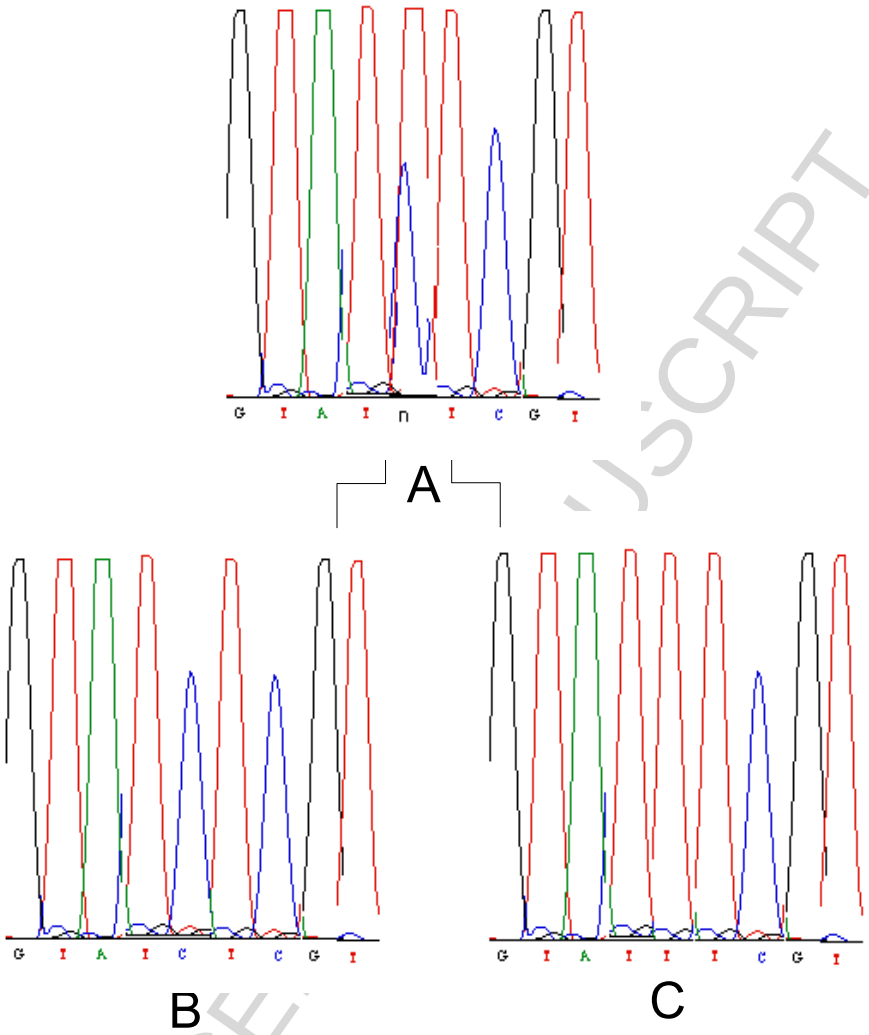

Fig. 2