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ORIGINAL ARTICLE

Single lymphocytes from two healthy individuals with mitochondrial point heteroplasmy are mainly homoplasmic

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Abstract The nature of mitochondrial DNA heteroplasmy is still unclear. It could either be caused by two mitochondrial DNA (mtDNA) haplotypes coexisting within a single cell or by an admixture of homoplasmic cells, each of which contains only one type of mtDNA molecule. To address this question, single lymphocytes were separated by flow cytometry assisted cell sorting and analyzed by cycle sequencing or minisequencing. To attain the required PCR sensitivity, the reactions were carried out on the surface of chemically structured glass slides in a reaction volume of 1–2 μl. In this study, blood samples from two healthy donors showing mitochondrial point heteroplasmy in direct sequencing (195Y and 234R, respectively) were analyzed. Nearly 96% of single lymphocytes tested were found to be in a homoplasmic state, but heteroplasmic cells were also detected. These results suggest that mitochondrial point heteroplasmy in blood may well be mainly due to the mixture of homoplasmic cells.

Keywords Mitochondrial DNA · Sequence heteroplasmy · Single cells · Flow cytometry

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Introduction

Mitochondrial heteroplasmy is usually defined as the presence of more than one type of mitochondrial DNA (mtDNA) within an individual [1, 2]. It can be differentiated into both sequence and length heteroplasmy [3–5]. A sequence, point, or site heteroplasmy is defined as sequences presenting different bases at the same base position, and the sequence electropherogram usually shows superimposition of two different bases at this base position.

In the early days of using mtDNA analysis for forensic casework, it was assumed that with the exception of mitochondrial diseases [6, 7], only a single mtDNA variant is present in an individual [8–10]. With the growing number of samples typed, the analytical methodology and thus the sensitivity and sequence quality improved, and it became obvious that the frequency of sequence heteroplasmy had been underestimated [11–15].

The occurrence and frequency of mitochondrial heteroplasmy is still poorly understood. First of all, it is not yet clear at which one of three possible levels mitochondrial heteroplasmy occurs within an individual. These are:

1. The tissue: each cell carries only a single mtDNA haplotype, but different cells carry different mtDNA haplotypes.
2. The cell: a cell carries different mtDNA haplotypes, but each single mitochondrion is homoplasmic.
3. The mitochondrion: the mitochondrion itself carries different mtDNA haplotypes.

In this study, we analyzed point heteroplasmy at the single cell level. To attain the required PCR sensitivity, the reaction volume was reduced to 1 µl. This low volume (LV) amplification was performed on chemically structured glass slides [16]. In combination with flow cytometry, it was possible to sort and deposit single lymphocytes on the glass slide and analyze them individually by PCR-based mtDNA typing. To study mitochondrial point heteroplasmy in single cells, two different techniques were applied, which included amplification of mtDNA followed by either cycle sequencing or minisequencing. As control experiments, PCR products amplified from either single cells or DNA extracted from leukocyte suspension were cloned, and different clones were sequenced. All experiments were performed with either DNA extracted from isolated leukocytes (also referred to as "leukocyte suspension" or "leukocyte extract") or with single lymphocytes separated from the leukocyte suspension by flow cytometry (referred to as "single cells" or "single lymphocytes").

This study contributes to a better understanding of the phenomenon of mitochondrial heteroplasmy, which can be crucial in the interpretation of forensic casework data.

Materials and methods

Samples

Blood samples were taken from two adults with informed consent and treated with ethylenediaminetetraacetic acid (EDTA). Subject 1 exhibits a T/C point heteroplasmy at position 195 and subject 2 a 234 A/G point heteroplasmy (see Table 1; numbering of nucleotides according to the revised Cambridge reference sequence, rCRS) [17].

Table 1 Differences to the revised Cambridge reference sequence (rCRS; [17]) within the control region of the two subjects

Individual	Differences to rCRS
Subject 1	16129A, 16223T, 16227C, 16290T, 16311C, 16319A, 64T, 73G, 195Y, 235G, 263G, 315.1C, 523del, 524del
Subject 2	16189C, 16193.1C, 16356C, 16362C, 16519C, 234R, 263G, 315.1C, 523del, 524del, 573.1C, 573.2C

Chips

Chemically structured glass slides (AmpliGrid AG480F, Advalytix, Brunnthal, Germany) were used for this study as described previously but with 48 instead of 60 hydrophilic reaction sites arranged in four rows (A–D) of 12 sites [16, 18]. Each reaction site can be supplied with up to 2 µl of reagents, e.g., components of an amplification reaction. Individual reaction mixes are covered with 5 µl of mineral oil to prevent evaporation and cross-contamination of samples.

Isolation of leukocytes from blood

Leukocytes from peripheral blood samples were isolated using Leucosep centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) following the manufacturer's recommendations.

Analysis of mtDNA extracted from isolated leukocytes by in-tube cycle sequencing

DNA from isolated leukocytes was extracted as described [19]. To determine the mtDNA control region sequences of subjects 1 and 2, conventional DNA amplification in PCR reaction tubes and subsequent sequencing were performed as described previously but with primers L15908/H16432, L16268/H159, L16450/H429, and L314/H611 for PCR and sequencing [16] (see Table 2). For additional sequencing of

Table 2 Sequences of the primers used for mitochondrial DNA amplification, sequencing and minisequencing

Primer	5′-3′-Nucleotide sequence
L15908	TACACCAGTCTTGTAAACC
L16268	CACTAGGATACCAACAAACC
H16432	TGTGCGGGATATTGATTTCA
L16450	GCTCCGGGCCCATAACACTTG
L16517	GGTCATAAAGCCTAAATAGCC
L15	CACCCTATTAACCACTCACG
L29	CTCACGGGAGCTCTCCATGC
L162	CGCACCTACGTTCAATATTACAGGCGAACATAC
L162snap	CGCACCTACGTTCAATATTACAGGCGAACATAC
L203snap	GTGTGTTAATTAATTAATGCTTGTAGGACAT
L314	CCGCTTCTGGCCACAGCACT
H159	AAATAATAGGATGAGGCAGGAATC
H280	GATGTCTGTGTGGAAAGTGGCT
H381	GCTGGTGTTAGGGTTCTTTG
H429	CTGTTAAAAGTGCATACCGC
H611	CAGTGTATTGCTTTGAGGAGG

the respective heteroplasmic positions, the sequencing primers L16517, L15, L29, L162, H280, and H381 were used.

Analysis of single cells

Flow cytometry and deposition of single cells Single lymphocytes were deposited on individual reaction sites of the chips using a MoFlo cell sorter (Dako-Cytomation, Ft Collins, CO). The lymphocytes were sorted using forward (low angle, FSC) vs. side scatter (90° scatter, SSC) signals to distinguish between live and dead cells, debris, and other cell types [20], and the area of forward scatter vs. pulse width to distinguish between single cells and cells attached to each other [21]. For sorting out the lymphocytes from the separated blood cells, we selected the population of lymphocytes in the scatter distribution by an ellipse (see Fig. 1a). Doublets were excluded simultaneously in the second distribution by a rectangular sort window (see Fig. 1b). The instrument settings for sorting were nozzle diameter 70 µm, sheath pressure 60 psi, and drop drive frequency 103,000 Hz. A drop volume of 1.4 nl resulted. Additionally, for the single cell sort, a low cell concentration and a slow sort rate were used to reduce the incidence of doublets. As sheath fluid, PBS or Hank's balanced salt solution without Ca^{++} and Mg^{++} and phenol red was used. No cells were placed on row D, and these spots were used for PCR positive and negative controls.

Accuracy of sorting and spotting was checked exemplarily by spotting cells stained with Hoechst-33342 dye (bisbenzimide trihydrochloride, Sigma, St. Louis, MO) and fluorescence microscopy.

On-chip mtDNA amplification and direct sequencing On-chip amplification with primer pair L29/H381 and direct sequencing with primer L29 were performed as described previously [16] but with spotted single lymphocytes instead of diluted DNA template.

Negative controls were performed on six to ten spots from row D using the same reagent solutions. Accordingly, two to six positive controls were carried out using 100 pg of human female DNA 9947A (Promega, Mannheim, Germany).

On-chip mtDNA amplification and minisequencing For minisequencing analysis, the amplification step was performed with PCR primers L162 and H280 (cf. Table 2) and with the following thermal cycling conditions: 95°C for 10 min, 32 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 min. Positive and negative PCR controls were performed as before. To deactivate residual primers and nucleotides, 0.4 µl ExoSap-IT (USB, Cleveland, OH) was added to each reaction spot and incubated at 37°C for 60 min and at 75°C for 20 min.

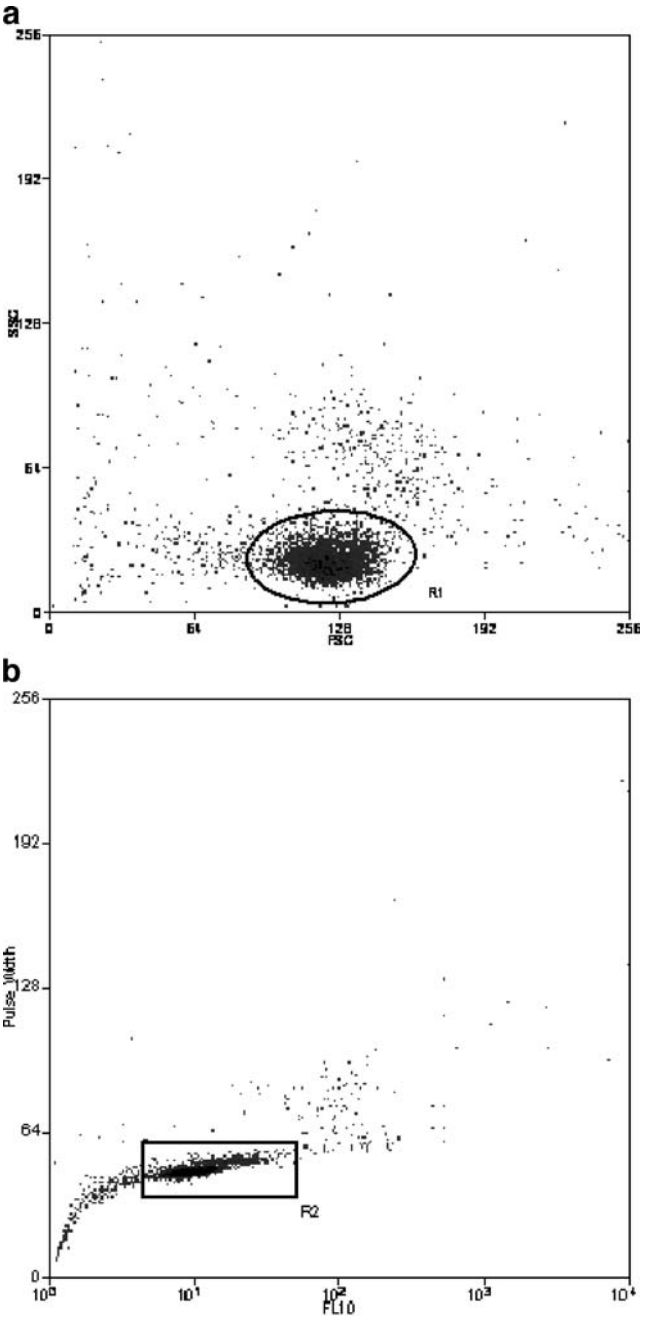

Fig. 1 Representative examples of flow cytometric sort criteria. **a** Forward scatter (*FSC*) and side scatter (*SSC*) are displayed on the *x*- and *y*-axis, respectively. The population of lymphocytes (main group) is selected by an elliptic sort window. **b** FCS-area (*Fl10*) and FCS-width (pulse width) are displayed on the *x*- and *y*-axis, respectively. Outside the sort rectangle we see the doublets (*top right*) and debris (*down left*)

The minisequencing reaction was performed by pipetting 0.5 µl ABI Prism SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA) and 0.1 µl primer L162snap (5 pmol/µl) for subject 1, or L203snap (5 pmol/µl) for subject 2, respectively (cf. Table 2). Reaction conditions for both primers were 25 PCR cycles with 20 s at 96°C, 10 s at 50°C,

and 40 s at 60°C. After primer extension, the samples were removed from the chip and transferred into tubes for treatment with 1 U SAP (USB; at 37°C for 60 min, at 75°C for 15 min). Separation of the products and data analysis was carried out on an ABI Prism 3100 Avant Genetic Analyzer using LIZ120 internal size standard (Applied Biosystems) and GeneMapper ID software v. 3.2 (Applied Biosystems).

Analysis of mtDNA as plasmid insert

Cloning After treatment with ExoSap-IT (see previous paragraph "*On-chip mtDNA amplification and minisequencing*"), amplification products generated from single cells were removed from the chip and cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. In addition, three amplification products per subject were independently generated from extracted leukocyte DNA and were cloned. Plasmids were isolated, purified, and sequenced as previously described [22]. For each cloned amplification product, at least 48 plasmids were sequenced.

Testing PCR amplification efficiency To determine the PCR amplification efficiency for the respective wild-type and mutant mtDNA, four bacterial clones, i.e., one clone per haplotype in subject 2, were chosen. Equimolar mixtures of the mutant and wild-type mtDNA were made by spotting identical numbers of bacterial cells onto 96-well PCR reaction plates. These mixtures were used for amplification of mtDNA followed by cycle sequencing or minisequencing. Techniques were carried out as described for the chip but within a 5-μl reaction volume and corresponding amounts of reagents.

Results

Detection of point heteroplasmy in mtDNA extracted from leukocyte suspension

Detection of point heteroplasmy by direct sequencing

MtDNA sequencing with different primers resulted in high quality electropherograms but with varying ratios of the major and the minor bases at the respective positions (see Fig. 2).

Detection of point heteroplasmy by cloning of amplified mtDNA fragments and sequencing of individual clones

Sequencing of single plasmid inserts of subjects 1 and 2 revealed a 195T:C ratio and a 234A:G ratio of approximately 7:1 (Table 3).

Detection of point heteroplasmy by single cell analysis

Detection of point heteroplasmy by direct sequencing of mtDNA from single cells

Direct on-chip sequencing of amplified mtDNA fragments from single cells (Fig. 3) of subject 1 revealed high quality electropherograms of at least 350 bp in length. There was a T/C/Y ratio of approximately 12:3:1 with a proportion of heteroplasmic cells of 7%. Single cells of subject 2 showed an average A/G ratio of approximately 3:1. Only a proportion of heteroplasmic cells of 1% was detected in this sample (Table 3).

Detection of point heteroplasmy by minisequencing of mtDNA from single cells

Minisequencing analysis of single cells from subject 1 resulted in a T/C/Y ratio of 35:7:1 with a proportion of heteroplasmic cells of 2%. For subject 2, an A/G/R ratio of approximately 17:4:1 with a proportion of heteroplasmic cells of 5% was found (Table 3).

Detection of point heteroplasmy by cloning of amplified mtDNA fragments from single cells and sequencing of individual clones

All sequenced colonies from an individual cell showed only one single haplotype. This was observed for randomly selected single lymphocytes from subjects 1 and 2. All possible haplotypes were analyzed, and up to 90 clones were sequenced.

Prevention of artifacts and contamination

To identify possible artifacts and contaminations, several quality checks were performed.

(a) "Accuracy and quantity check": to reassure that indeed single lymphocytes were placed onto each reaction site, the accuracy of the deposition and the number of the deposited cells was exemplarily tested by microscopy. In Fig. 4, one single spotted cell is located on the circular reaction site. The insert shows a magnification of the respective cell.
(b) "Device zero check": to detect any pre-existing contamination within the cell sorter, the sorting buffer used by the MoFlo was spotted onto one chip before the actual deposition of cells. Amplification with primer pair L29/H381 and electrophoresis on a 2% agarose gel yielded no detectable product.
(c) "Medium zero check": to detect any extracellular mitochondrial DNA within the cell suspension, the

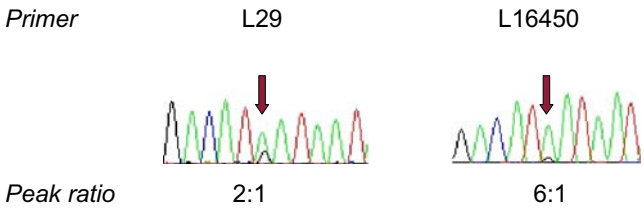

Fig. 2 Sequence electropherograms showing different degrees of point heteroplasmy in leukocyte suspension. Different peak ratios of the bases affected are being displayed depending on the sequencing primer used

rest of the respective sample was centrifuged after sorting, and the supernatant was spotted on one chip. PCR amplification with primer pair L29/H381 and electrophoresis on a 2% agarose gel yielded weak signals in 2 out of 36 samples (rows A–C) at most. No sequence data could be obtained from these amplification products. Altogether, six "medium zero chips" were analyzed.

(d) "Negative and positive controls": positive and no template controls were performed as described above. Out of 144 negative controls, 4 were contaminated. All samples from chips with contaminated negative controls were excluded from the analysis.

(e) "Identity check": in this study, two individuals with the rare phenomenon of point heteroplasmy were studied. Additionally, subject 1 showed polymorphisms within the sequenced region found in the donor but not in the laboratory staff involved. In accordance with preceding experiments, amplified products obtained from the chips always showed the expected base sequence.

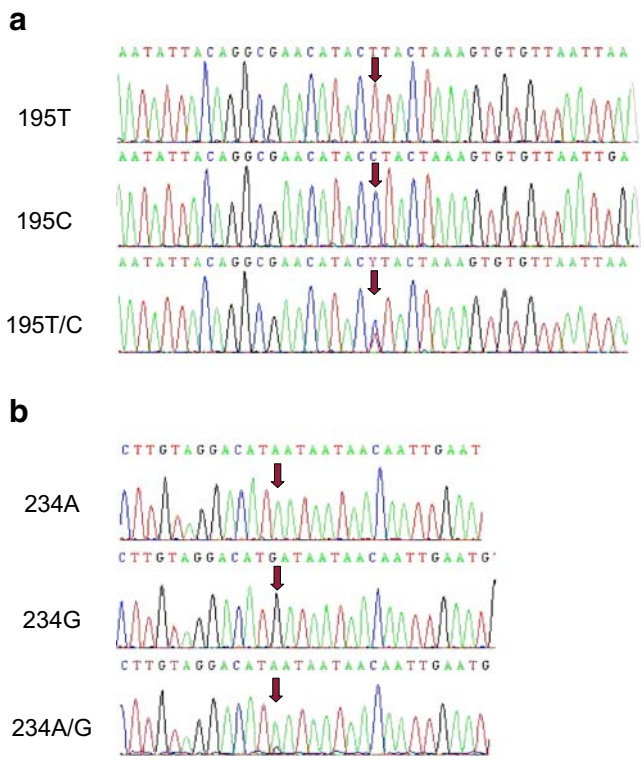

Fig. 3 Representative examples of electropherograms showing base sequences derived from single cells. The respective nucleotide positions, 195 in subject 1 (**a**) and 234 in subject 2 (**b**), are marked by arrows

Table 3 Results of mtDNA analysis from leukocyte suspension (extracted mtDNA) and from single cells; nd: not detected

Subject 1	Extracted mtDNA	Single lymphocytes	
	Cloned PCR fragments	Direct sequencing	Minisequencing
195 T	156	93	105
195 C	22	20	21
195 C/T	nd	8	3
Total	178	121	129
Subject 2			
234 A	200	81	99
234 G	28	31	26
234 A/G	nd	1	6
Total	228	113	131

Amplification bias

Analysis of the mutant and wild-type mtDNA artificial mixtures by cycle sequencing and minisequencing revealed no sequence-related preferential amplification (data not shown). Directional difference in amplification efficiency of the either 234A or 234G containing sequences of subject 2 was not observed.

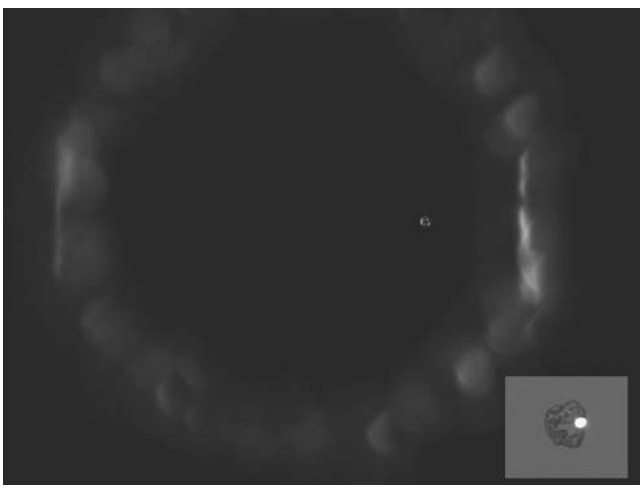

Fig. 4 Micrographs of the surface of a single reaction site supplied with one lymphocyte by fluorescence activated cell sorting (40× magnification). The main image shows an overview of the reaction site area indicated by the glass etching on the bottom of the chip. The insert shows the single discrete signal detected by fluorescence microscopy (100× magnification). The size of the fluorescent spot in the center of the structure is approximately 10 μm

Discussion

With increasing sensitivity of detection methods and improving quality of direct sequencing techniques, mitochondrial point heteroplasmy is being detected more frequently. However, it is still open to discussion whether heteroplasmy occurs because mutant and normal mtDNA molecules coexist within a single cell (intracellular heteroplasmy) or because there is a mixture of cells, each of which contains either normal or mutant mtDNA (intercellular heteroplasmy) [23].

Heteroplasmy in leukocyte suspension

In the present study, blood samples from two healthy donors showing a mitochondrial point heteroplasmy in direct sequencing (195Y and 234R, respectively) were analyzed. The ratio of the respective haplotypes was determined by conventional amplification of DNA extracted from isolated leukocytes and cycle sequencing or minisequencing. Depending on the sequencing primers and on the method used, highly variable ratios of the different mtDNA haplotypes were found in both individuals. An example is given in Fig. 2. The results make clear that conventional amplification of leukocyte extracts followed by direct sequencing or minisequencing does not reflect the heteroplasmic *in vivo* status correctly in either case. In addition, on-chip sequencing or minisequencing reactions of DNA extracted from leukocyte suspension resulted in varying heteroplasmy ratios (data not shown). The considerable difference in haplotype ratios of a given heteroplasmic position found here corresponds to the observations made by other authors [14, 15, 24–26], which can be summarized: "different PCR and sequencing conditions may lead to varying results concerning the level of detectable length and point heteroplasmy, i.e., in the way how the admixtures of the components are displayed" [25].

To get a more realistic impression of the actual (*in vivo*) haplotype ratio for both subjects, three independently generated PCR products from each leukocyte extract were cloned. A number of at least 48 clones from each PCR product were sequenced, respectively. With this method, the ratios were approximately 7:1 for 195Y (T/C; subject 1) as well as for 234R (A/G; subject 2). This procedure seems to be more accurate because, here, single fragments from mitochondrial genomes are being randomly selected from the haplotype admixture present in the respective sample. Thus, an actual number of haplotypes is directly detected without any influence of fluorescence intensities. Of course, the accuracy of the cloning method grows with increasing number of analyzed clones. In fact, this is to date the most exact method to determine a heteroplasmy ratio at the mitochondrial genome level. However, it is not possible to look at the composition of wild-type and mutant haplotypes within one cell.

Heteroplasmy in single lymphocytes

To systematically analyze the distribution of variant mtDNA molecules at the single cell level, a rapid and exact method for deposition of cells without contamination was developed using flow cytometry. Single lymphocytes were spotted onto chemically structured chips. This technique allows for various downstream applications, some of which have been previously presented [27–30]. In this study, a MoFlo cell sorter was used, which identifies cells by their specific features (e.g., cell volume and inner complexity of the particles) and deposits them within a small drop of liquid medium. Chips are supplied with single cells within a few seconds without any manual contact. However, adjustment of instrument settings is not easy to accomplish and needs time and experience. Whenever cells were not placed exactly within the hydrophilic reaction sites of a chip, mtDNA amplification failed. This is the most probable explanation for the observed number of unsuccessful amplification reactions (269 out of 720), which occurred in the processing of 20 chips. MtDNA molecules not being fully accessible to the polymerase must also be discussed, as an extra DNA extraction step before amplification was omitted. In fact, subjecting chips to a freeze-thaw treatment seems to reduce the number of unsuccessful amplifications. Further systematic experiments have to be performed.

The most important finding of the present study is that the vast majority of single lymphocytes analyzed (roughly 96%) exhibited homoplasmic haplotypes. Single lymphocytes from subjects 1 and 2 were analyzed by direct sequencing and, to confirm these results by an independent method, were also subjected to minisequencing. Heteroplasmy of single cells was only observed in 5% in subject 1 and 3% in subject 2. Regarding homoplasmic single lymphocytes only, those were present in ratios of approximately 5:1 (195T:C in subject 1) and 3:1 (234A:G in subject 2). These ratios differ from the respective numbers of sequenced clones prepared from extracted leukocyte mtDNA (7:1 in both subjects; cf. Table 3). This difference in haplotype ratios between single cells and lysates might result from the fact that with flow cytometry only lymphocytes were selected from the leukocyte suspension, whereas analysis of mtDNA from leukocyte extract comprises detection of mitochondrial genomes from several different types of leukocytes present in the respective sample. Clonal proliferation of progenitors is known to play a role in mtDNA sequence variation [29–31].

Studies on disease related heteroplasmy in single cells

Studies conducted on mitochondrion-related diseases show that mutant DNA and wild-type DNA coexist in the same cell [23, 27, 32–35]. Several authors proposed clonal proliferation and genetic drift due to a rapid proliferation rate as mechanisms of a development of homoplasmy [29, 30, 36, 37].

To date, most studies on mitochondrial heteroplasmy at the single cell level have not dealt with healthy individuals [29, 38–40]. In most cases, individuals suffering from inherited mitochondrial diseases like Lebers hereditary optic neuropathy (LHON) and myoclonic epilepsy and ragged-red fibers (MERRF) were examined [23, 27, 30–33, 41, 42]. In this study, the distribution of mtDNA copies was additionally influenced by the selective pressure on the mutant genome, whose dominance is usually associated with considerable damage to the respiratory chain. Thus, formation of a homoplasmic mutant genotype is normally prevented. To study the distribution of mtDNA haplotypes independent of such selection pressure, the inheritance of mitochondrial genomes has to be analyzed in a selectively neutral context. The heteroplasmic positions studied here do not cause a mutant phenotype, which is why they were well suited for the experiments presented.

Prevention of artifacts and contamination

The applied complex methodology for analysis of rather small amounts of DNA holds several sources of error. That is why a number of different steps were taken to prevent artifacts (cf. "Results"). Contamination originating from "open" amplification on the chip and from the procedure of cell sorting itself could be excluded. Furthermore, contamination of the sheath fluid by damaged cells is not problematic. The identity of the obtained sequences could be assigned by detecting heteroplasmy at the respective base positions. Homoplasmic sequences of subjects 1 and 2 were distinguished from sequences of the laboratory staff involved by additional base exchanges within the different sequenced regions.

Our own extensive microscopic examinations (cf. Fig. 4) and the studies of Heinzel et al. [43] have shown that the possibility of an erroneous spotting of additional cells in FACS applications is negligible. Therefore, the few detected heteroplasmic cells do not represent artifacts produced by inaccurate spotting. Results of "medium zero" and "device zero" checks (cf. "Results") also show that coamplification of extracellular mitochondrial DNA from damaged cells does not imitate single cell heteroplasmy because such extracellular mtDNA does not exist in amounts sufficient for successful sequencing reactions.

The sensitivities of single cell sequencing and minisequencing were tested by cloning of randomly selected amplification products from single cells. All the clones originating from one single-cell amplification reaction exhibited the same base sequence at the respective heteroplasmic positions. In addition, preferential amplification of a certain template as another way to erroneously overlook heteroplasmy could be excluded by testing equimolar mixtures of the cloned major and minor mtDNA type.

The sensitivity of the employed technique was additionally highlighted by the finding of another nucleotide position in subject 1, which repeatedly showed a base exchange in on-chip amplification of single lymphocytes. This was a 148Y heteroplasmy, which had not been detected in direct sequencing of extracted mtDNA due to its sporadic occurrence (7 findings in 103 successful amplifications). Nucleotide positions other than 148 in subject 1 were not affected. This strongly suggests that an artifact by reading errors of the *Taq* DNA polymerase can be excluded as a source for this finding.

Segregation of heteroplasmy

For both individuals, the presence of heteroplasmy in varying degrees could also be verified in hair and buccal swabs (data not shown). Thus, the respective mutation can be found in tissues of different origin (mesodermal blood cells, ectodermal buccal cells, and hair). Therefore, it seems probable that the underlying mutation has been either inherited or that it occurred in the individuals themselves at an early stage of zygote development. Alternatively, it may have resulted from germ line mutations that took place at the oocyte from which the heteroplasmic individual developed. In all three cases, an intracellular heteroplasmy occurs. The results presented in this paper show that most single cells analyzed exhibited homoplasmic haplotypes. Therefore, questions arise about the mechanism responsible for this predominant intracellular homoplasmy.

A number of authors have shown that random processes are sufficient to explain the clonal expansion of somatic mtDNA mutations [36, 44]. Thus, when heteroplasmic cells have gone through many cycles of mitotic cell division and assuming that two types of mtDNA are selectively neutral, genetic drift will lead to the emergence of homoplasmic cells of the two respective haplotypes. Reducing the effective number of mtDNA molecules being transferred (bottleneck) will increase the chance of generating homoplasmic cells.

The bottleneck hypothesis was proposed to explain the homogeneity of mitochondrial genomes within organisms despite the high mutation rate and a lack of effective repair mechanisms and recombination [45]. The mitochondrial

bottleneck leads to a homoplasmic mtDNA content early during oocyte formation. A widespread hypothesis is that a massive reduction in mtDNA content during early oogenesis leads to this bottleneck [46, 47]. Less than ten mitochondria were found in the early primordial germ cells of 3-week-old human embryos [46]. Thus, most individuals are likely to start with a nearly homoplasmic set of mitochondria [48, 49]. On the other hand, the study by Cao et al. [50] estimating the mtDNA copy number in single germ line cells and in single somatic cells of early embryos in mice, leads to the conclusion that the mitochondrial bottleneck is rather due to a small number of segregation units for mtDNA in germ cells than to a decline in mtDNA copy number in early oogenesis. Making allowance for the very slow rates of mitotic segregation observed in cultured heteroplasmic cell lines, Lehtinen et al. [51] proposed a model in which errorless replicated mitochondrial nucleoids are the unit of segregation.

Conclusion and outlook

In this study, samples from two healthy individuals exhibiting the relatively rare phenomenon of mitochondrial point heteroplasmy were analyzed. Both the major and the minor haplotypes of the subjects in this study were predominantly found in a homoplasmic state at the single cell level. This might point to a reduction of the number of mitochondrial genomes at the cellular level at least in a precursor cell of blood stem cells during early development. However, to test this hypothesis, a reliable analysis of single nucleoids or of single mitochondria would be essential.

The study presented in this paper reveals the degree of a heteroplasmic state in two healthy donors at a given point of time. It would be very intriguing to repeat the analysis in a few years to get information on longitudinal changes in mitochondrial heteroplasmy. Furthermore, mtDNA analyses at the single cell level should be conducted in other tissue types to shed further light on the hitherto unknown mechanisms of mtDNA distribution. Of course, studies on tissue samples cannot be performed without any preparative steps.

MtDNA analysis in single cells has many potential applications in a variety of fields of biomedical research. The analysis of single mitochondria and their pool of genomes would be a very important goal, especially for the understanding of mitochondrial heteroplasmy, which is believed to play a role in many contexts, e.g., inherited disease, and techniques using flow cytometry may hold the key to making this problem accessible to research. However, two major obstacles have yet to be overcome. One is the isolation of single mitochondria out of their networks; the other will be access to powerful machinery enabling a better resolution and sorting of such small structures as mitochondria.

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