REGULAR ARTICLE

Accumulation of mtDNA variations in human single CD34+ cells from maternally related individuals: Effects of aging and family genetic background

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Abstract   Marked sequence variation in the mtDNA control region has been observed in human single CD34+ cells, which persist *in vivo* and are present also in differentiated hematopoietic cells. In this study, we analyzed 5071 single CD34+ cells from 49 individuals (including 31 maternally related members from four families and 18 unrelated donors) in order to determine the mutation spectrum within the mtDNA control region in single cells, as related to aging and family genetic background. Many highly mutated sites among family members were hypervariable sites in the mtDNA control region. Further, CD34+ cells from members of the same family also shared several unique mtDNA variants, suggesting pedigree-specific occurrence of these variants. Overall age-related accumulation of mtDNA mutations in CD34+ cells varied in different families, suggesting a specific accumulation pattern, which might be modulated by family genetic background. Our current findings have implications for the occurrence of mtDNA mutations in hematopoietic stem cells and progenitors.

Introduction

Mitochondria play a central role in energy metabolism, in which mitochondrial oxidative phosphorylation is an extremely efficient system to produce energy required for the body (Wallace et al., 2010). Although *reactive oxygen species* (ROS) are a natural byproduct during the normal oxidative phosphorylation process, increased production of ROS and decline of mitochondrial respiratory capacity is observed during aging (Finkel and Holbrook, 2000; Vendelbo and Nair,

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2011). Oxidation of cell components by ROS affects the function of various tissues and organs with age (Finkel and Holbrook, 2000; Vendelbo and Nair, 2011). Under the mitochondrial theory of aging, aging is a vicious cycle between elevated ROS and mitochondrial dysfunction, leading to mtDNA damage and age-related cell death or senescence (Harman, 1972; Nagley and Wei, 1998; Trifunovic, 2006). This theory has been broadly supported by previous reports of accumulation of mtDNA mutations with aging (Greaves and Turnbull, 2009; Trifunovic, 2006), as well as molecular genetic and evolutionary studies (Agarwal and Sohal, 1996; Barja, 2004; Beckman and Ames, 1998; Finkel and Holbrook, 2000).

In our previous studies, we employed mtDNA sequence analysis of single hematopoietic cells to assess aging effects on the accumulation of mutations (Shin et al., 2004; Yao et al., 2007c). In particular, we found that human hematopoietic stem cells (HSCs) and progenitors, as represented by CD34+ cell surface staining, showed striking intercellular and intracellular mtDNA sequence variations (Ogasawara et al., 2005; Yao et al., 2007b, 2007d). Some of these sequence variations in single CD34+ cells persisted *in vivo* over time (Yao et al., 2007b) and were present in CD34+ cell progeny, circulating white blood cells (Ogasawara et al., 2005). In transplanted patients, CD34+ cells from the donor could be tracked by their distinctive mtDNA mutations as patients became full myeloid chimeras (Yao et al., 2007b). mtDNA mutation analysis of murine HSCs and progenitors showed age-related accumulation of mutations, dependent on the nuclear genetic background (Yao et al., 2007c). However, as we also reported (Yao et al., 2007b), a preliminary analysis of human mtDNA variation in individual CD34+ cells from the healthy adults (aged from 25 to 65 years) revealed no obvious correlation between the mtDNA mutation number and the age of the individual, and the mutation levels in CD34+ cells from the adult samples could be seemingly classified into two groups defined by 6.5−12.8 haplotypes per 100 cells and 29.7−42.6 haplotypes per 100 cells. These results are reminiscent of potential modulation from the nuclear genetic background as observed in mice (Yao et al., 2007c). In one intriguing finding, based on comparison of CD34+ cells from the maternally related donors and recipients, a very small portion of CD34+ cells shared certain nucleotide substitutions that appeared to be exclusive to the family (Yao et al., 2007b). The nature of these "family-specific" mutations in HSCs and progenitors from siblings, perhaps transmitted with the mother's mitochondria, or occurring somatically in parallel, or otherwise modulated by the nuclear genetic background, remained to be validated and clarified.

In the current work, we have analyzed 5071 single CD34+ cells from 49 persons. Among them, 32 individuals were newly collected from four families, in age ranging from 13 to 89 years, and three samples were cord blood. We had two aims: (1) to examine the mutation spectra of the noncoding mtDNA control region in single CD34+ cells from maternally related individuals and to characterize the family-specific occurrence of mtDNA mutations; (2) to discern an aging effect on mutation accumulation in the mtDNA control region in single CD34+ cells. With an enlarged sample size and members from a family, we were able to obtain a comprehensive characterization of the mtDNA mutation spectrum, providing some insight into age, family genetic background and mtDNA mutations in human cells.

Materials and methods

Subjects

A total of 32 healthy individuals from four families were recruited for this study (Table 1 and Fig. 1). For each individual, peripheral blood (PB, 8−10 mL) was collected in a heparinized tube and mononuclear cells (MNCs) were isolated by Ficoll density gradient centrifugation after overnight shipping and delivery. For donors in Family B living in Italy (N=11), the same amount of PB was collected and processed locally within 24 h of phlebotomy for MNC separation, freezing, and storage; frozen MNCs were shipped to our laboratory at Bethesda on dry ice and then transferred to liquid nitrogen until they were subjected to single cell sorting. A control group was composed of 18 unrelated individuals: three cord blood (CB) samples and one member (C-2) from Family C (this study); 14 healthy individuals reported in our previous studies (Ogasawara et al., 2005; Yao et al., 2007b, 2007d). A total of 20 samples (1952 CD34+ cells) were obtained from these 18 unrelated individuals as two of the 14 reported healthy individuals were sampled twice at different time points (Yao et al., 2007b). All donors gave informed consent, and this study was approved by the institutional review board of the National Heart, Lung, and Blood Institute.

Single CD34+ cell sorting and PCR amplification

Single CD34+ cells were isolated and analyzed using the same procedure described in our previous studies (Ogasawara et al., 2005; Yao et al., 2007b, 2007d). In brief, after thawing and washing with phosphate buffered saline (PBS), cells were suspended in 100 μL of PBS containing 0.5% bovine serum albumin (BSA) and incubated with anti-CD34 phyco-erythrin (PE)-conjugated monoclonal antibody (BD Bioscience, San Jose, CA) for 30 min at 4 °C. Cells were then washed, resuspended in 600 μL of PBS with 0.5% BSA, and added 4 μL of 7-amino-actinomycin D (7-AAD). Cell sorting was performed on the MoFlo Legacy high-performance cell sorter (Dako-Cytomation, Ft Collins, CO), using 100 mW of the 488 nm line of an argon laser (I-90, Coherent, Palo Alto, CA) for excitation. Forward scatter was the triggering parameter (Fig. S3). Single cell deposition was accomplished using the CyClone Automated Cloner (Dako-Cytomation, Ft Collins, CO) in a 0.5 single drop mode with gating, based on forward scatter and fluorescence. Single CD34+ cells were sorted into each well of an optical 96-well reaction plate containing 50 μL of lysis buffer (10 mM Tris−HCl [pH 8.0], 50 mM KCl, 100 μg/mL Proteinase K, and 1% Triton X-100). The plate was incubated at 56 °C for 30 min, followed by an additional incubation at 96 °C for 8 min.

Two-step nested PCR was used to amplify the entire mtDNA control region in single CD34+ cells, following the same PCR condition as reported previously (Yao et al., 2007b, 2007d). In brief, the first PCR was performed in a total volume of 30 μL, which contains 400 μM of each dNTP, 1 unit of TaKaRa LA Taq™ (which has proof reading activity, with a relative error rate of

Table 1 The level of mtDNA sequence variation in single CD34+ cell populations from 49 healthy donors with different ages.

Sample	Age	Sex	No. of cells	No. of haplotypes	No. of nonaggregate haplotypes by nucleotide substitutions	No. of cells with nonaggregate sequences	No. of cells with nonaggregate sequences defined by substitution	Reference
A-1	85	F	91	50	43	52	43	This study
A-2	55	M	92	35	28	40	28	This study
A-3	59	F	93	25	20	38	22	This study
A-4	35	M	93	13	8	36	10	This study
A-5	30	M	93	27	21	40	21	This study
A-6	25	F	95	12	5	32	14	This study
B-1	83	M	95	39	33	74	33	This study
B-2	81	M	94	42	36	73	36	This study
B-3	89	F	86	48	42	71	42	This study
B-4	79	F	96	35	29	74	29	This study
B-5	87	F	95	49	40	70	41	This study
B-6	60	F	95	47	38	73	41	This study
B-7	47	M	95	38	29	73	29	This study
B-8	51	M	94	35	28	64	29	This study
B-9	54	F	94	52	43	74	43	This study
B-10	57	F	93	37	30	70	30	This study
B-11	27	M	91	35	27	69	27	This study
B-12	21	M	185	76	70	160	90	This study
B-13	20	M	191	77	70	132	70	This study
B-14	13	M	96	54	43	85	43	This study
B-15	32	F	96	50	41	76	42	This study
B-16	28	F	96	31	23	71	23	This study
C-1	80	F	93	51	45	61	46	This study
C-2	79	M	94	45	37	65	37	This study
C-3	76	F	96	17	15	19	17	This study
C-4	46	F	96	48	43	56	44	This study
C-5	49	M	112	14	13	13	13	This study
C-6	46	F	95	8	5	7	5	This study
C-7	18	M	94	29	24	41	25	This study
C-8	21	M	92	16	14	17	14	This study
D-1	65	F	96	37	32	39	34	This study
D-2	42	M	96	21	19	20	19	This study
Donor 1	48	F	85	14	8	32	10	Ogasawara et al., 2005
Donor 2	44	M	92	16	11	32	11	Ogasawara et al., 2005
Donor 3	36	M	93	12	6	34	11	Ogasawara et al., 2005
Donor 4	55	M	93	14	8	41	23	Ogasawara et al., 2005
Donor 5	35	M	93	13	10	34	11	Ogasawara et al., 2005
Donor 6	35	M	95	15	10	40	10	Yao et al., 2007d
Donor 7	32	M	93	40	35	53	35	Yao et al., 2007d
Donor 8	25	M	93	38	35	56	35	Yao et al., 2007d
Donor 9	57	M	93	31	28	38	35	Yao et al., 2007b, 2007d
Donor 10	39	M	94	17	12	43	12	Yao et al., 2007b, 2007d
QUIDC-3-14-5	65	F	91	39	34	48	39	Yao et al., 2007b
HERJC-2-16-5	48	F	93	40	31	73	31	Yao et al., 2007b
CABO-2-5-98	34	M	91	32	27	57	27	Yao et al., 2007b
CABO-1-31-6	42	M	94	43	37	64	37	Yao et al., 2007b
KIRGC-12-10-2	53	F	94	43	41	43	41	Yao et al., 2007b
KIRGC-2-2-6	57	F	96	39	38	39	39	Yao et al., 2007b
CB-1	0	–	93	6	4	7	4	This study

(continued on next page)

Table 1 (*continued*)

Sample	Age	Sex	No. of cells	No. of haplotypes	No. of nonaggregate haplotypes by nucleotide substitutions	No. of cells with nonaggregate sequences	No. of cells with nonaggregate sequences defined by substitution	Reference
CB-2	0	−	187	30	23	85	29	This study
CB-3	0	−	95	18	15	49	16	This study
Total	−	−	5071	1693	1408	2753	1496	−

Note — Donors 9 and 10 that were reported in Yao et al. (2007d) refer to BURRC-9-23-4 and GREHC-2-10-5 in Yao et al. (2007b), respectively. We counted the number of haplotypes (including the aggregate mtDNA haplotype [which has the highest frequency in a population of cells and bears the consensus sequence; Table S3] and nonaggregate haplotypes that differed from the aggregate sequence by nucleotide substitutions or indels [insertions and deletions] or both) and the number of nonaggregate haplotypes that differed from the aggregate type only by nucleotide substitutions, based on the mutations detected in a population of single CD34^{+} cells from each donor (Table S4). As the nonaggregate haplotype may be shared by several single cells, which reflects the clonal expansion of the clone, we then counted the number of cells harboring the nonaggregate haplotypes or nonaggregate haplotypes by nucleotide substitutions for each donor.

0.16 as compared to conventional Taq DNA polymerase; Takara Bio. Inc.), 0.5 μM of each forward and reverse outer primer (L15594: 5′-CGCCTACACAATTCTCCGATC-3′ and H901: 5′-ACTTGGGTTAATCGTGTGACC-3′), and 5 μL of cell lysates. PCR amplification was run on the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) with the following thermal conditions: one denaturation cycle of 94 °C for 3 min; 35 amplification cycles of 94 °C for 30 s, 52 °C for 40 s and 72 °C for 1 min with a 5-second increase per cycle; and a full extension cycle of 72 °C for 10 min. The second PCR was performed in 50 μL of reaction mixture containing 400 μM of each dNTP, 2 units of TaKaRa LA Taq™, 0.5 μM of each forward and reverse inner primers (L15990: 5′-TTAACTCCACCATTAGCACC-3′ and H650: 5′-GAAAGGCTAGGACCAAACCTA-3′), and 5 μL of first PCR product under the same amplification conditions as the first PCR but with an extension time of 90 s at 72 °C per cycle. Second PCR products were purified and then sequenced by using the BigDye Terminator v3.1 Cycle Sequencing Kit on the 3100 DNA Sequencer (Applied Biosystems). The same sequencing primers described previously (Yao et al., 2007b, 2007d) were used to determine the entire mtDNA control region sequence.

Identification of mtDNA sequence variation and statistical analysis

Germline sequence variants in the consensus/aggregate sequences of single CD34^{+} cells from each subject were scored relative to the revised Cambridge Reference Sequence (rCRS) (Andrews et al., 1999). Sequencing electrophoregram of each single cell was proof-read, as reported previously (Yao et al., 2007b, 2007d), and mutation was scored by referring to the aggregate sequence of the donor. Cells with contamination were recognized using the same strategy as described in our previous study (Yao et al., 2007a). In brief, if the mtDNA sequence of a single cell from a particular subject did not contain or had heteroplasmy at all of the variants detected in the aggregate sequence for that subject, it was regarded as being contaminated. The overall contamination rate was very low (around 0.4% of total single cells) in this study and exclusion of these contaminated cells did not influence the result. We scored for heteroplasmy (co-existence of wild-type

and mutant alleles) of certain mtDNA variants, based on the electrophoregram in which mutant alleles were present at >10% of the highest peak. As nearly all identified mtDNA mutations were heteroplasmic in cells (with a mutant ratio varying from >10% to 100%; Fig. S2), we counted the mutation regardless of its exact level of heteroplasmy. Length variation of the poly C-tract in region 303–309 was scored by direct counting of the base shift of T at nucleotide 310. A length mutation of the AC repeat in region 515–524 in the third hypervariable segment was also scored on the basis of the sequencing electrophoregram. But, a length variation of the poly C-tract in region 16184–16193, caused by variant 16189 T>C, was not considered in this study because most of them were heteroplasmic of multiple poly C-tracts (>10 Cs) and could not be conservatively counted according to sequencing chromatographs.

As the majority of single cells from each donor shared the same consensus mtDNA sequence (which we defined as the aggregate haplotype), we define a cell with a unique haplotype if this cell differed from the aggregate haplotype by containing extra nucleotide alteration(s). We used the number of haplotypes in a population of 100 cells from each sample as an index to compare frequencies of mtDNA sequence variation among different samples. This index reflects the total number of mutations that occurred or retained within a given number of cells and not the frequency of the cell clone harboring certain mutation(s) (Yao et al., 2007b, 2007d). As polynucleotide length mutations have different mutation mechanisms compared to nucleotide substitutions, we mainly focused on analysis of nucleotide substitutions, as represented by the number of haplotypes defined by nucleotide substitutions per 100 cells in our analysis. An aging effect on an mtDNA variation level in a cell population was evaluated by the linear regression analysis using the GraphPad Prism 4.0.

Results

Data quality control

Single cell analysis has a very high sensitivity to detect mtDNA mutations present at low levels. However, this technique also poses challenges, especially to eliminate
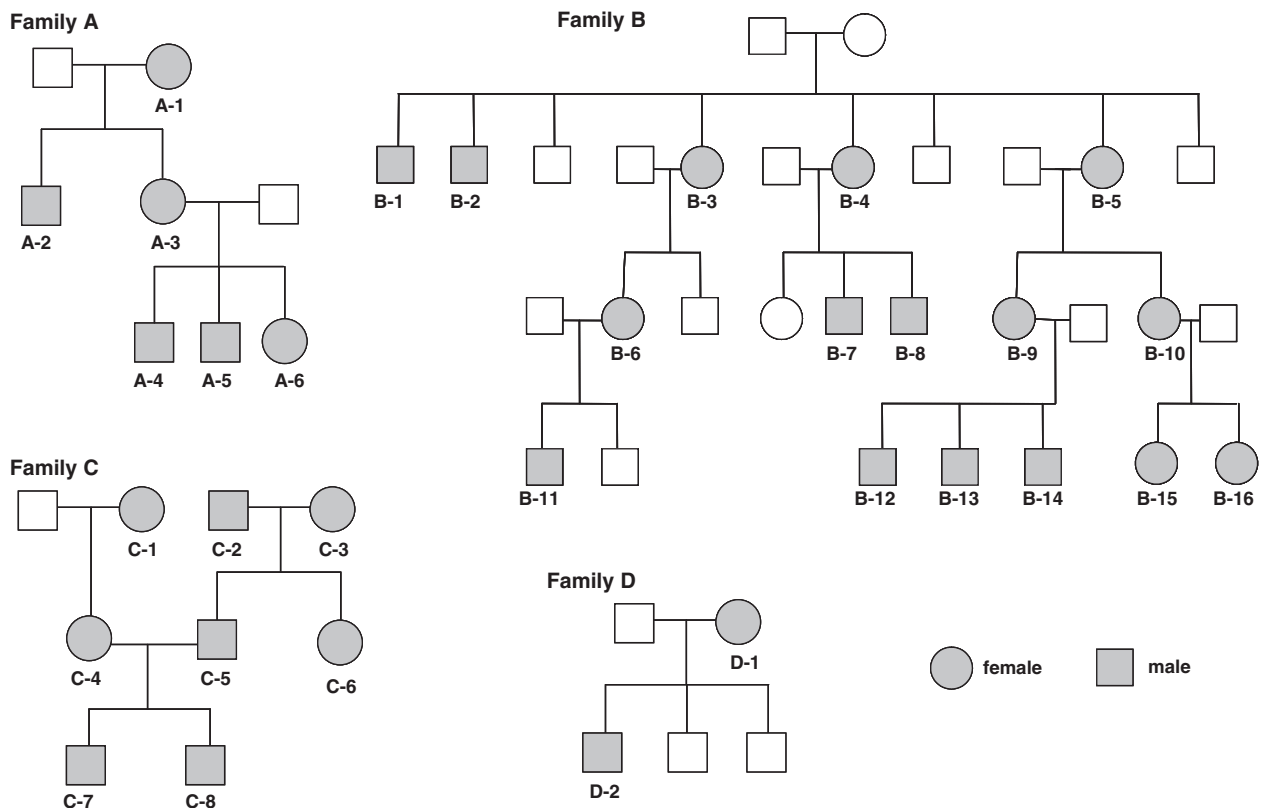
Figure 1 Pedigrees of the four families analyzed in this study. Members of four families (A, B, C, and D) were recruited for examination of mtDNA control region sequence in single CD34⁺ cells. Individuals who provided blood samples are marked in gray; ages are listed in Table 1.

artifacts caused by multiple-step PCR and contamination (Reeve et al., 2009; Yao et al., 2007a). We benefited from our previous experience of single cell analysis (Ogasawara et al., 2005; Yao et al., 2007b, 2007d), and we believe that our strategy was sufficiently stringent to maintain data reliability. First, we used the same high fidelity TaKaRa LA Taq™ DNA polymerase for PCR and negative controls during the amplification. Application of the same reagents for all samples should yield similar levels of background sequence variation inevitably arising from PCR amplification, and should not affect the overall pattern of mutation rate. Second, we were able to consistently reproduce the majority of mutations (~80%) in single cells among duplicates in independent amplifications of single cells from donors D-2 and B-11. Moreover, the levels of mtDNA mutation in each single cell population of the duplicates from the same donor were similar. Finally, we employed the same phylogenetic approach as described for single cell analysis (Yao et al., 2007a) in order to identify and eliminate potential cases of contamination.

Aging effect on the accumulation of mtDNA variations in single CD34⁺ cells

A total of 5071 single CD34⁺ cells sorted from 51 PB samples collected from 49 healthy donors with age ranging from 0 to 89 years were (re-)analyzed in this study (Table 1). Among them, 32 individuals were from four families (6 from Family

A, 16 from Family B, 8 from Family C, and 2 from Family D). Pedigrees of the families and selected family members are illustrated in Fig. 1. The other 17 individuals were as follows: 14 healthy individuals (two donors were sampled twice at different time points Yao et al., 2007b) reported in our previous studies (Yao et al., 2007b, 2007d); three CB samples also were included due to the paucity of young individuals in our families (only one donor<18 years old; Table 1). When variation levels of the mtDNA control region (the number of haplotypes defined by nucleotide substitutions per 100 cells) were plotted against ages of individual donors, we observed a roughly age-related accumulation pattern of mtDNA sequence variations in single CD34⁺ cells, although the correlation was moderate ($r^2=0.19$, $P=0.001$; Table 1, Fig. 2A and Table S1). We also performed the analysis using just the adult samples, to exclude potential bias of three CB samples and one young donor (age 13), a similar result was observed ($r^2=0.17$, $P=0.004$; Table S1). As different families had different number of donors, we included "Family" as a control variable, but the result was not changed ($r=0.40$, $P=0.004$; Table S1). We obtained same results when we considered the number of total haplotypes (defined by nucleotide substitutions and insertions/deletions) per 100 single cells (Table S2). Overall, a high frequency of mtDNA mutation was not always present in CD34⁺ cells from donors older than 75 years, as compared to donors younger than 75 years old or even much younger donors less than 30 years old. This pattern may reflect the heterogeneity within and the dynamics of CD34⁺ cell proliferation *in vivo*,

and we cannot exclude a bias due to sampling of comparatively few younger donors. The high level of mtDNA variation in CD34+ cells from CB or donors with young age seemed to suggest that there is not necessarily a low mutation rate at young age and mtDNA mutations accrue in CD34+ cells not only after birth.

Family-specific pattern for age-dependent accumulation of mtDNA variations in single CD34+ cells

Our previous work demonstrated that the level of mtDNA sequence heterogeneity in murine HSCs and progenitors was modulated by the nuclear genetic background (Yao et al., 2007c), and we speculated that this might also be observable in human. Although the best samples to address this hypothesis should be monozygotes, maternally related members from the same family may be helpful. Plots of mtDNA mutation levels against ages in the families with at least 5 members displayed remarkably difference between families: there was a significant correlation between the age and the mtDNA mutation level in Family A ($r^2 = 0.79$; $P = 0.018$; Figs. 2B and S1), whereas the mutation levels in the 16 donors in Family B ($r^2 = 0.04$; $P = 0.485$) showed no clear tendency for

age-dependent accumulation of mtDNA mutations in single CD34+ cells, mainly because younger donors had surprisingly high levels of mtDNA mutations (Fig. 2C). In four maternally related members in Family C, an age-related pattern was evident but not statistically significant ($r^2 = 0.76$; $P = 0.128$) (Fig. 2D). The results indicated that the accumulation of mtDNA mutations in single CD34+ cells was indeed influenced by the family genetic backgrounds of families, as the accumulation rate differed in various pedigrees. Note that our observation for families A and C was based on limited number of donors, and larger cohorts will be necessary to further validate our result.

Family-specific variants or recurrent mutations in single CD34+ cells?

The age-related accumulation of specific variants, such as C150T, A189G, T408A, and T414G, has been reported in leukocytes, muscle tissues, and fibroblasts (Michikawa et al., 1999; Wang et al., 2001; Zhang et al., 2003). Some of these variants are highly hypervariable (e.g. C150T and A189G), according to the estimation of the available worldwide populations (Soares et al., 2009; Stoneking, 2000), but other sites appear to be less mutable and may be tissue or cell-specific. In our previous experiments aimed to trace donor CD34+ clones in a recipient after transplantation, we found
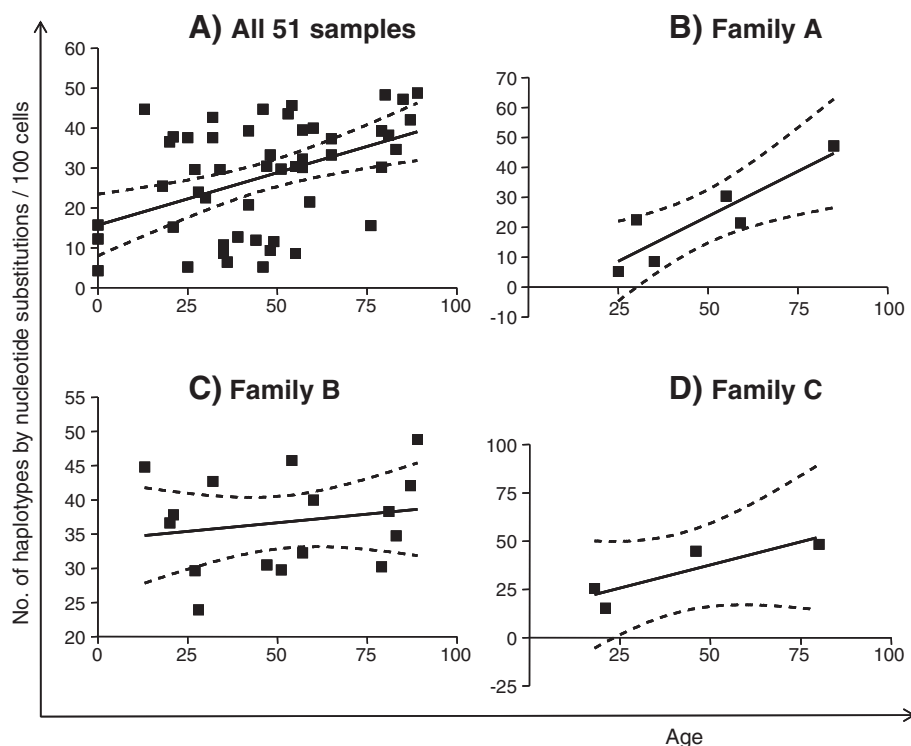
Figure 2 Number of mtDNA control region haplotypes in a population of CD34+ cells. Haplotype frequencies were defined by the number of haplotypes (characterized by nucleotide substitutions)/100 single CD34+ cells from unrelated healthy individuals and family members. (A) All 51 samples were derived from 49 healthy donors in the age range of 0–89, in which two donors were sampled twice at different time points (Yao et al., 2007b). Among them, 32 individuals were newly recruited from four families (6 from family A, 16 from Family B, 8 from Family C, and 2 from Family D). The other 17 individuals consisted of previously reported 14 healthy donors (Ogasawara et al., 2005; Yao et al., 2007b, 2007d) and three cord blood donors (this study). The number of haplotypes in each donor was shown relative to the donors' age: (B) 6 members of Family A, (C) 16 members of Family B, and (D) 4 members (C-1, C-4, C-7 and C-8) of Family C.

that a small portion of CD34⁺ cells from the sibling samples shared certain mtDNA variants, suggesting potential family-specific variants if not recurrent mutations (Yao et al., 2007b).

To further examine this pattern for a potentially high prevalence of somatic mutations in CD34⁺ cells, we first compiled the mtDNA control region mutation spectrum of 1952 CD34⁺ cells from 20 PB samples collected from 18 unrelated individuals (Table 1 and Fig. 3A): 16 samples from 14 healthy donors reported previously (Ogasawara et al., 2005; Yao et al., 2007b, 2007d); three CB samples; one sample from a member (C-2) of Family C. The mutation pattern from these 18 unrelated individuals was then compared with those from four different families (A, B, C, and D). An occurrence of each variant (in heteroplasmic or homoplasmic status) was scored according to its presence in a donor, but the variant frequency in a cell population from the donor was neglected. The top five highly mutational sites were 146, 16129, 204, 16311, and 16131 in these 18 unrelated individuals (Figs. 3A and S2). Except for 16131, the positions were all listed as hypervariable sites in the mutational hotspot list compiled by Soares et al. (2009) on the basis of 2196 complete mtDNA genomes from worldwide populations (Fig. 3B). We also identified several variable sites, such as 42, 64, 16038, and 16086, which had a modest or low rate in the worldwide human mtDNA population data, but they had a relatively high frequency in the data from the 18 unrelated individuals (Fig. 3A). Further studies are required to address why these variants are so highly prevalent in CD34⁺ cells.

Figs. 3C and 3D represent the highest mutational hits in the maternally related members from Families A and B, respectively. In Family A, a total of 15 sites had mutation hits at least twice, among which 16192 and 204 were most frequent, and the other 12 sites showed a similar mutational frequency. Family B displayed a total of 16 sites with mutation hits at least four times, among which 146, 204, 16129, and 16131 were the top four, followed by sites 16093, 16150, and 16187. Of interest, the top four sites (146, 204, 16129, and 16131) in Family B matched four of the top five hypervariable sites (146, 16129, 204, 16311, and 16131) present in the 18 unrelated individuals (Fig. 3A). In particular, the occurrence of variation at site 16131 ranked fourth in both Family B and the 18 unrelated individuals, suggesting that this variant might be more prone to undergo somatic change in single CD34⁺ cells.

We also observed a relatively high frequency of apparently family-unique variation at sites 16192, 16079, 215 and 217 in members from Family A (Figs. 3C and S1) and at sites 16150, 16187 and other sites in members from Family B (Fig. 3D). These mutations were present at either low frequency or were absent in 1952 single CD34⁺ cells from the 18 unrelated individuals and in the complete mtDNA database from worldwide populations. Although the frequency of a certain variant may be biased, due to the limited number of donors in each family, we inferred unique mutation distribution patterns in single CD34⁺ cells from different families.

Discussion

There is accumulating evidence that mtDNA mutations play important roles in human disease and in physiologic aging (Taylor and Turnbull, 2005; Wallace et al., 2010) and mtDNA mutations are common in normal and tumor tissues (Greaves

and Turnbull, 2009; He et al., 2010; Yao et al., 2007d). The aging of hematopoietic stem cells (HSCs) is complex and multifactorial (Sahin and Depinho, 2010), accompanying with age-dependent accumulation of mtDNA mutations (Shin et al., 2004). However, whether accumulated mtDNA mutations in HSCs and progenitors affect their developmental or function, has not been sufficiently resolved (Sahin and Depinho, 2010; Shin et al., 2004; Yao et al., 2007c). Recently, Norddahl et al. (2011) demonstrated that hematopoietic defects in mice carrying a proofreading-defective mtDNA polymerase is reminiscent of premature HSC aging. They further showed that rapid accumulation of mtDNA mutations had little functional effect on the HSC pool, but would cause distinct differentiation blocks for downstream progenitor cells (Norddahl et al., 2011).

In the current work, there was a tendency for age-associated mutation accumulation in human CD34⁺ cells, but the mutation levels in certain young donors were not necessarily low (Fig. 2 and Table 1), suggesting that CD34⁺ cells accumulated mutations at all stages of growth. Furthermore, age-related mutation accumulation seemed to be affected by family genetic background, varying substantially from one family to another. Of note, various mutational hotspots in the maternally related individuals (Family A or B) were also observed as hypervariable mutations in single cell populations from the 18 unrelated individuals and in 2196 complete mtDNAs from the worldwide populations (Soares et al., 2009). Therefore, it seems probable that highly frequent variants/mutations in single CD34⁺ cells do not occur randomly. Forster et al. (2002) analyzed the mtDNA mutations in 248 pedigrees living in the high-radiation peninsular and in nearby low-radiation islands in Kerala, India, to characterize radiation-associated mtDNA mutations; they surmised that radioactive conditions significantly accelerated mtDNA mutations in evolutionarily hot spots between mothers and their offspring. Our current study of mutational hotspots in single CD34⁺ cells is consistent with their observation.

In *Drosophila*, mtDNA haplotypes' influence on the longevity has varied on different nuclear backgrounds, suggesting the mtDNA effects could be masked or exaggerated by significant interactions with the nuclear allelic variation (Rand et al., 2006). In humans, there also appeared to be an effect of inherited mtDNA variants on longevity, dependent on the population, probably due to different nuclear genetic background (Alexe et al., 2007; Dato et al., 2004; Santoro et al., 2006). Therefore, aging and longevity may be regulated by nuclear gene variants associated with inherited and somatic mtDNA variations/mutations in nuclear-mitochondrial interaction (Santoro et al., 2006; Vendelbo and Nair, 2011). As more than 98% of the mitochondria's proteins are encoded by nuclear genes, and coordinated expression of nuclear and mitochondrial genes is indispensable for mitochondrial function (Calvo and Mootha, 2010), it is highly plausible that nuclear-mitochondrial epistatic interactions in different families account for the diverse pattern of mtDNA mutation accumulation in single CD34⁺ cells. The lack of aging-related accumulation of mtDNA mutation in Family B, but not in families A and C, was simply caused by an elevated level of mtDNA mutation in donors at young age (Fig. 2). Does Family B harbor a "mutagenic background" that accounts for their high load of mutations? Apparently, we have no clear answer to this question based on the current observation. As CD34⁺ cells are very heterogeneous and replenish during hematopoiesis
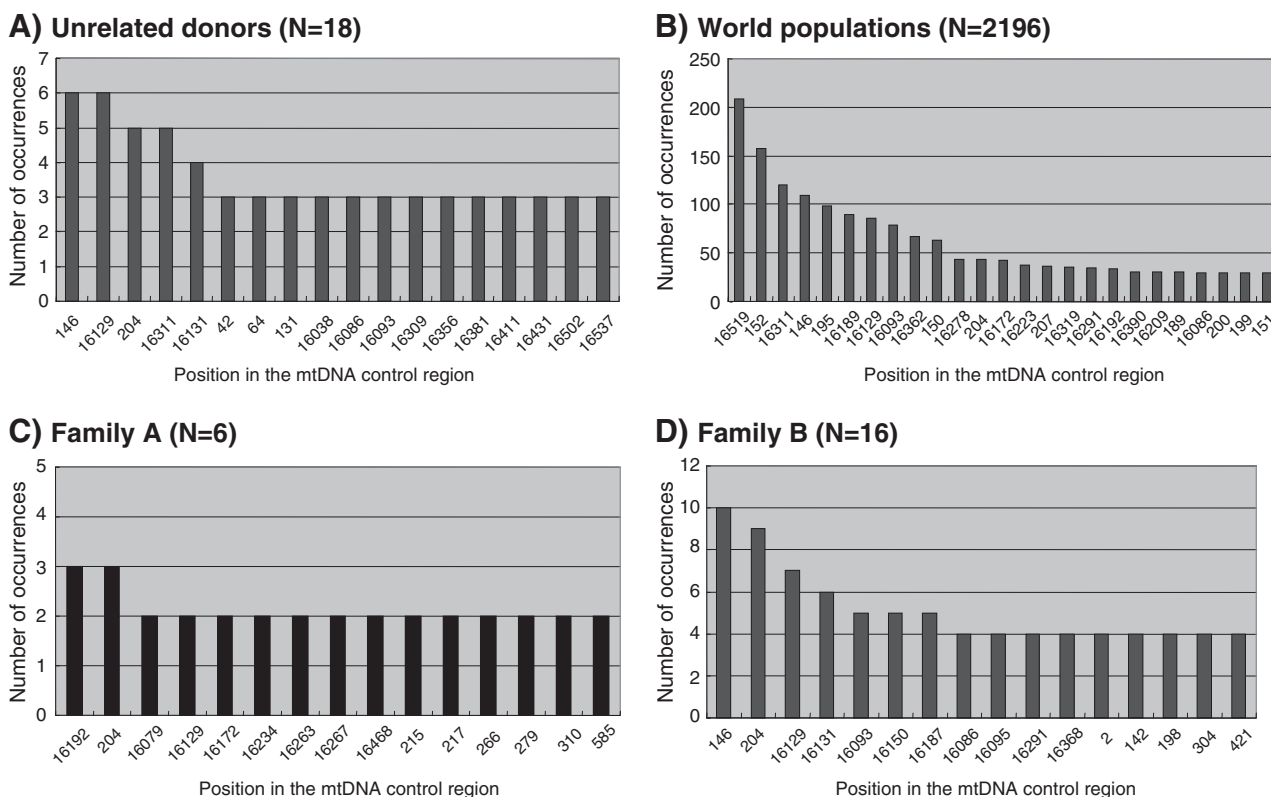
Figure 3   Mutational hot spots of the mtDNA control region in single CD34$^+$ cells and in reported complete mtDNAs from worldwide populations. Mutational hot spots were scored, according to their occurrences in the respective individual, but with disregarding their frequencies in a cell population from the individual. As the number of donors in each family varied, we arbitrarily regarded a certain site as a mutational hot spot if mutations hit the site at least in 3 donors in 18 unrelated healthy individuals (A), 2 donors in Family A (N=6) (C), and 4 donors in Family B (N=16) (D). The 18 unrelated healthy individuals included one member (C-2) of Family C in addition to 17 unrelated donors (14 reported healthy individuals (Ogasawara et al., 2005; Yao et al., 2007b, 2007d) and three cord blood donors). The mutational hot spots in worldwide mtDNAs are listed (B) when mutations appear at least 30 times in the global tree of 2196 complete mtDNA sequences (Soares et al., 2009).

(Doulatov et al., 2012), it is possible that mutation level will finally reach a platform due to the balance of accumulation and differentiation/disappearance of mutated cells in those donors with a "mutagenic background", irrespective of age. Considering the results of functional assays of HSCs and progenitors with mtDNA mutations (Yao et al., 2007c), we would expect no tight connection between mutation frequency and functional consequence in the mutated CD34$^+$ cells, or in the hematopoietic systems of these young individuals with a high level of mtDNA mutation.

In this study, besides the high hits for the evolutionarily hypervariable sites, we also noticed that certain mtDNA variants were shared by family members, pedigree-specific occurrence of mtDNA variants. This observation was unexpected but confirmed our previous speculation for a family-specific occurrence of mtDNA variants among siblings (Yao et al., 2007b). We hypothesized that this variation was shaped by family genetic background. Indeed, others have recently shown that somatic point mutations in the mtDNA control region are influenced by genetic background and associated with healthy aging (Rose et al., 2010). How this seemingly family-specific mtDNA mutation occurs and accumulates and whether it is restricted to CD34$^+$ cells but not other tissues are unknown. As the family differences for mutation

accumulation and presence of family-specific variants are obvious in this study, one likely explanation for this observation is the maternal inheritance of low-level variants or maternal influence on occurrence of low-level variants. Direct experimental data is needed to confirm this speculation. Nonetheless, our observations contribute to an inference of complexity in the mechanism of mtDNA mutations in single cells. The pedigree-specific mtDNA variants could have potential forensic consequences and individual patterns of mutations could help to identify individuals instead of matri-lineages (Salas et al., 2007).

The mtDNA control region plays a crucial role in regulating mtDNA replication and transcription (Falkenberg et al., 2007; Shadel and Clayton, 1997). Deleterious mutations in this region would be expected to have functional consequences (Bi et al., 2010). We do not know the exact role of the mutations that were frequently observed in the mtDNA control region of CD34$^+$ cells. As some of these highly mutated sites, such as 146, 204 and 16129, are also hypervariable in the general populations, indicating that they are unlikely to be of deleterious significance. However, the accumulation of these mutations is unlikely to be a simple byproduct of oxidative damage, as we observed high frequencies of various mutations at both evolutionarily hypervariable sites and

seemingly family-specific sites. For instance, the frequency of variant A215G is relatively high in pedigrees from a high radiation area (Forster et al., 2002), but this variant also was present in two out of six individuals in Family A. The age-associated variant T414G (Michikawa et al., 1999) was only found in two CD34⁺ cells from a 48-year old healthy donor in our previous study (Yao et al., 2007b) and in one 79-year old donor (C-2) in this study. Further work is required to clarify putative functional effects of these variants on mtDNA replication and mtDNA copy number within a cell (Falkenberg et al., 2007; Shadel and Clayton, 1997).

Our current study has some limitations. First, due to the relatively low number of subjects per family and limited number of families analyzed, the family-related patterns as we observed here need to be further confirmed. Second, we did not perform any functional characterizations for those CD34⁺ cells with and without mtDNA mutations, to show the potential effect of mtDNA variants. It remains unknown whether there is a family-specific mutation accumulation in mtDNA coding region in CD34⁺ cells. Further study should be carried out to answer these questions.

In summary, we provide, to the best of our knowledge, the mutation spectra of the human mtDNA control region in CD34⁺ hematopoietic cells from a large cohort of maternally related and unrelated individuals. The results in general support age-related accumulation of mtDNA mutations, but further discerned a family-specific rate of mtDNA mutation accumulation and mutational sites. The exact biological significance of accumulation of aging-related and family-specific mtDNA mutations in CD34⁺ cells awaits future study.

Acknowledgments

Authorship Contributions

Y.-G.Y. designed research, performed research, analyzed data and wrote the paper. S.K. designed research and wrote the paper. X.F., L.S. and J.P.M. performed research. G.T. contributed vital samples. N.S.Y. designed research and wrote the paper.

Disclosure of Conflicts of Interest

The authors declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.01.006.

References

Agarwal, S., Sohal, R.S., 1996. Relationship between susceptibility to protein oxidation, aging, and maximum life span potential of different species. Exp. Gerontol. 31, 365–372.

Alexe, G., Fuku, N., Bilal, E., Ueno, H., Nishigaki, Y., Fujita, Y., Ito, M., Arai, Y., Hirose, N., Bhanot, G., Tanaka, M., 2007. Enrichment of longevity phenotype in mtDNA haplogroups D4b2b, D4a, and D5 in the Japanese population. Hum. Genet. 121, 347–356.

Andrews, R.M., Kubacka, I., Chinnery, P.F., Lightowlers, R.N., Turnbull, D.M., Howell, N., 1999. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat. Genet. 23, 147.

Barja, G., 2004. Free radicals and aging. Trends Neurosci. 27, 595–600.

Beckman, K.B., Ames, B.N., 1998. The free radical theory of aging matures. Physiol. Rev. 78, 547–581.

Bi, R., Zhang, A.-M., Zhang, W., Kong, Q.-P., Wu, B.-L., Yang, X.-H., Wang, D., Zou, Y., Zhang, Y.-P., Yao, Y.-G., 2010. The acquisition of an inheritable 50-bp deletion in the human mtDNA control region does not affect the mtDNA copy number in peripheral blood cells. Hum. Mutat. 31, 538–543.

Calvo, S.E., Mootha, V.K., 2010. The mitochondrial proteome and human disease. Annu. Rev. Genomics Hum. Genet. 11, 25–44.

Dato, S., Passarino, G., Rose, G., Altomare, K., Bellizzi, D., Mari, V., Feraco, E., Franceschi, C., De Benedictis, G., 2004. Association of the mitochondrial DNA haplogroup J with longevity is population specific. Eur. J. Hum. Genet. 12, 1080–1082.

Doulatov, S., Notta, F., Laurenti, E., Dick, J.E., 2012. Hematopoiesis: a human perspective. Cell Stem Cell 10, 120–136.

Falkenberg, M., Larsson, N.G., Gustafsson, C.M., 2007. DNA replication and transcription in mammalian mitochondria. Annu. Rev. Biochem. 76, 679–699.

Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247.

Forster, L., Forster, P., Lutz-Bonengel, S., Willkomm, H., Brinkmann, B., 2002. Natural radioactivity and human mitochondrial DNA mutations. Proc. Natl. Acad. Sci. U. S. A. 99, 13950–13954.

Greaves, L.C., Turnbull, D.M., 2009. Mitochondrial DNA mutations and ageing. Biochim. Biophys. Acta 1790, 1015–1020.

Harman, D., 1972. The biologic clock: the mitochondria? J. Am. Geriatr. Soc. 20, 145–147.

He, Y., Wu, J., Dressman, D.C., Iacobuzio-Donahue, C., Markowitz, S.D., Velculescu, V.E., Diaz Jr., L.A., Kinzler, K.W., Vogelstein, B., Papadopoulos, N., 2010. Heteroplasmic mitochondrial DNA mutations in normal and tumour cells. Nature 464, 610–614.

Michikawa, Y., Mazzucchelli, F., Bresolin, N., Scarlato, G., Attardi, G., 1999. Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. Science 286, 774–779.

Nagley, P., Wei, Y.H., 1998. Ageing and mammalian mitochondrial genetics. Trends Genet. 14, 513–517.

Norddahl, G.L., Pronk, C.J., Wahlestedt, M., Sten, G., Nygren, J.M., Ugale, A., Sigvardsson, M., Bryder, D., 2011. Accumulating mitochondrial DNA mutations drive premature hematopoietic aging phenotypes distinct from physiological stem cell aging. Cell Stem Cell 8, 499–510.

Ogasawara, Y., Nakayama, K., Tarnowka, M., McCoy Jr., J.P., Kajigaya, S., Levin, B.C., Young, N.S., 2005. Mitochondrial DNA spectra of single human CD34+ cells, T cells, B cells, and granulocytes. Blood 106, 3271–3284.

Rand, D.M., Fry, A., Sheldahl, L., 2006. Nuclear-mitochondrial epistasis and drosophila aging: introgression of *Drosophila* simulans mtDNA modifies longevity in D. melanogaster nuclear backgrounds. Genetics 172, 329–341.

Reeve, A.K., Krishnan, K.J., Taylor, G., Elson, J.L., Bender, A., Taylor, R.W., Morris, C.M., Turnbull, D.M., 2009. The low abundance of clonally expanded mitochondrial DNA point mutations in aged substantia nigra neurons. Aging Cell 8, 496–498.

Rose, G., Romeo, G., Dato, S., Crocco, P., Bruni, A.C., Hervonen, A., Majamaa, K., Sevini, F., Franceschi, C., Passarino, G., 2010. Somatic point mutations in mtDNA control region are influenced by genetic background and associated with healthy aging: a GEHA study. PLoS One 5, e13395.

Sahin, E., Depinho, R.A., 2010. Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature 464, 520–528.

Salas, A., Bandelt, H.-J., Macaulay, V., Richards, M.B., 2007. Phylogeographic investigations: the role of trees in forensic genetics. Forensic Sci. Int. 168, 1–13.

Santoro, A., Salvioli, S., Raule, N., Capri, M., Sevini, F., Valensin, S., Monti, D., Bellizzi, D., Passarino, G., Rose, G., De Benedictis, G., Franceschi, C., 2006. Mitochondrial DNA involvement in human longevity. Biochim. Biophys. Acta 1757, 1388–1399.

Shadel, G.S., Clayton, D.A., 1997. Mitochondrial DNA maintenance in vertebrates. Annu. Rev. Biochem. 66, 409–435.

Shin, M.G., Kajigaya, S., McCoy Jr., J.P., Levin, B.C., Young, N.S., 2004. Marked mitochondrial DNA sequence heterogeneity in single CD34+ cell clones from normal adult bone marrow. Blood 103, 553–561.

Soares, P., Ermini, L., Thomson, N., Mormina, M., Rito, T., Röhl, A., Salas, A., Oppenheimer, S., Macaulay, V., Richards, M.B., 2009. Correcting for purifying selection: an improved human mitochondrial molecular clock. Am. J. Hum. Genet. 84, 740–759.

Stoneking, M., 2000. Hypervariable sites in the mtDNA control region are mutational hotspots. Am. J. Hum. Genet. 67, 1029–1032.

Taylor, R.W., Turnbull, D.M., 2005. Mitochondrial DNA mutations in human disease. Nat. Rev. Genet. 6, 389–402.

Trifunovic, A., 2006. Mitochondrial DNA and ageing. Biochim. Biophys. Acta 1757, 611–617.

Vendelbo, M.H., Nair, K.S., 2011. Mitochondrial longevity pathways. Biochim. Biophys. Acta 1813, 634–644.

Wallace, D.C., Fan, W., Procaccio, V., 2010. Mitochondrial energetics and therapeutics. Annu. Rev. Pathol. 5, 297–348.

Wang, Y., Michikawa, Y., Mallidis, C., Bai, Y., Woodhouse, L., Yarasheski, K.E., Miller, C.A., Askanas, V., Engel, W.K., Bhasin, S., Attardi, G., 2001. Muscle-specific mutations accumulate with aging in critical human mtDNA control sites for replication. Proc. Natl. Acad. Sci. U. S. A. 98, 4022–4027.

Yao, Y.-G., Bandelt, H.-J., Young, N.S., 2007a. External contamination in single cell mtDNA analysis. PLoS One 2, e681.

Yao, Y.-G., Childs, R.W., Kajigaya, S., McCoy Jr., J.P., Young, N.S., 2007b. Mitochondrial DNA sequence heterogeneity of single CD34$^+$ cells after nonmyeloablative allogeneic stem cell transplantation. Stem Cells 25, 2670–2676.

Yao, Y.-G., Ellison, F.M., McCoy, J.P., Chen, J., Young, N.S., 2007c. Age-dependent accumulation of mtDNA mutations in murine hematopoietic stem cells is modulated by the nuclear genetic background. Hum. Mol. Genet. 16, 286–294.

Yao, Y.-G., Ogasawara, Y., Kajigaya, S., Molldrem, J.J., Falcão, R.P., Pintão, M.-C., McCoy Jr., J.P., Rizzatti, E.G., Young, N.S., 2007d. Mitochondrial DNA sequence variation in single cells from leukemia patients. Blood 109, 756–762.

Zhang, J., Asin-Cayuela, J., Fish, J., Michikawa, Y., Bonafe, M., Olivieri, F., Passarino, G., De Benedictis, G., Franceschi, C., Attardi, G., 2003. Strikingly higher frequency in centenarians and twins of mtDNA mutation causing remodeling of replication origin in leukocytes. Proc. Natl. Acad. Sci. U. S. A. 100, 1116–1121.

Table S1 Correlation of the number of nonaggregate haplotypes by nucleotide substitutions per 100 single cells with age

Samples	r	r^2	P-value
All 51 samples from 49 subjects	0.44	0.19	0.001
All samples excluding the three cord blood samples and one adolescent sample	0.41	0.17	0.004
All samples (controlling for family)[1]	0.40	—	0.004
All samples excluding the three cord blood samples and one adolescent sample (controlling for family)[1]	0.40	—	0.007
Family A only	0.89	0.79	0.018
Family B only	0.19	0.04	0.485
Family C only	0.87	0.76	0.128

[1] "Family" as a control variable for all subjects

Table S2 Correlation of the number of haplotypes per 100 single cells with age

Samples	r	r^2	P-value
All 51 samples from 49 subjects	0.44	0.20	0.001
All samples excluding the three cord blood samples and one adolescent sample	0.42	0.17	0.003
All samples (controlling for family)[1]	0.40	—	0.005
All samples excluding the three cord blood samples and one adolescent sample (controlling for family)[1]	0.40	—	0.006
Family A only	0.88	0.77	0.022
Family B only	0.16	0.03	0.554
Family C only	0.87	0.75	0.135

[1] "Family" as a control variable for all subjects

Table S3. Aggregate mtDNA sequence in single CD34$^+$ cells from members in the four families and three cord blood samples

Family / Donor	Sequence variation	Haplogroup
Family A	16168-16192-16256-16266-16294-16526-73-263-455delT-(523-524)insAC	U5a
Family B	16150-16189-16311-152-195-263-270	H11
Family C / C01	16192-16256-16270-16311-16399-73-199-263	U5a1
Family C / C02	16145-16176A-16223-16390-16519-73-152-263	N1b
Family C / C03	16126-16294-16296-16304-16519-73-263	T2b
Family D	16192-16256-16270-16362-16399-16428-73-249-263	U5a1
CB-1	16129-16223-16391-16519-73-152-199-204-207-250-263-573+CCC	I2'3
CB-2	16111-16223-16290-16319-16360-16362-97-(106-111)del-146-153-235-263	A2
CB-3	16092-16172-16182C-16183C-16189-16223-16266-16362-73-150-263-489-(523-524)delAC	D5a2a

Note: These maternally related members in each family had same aggregate sequence. Family C has three matrilines, as represented by C-1, C-2, and C-3, respectively. Sequence variation was scored according to the consensus sequence of the single CD34$^+$ cells. Positions were numbered according to the revised Cambridge Reference Sequence (CRS) [1]. Suffixes C and G indicate transversions and ''ins'' indicates insertions. Insertions were recorded at the last possible site. The length mutations of the C-tract in regions 16184-16193 and 303-309 were not included. All samples had 315insC in the second hypervariable segment of mtDNA control region. Each mtDNA was assigned to respective haplogroup based on the classification system of world mtDNA phylogeny [2].

Supplementary references:

[1] Andrews RM, Kubacka I, Chinnery PF, et al. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet 1999;23:147.

[2] van Oven M, Kayser M. Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. Hum Mutat 2009;30:E386-394

Table S4. mtDNA sequence variation in single CD34^{+} cells from healthy donors in four families

Sample	Mutation	No.	Haplotype	Haplotype defined by substitutions
Family A				
A-1	Aggregate sequence	39	1	
	16076C>Y, 16174C>Y	1	2	1
	16079C>Y, 523-524insAC/non-ins	1	3	2
	16129G>A, 16310G>R	1	4	3
	16129G>R	1	5	4
	16167C>Y, 16392T>Y, 371C>Y	1	6	5
	16185C>Y, 7C	1	7	6
	16192C>Y, 8C/9C	1	8	7
	16224T>Y	1	9	8
	16234C>Y, 40T>Y, 523-524insAC/non-ins	1	10	9
	16275A>R	1	11	10
	16283A>R	1	12	11
	16286C>T	1	13	12
	16287C>Y, 16336G>R	1	14	13
	16295C>Y, 123A>R	1	15	14
	16299A>R	1	16	15
	16304T>Y, 78C>Y, 8C/9C, 523-524insAC/non-ins	1	17	16
	16308T>Y, 16545T>Y, 91C>Y	1	18	17
	16338A>R	1	19	18
	16381T>Y, 16449C>Y, 523-524ins2AC/insAC	1	20	19
	16445T>Y, 436C>Y	1	21	20
	16447C>A	1	22	21
	16468T>Y, 523-524insAC/non-ins	1	23	22
	16476A>R, 17C>Y	1	24	23
	16484T>Y	1	25	24
	16536C>Y	1	26	25
	72T>Y	1	27	26
	127T>Y	1	28	27
	129T>Y, 8C/9C	1	29	28
	204T>C	1	30	29
	217T>Y	1	31	30
	220T>Y	1	32	31
	233T>Y, 412G>R, 7C/8C	1	33	32
	266T>Y, 8C/9C	1	34	33
	279T>G	1	35	34
	368A>R	1	36	35
	391T>C	1	37	36
	430T>T/A, 437C>Y	1	38	37
	438C>T, 556A>G	1	39	38
	455non-del	1	40	
	460T>Y, 8C/9C	1	41	39
	469C>Y	1	42	40
	572C>Y, 594C>Y	1	43	41
	579T>Y	1	44	42
	628C>Y	1	45	43
	523-524insAC/non-ins	1	46	
	523-524non-ins	1	47	
	7C	1	48	
	7C/8C	1	49	
	8C/9C	4	50	
A-2	Aggregate sequence	52	1	
	16017T>Y, 16132A>R	1	2	1
	16064T>Y	1	3	2

	16075T>Y	1	4	3
	16121T>Y	1	5	4
	16153G>R	1	6	5
	16159C>Y, 99T>Y, 7C/8C	1	7	6
	16172T>Y	1	8	7
	16175A>R, 71G>A, 8C/9C	1	9	8
	16198T>Y, 16234C>Y, 214A>G	1	10	9
	16209T>Y, 141C>Y, 495C>T	1	11	10
	16298T>Y	1	12	11
	16356T>Y	1	13	12
	16378C>Y, 387C>Y, 523-524insAC/non-ins	1	14	13
	16549C>Y, 523-524insAC/non-ins	1	15	14
	16559A>R, 292T>C	1	16	15
	109G>R, 8C/9C	1	17	16
	172T>Y, 225G>R, 7C/8C	1	18	17
	187G>G/C	1	19	18
	197A>R	1	20	19
	215A>G, 8C/9C	1	21	20
	290-291delAA/non-del	1	22	
	305C>Y, 7C	1	23	21
	323G>R	1	24	22
	333T>Y	1	25	23
	393T>Y	1	26	24
	426A>A/T	1	27	25
	488A>G	1	28	26
	574A>R	1	29	27
	578T>Y	1	30	28
	523-524insAC/non-ins	2	31	
	7C	2	32	
	7C/8C	3	33	
	7C/8C/9C	1	34	
	8C/9C	3	35	
A-3	Aggregate sequence	55	1	
	16034G>R	1	2	1
	16051A>R	1	3	2
	16052C>Y, 16263T>Y	1	4	3
	16083C>Y, 7C/8C/9C	1	5	4
	16189T>C, 16191insC/non-ins, 7C	1	6	5
	16189T>C, 7C	1	7	
	16189T>Y, 7C	1	8	6
	16192C>Y, 112C>Y	1	9	7
	16192C>Y, 8C/9C	1	10	8
	16221C>Y	1	11	9
	16267C>Y, 80C>Y	1	12	10
	16311T>C	1	13	11
	16324T>Y, 204T>C, 7C	1	14	12
	16380C>Y	1	15	13
	16468T>Y	1	16	14
	16568T>Y, 523-524insAC/non-ins	1	17	15
	12T>Y, 523-524insAC/non-ins	1	18	16
	310T>Y (C-tract not counted)	2	19	17
	385A>R, 8C/9C	1	20	18
	512A>R	1	21	19
	585A>R, 8C/9C	1	22	20
	7C/8C	5	23	
	7C/8C/9C	3	24	
	8C/9C	8	25	

A-4	Aggregate sequence	57	1	
	16129G>R, 466T>C	1	2	1
	16250C>Y	1	3	2
	16267C>Y	1	4	3
	16544T>Y, 523-524non-ins	1	5	4
	215A>G	2	6	5
	215A>G, 523-524insAC/non-ins	1	7	
	217T>Y, 523-524insAC/non-ins	1	8	6
	227A>R	1	9	7
	279T>G/T	1	10	8
	523-524insAC/non-ins	10	11	
	7/8C	3	12	
	8/9C	13	13	
A-5	Aggregate sequence	53	1	
	16172T>Y	1	2	1
	16192C (same to CRS), 189A>G	1	3	2
	16197C>Y, 8/9C	1	4	3
	16249T>Y, 523-524insAC/non-ins	1	5	4
	16251C>Y, 523-524insAC/non-ins	1	6	5
	16272A>R	1	7	6
	16309A>R, 523-524non-ins	1	8	7
	16325T>Y	1	9	8
	16452T>Y, 8/9C	1	10	9
	16482insA/non-ins, 204T>Y	1	11	10
	10T>Y	1	12	11
	18C>Y	1	13	12
	146T>Y	1	14	13
	200A>R, 8/9C	1	15	14
	265T>Y, 266T>Y	1	16	15
	298C>Y	1	17	16
	310T>Y	1	18	17
	346T>Y	1	19	18
	401T>Y	1	20	19
	472A>R	1	21	20
	627G>R, 8C/9C	1	22	21
	7/8C	1	23	
	7/8C/9C	2	24	
	8/9C	10	25	
	523-524insAC/ins2AC	1	26	
	523-524insAC/non-ins	5	27	
A-6	Aggregate sequence	63	1	
	16079C>Y, 1G>R	1	2	1
	16145G>A	2	3	2
	16145G>R	8	4	3
	16145G>R, 8C/9C	1	5	
	16263delT/non-del	1	6	
	150C>Y	1	7	4
	585A>R	1	8	5
	7C/8C	1	9	
	8C/9C	10	10	
	8C/9C, 523-524insAC/non-ins	1	11	
	523-524insAC/non-ins	5	12	
		95		
Family B				
B-1	Aggregate sequence (9C/10C/11C/12C)	21	1	
	16049G>R, 182C>Y, 9C/10C/11C	1	2	1
	16093T>Y, 9C/10C/11C	1	3	2

	16110G>R, 9C/10C/11C	1	4	3
	16129G>A, 16540C>Y, 9C/10C	1	5	4
	16131T>C, 9C/10C	1	6	5
	16187C>Y, 9C/10C/11C/12C	1	7	6
	16264C>Y, 570C>Y, 9C/10C	1	8	7
	16295C>Y, 9C/10C/11C	1	9	8
	16319G>R, 8C/9C/10C	1	10	9
	16321C>Y, 9C/10C/11C/12C	1	11	10
	16400C>Y, 9C/10C/11C	1	12	11
	16408C>Y, 540A>R, 8C/9C/10C	1	13	12
	16418T>Y, 466T>Y, 9C/10C/11C	1	14	13
	16475T>Y, 9C/10C	1	15	14
	16529T>Y, 9C/10C	1	16	15
	16544T>Y, 9C/10C/11C/12C	1	17	16
	14T>Y, 9C/10C/11C	1	18	17
	48C>Y, 9C/10C	1	19	18
	64C>T, 9C/10C/11C/12C	1	20	19
	112C>Y, 9C/10C/11C	1	21	20
	113C>Y, 9C/10C/11C	1	22	21
	133T>Y, 204T>C, 9C/10C/11C	1	23	22
	181A>R, 9C/10C/11C/12C	1	24	23
	182C>Y, 8C/9C/10C	1	25	24
	182C>Y, 9C/10C/11C/12C	1	26	
	230A>R, 9C/10C/11C/12C	1	27	25
	305C>Y, 9C/10C/11C	1	28	26
	368A>R, 492A>R, 9C/10C/11C	1	29	27
	436C>Y, 9C/10C/11C	1	30	28
	470A>R, 9C/10C	1	31	29
	490A>R, 9C/10C/11C	1	32	30
	509C>Y, 9C/10C/11C	1	33	31
	630C>Y, 9C/10C/11C/12C	1	34	32
	8C/9C	1	35	
	8C/9C/10C	2	36	
	8C/9C/10C/11C	3	37	
	9C/10C	15	38	
	9C/10C/11C	20	39	
B-2	Aggregate sequence (9C/10C)	21	1	
	16024T>Y, 16381T>Y, 9C/10C	1	2	1
	16068T>Y, 9C/10C	1	3	2
	16093T>Y, 9C/10C/11C	1	4	3
	16100A>R, 9C/10C	1	5	4
	16131T>Y, 9C/10C/11C/12C	1	6	5
	16147C>Y, 9C/10C/11C/12C	1	7	6
	16150C>Y, 291delA/non-del, 9C/10C/11C	1	8	7
	16187C>Y, 9C/10C/11C/12C	1	9	8
	16256C>Y, 9C/10C/11C/12C	1	10	9
	16258A>R, 9C/10C/11C/12C	1	11	10
	16274G>R, 405T>C, 9C/10C/11C	1	12	11
	16278C>T, 9C/10C	1	13	12
	16298T>Y, 422T>Y, 9C/10C/11C	1	14	13
	16318A>R, 9C/10C/11C/12C	1	15	14
	16356T>Y, 296C>Y, 9C/10C/11C	1	16	15
	16388G>R, 16466A>R, 521A>R, 9C/10C/11C/12C	1	17	16
	16430A>R, 9C/10C/11C	1	18	17
	16438G>R, 9C/10C/11C/12C	1	19	18
	16440T>Y, 8C/9C	1	20	19
	16443T>Y, 9C/10C/11C/12C	1	21	20
	16473G>R, 8C/9C/10C	1	22	21

	67G>R, 9C/10C/11C/12C	1	23	22
	69G>R, 9C/10C	1	24	23
	105C>Y, 298C>Y, 9C/10C/11C	1	25	24
	111A>R, 9C/10C/11C/12C	1	26	25
	151C>Y, 581A>R, 8C/9C/10C/11C	1	27	26
	204T>Y, 9C/10C/11C	1	28	27
	371C>Y, 9C/10C/11C/12C	1	29	28
	392T>Y, 9C/10C	1	30	29
	419A>R, 9C/10C	1	31	30
	448A>R, 9C/10C/11C	1	32	31
	453T>Y, 9C/10C/11C/12C	1	33	32
	483C>Y, 9C/10C	1	34	33
	515A>R, 9C/10C	1	35	34
	520C>Y, 9C/10C/11C/12C	1	36	35
	573delC/non-del, 9C/10C	1	37	
	595C>Y, 9C/10C/11C	1	38	36
	8C/9C/10C	2	39	
	8C/9C/10C/11C	3	40	
	9C/10C/11C	18	41	
	9C/10C/11C/12C	13	42	
B-3	Aggregate sequence (9C/10C/11C)	15	1	
	16014C>Y, 272A>R, 9C/10C/11C	1	2	1
	16024T>Y, 16086T>Y, 9C/10C/11C	1	3	2
	16032T>Y, 308C>Y, 9C/10C	1	4	3
	16062A>A/T, 9C/10C/11C	1	5	4
	16073C>Y, 9C/10C/11C/12C	1	6	5
	16081A>R, 9C/10C/11C/12C	1	7	6
	16093T>C, 9C/10C/11C/12C	1	8	7
	16105T>Y, 16396T>Y, 9C/10C/11C/12C	1	9	8
	16110G>R, 9C/10C/11C	1	10	9
	16129G>R, 152T>Y, 9C/10C	1	11	10
	16129G>R, 9C/10C/11C/12C	1	12	11
	16131T>C, 146T>Y, 9C/10C/11C/12C	1	13	12
	16131T>C, 8C/9C	1	14	13
	16181A>C, 9C/10C/11C/12C	1	15	14
	16184C>Y, 183A>R, 9C/10C/11C/12C	1	16	15
	16187C>Y, 198C>Y, 9C/10C/11C	1	17	16
	16218C>Y, 523-524delAC/non-del, 9C/10C/11C	1	18	17
	16234C>Y, 9C/10C	1	19	18
	16264C>Y, 9C/10C/11C/12C	1	20	19
	16336G>R, 16563C>Y, 71G>R, 9C/10C/11C/12C	1	21	20
	16362T>Y, 9C/10C/11C	1	22	21
	16488C>T, 10T>Y, 111A>G, 10C/11C/12C	1	23	22
	16513T>Y, 9C/10C	1	24	23
	7A>R, 9C/10C/11C/12C	1	25	24
	22T>Y, 178A>R, 573insC, 8C/9C/10C	1	26	25
	110C>Y, 9C/10C/11C/12C	1	27	26
	125T>T/A, 8C/9C/10C/11C	1	28	27
	133T>Y, 453T>Y, 9C/10C	1	29	28
	146T>Y, 9C/10C/11C/12C	1	30	29
	153A>A/C, 9C/10C	1	31	30
	157T>Y, 9C/10C/11C/12C	1	32	31
	169A>R, 9C/10C/11C	1	33	32
	214A>R, 9C/10C/11C	1	34	33
	304C>A, 11C/12C	1	35	34
	304C>Y, 544C>Y, 8C/9C/10C/11C	1	36	35
	307C>Y, 9C/10C	1	37	36
	310T>Y, 540A>R, 9C/10C	1	38	37

	370C>Y, 9C/10C/11C/12C	1	39	38
	439A>A/T, 9C/10C	1	40	39
	454T>Y, 9C/10C/11C	1	41	40
	489T>C, 8C/9C/10C/11C	1	42	41
	573insC/non-ins, 9C/10C	1	43	
	609C>Y, 9C/10C/11C	1	44	42
	8C/9C	4	45	
	8C/9C/10C	1	46	
	9C/10C	9	47	
	9C/10C/11C/12C	14	48	
B-4	Aggregate sequence (9C/10C/11C)	22	1	
	16026C>T, 9C/10C/11C	1	2	1
	16086T>C, 454T>Y, 9C/10C/11C/12C	1	3	2
	16099C>Y, 8C/9C/10C	1	4	3
	16115C>Y, 9C/10C/11C	1	5	4
	16150C>Y, 511C>Y, 8C/9C/10C/11C	1	6	5
	16179C>T, 9C/10C/11C	1	7	6
	16181A>C, 9C/10C/11C	1	8	7
	(16194-16195)AT>CC, 9C/10C/11C	1	9	8
	16214C>T, 9C/10C/11C/12C	1	10	9
	16256C>Y, 8C/9C/10C/11C	1	11	10
	16321C>Y, 9C/10C/11C/12C	1	12	11
	16368T>C, 8C/9C/10C	1	13	12
	16381T>Y, 9C/10C/11C/12C	1	14	13
	16387A>R, 9C/10C/11C	1	15	14
	16542C>Y, 9C/10C/11C	1	16	15
	7A>R, 424T>Y, 9C/10C	1	17	16
	43C>Y, 9C/10C/11C	1	18	17
	107G>R, 210A>R, 9C/10C/11C/12C	1	19	18
	161T>Y, 9C/10C/11C/12C	1	20	19
	162C>Y, 9C/10C/11C	1	21	20
	178A>R, 8C/9C/10C/11C	1	22	21
	195T>Y, 9C/10C/11C	1	23	22
	198C>Y, 9C/10C/11C/12C	1	24	23
	204T>C, 9C/10C/11C	1	25	24
	421T>Y, 9C/10C	1	26	25
	508A>R, 8C/9C/10C/11C	1	27	26
	514C>Y, 9C/10C/11C/12C	1	28	27
	593T>C, 8C/9C/10C	1	29	28
	595C>Y, 9C/10C/11C/12C	1	30	29
	523-524delAC/non-del, 9C/10C/11C	1	31	
	8C/9C/10C	4	32	
	8C/9C/10C/11C	2	33	
	9C/10C	18	34	
	9C/10C/11C/12C	20	35	
B-5	Aggregate sequence (9C/10C/11C/12C)	25	1	
	16042G>A, 2A>R, 463C>Y, 8C/9C/10C/11C	1	2	1
	16069C>Y, 16129G>R, 9C/10C/11C/12C	1	3	2
	16087A>R, 16268C>Y, 16283A>R, 8C/9C	1	4	3
	16093T>C, 9C/10C/11C	1	5	4
	16129G>A, 9C/10C/11C	1	6	5
	16129G>R, 9C/10C/11C/12C	1	7	6
	16140T>Y, 9C/10C/11C/12C	1	8	7
	16190C>Y, 9C/10C	1	9	8
	16214C>Y, 16463A>R, 149T>Y, 9C/10C/11C/12C/13C	1	10	9
	16261C>Y, 16559A>R, 8C/9C	1	11	10
	16262C>Y, 9C/10C/11C	1	12	11

	16269A>R, 146T>C, 9C/10C/11C/12C	1	13	12
	16282C>Y, 9C/10C	1	14	13
	16291C>Y, 9C/10C/11C/12C	1	15	14
	16328C>Y, 16526G>R, 9C/10C	1	16	15
	16353C>Y, 16548C>Y, 242C>Y, 9C/10C/11C/12C	1	17	16
	16374A>R, 9C/10C	1	18	17
	16411C>Y, 9C/10C/11C/12C	1	19	18
	2A>R, 71delG/non-del, 9C/10C/11C/12C, 523-524delAC/non-del	1	20	19
	21A>R, 9C/10C/11C	1	21	20
	24A>R, 9C/10C	1	22	21
	51T>Y, 9C/10C/11C	1	23	22
	63T>Y, 8C/9C	1	24	23
	97G>A, 9C/10C/11C	1	25	24
	142T>Y, 9C/10C/11C	1	26	25
	143G>R, 9C/10C/11C	1	27	26
	144C>Y, 9C/10C, 523-524delAC/non-del	1	28	27
	146T>C, 220T>Y, 9C/10C/11C/12C	1	29	28
	151C>Y, 9C/10C	1	30	29
	155T>Y, 9C/10C/11C/12C	1	31	30
	204T>C, 9C/10C	1	32	31
	204T>C, 9C/10C/11C	1	33	
	217T>C, 9C/10C	1	34	32
	246T>Y, 9C/10C/11C	1	35	33
	247G>R, 9C/10C/11C	1	36	34
	435C>Y, 500C>Y, 9C/10C/11C	1	37	35
	436C>Y, 9C/10C	1	38	36
	462C>Y, 9C/10C	1	39	37
	519A>A/T, 9C/10C/11C	1	40	38
	521A>R, 9C/10C/11C	1	41	39
	525C>Y, 9C/10C/11C/12C	1	42	40
	8C/9C	1	43	
	8C/9C/10C	1	44	
	8C/9C/10C/11C	1	45	
	8C/9C/10C/11C/12C	1	46	
	9C/10C	12	47	
	9C/10C/11C	12	48	
	9C/10C/11C/12C/13C	1	49	
B-6	Aggregate sequence (9C/10C/11C)	22	1	
	16020T>Y, 195T>Y, 9C/10C/11C/12C	1	2	1
	16029T>Y, 182C>T, 9C/10C/11C/12C	1	3	2
	16029T>Y, 577G>R, 9C/10C	1	4	3
	16036G>R, 9C/10C	1	5	4
	16135delA/non-del, 9C/10C	1	6	
	16150C>Y, 9C/10C/11C/12C	1	7	5
	16151C>Y, 16256C>Y, 9C/10C	1	8	6
	16165A>R, 16374A>R, 9C/10C	1	9	7
	16173C>Y, 9C/10C/11C	1	10	8
	16177A>R, 9C/10C/11C	1	11	9
	16235A>R, 9C/10C	1	12	10
	16249T>Y, 9C/10C/11C	1	13	11
	16278C>Y, 9C/10C/11C/12C	1	14	12
	16293A>R, 9C/10C/11C	1	15	13
	16293A>R, 9C/10C/11C, 523-524delAC/non-del	1	16	
	16312A>R, 2A>R, 152T>Y, 182C>Y, 9C/10C/11C/12C	1	17	14
	16314A>R, 16423A>R, 9C/10C/11C	1	18	15
	16368T>C, 9C/10C/11C/12C	1	19	16
	16389G>R, 8C/9C/10C	1	20	17
	16459C>Y, 500C>Y, 507T>T/A, 9C/10C/11C	1	21	18

	16469T>T/G, 9C/10C/11C/12C	1	22	19
	16482delA/non-del, 9C/10C/11C/12C	1	23	
	13A>R, 9C/10C/11C	1	24	20
	59T>Y, 8C/9C/10C	1	25	21
	70G>R, 9C/10C/11C	1	26	22
	142T>Y, 9C/10C	1	27	23
	146T>Y, 9C/10C/11C/12C	1	28	24
	149T>Y, 9C/10C/11C	1	29	25
	157T>Y, 9C/10C/11C/12C	1	30	26
	182C>T, 9C/10C/11C	1	31	27
	182C>Y, 9C/10C/11C	2	32	28
	182C>Y, 9C/10C/11C			
	182C>T, 9C/10C/11C/12C	1	33	
	198C>Y, 9C/10C/11C	1	34	29
	208T>Y, 9C/10C/11C/12C	1	35	30
	266T>Y, 9C/10C	1	36	31
	303C>Y, 9C/10C	1	37	32
	306C>Y, 9C/10C/11C	1	38	33
	484A>R, 9C/10C/11C	1	39	34
	487A>G, 9C/10C/11C	1	40	35
	509C>Y, 9C/10C/11C	1	41	36
	553C>Y, 9C/10C/11C	1	42	37
	605T>C, 9C/10C/11C	1	43	38
	8C/9C/10C	5	44	
	9C/10C	14	45	
	9C/10C/11C/12C	10	46	
	9C/10C/11C/12C/13C	1	47	
B-7	Aggregate sequence (9C/10C/11C)	22	1	
	16034G>R, 16105T>Y, 16430A>R, 16487A>R, 9C/10C	1	2	1
	16037A>R, 9C/10C/11C	1	3	2
	16046T>Y, 9C/10C/11C	1	4	3
	16073C>Y, 127T>Y, 9C/10C	1	5	4
	16103A>R, 9C/10C/11C	1	6	5
	16114C>Y, 8C/9C/10C/11C	1	7	6
	16129G>R, 9C/10C/11C	1	8	7
	16129G>R, 9C/10C/11C/12C	1	9	8
	16175A>R, 16183A>Y, 9C/10C	1	10	9
	16226A>R, 629T>Y, 9C/10C	1	11	10
	16254A>R, 9C/10C	1	12	11
	16286C>Y, 113C>Y, 635C>Y, 9C/10C/11C	1	13	12
	16482A>R, 9C/10C/11C/12C	1	14	13
	16545T>Y, 9C/10C	1	15	14
	18C>Y, 9C/10C/11C/12C	1	16	15
	91C>Y, 9C/10C/11C/12C	1	17	16
	197A>R, 9C/10C/11C/12C	1	18	17
	272A>R, 9C/10C/11C	1	19	18
	302A>A/C, 9C/10C/11C	1	20	19
	302delA, 9C/10C/11C	1	21	
	345C>Y, 9C/10C/11C/12C	1	22	20
	392T>Y, 9C/10C	1	23	21
	489T>Y, 9C/10C/11C	1	24	22
	494C>Y, 9C/10C/11C	1	25	23
	502C>Y, 9C/10C/11C	1	26	24
	516C>Y, 8C/9C/10C	1	27	25
	522C>Y, 9C/10C/11C	1	28	26
	537C>Y, 8C/9C/10C/11C	1	29	27
	548C>Y, 9C/10C/11C	1	30	28
	635C>Y, 9C/10C/11C	1	31	29

	8C/9C/10C	2	32	
	8C/9C/10C/11C	1	33	
	8C/9C/10C/11C/12C	1	34	
	8C/9C/10C/11C/12C, 523-524delAC/non-del	1	35	
	9C/10C	22	36	
	9C/10C, 523-524delAC/non-del	1	37	
	9C/10C/11C/12C	15	38	
B-8	Aggregate sequence (9C/10C/11C)	30	1	
	16053C>Y, 560C>Y, 9C/10C/11C	1	2	1
	16073C>Y, 9C/10C/11C/12C	1	3	2
	16119A>R, 16455G>R, 633A>R, 9C/10C/11C/12C	1	4	3
	16129G>R, 9C/10C	1	5	4
	16129G>R, 9C/10C/11C	1	6	
	16131T>Y, 9C/10C/11C	1	7	5
	16171A>R, 16311T>Y, 9C/10C/11C/12C	1	8	6
	16214C>T, 9C/10C/11C	1	9	7
	16234C>Y, 9C/10C/11C	1	10	8
	16250C>A/C, 16374A>R, 9C/10C/11C	1	11	9
	16260C>Y, 9C/10C/11C/12C	1	12	10
	16262C>Y, 40T>Y, 8C/9C	1	13	11
	16269A>R, 9C/10C	1	14	12
	16345A>A/T, 16354C>Y, 9C/10C/11C	1	15	13
	16367A>R, 146T>Y, 9C/10C/11C/12C	1	16	14
	16370G>R, 9C/10C/11C	1	17	15
	16469T>Y, 9C/10C/11C	1	18	16
	16509T>Y, 9C/10C/11C	1	19	17
	11C>Y, 9C/10C/11C	1	20	18
	22T>Y, 9C/10C/11C	1	21	19
	89T>Y, 542C>Y, 9C/10C/11C/12C	1	22	20
	102A>R, 9C/10C/11C/12C	1	23	21
	146T>Y, 9C/10C/11C/12C	1	24	22
	156A>R, 9C/10C	1	25	23
	178A>R, 9C/10C/11C	1	26	24
	182C>T, 9C/10C/11C/12C	1	27	25
	422T>Y, 8C/9C	1	28	26
	483C>Y, 9C/10C	1	29	27
	540A>R, 9C/10C	1	30	28
	8C/9C	3	31	
	8C/9C/10C	1	32	
	9C/10C	23	33	
	9C/10C/11C, 523-524delAC/non-del	1	34	
	9C/10C/11C/12C	7	35	
B-9	Aggregate sequence (9C/10C/11C/12C)	20	1	
	16052C>Y, 9C/10C/11C/12C	1	2	1
	16057C>Y, 9C/10C/11C/12C	1	3	2
	16084G>R, 16322A>R, 529G>A, 10C/11C/12C	1	4	3
	16088T>Y, 483C>Y, 611G>R, 9C/10C/11C/12C	1	5	4
	16096G>R, 16134C>Y, 9C/10C/11C/12C	1	6	5
	16140T>Y, 9C/10C/11C/12C	1	7	6
	16146A>R, 9C/10C/11C/12C	1	8	7
	16167C>Y, 9C/10C	1	9	8
	16169C>Y, 9C/10C/11C/12C	1	10	9
	16230A>R, 9C/10C	1	11	10
	16239C>Y, 9C/10C	1	12	11
	16265A>R, 9C/10C	1	13	12
	16290C>Y, 193A>R, 9C/10C/11C/12C	1	14	13
	16291C>Y, 9C/10C/11C	1	15	14

	16309A>R, 9C/10C/11C	1	16	15
	16328C>Y, 9C/10C/11C	1	17	16
	16333A>R, 619T>Y, 9C/10C/11C	1	18	17
	16337C>Y, 9C/10C/11C/12C	1	19	18
	16399A>R, 16509T>Y, 9C/10C	1	20	19
	16443T>Y, 9C/10C/11C	1	21	20
	16468T>Y, 9C/10C	1	22	21
	16497A>R, 9C/10C/11C/12C	1	23	22
	60T>Y, 9C/10C/11C/12C	1	24	23
	142T>Y, 9C/10C/11C/12C	1	25	24
	146T>Y, 9C/10C/11C/12C	1	26	25
	161T>Y, 9C/10C	1	27	26
	169A>R, 527C>Y, 9C/10C/11C	1	28	27
	173T>Y, 9C/10C/11C	1	29	28
	184G>R, 9C/10C/11C	1	30	29
	198C>Y, 8C/9C/10C	1	31	30
	204T>C, 9C/10C/11C/12C	1	32	31
	208T>T/A, 523-524delAC/non-del, 9C/10C/11C/12C	1	33	32
	219A>R, 9C/10C/11C/12C	1	34	33
	230A>R, 9C/10C/11C	1	35	34
	242+TAA/non-ins, 9C/10C/11C/12C	1	36	
	248A>R, 9C/10C	1	37	35
	310T>C, long Cs	1	38	36
	390A>R, 446A>R, 523-524delAC/non-del, 9C/10C/11C	1	39	37
	404C>Y, 8C/9C	1	40	38
	419A>R, 9C/10C/11C/12C	1	41	39
	466T>Y, 9C/10C/11C	1	42	40
	497C>Y, 9C/10C/11C	1	43	41
	540A>R, 9C/10C/11C	1	44	42
	612A>R, 10C/11C/12C	1	45	43
	523-524delAC/non-del, 8C/9C/10C	1	46	
	523-524delAC/non-del, 9C/10C	1	47	
	8C/9C/10C	2	48	
	8C/9C/10C/11C	2	49	
	9C/10C	8	50	
	10C/11C/12C	2	51	
	9C/10C/11C	14	52	
B-10	Aggregate sequence (9C/10C/11C)	23	1	
	16056C>Y, 9C/10C/11C/12C	1	2	1
	16086T>Y, 16122A>R, 8C/9C/10C	1	3	2
	16093T>C, 9C/10C/11C/12C	1	4	3
	16124T>Y, 9C/10C	1	5	4
	16131T>Y, 179T>Y, 9C/10C/11C/12C	1	6	5
	16186C>Y, 9C/10C/11C	1	7	6
	16272A>R, 8C/9C/10C	1	8	7
	16277A>R, 16547C>Y, 8C/9C/10C	1	9	8
	16311T>Y, 8C/9C	1	10	9
	16317A>R, 9C/10C/11C/12C	1	11	10
	16368T>Y, 9C/10C/11C	1	12	11
	16533T>Y, 355C>Y, 9C/10C/11C	1	13	12
	42T>Y, 9C/10C/11C/12C	1	14	13
	56A>R, 523-524delAC/non-del, 9C/10C	1	15	14
	58T>Y, 9C/10C/11C/12C	1	16	15
	142T>Y, 172T>T/G, 9C/10C	1	17	16
	146T>C, 8C/9C	1	18	17
	185G>R, 9C/10C/11C	1	19	18
	190C>Y, 8C/9C/10C	1	20	19
	224T>Y, 8C/9C	1	21	20

	235A>R, 9C/10C	1	22	21
	238A>R, 250T>Y, 8C/9C/10C/11C	1	23	22
	291A>R, 8C/9C/10C	1	24	23
	403T>Y, 587C>Y, 9C/10C/11C	1	25	24
	405T>Y, 9C/10C	1	26	25
	420C>Y, 564G>R, 8C/9C/10C	1	27	26
	421T>Y, 9C/10C/11C/12C	1	28	27
	497C>Y, 9C/10C/11C/12C	1	29	28
	536C>Y, 9C/10C/11C	1	30	29
	557C>Y, 9C/10C/11C/12C	1	31	30
	523-524insAC/non-ins, 9C/10C/11C	1	32	
	8C/9C	5	33	
	8C/9C/10C	4	34	
	8C/9C/10C/11C	3	35	
	9C/10C	7	36	
	9C/10C/11C/12C	20	37	
B-11	Aggregate sequence (9C/10C/11C)	22	1	
	16066A>G, 16123T>Y, 9C/10C/11C	1	2	1
	16103A>R, 421T>Y, 512A>R, 9C/10C/11C/12C	1	3	2
	16150C>Y, 9C/10C	1	4	3
	16182A>Y, 9C/10C	1	5	4
	16222C>Y, 9C/10C/11C	1	6	5
	16226A>R, 9C/10C/11C	1	7	6
	16251C>Y, 9C/10C/11C	1	8	7
	16262delC/non-del, 9C/10C/11C	1	9	
	16291C>Y, 8C/9C/10C/11C	1	10	8
	16368T>Y, 8C/9C	1	11	9
	16386T>Y, 8C/9C	1	12	10
	16411C>Y, 9C/10C/11C	1	13	11
	16425C>Y, 9C/10C	1	14	12
	16442C>Y, 9C/10C/11C	1	15	13
	16459C>Y, 9C/10C/11C	1	16	14
	16490G>R, 9C/10C	1	17	15
	16495C>Y, 9C/10C/11C/12C	1	18	16
	16534A>R, 8C/9C/10C	1	19	17
	16556A>R, 8C/9C	1	20	18
	19C>Y, 9C/10C/11C	1	21	19
	114C>Y, 449T>Y, 9C/10C	1	22	20
	146T>Y, 9C/10C	1	23	21
	174C>Y, 9C/10C/11C	1	24	22
	234A>R, 9C/10C/11C/12C	1	25	23
	304C>Y, 9C/10C	1	26	24
	404C>Y, 9C/10C/11C	1	27	25
	482T>Y, 8C/9C/10C	1	28	26
	538A>R, 9C/10C	1	29	27
	8C/9C	5	30	
	8C/9C/10C	5	31	
	8C/9C/10C/11C	2	32	
	8C/9C/10C/11C/12C	1	33	
	9C/10C	20	34	
	9C/10C/11C/12C	8	35	
B-12	Aggregate sequence (9C/10C)	25	1	
	16029T>Y, 153A>R, 8C/9C/10C	1	2	1
	16062A>R, 9C/10C/11C	1	3	2
	16086T>Y, 16129G>A, 8C/9C	1	4	3
	16095C>Y, 9C/10C	1	5	4
	16114C>Y, 8C/9C	1	6	5

16124T>C, 9C/10C/11C/12C	1	7	6
16129G>A, 16278C>Y, 8C/9C/10C	1	8	7
16129G>A, 16476A>R, 9C/10C	1	9	8
16129G>A, 204T>C, 9C/10C	1	10	9
16129G>A, 462C>Y, 8C/9C/10C	1	11	10
16129G>A, 8C/9C	5	12	11
16129G>A, 8C/9C/10C	2	13	
16129G>A, 8C/9C/10C/11C	1	14	
16129G>A, 9C/10C	6	15	
16129G>A, 9C/10C/11C	2	16	
16129G>R, 8C/9C	5	17	12
16129G>R, 8C/9C/10C	1	18	
16129G>R, 8C/9C/10C/11C	1	19	
16129G>R, 9C/10C	4	20	
16129G>R, 9C/10C/11C	3	21	
16129G>R, 9C/10C/11C/12C	1	22	
16131T>Y, 8C/9C	1	23	13
16136T>T/A, 9C/10C	1	24	14
16137A>R, 9C/10C/11C/12C	1	25	15
16152T>Y, 9C/10C/11C	1	26	16
16158A>R, 16272A>R, 8C/9C/10C	1	27	17
16167C>Y, 9C/10C/11C/12C	1	28	18
16184C>Y, 8C/9C	1	29	19
16187C>Y, 8C/9C	1	30	20
16203A>R, 9C/10C/11C	1	31	21
16211C>Y, 8C/9C/10C	1	32	22
16228C>T, 9C/10C	1	33	23
16250C>Y, 71delG/non-del, 8C/9C	1	34	24
16263delT/non-del, 4C>Y, 9C/10C/11C	1	35	25
16307A>R, 8C/9C/10C, 523-524delAC/non-del	1	36	26
16320C>Y, 8C/9C	1	37	27
16336G>R, 8C/9C/10C/11C	1	38	28
16377C>Y, 8C/9C/10C	1	39	29
16423A>R, 143G>R, 8C/9C/10C	1	40	30
16427C>Y, 9C/10C	1	41	31
16449C>Y, 9C/10C	1	42	32
16463A>R, 9C/10C	1	43	33
16464A>R, 9C/10C/11C	1	44	34
16486A>R, 8C/9C	1	45	35
16519T>C, 8C/9C/10C	1	46	36
39C>Y, 8C/9C/10C	1	47	37
54G>R, 8C/9C	1	48	38
60T>Y, 8C/9C	1	49	39
70G>R, 9C/10C/11C/12C, 523-524delAC/non-del	1	50	40
93A>R, 9C/10C	1	51	41
112C>Y, 9C/10C/11C	1	52	42
133T>Y, 9C/10C/11C	1	53	43
156A>R, 9C/10C/11C/12C	1	54	44
181A>R, 9C/10C	1	55	45
226T>Y, 9C/10C	1	56	46
246T>Y, 8C/9C/10C	1	57	47
266T>Y, 8C/9C	1	58	48
296C>Y, 9C/10C	1	59	49
307C>Y, 9C/10C	1	60	50
381C>Y, 9C/10C	1	61	51
420C>Y, 9C/10C/11C	1	62	52
434C>Y, 9C/10C	1	63	53
439A>R, 8C/9C/10C	1	64	54
468C>Y, 9C/10C	1	65	55

	515A>R, 9C/10C	1	66	56
	519A>A/T, 9C/10C/11C/12C	1	67	57
	544C>Y, 9C/10C/11C	1	68	58
	570C>Y, 8C/9C	1	69	59
	605T>Y, 8C/9C	1	70	60
	626G>R, 9C/10C/11C	1	71	61
	8C/9C	22	72	
	8C/9C/10C	16	73	
	8C/9C/10C/11C	6	74	
	9C/10C/11C	16	75	
	9C/10C/11C/12C	10	76	
B-13	Aggregate sequence (9C/10C/11C)	59	1	
	16022T>Y, 9C/10C	1	2	1
	16036G>R, 470A>R, 9C/10C/11C/12C	1	3	2
	16037A>R, 9C/10C	1	4	3
	16052C>Y, 9C/10C/11C	1	5	4
	16095C>Y, 9C/10C/11C	1	6	5
	16111C>Y, 9C/10C	1	7	6
	16129G>A, 9C/10C	1	8	7
	16129G>R, 215A>G, 9C/10C/11C	1	9	8
	16134C>Y, 9C/10C	1	10	9
	16137A>R, 9C/10C/11C	1	11	10
	16142C>Y, 204T>Y, 10C/11C/12C	1	12	11
	16154T>Y, 9C/10C/11C/12C	1	13	12
	16176C>Y, 298C>Y, 9C/10C/11C	1	14	13
	16178T>A, 16264C>Y, 8C/9C	1	15	14
	16187C>Y, 9C/10C/11C/12C	1	16	15
	16231T>Y, 16281A>R, 57T>Y, 9C/10C/11C	1	17	16
	16242C>Y, 9C/10C	1	18	17
	16246A>R, 9C/10C/11C	1	19	18
	16265A>R, 9C/10C/11C/12C	1	20	19
	16266C>Y, 9C/10C/11C	1	21	20
	16281A>R, 9C/10C/11C/12C	1	22	21
	16342T>Y, 9C/10C/11C	1	23	22
	16371A>R, 9C/10C/11C/12C	1	24	23
	16381delT/non-del, 9C/10C	1	25	
	16449C>Y, 9C/10C/11C/12C	1	26	24
	16487A>R, 230A>R, 8C/9C	1	27	25
	16507C>Y, 9C/10C/11C	1	28	26
	16512T>Y, 9C/10C/11C	1	29	27
	16518G>R, 9C/10C/11C/12C	1	30	28
	16520C>T, 8C/9C	1	31	29
	16533T>Y, 189A>R, 9C/10C	1	32	30
	16534A>R, 9C/10C	1	33	31
	16540C>Y, 114C>Y, 9C/10C/11C	1	34	32
	16544T>Y, 9C/10C	1	35	33
	16559A>R, 9C/10C/11C	1	36	34
	2A>R, 9C/10C/11C	1	37	35
	3T>Y, 9C/10C/11C/12C	1	38	36
	3T>Y, 9C/10C/11C/12C	1	39	37
	10T>Y, 16A>R, 9C/10C/11C/12C	1	40	38
	12T>Y, 9C/10C/11C/12C	1	41	39
	41C>Y, 431C>Y, 9C/10C/11C/12C	1	42	40
	55T>Y, 9C/10C/11C/12C	1	43	41
	88T>Y, 9C/10C/11C	1	44	42
	91C>Y, 305C>Y, 9C/10C/11C/12C	1	45	43
	97G>G/T, 8C/9C/10C	1	46	44
	98C>Y, 9C/10C/11C	1	47	45

	102A>R, 9C/10C	1	48	46
	143G>R, 9C/10C/11C/12C	1	49	47
	146T>Y, 9C/10C	1	50	48
	162C>Y, 9C/10C/11C/12C	1	51	49
	172T>Y, 9C/10C	1	52	50
	216T>Y, 9C/10C	1	53	51
	227A>R, 9C/10C/11C/12C	1	54	52
	239T>Y, 9C/10C/11C	1	55	53
	240A>R, 9C/10C/11C	1	56	54
	258C>Y, 9C/10C	1	57	55
	265T>Y, 9C/10C	1	58	56
	269C>Y, 8C/9C/10C/11C	1	59	57
	270A>R, 9C/10C/11C/12C	1	60	58
	276A>R, 10C/11C/12C	1	61	59
	278A>R, 439A>R, 9C/10C/11C/12C	1	62	60
	279T>Y, 8C/9C/10C/11C	1	63	61
	308C>Y, 9C/10C	1	64	62
	309C>Y, 9C/10C/11C/12C	1	65	63
	399T>Y, 9C/10C	1	66	64
	405T>Y, 8C/9C/10C/11C/12C	1	67	65
	421T>Y, 506C>Y, 9C/10C/11C/12C	1	68	66
	438C>Y, 10C/11C/12C	1	69	67
	454T>Y, 9C/10C	1	70	68
	466T>Y, 9C/10C/11C	1	71	69
	552C>Y, 9C/10C/11C	1	72	70
	8C/9C	7	73	
	8C/9C/10C	5	74	
	9C/10C	22	75	
	9C/10C/11C/12C	25	76	
	10C/11C/12C	2	77	
B-14	Aggregate sequence (8C/9C/10C)	13	1	
	16034G>R, 9C/10C	1	2	1
	16096G>R, 9C/10C/11C/12C	1	3	2
	16102T>Y, 9C/10C	1	4	3
	16109A>A/T, 146T>C, 8C/9C	1	5	4
	16121T>Y, 9C/10C	1	6	5
	16150C>Y, 16377C>Y, 8C/9C	1	7	6
	16169C>T, 16184C>Y, 416T>Y, 9C/10C/11C	1	8	7
	16229T>Y, 9C/10C	1	9	8
	16267C>Y, 113C>Y, 9C/10C/11C/12C	1	10	9
	16275A>R, 8C/9C/10C/11C	1	11	10
	16290C>Y, 8C/9C	1	12	11
	16312A>R, 9C/10C/11C/12C	1	13	12
	16418T>Y, 9C/10C/11C/12C	1	14	13
	16457G>R, 8C/9C	1	15	14
	16481A>A/T, 386C>Y, 9C/10C	1	16	15
	16486A>R, 9C/10C	1	17	16
	16531A>R, 223T>Y, 9C/10C	1	18	17
	16544T>Y, 9C/10C/11C	1	19	18
	12T>Y, 9C/10C	1	20	19
	15C>T, 9C/10C	1	21	20
	136G>R, 9C/10C	1	22	21
	146T>C, 8C/9C	1	23	22
	146T>Y, 497C>Y, 9C/10C/11C	1	24	23
	146T>Y, 8C/9C	1	25	24
	146T>Y, 8C/9C/10C	1	26	
	146T>Y, 9C/10C	1	27	
	173T>Y, 9C/10C	1	28	25

	200A>R, 8C/9C	1	29	26
	204T>C, 9C/10C/11C/12C	1	30	27
	204T>Y, 8C/9C	1	31	28
	204T>Y, 9C/10C/11C	1	32	
	252T>Y, 8C/9C	1	33	29
	295C>Y, 606A>R, 9C/10C	1	34	30
	297A>R, 8C/9C/10C	1	35	31
	302A>R, 8C/9C/10C	1	36	32
	302delA, 303C>C/A, 9C/10C/11C	1	37	33
	302delA, 304C>Y, 405T>Y, 9C/10C/11C/12C	1	38	34
	302delA, 9C/10C	1	39	
	369C>Y, 9C/10C/11C/12C	1	40	35
	431C>Y, 8C/9C	1	41	36
	440delA/non-del, 9C/10C/11C	1	42	
	453T>Y, 8C/9C	1	43	37
	523-524delAC/non-del, 9C/10C/11C	1	44	
	534C>Y, 8C/9C/10C/11C	1	45	38
	573insC/non-ins, 9C/10C	1	46	
	587C>Y, 9C/10C	1	47	39
	606A>R, 8C/9C	1	48	40
	8C/9C	8	49	
	8C/9C/10C/11C	3	50	
	8C/9C/11C	1	51	
	9C/10C	11	52	
	9C/10C/11C	10	53	
	9C/10C/11C/12C	3	54	
B-15	Aggregate sequence (9C/10C/11C)	20	1	
	16038insA/non-ins, 9C/10C/11C	1	2	
	16049G>R, 16114C>Y, 9C/10C	1	3	1
	16053C>Y, 8C/9C/10C	1	4	2
	16062A>R, 16347T>Y, 9C/10C	1	5	3
	16068T>Y, 16218C>T, 416T>Y, 8C/9C	1	6	4
	16084G>R, 146T>Y, 8C/9C	1	7	5
	16109A>A/T, 8C/9C/10C	1	8	6
	16144T>Y, 8C/9C/10C	1	9	7
	16151C>Y, 9C/10C	1	10	8
	16162A>G, 9C/10C/11C	1	11	9
	16179C>Y, 9C/10C/11C/12C	1	12	10
	16237A>R, 8C/9C/10C	1	13	11
	16249T>Y, 9C/10C	1	14	12
	16268C>Y, 423T>Y, 9C/10C/11C/12C	1	15	13
	16274G>R, 353C>Y, 9C/10C	1	16	14
	16379C>Y, 9C/10C	1	17	15
	16395C>Y, 9C/10C/11C	1	18	16
	16403C>Y, 9C/10C/11C	1	19	17
	16453C>Y, 8C/9C/10C/11C	1	20	18
	16506T>Y, 59T>Y, 9C/10C/11C/12C	1	21	19
	16529T>Y, 8C/9C	1	22	20
	13A>R, 9C/10C/11C/12C	1	23	21
	33C>Y, 9C/10C	1	24	22
	54G>R, 8C/9C/10C	1	25	23
	68G>R, 9C/10C/11C/12C	1	26	24
	74T>Y, 9C/10C/11C	1	27	25
	121G>R, 146T>C, 9C/10C/11C/12C	1	28	26
	125T>Y, 9C/10C	1	29	27
	199T>Y, 490A>R, 9C/10C/11C	1	30	28
	227A>A/T, 384A>R, 9C/10C/11C/12C	1	31	29
	245T>Y, 9C/10C	1	32	30

	285C>Y, 8C/9C/10C/11C	1	33	31
	298C>Y, 9C/10C/11C	1	34	32
	302delA, 9C/10C	1	35	
	356C>Y, 8C/9C	1	36	33
	382C>Y, 8C/9C	1	37	34
	422T>Y, 9C/10C/11C	1	38	35
	435C>Y, 8C/9C	1	39	36
	442T>Y, 607C>Y, 9C/10C/11C/12C	1	40	37
	454T>Y, 8C/9C/10C	1	41	38
	482T>Y, 8C/9C	1	42	39
	605T>Y, 9C/10C	1	43	40
	605T>Y, 8C/9C/10C	1	44	
	626G>R, 8C/9C/10C	1	45	41
	8C/9C	4	46	
	8C/9C/10C	3	47	
	8C/9C/10C/11C	2	48	
	9C/10C	18	49	
	9C/10C/11C/12C	5	50	
B-16	Aggregate sequence (9C/10C/11C)	25	1	
	16095C>C/G, 9C/10C/11C	1	2	1
	16095C>Y, 9C/10C/11C	1	3	2
	16158A>R, 9C/10C/11C/12C	1	4	3
	16167C>Y, 16185C>Y, 9C/10C	1	5	4
	16173C>Y, 9C/10C	1	6	5
	16176C>Y, 9C/10C/11C/12C	1	7	6
	16177A>A/T, 8C/9C	1	8	7
	16180A>R, 9C/10C/11C	1	9	8
	16260C>Y, 8C/9C	1	10	9
	16286C>Y, 9C/10C/11C/12C	1	11	10
	16291C>Y, 9C/10C	1	12	11
	16384G>R, 9C/10C	1	13	12
	16478C>Y, 9C/10C/11C	1	14	13
	151C>T, 9C/10C/11C/12	1	15	14
	167C>Y, 9C/10C	1	16	15
	179T>Y, 9C/10C/11C	1	17	16
	204T>Y, 8C/9C	1	18	17
	252T>Y, 9C/10C/11C	1	19	18
	296C>Y, 9C/10C/11C	1	20	19
	303C>Y, 9C/10C/11C/12C	1	21	20
	398T>Y, 9C/10C/11C	1	22	21
	467C>Y, 9C/10C	1	23	22
	569C>Y, 9C/10C	1	24	23
	523-524delAC/non-del, 9C/10C/11C/12C	1	25	
	8C	1	26	
	8C/9C	3	27	
	8C/9C/10C	3	28	
	8C/9C/10C/11C	1	29	
	9C/10C	18	30	
	9C/10C/11C/12C	21	31	
Family C				
C-1	aggregate sequence	32	1	
	16032T>Y	1	2	1
	16055C>Y, 16541A>R	1	3	2
	16129G>A, 16471G>A	1	4	3
	16131T>C	1	5	4
	16131T>C, 452T>Y	1	6	5
	16137A>R, 305C>Y, 7C/8C	1	7	6

	16140T>Y, 162C>C/A, 519A>A/T	1	8	7
	16149A>A/T, 152T>C	1	9	8
	16171A>A/T, 204T>C	1	10	9
	16189T>Y, 16252A>R	1	11	10
	16189T>Y, 16559A>R	1	12	11
	16210A>R	1	13	12
	16277A>R	1	14	13
	16292delC/non-del	1	15	
	16297T>Y	1	16	14
	16319G>A	1	17	15
	16323T>Y	1	18	16
	16346G>R, 278A>R, 8C/9C	1	19	17
	16353C>Y, 8C/9C	1	20	18
	16409T>Y, 385A>G	1	21	19
	16411C>Y	1	22	20
	16413T>T/G, 204T>C	1	23	21
	16448T>Y, 109G>R, 8C/9C	1	24	22
	16463A>R	1	25	23
	16474insG/non-ins	1	26	
	16521A>R	1	27	24
	16538C>Y	1	28	25
	13A>R	1	29	26
	37A>R	1	30	27
	53G>G/T	1	31	28
	55T>Y	1	32	29
	113C>Y, 8C/9C	1	33	30
	126A>A/T, 394C>Y, 471T>Y	1	34	31
	153A>G	1	35	32
	204T>C	2	36	33
	204T>Y, 291delA	1	37	34
	234A>R	1	38	35
	312C>Y	1	39	36
	398T>Y	1	40	37
	438delC/non-del	1	41	
	453T>Y	1	42	38
	478A>R	1	43	39
	490A>R	1	44	40
	512A>A/C, 7C/8C	1	45	41
	512A>R	1	46	42
	532A>R	1	47	43
	541C>Y	1	48	44
	558C>Y	1	49	45
	7C/8C	2	50	
	8C/9C	10	51	
C-2	Aggregate sequence	29	1	
	16075T>Y	1	2	1
	16092T>Y	1	3	2
	16129G>A	1	4	3
	16151C>Y, 16324T>T/A	1	5	4
	16166A>C	1	6	5
	16213G>A, 5A>R, 71delG	1	7	6
	16219A>R	1	8	7
	16221C>Y	1	9	8
	16251C>Y	1	10	9
	16291C>Y, 16435A>R, 462C>Y	1	11	10
	16302A>R, 617G>R	1	12	11
	16311T>Y, 71delG, 523-524delAC/non-del	1	13	12
	16333A>R	1	14	13

	16365C>Y, 71delG	1	15	14
	16369G>A, 146T>C	1	16	15
	16377C>T, 71delG	1	17	16
	16406T>Y	1	18	17
	16425C>Y	1	19	18
	16439C>Y	1	20	19
	16476A>R, 72T>Y	1	21	20
	16479T>Y	1	22	21
	16489T>Y, 71delG	1	23	22
	16540C>T	1	24	23
	16563C>Y	1	25	24
	59T>T/G, 71delG	1	26	25
	71delG	15	27	
	71delG, (311-315)6C/7C	1	28	
	71delG, 131T>Y, 497C>Y	1	29	26
	71delG, 220T>Y	1	30	27
	71delG, 452T>Y	1	31	28
	71delG, 8C/9C	1	32	
	71delG/non-del	2	33	
	72T>Y, 193A>R	1	34	29
	113C>Y	1	35	30
	206T>Y	1	36	31
	242C>Y	1	37	32
	247G>A	1	38	33
	373A>T, 8C/9C	1	39	34
	393T>Y, 414T>G, 7C/8C	1	40	35
	612A>R, 8C/9C	1	41	36
	627G>R	1	42	37
	523-524delAC/non-del	2	43	
	7C/8C/9C	3	44	
	8C/9C	4	45	
C-3	aggregate sequence	77	1	
	16129G>R	1	2	1
	16221C>Y	1	3	2
	16265A>C	1	4	3
	16266C>Y	1	5	4
	16287C>Y	1	6	5
	16304T (same as CRS)	1	7	6
	146T>Y	1	8	7
	150C>T, 7C/8C	1	9	8
	152T>Y	2	10	9
	189A>R	2	11	10
	215A>R	1	12	11
	222C>Y	1	13	12
	491C>Y	1	14	13
	522C>Y	1	15	14
	593T>Y	1	16	15
	523-524delAC/non-del	2	17	
C-4	aggregate sequence	40	1	
	16023G>R	1	2	1
	16053C>Y, 523-524delAC/non-del	1	3	2
	16062A>R	1	4	3
	16083C>Y, 172T>Y	1	5	4
	16086T>Y	1	6	5
	16126T>Y	1	7	6
	16129G>R, 8C/9C/10C	1	8	7
	16132A>R, 8C/9C	1	9	8

	16148C>Y	1	10	9
	16151C>Y	1	11	10
	16183A>R	1	12	11
	16189T>Y	1	13	12
	16192C>Y, 529G>R	1	14	13
	16214C>Y	1	15	14
	16254A>R	1	16	15
	16266C>Y	1	17	16
	16278C>Y	1	18	17
	16327C>Y, 495C>Y	1	19	18
	16345A>R, 7C/8C	1	20	19
	16368T>C	1	21	20
	16397T>Y	2	22	21
	16457G>R, 124G>R	1	23	22
	16546C>Y, 7C/8C	1	24	23
	16567A>R	1	25	24
	5A>R	1	26	25
	26C>Y	1	27	26
	29C>Y	1	28	27
	31C>Y, 185G>R	1	29	28
	57T>Y	1	30	29
	71insG/non-ins	1	31	
	103G>R	1	32	30
	152T>Y	1	33	31
	153A>R, 542C>Y, 7C/8C, 523-524delAC	1	34	32
	204T>C	1	35	33
	234A>R	1	36	34
	354C>Y	1	37	35
	367A>R	1	38	36
	427C>Y, 8C/9C	1	39	37
	437C>Y	1	40	38
	471T>Y	1	41	39
	503A>R	1	42	40
	549C>Y	1	43	41
	567A>R, 8C/9C	1	44	42
	593T>Y	1	45	43
	7C/8C	5	46	
	8C/9C	5	47	
	523-524delAC/non-del	1	48	
C-5	aggregate sequence	99	1	
	16086T>Y	1	2	1
	16129G>R	1	3	2
	16289A>R	1	4	3
	16399A>R	1	5	4
	16407C>Y	1	6	5
	16497A>R	1	7	6
	16519T	1	8	7
	182C>Y	1	9	8
	204T>C	1	10	9
	204T>Y	1	11	10
	251G>A	1	12	11
	385A>G	1	13	12
	513G>R	1	14	13
C-6	aggregate sequence	88	1	
	16380insC/non-ins	1	2	
	36delG/non-del	1	3	
	116A>R	1	4	1

	178A>R	1	5	2
	189A>R	1	6	3
	204T>C	1	7	4
	524C>Y	1	8	5
C-7	aggregate sequence	53	1	
	16032T>Y, 460T>Y	1	2	1
	16103A>R	1	3	2
	16131T>Y, 16442C>Y	1	4	3
	16149A>G	1	5	4
	16277A>R	1	6	5
	16282C>Y	1	7	6
	16291C>Y, 8C/9C	1	8	7
	16362T>Y	1	9	8
	16363C>Y	1	10	9
	16367A>R, 564G>R	1	11	10
	16368T>T/G, 146T>Y	1	12	11
	16462T>T/A, 227A>R	1	13	12
	16502T>Y	1	14	13
	16517G>R	2	15	14
	16538C>Y, 8C/9C	1	16	15
	71delG/non-del	1	17	
	173T>Y	1	18	16
	258C>Y	1	19	17
	303C>Y	1	20	18
	340C>Y	1	21	19
	408T>Y	1	22	20
	443A>R	1	23	21
	497C>Y	1	24	22
	517A>R	1	25	23
	572C>Y	1	26	24
	8C/9C, 573delC/non-del	1	27	
	7C/8C	4	28	
	8C/9C	10	29	
C-8	aggregate sequence	75	1	
	16122A>R, 228G>R	1	2	1
	16129G>R	1	3	2
	16135A>R	1	4	3
	16139A>R	1	5	4
	16159C>Y	1	6	5
	16395C>Y	1	7	6
	16399A>R	1	8	7
	16399A>R, 16405A>R, 142T>Y	1	9	8
	16399A>R, 16488C>Y	1	10	9
	16417A>R, 16433A>R	1	11	10
	16440T>Y	1	12	11
	445C>Y	1	13	12
	469C>Y	1	14	13
	570C>Y	1	15	14
	8C/9C	3	16	
Family D				
D-1	aggregate sequence	57	1	
	16036delG/non-del, 180T>Y	1	2	1
	16099C>Y, 15C>Y	1	3	2
	16106G>R	1	4	3
	16119A>R	1	5	4
	16122A>R	2	6	5

	16129G>R	1	7	6
	16130G>R	1	8	7
	16132A>R, 16465C>Y	1	9	8
	16189T>Y	1	10	9
	16190C>Y, 7C/8C	1	11	10
	16192C>Y, 16477G>R, 59T>Y, 7C/8C	1	12	11
	16272A>R, 533A>R	1	13	12
	16335A>R	1	14	13
	16352T>Y, 438C>Y	1	15	14
	16428G (same to CRS)	2	16	15
	16439C>Y	1	17	16
	16515A>R	1	18	17
	109G>R, 526G>R, 533A>A/T	1	19	18
	142T>Y	1	20	19
	146T>Y, 456C>Y	1	21	20
	234A>R, 6C/7C	1	22	21
	242C>Y	1	23	22
	244A>R	1	24	23
	253C>Y, 356C>Y	1	25	24
	262C>Y	1	26	25
	260G>A	1	27	26
	399T>Y, 506C>Y	1	28	27
	490A>R	1	29	28
	517A>R	1	30	29
	540A>G	1	31	30
	540A>R	1	32	31
	561A>A/T	1	33	32
	573delC/non-del	2	34	
	523-524delAC/non-del	1	35	
	6C/7C	1	36	
	7C/8C	1	37	
D-2	aggregate sequence	76	1	
	16051A>R, 16430A>A/T, 227A>R	1	2	1
	16068T>Y, 40T>Y	1	3	2
	16162A>R	1	4	3
	16168C>Y, 16335A>R	1	5	4
	16172T>Y	1	6	5
	16192C>Y, 16435A>R	1	7	6
	16397T>Y, 16547C>Y	1	8	7
	16399A>R	1	9	8
	16404C>Y	1	10	9
	16408C>Y	1	11	10
	47G>R	1	12	11
	91C>Y	1	13	12
	195T>Y	1	14	13
	204T>C	1	15	14
	204T>Y	1	16	15
	419A>R	1	17	16
	439A>R	1	18	17
	490A>R	1	19	18
	515A>A/T	1	20	19
	523-524delAC/non-del	1	21	20

Cord blood

CB-1	aggregate sequence	86	1	
	16134C>Y	1	2	1
	16201C>Y	1	3	2

	194C>Y	1	4	3
	247G>R	1	5	4
	7C/8C	3	6	
CB-2	aggregate sequence (8C/9C)	102	1	
	16022T>Y, 8C/9C	1	2	1
	16038delA/non-del, 8C/9C/10C	1	3	
	16100A>R, 8C/9C	1	4	2
	16102T>Y, 265T>Y, 8C/9C	1	5	3
	16111C>Y, 8C/9C	1	6	4
	16179C>Y, 8C/9C	1	7	5
	16215A>R, 16431C>Y, 8C/9C	1	8	6
	16301C>Y, 8C/9C/10C	1	9	7
	16432A>R, 8C/9C	1	10	8
	16519T>Y, 8C/9C	1	11	9
	85G>A, 200A>R, 8C/9C	1	12	10
	86C>Y, 8C/9C	1	13	11
	131T>Y, 8C/9C	1	14	12
	143G>R, 8C/9C	1	15	13
	152T>T/A, 8C/9C	1	16	14
	166C>Y, 8C/9C/10C	1	17	15
	178A>R, 8C/9C	5	18	16
	178A>R, 8C/9C/10C	2	19	
	193A>R, 8C/9C	1	20	17
	308C>Y, 8C/9C/10C	1	21	18
	416T>Y, 8C/9C	1	22	19
	467C>Y, 8C/9C/10C	1	23	20
	494C>Y, 8C/9C	1	24	21
	498delC/non-del, 8C/9C	1	25	
	559C>Y, 8C/9C	1	26	22
	573delC/non-del, 8C/9C	1	27	
	609C>Y, 8C/9C	1	28	23
	8C/9C, 523-524delAC/non-del	1	29	
	8C/9C/10C	52	30	
CB-3	aggregate sequence	46	1	
	16274G>R	1	2	1
	16278C>Y, 8C/9C	1	3	2
	16343A>R, 16470G>R	1	4	3
	16540C>Y, 8C/9C	1	5	4
	16545T>Y, 8C/9C	1	6	5
	5A>R	1	7	6
	24A>R, 8C/9C	1	8	7
	27C>Y	2	9	8
	44C>Y, 8C/9C	1	10	9
	72T>Y	1	11	10
	116A>A/T, 229G>R	1	12	11
	222C>Y	1	13	12
	516C>Y	1	14	13
	572C>Y	1	15	14
	587C>Y	1	16	15
	8C/9C	29	17	
	8C/9C/10C	4	18	

Note: For each donor, mutation in each single cell was scored relative to the aggregate sequence of all single cells, and mutation was numbered according to the revised Cambridge reference sequence. A site heterogeneous for both thymidine (T) and cytidine (C) was abbreviated as Y, and heterogeneous for adenosine (A) and guanosine (G) was abbreviated as R. A site heterogeneous for a transversion was highlighted by listing all status, e.g. 515A>A/T means site 515 has both A and T. We highlight the status of the length mutations of the C-tract at region 303-309 and the dinucleotide AC repeat at region 515-524 in these cells differ from the aggregate sequence by listing all the status, e.g. 8C means region 303-315 has CCCCCCCCTCCCCCC in the cell, 8C/9C means region 303-315 has a heteroplasmy of CCCCCCCCTCCCCCC and CCCCCCCCCTCCCCCC in the cell, 523-524delAC means this cell contains 4 repeats of AC at region 515-524, while 523-524insAC/non-ins means co-existence of 6 and 5 repeats of AC at region 515-524 in the cell. Back mutations relative to the sequence variation in aggregate sequence are denoted by adding "(same to CRS)" .

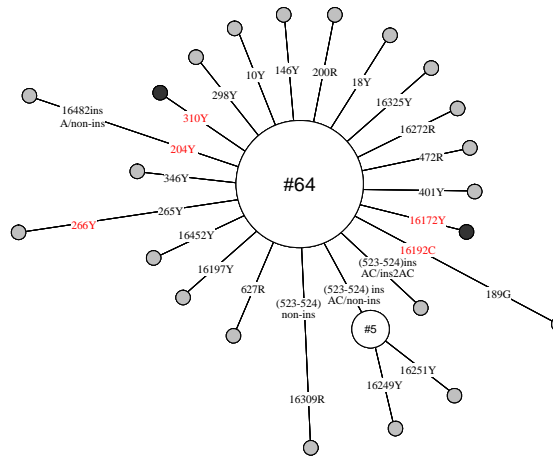**Supplementary Figures**

There are three supplementary figures.

A-1 #45

A-2 #63

A-3 #71

A-4 #73

A-5 #64

A-6 #74
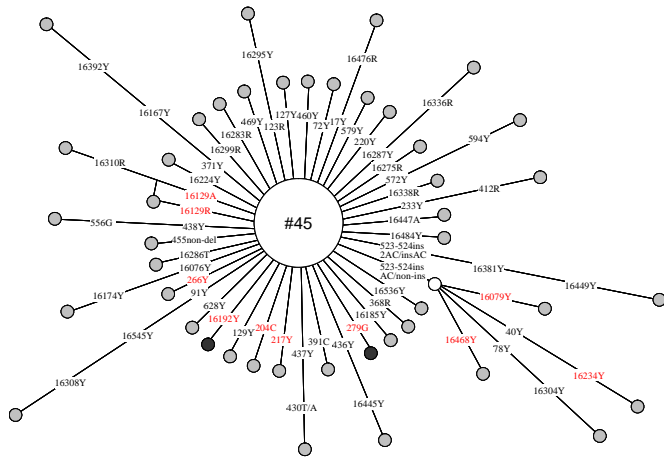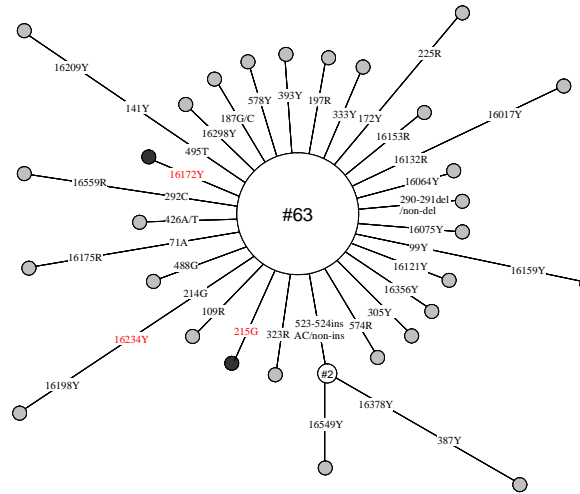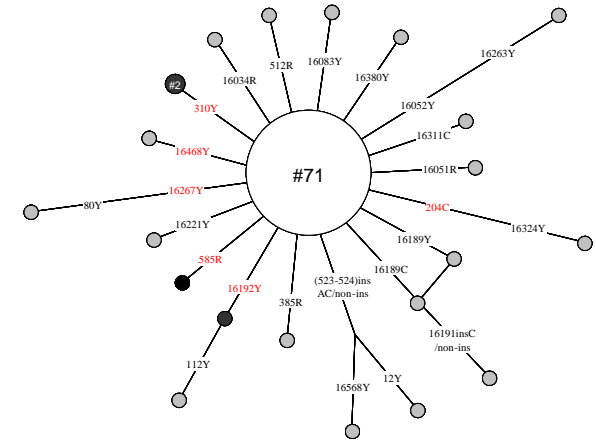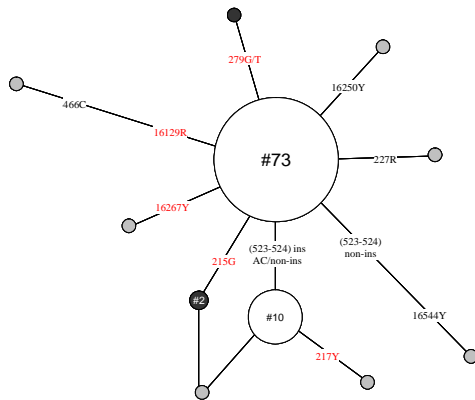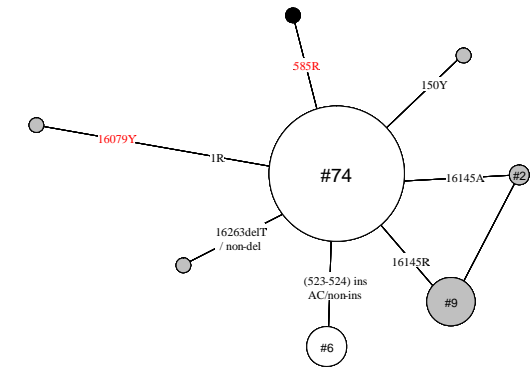
Figure S1 Network profile of mtDNA haplotypes (CD34$^+$ cell clones) observed in single CD34$^+$ cell populations from the six donors in family A. The networks were drawn according to the median-joining method. The length mutation of C-tract in region 303-309 in the mtDNA control region was not considered. The order of mutations on the branch is arbitrary. Each circle represents an mtDNA haplotype or a CD34$^+$ cell clone as recognized by mtDNA mutation(s) in a population of CD34$^+$ cells. The area of the circle is proportional to the frequency of the haplotype and is further specified by the number of cells sharing that haplotype. For instance, "#45" means this haplotype was found in 45 single cells in that sample. In each network, the haplotype in the center is the major type that has the consensus or aggregate sequence. mtDNA variants shared by cells from different donors are marked in red. These haplotypes / CD34$^+$ cell clones that were observed in different donors are marked by filled circles.
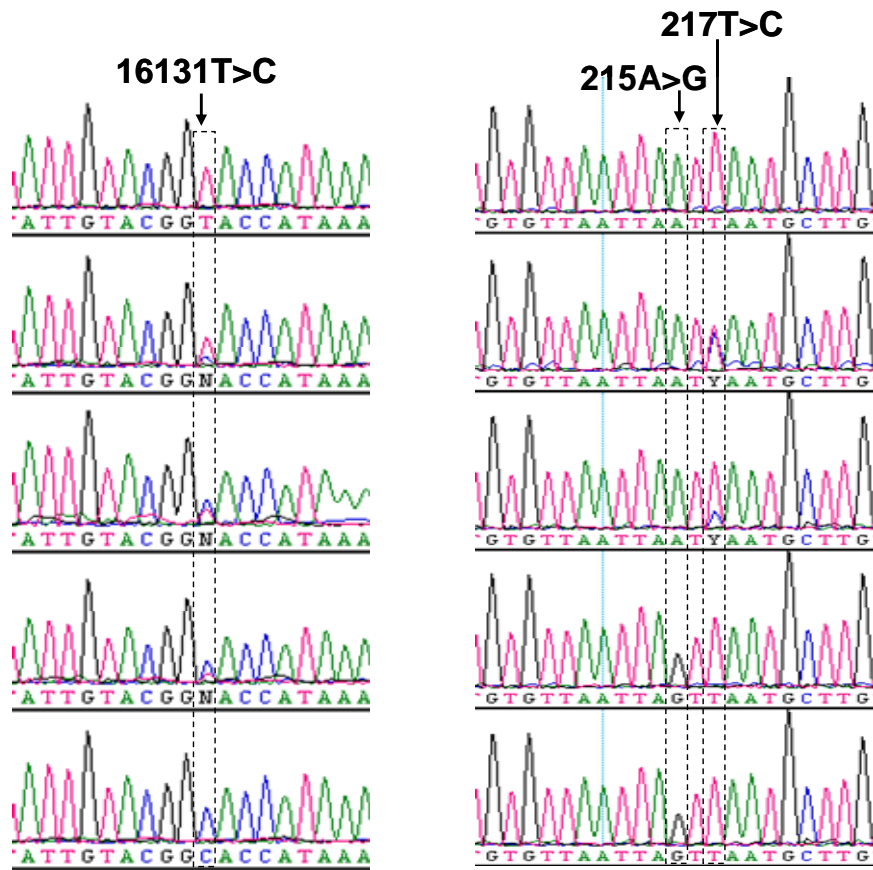
Figure S2 mtDNA mutations in single CD34$^+$ cells from different healthy donors at positions 16131 (Family B) and 215 and 217 (Family A). Each sequencing electropherogram refers to one single cell.
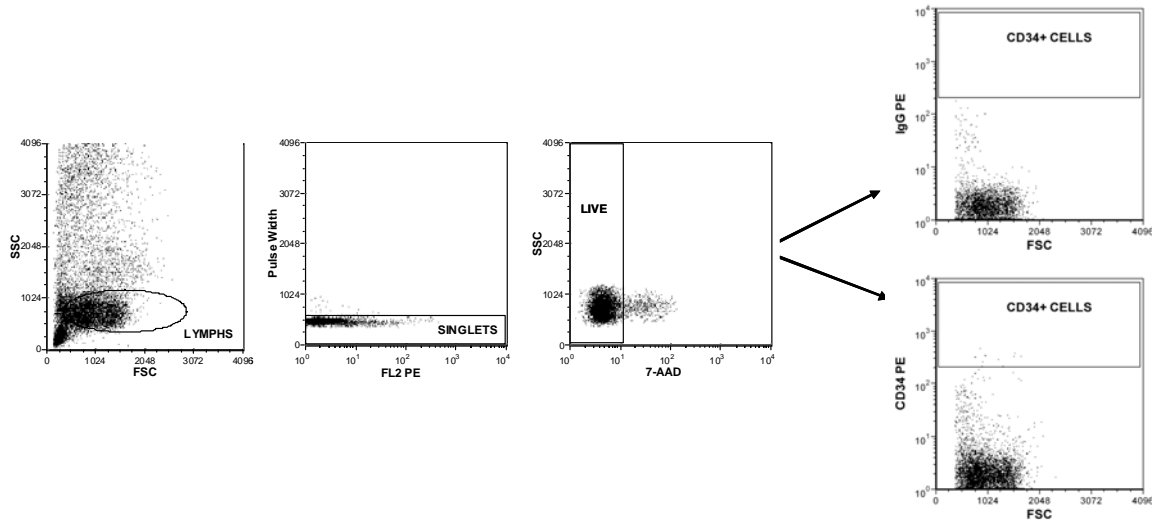
Figure S3 Representative staining and gating for single CD34⁺ cell sorting.
Mononuclear cells from whole blood were isolated by Ficoll density gradient
centrifugation and were stained with anti-CD34 phycoerythrin (PE)-conjugated
monoclonal antibody (BD Bioscience, San Jose, CA). Cell sorting was performed
on the MoFlo Legacy high-performance cell sorter (Dako-Cytomation, Ft Collins,
CO) and single cell deposition was accomplished using the CyClone Automated
Cloner (Dako-Cytomation, Ft Collins, CO) in a 0.5 single drop mode.