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ARTICLE

The level of the mitochondrial mutation A3243G decreases upon ageing in epithelial cells from individuals with diabetes and deafness

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We have in a longitudinal study determined the proportion of the mitochondrial A3243G mutation in DNA obtained from cervical cell samples collected from three individuals affected with mitochondrial diabetes and hearing loss during a period of up to 18 years. Using the minisequencing method we were able to sensitively determine the proportion between mutant and normal mitochondrial DNA. Our results demonstrate a constant decrease in the levels of the pathogenic mutation in mitotic tissues of affected individuals with time.
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Introduction

Mutations in mitochondrial DNA (mtDNA) have been implicated in the pathogenesis of several clinical syndromes.[1] Both large rearrangements and point mutations have been shown to cause disease. Point mutations in the mitochondrial tRNA genes are particularly frequent, since over 20 pathogenic mtDNA mutations have been described in various tRNA genes.[2] In the study presented here we have analysed patients with a syndrome of maternally inherited diabetes and deafness, MIDD.[3,4] The patients exhibit an atypical form of non-insulin dependent diabetes mellitus and are nonobese. The sensorineural hearing loss is bilateral, symmetric and originates in the cochlea.[5] This disorder is caused by an A-to-G transition at position 3243 of the mtDNA. The mutation is located in the gene encoding $tRNA^{Leu}$, and results in impaired protein synthesis and electron transport chain dysfunction.[6,7] Approximately 1% of the patients with non-insulin dependent diabetes carry this mutation, as well as 80% of the patients with the MELAS syndrome (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes).[8–10]

Disease-causing mutations in the mtDNA are typically heteroplasmic with normal and mutant sequences coexisting at variable proportions in the same individual, tissue and cell.[11] The A3243G mutation is a well-known example of mitochondrial heteroplasmy[3,9] and it shows a widespread tissue distribution with levels of heteroplasmy that vary between tissues.[12–14] We and others have previously reported that the percentage level of heteroplasmy of the A3243G mutation appears to be correlated with the severity and/or the age at onset of disease.[4,14,15]

To address the question of how heteroplasmy of the A3243G mutation changes *in vivo*, we determined the percentage level of the heteroplasmic A3243G mitochondrial point mutation in patients affected by MIDD over time periods of up to two decades.

Material and methods

Subjects

Three female patients with MIDD carrying the A3243G mutation were investigated. The patients were recruited from an earlier study, where they had been shown to carry the A3243G mutation.[4] Clinical data of the three patients was collected in 1996. Patient 1 had a serum C-peptide level

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of 0.58 nM (baseline, fasting), body mass index (BMI) of 22 kg/m^2, sensorineural hearing loss (SNHL) from the age of 20, and had been treated with insulin from the age of 52. Patient 2 had a C-peptide level of 0.78 nM, BMI of 22 kg/m^2, SNHL from the age of 65, and had been treated with oral hypoglycaemic agents from the age of 70. Patient 3 had a C-peptide level of 0.51 nM, BMI of 22 kg/m^2, SNHL and impaired glucose tolerance from the age of 39. Informed consent was obtained from the patients for the current study. Multiple cervical smear samples from the patients were localised and collected from pathology departments.

Extraction of DNA

DNA was extracted from the Papanicolaou stained cell smears as described by Josefsson *et al.*[16] The slides were soaked in xylene for 5–6 days to remove the cover slips. To destain, the slides were further soaked in ethanol for 30 min. After drying at room temperature, 150 μl of buffer consisting of equal parts of lysis buffer (1% Sarcosyl, 8 M Urea, 20 mM EDTA, 0.4 M NaOH, 0.2 M Tris, pH 8.0) and suspension buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) together with 25 μl proteinase K (20 mg/ml) per ml, were added to each slide. The slides were then incubated in a humidified chamber at 37°C for 20 min. The cells were dislodged from the glass surface with a pipette tip, and transferred to an Eppendorf tube. The dislodging of the cells was repeated, and the suspensions were combined. Ten μl of proteinase K (20 mg/ml) was added, followed by incubation at 60°C for 1 h. Finally, DNA was recovered by ethanol precipitation.

PCR amplification

The region of mtDNA encompassing the A3243G site was amplified with the forward primer 5′-CCA CCC AAG AAC AGG GTT TGT TAA-3′ at 1 μM and the reverse biotinylated primer 5′-TTA GGA ATG CCA TTG CGA TTA GAA-3′ at 0.1 μM, in 100 μl of buffer containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 12.5 μg/ml bovine serum albumin (Applied Biosystems, USA), using ≈50 ng of DNA, 0.2 mM dNTPs, and 2.5 units of *AmpliTaq Gold* DNA polymerase (Applied Biosystems, USA). After initial activation of the enzyme at 95°C for 10 min, the temperature was varied during 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min.

Minisequencing

Four 10 μl aliquots of each PCR product and 40 μl of phosphate buffered saline with 0.1% Tween 20, were added to streptavidin-coated microtiter plate wells (Combiplate 8, Labsystems, Finland). The plates were incubated at 37°C for 1.5 h in a shaker (Labsystems Thermomix 1415, Finland). After washing six times with 40 mM Tris-HCl, pH 8.8, 1 mM EDTA, 50 mM NaCl and 0.1% Tween 20 in a plate washer, the non-biotinylated strand of the PCR-product was removed by denaturation with 60 μl of 0.1 M NaOH for 3 min. After washing as above, 50 μl of a minisequencing reaction mix containing 50 mM Tris-HCl, pH 8.8, 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton and 0.01% gelatine, 0.2 U of *AmpliTaq* DNA polymerase (Applied Biosystems, USA), 0.1 μCi of [^{3}H]-ATP (TRK 633, 57–76 Ci/mmol) and [^{3}H]-GTP (TRK 627, 24–34 Ci/mmol) (Amersham Pharmacia Biotech, England) and 10 pmol of the detection primer 5′-AGG GTT TGT TAA GAT GGC AG-3′ were added to the wells. The plates were incubated at 50°C for 10 min. Unincorporated label was removed by washing as above, and the detection primers were released with 100 μl of 0.1 M NaOH, and measured in a liquid scintillation counter (1414, Wallac, Finland).

Quantification

PCR products representing normal and mutant sequences spanning the A3243G site were cloned into a plasmid vector using the TA cloning kit (Invitrogen, USA). Sequence verified plasmids containing the mutant and normal sequences were mixed in different proportions before amplification. The A3243G site was analysed in the PCR products by minisequencing as described above. A linear regression curve with the ratio between the mutant and normal nucleotides incorporated in the minisequencing reactions on the y-axis (G_{cpm}/A_{cpm}) and the initial ratio between the mutant and normal sequences on the x-axis was constructed (Figure 1A). Quadruplicate samples of each PCR product were analysed. The regression function of the standard curve was used to calculate the percentage level of mutant mtDNA in the clinical samples.

Results

To analyse the heteroplasmy level of the A3243G mutation over long time periods we made use of DNA from cervical smear samples that in Sweden are collected approximately every third year from women over the age of 23. This program was initiated in the 1960s for the purpose of screening for precursors of invasive cervical cancer. Some of these cell samples have been stored at pathology departments throughout Sweden. We were able to retrieve several cervical smears collected during 18, 8 and 4 years, respectively, from three study subjects affected with MIDD carrying the A3243G mutation.[4] We analysed the heteroplasmy levels of the A3243G mutation in DNA samples from 10 cervical smears and three blood samples using the minisequencing method.[14,17] Accurate heteroplasmy level determinations were obtained by comparing the G_{cpm}/A_{cpm} values from the cervical cell samples to a quantification standard curve prepared in parallel by mixing two plasmids with the mutant and normal sequences, respectively, in known ratios. As can be seen in Figure 1A, the standard curve is linear between 0.5 and 99.5% of mutant mtDNA.

We found that the proportion of mtDNA with the A3243G mutation decreased over time in the samples from all three patients (Figure 1B). Patient 1 had 32% of mutant mtDNA in

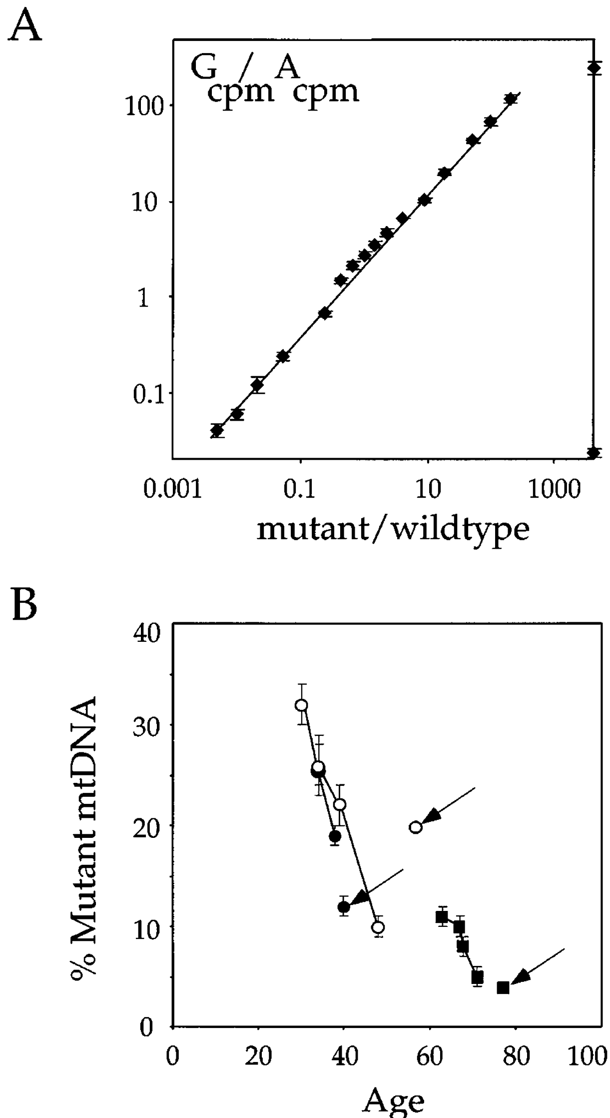

Figure 1 (**A**) Minisequencing standard curve for determining the level of heteroplasmy of the A3243G mutation of mtDNA. The ratios between the cpm-values from minisequencing reactions corresponding to the mutant and normal nucleotides are plotted as a function of the ratio between the two sequences in the original mixture. The mean values of quadruplicate assays from the same PCR product with standard deviations are plotted. The signal ratios obtained in control samples containing only one of the sequence variants are given on the vertical axis on the right. The regression function of the standard curve was used to calculate the level of heteroplasmy in the clinical samples. (**B**) Changes over time in the level of heteroplasmy of the A3243G mutation in cervical cell samples from three patients with diabetes and hearing loss. The percentage level of mutant mtDNA is plotted as a function of the ages of Patient 1 (open circles), Patient 2 (squares), and Patient 3 (filled circles), at the time of sampling. Arrows indicate the blood leukocyte sample taken from the patients in 1996. Each data point represents the mean value with standard deviation of four assays, in which quadruplicate samples of each PCR product were analysed.

her cervical DNA sample taken at the age of 30. Eighteen years later the level of the mutant mtDNA had decreased to 10%. Similarly in Patient 2, the mutant mtDNA decreased from 11 to 5% in 8 years, and in Patient 3, from 26 to 19% in 4 years. We also determined the level of mutant mtDNA in blood leukocytes taken from the patients in 1996, which was estimated to 20% in Patient 1, 4% in Patient 2, and 12% in Patient 3 (Figure 1B).

Discussion

It has been stated that in patients with the MELAS mutation, the level of heteroplasmy is higher in post mitotic tissue, such as muscle, than in blood.[18,19] In contrast, when studying the A3243G mutation in a 24 week old foetus, there was almost no differences in the heteroplasmy level between different tissues.[20] These observations indicate that the level of heteroplasmy increases in post mitotic tissue and/or decreases in mitotic tissue.[21] Heteroplasmic *de novo* deletions of the mtDNA are known to accumulate during life.[22,23] It has, however been less clear if the level of pathogenic mitochondrial point mutations increase or decrease with time. When performing a serial analysis of one patient with skeletal myopathy, the somatic heteroplasmic A12320G mutation in the $tRNA^{Leu}$ gene increased in skeletal muscle over a 12-year period from 69 to 90%.[24] According to our systematic longitudinal study presented here, the level of heteroplasmy of the disease-causing A3243G mutation decreased *in vivo* over time in the epithelial cell samples from all patients with MIDD (Figure 1B). Our results are supported by an observation of a decrease of mtDNA with the A3243G mutation in leukocytes in two DNA samplings within a time period of 1.5–6 years[25] and when comparing blood samples taken at birth (Guthrie cards) and at the time of diagnosis, in five patients with the MELAS syndrome.[26] A similar decrease in heteroplasmy level was observed when studying the 3460 LHON (Leber hereditary optic neuropathy) mtDNA mutation over a time period of 5–6 years.[27]

We have previously analysed cervical cell samples from four healthy women with heteroplasmic, non-pathogenic nucleotide variations at position 309 or 16189 of the control region of mtDNA. Using a similar approach as in the current study, we found that the proportion of the neutral sequence variants in epithelial cells remained stable for more than 20 years.[28]

The observed decrease in mutational load of the pathogenic A3243G mutation in mitotic tissue with time is probably the consequence of negative selection acting against high levels of mutation load either at the level of cells or mitochondria due to impairment of the oxidative phosphorylation. Selection can occur only if there is heterogeneity in fitness among cells or mitochondria. The heterogeneity in heteroplasmy level could be present in the stem cells where there might be slight or dramatic differences

in heteroplasmy level between individual cells, or at the mitochondrial level, if there is heterogeneity in heteroplasmy level among mitochondria in heteroplasmic stem cells. However, our data cannot elucidate on which level the selection acts.

In post mitotic tissue, at least in muscles, the cells seems to compensate for the insufficient oxidative phosphorylation by an increased mitochondrial proliferation, manifested as red ragged fibres.[29] Increased mtDNA replication has been modelled to increase the level of heteroplasmy.[30] In contrast to the observations made here for the pathogenic heteroplasmic A3243G mutation, there seems to be a lack of selection for non-pathogenic heteroplasmic variants.[27,28]

This is the first time a decrease in the level of heteroplasmy of a pathogenic mitochondrial mutation has been shown in mitotic tissue other than blood. We speculate that there is a constant rate of decline in the level of heteroplasmy with time, which is proportional to the heteroplasmy level such that a greater loss of mutant mtDNA in absolute numbers will occur at higher levels of heteroplasmy. A relative annual decline of 5.8% (coefficient of variance, CV=0.29) of the initial heteroplasmy level fits our data better than an absolute annual decline of 1.2% (CV=0.40). This hypothesis is in line with the observation in patient number 2, the oldest patient, in whom the level of heteroplasmy diminishes at a similar relative rate as in the other patients but with a markedly lower absolute value for each year. The rate of decrease may also be age dependent, perhaps due to lower rates of cell division with increasing age.

When comparing the results from two previous studies on peripheral blood by Rahman *et al.*,[26] and Howell *et al.*,[27] we find that a relative rate of decline in the level of heteroplasmy (average of 2.5% and 5.3% with CV:s of 0.34 and 0.48 respectively) better fit the data than an absolute rate of decline (average of 1.3% and 1.1% with CV:s of 0.37 and 0.60). This is not the case for a third study by, 't Hart *et al.*[25] where the relative rate of decline is 3.9% (CV, 1.44) in contrast to an absolute rate of decline of 0.69% (CV, 0.88).

Our results demonstrate that a decrease of the level of heteroplasmy of the A3243G mutation over time may explain the observations of molecular genetic anticipation seen in some pedigrees with mitochondrial disorders.[4,31,32] The percentage level of mutant mtDNA may be higher in the recent generation compared to the preceding one simply because the samplings were made on individuals of different ages in the pedigree. Our result is also of importance for diagnosis of MIDD, since analysis of leukocytes may give false-negative results. Predictive analyses should therefore be conducted early in life, or on tissue samples where the mutational load is high.[14,18]

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