New Insights into the Metabolic Consequences of Large-Scale mtDNA Deletions: 
A Quantitative Analysis of Biochemical, Morphological, and 
Genetic Findings in Human Skeletal Muscle

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Abstract. In order to study putative genotype-phenotype correlations in mitochondrial disorders due to large-scale mtDNA deletions, we performed a quantitative analysis of biochemical, morphological, and genetic findings in 20 patients. The size of the mtDNA deletions varied from 2 to 7.5 kb with a degree of heteroplasmy ranging from 16% to 78%. Applying improved methods for measuring respiratory chain enzyme activities, we found highly significant inverse correlations between the percentage of cytochrome c oxidase (COX)-negative fibers and citrate synthase (CS) normalized COX ratios. Significant correlations were also established between CS normalized complex I and complex IV ratios as well as between the degree of heteroplasmy of mtDNA deletions and the percentage of ragged red fibers, COX-negative fibers, and CS normalized complex I and complex IV ratios. Our results indicate that the degree of heteroplasmy of mtDNA deletions is mirrored on the histological as well as the biochemical level. Furthermore, our findings suggest that single large-scale deletions equally influence the activities of all mitochondrially encoded respiratory chain enzymes. Even low degrees of heteroplasmy of mtDNA deletions were found to result in biochemical abnormalities indicating the absence of any well-defined mtDNA deletion threshold in skeletal muscle.

Key Words: Cytochrome c oxidase; COX-negative fibers; Genotype-phenotype relations; Mitochondrial myopathy; mtDNA deletion; NADH:CoQ oxidoreductase; Ragged red fibers.

INTRODUCTION

Since the discovery of human diseases caused by mutations of the mitochondrial DNA (mtDNA) there has been considerable interest in unravelling the metabolic consequences of mtDNA defects (1). However, clear-cut genotype-phenotype correlations in chronic progressive ophthalmoplegia (PEO), Kearns-Sayre syndrome (KSS), and mitochondrial myopathies (MM), all of which are associated with large-scale mtDNA deletions, remained elusive (2). Furthermore, muscle biopsy studies of PEO or KSS patients provided incongruent results regarding the relationship between muscle morphology, mitochondrial enzyme activities in skeletal muscle homogenates, and the degree of heteroplasmy of the mtDNA mutation (3–8). These discrepancies may be attributed to the following reasons: (1) the unpredictable mosaic distribution of mtDNA mutations in the affected tissue; (2) unknown individual effects of various mutations; and (3) technical difficulties and inaccuracies in the quantification of respiratory chain enzyme activities and morphological changes in skeletal muscle specimens.

In cell cultures containing well-defined amounts of heteroplasmic mutant mtDNA, threshold values have been defined beyond which each mutation had an effect on the activity of respiratory chain enzymes. For various tRNA point mutations this threshold has been determined to be above 85% of mutated mtDNA (9). In contrast, the 4977 bps “common deletion” had a 50%–55% threshold (10, 11). Surprisingly, in muscle tissue of some CPEO patients with generalized muscle weakness, much lower degrees of mtDNA heteroplasmy have been observed (3–8).

The aim of the present study was to readdress the issue of putative correlations between genetic findings and morphological and biochemical abnormalities in patients with mitochondrial disorders due to large-scale mtDNA deletions. To this end, we employed improved enzyme assays and correlated the results with quantitative morphological and genetic findings in 20 patients with PEO, KSS, and MM.

MATERIALS AND METHODS

Patients

Between 1987 and 1999, 82 patients with PEO, KSS, and MM were seen at the Department of Neurology at the University Hospital in Bonn, Germany. Clinical diagnosis of KSS was
based on the following criteria: 1) progressive external ophthalmoplegia; 2) age of onset <20 years; 3) pigmentary retinopathy together with one or more of the following: i) cerebellar syndrome; ii) cardiac conduction defect; iii) cerebrospinal fluid protein concentration >1 g/l. Diagnostic criteria of PEO included progressive external ophthalmoplegia and histological evidence of a mitochondrial myopathy. Diagnostic criteria of MM included clinical evidence of myopathy, histological evidence of a mitochondrial myopathy, and absence of progressive external ophthalmoplegia.

Twenty patients met the following criteria for inclusion in the present study: 1) clinical and histological evidence for KSS, PEO, or MM; 2) presence of a large-scale mtDNA deletion in skeletal muscle tissue; and 3) sufficient amounts of residual muscle biopsy specimen for a combined quantitative histological, biochemical, and genetic analysis. Normal control specimens were obtained from 28 patients who underwent a muscle biopsy for diagnosis of neuromuscular symptoms, but were ultimately deemed to be normal by means of combined clinical, electrophysiological, and histological criteria.

**Genetic Analysis**

Genomic DNA was isolated as described previously (12). DNA was purified by ethanol precipitation and stored in aliquots at −80°C. Aliquots of 10 µg genomic DNA were subjected to the restriction endonucleases Bam HI or Pvu II according to the conditions of the manufacturer (AGS, Heidelberg, Germany). DNA samples were separated on a 0.5% agarose gel (15 × 15 cm, 40 V, 12 h) and alkali-transferred onto a Hybond N+ nylon membrane (Amersham Buchler, Braunschweig, Germany). The immobilized nucleic acids were hybridized at 65°C for 12 h to a radioactively labeled and purified mtDNA. DNA was finally visualized by autoradiography. Autoradiographic images were scanned utilizing a digital light-box scanner and imaging software (Agfa, Leverkusen, Germany). Digital images were quantified using the UTHSCSA Image Tool for Windows (version 1.28). Ratios of deleted versus intact mtDNA were determined by comparing the intensities of deleted and intact mtDNA bands minus background.

**Biochemical Analysis**

NADH:CoQ1 Oxidoreductase (Complex I): The activity of rotenone-sensitive NADH:CoQ1 oxidoreductase was measured at 30°C using a dual-wavelength spectrophotometer (Aminco DW 2000, SLM Instruments, Rochester, NY) at 340−380 nm (ε_{680nm} = 5.5 mM−1 cm−1), as recommended by Estornell et al. (13). The reaction medium contained 50 mM KCl, 1 mM EDTA, 10 mM TRIS-HCl (pH 7.4), 1 mM KCN, 100 µM CoQ1, and 150 µM NADH. Coenzyme Q1 was a kind gift from Eisai Co. (Tokyo, Japan). The assay was initiated by addition of the sample and the velocity of NADH oxidation was monitored. To determine the rotenone-insensitive NADH oxidation rate after 2 min, 20 µM rotenone were added to the assay mixture. The given activities are differences between the total NADH oxidation rate and the rotenone-insensitive NADH oxidation rate.

Cytochrome C Oxidase (COX, Complex IV): The COX activities were measured at 30°C in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.02% laurylmaltoside (Sigma, Germany) monitoring the oxidation of ferrocytochrome c in its β-band at the wavelength pair 510−535 nm using a dual wavelength spectrophotometer (Aminco DW 2000, SLM Instruments, Rochester, NY). To obtain reduced cytochrome c, oxidized bovine heart cytochrome c (purity 99%, Sigma, Germany) was reduced with ascorbate, desalted on a Sephadex-G25 column, and stored in liquid nitrogen until use. Routinely, the content of ferrocytochrome c in our preparations was lower than 3% (determined by the addition of active fibres of soybean dithionite). The reduced oxidized extinction coefficient of cytochrome c at 510−535 nm was determined by titration experiments. After addition of a defined amount of ferrocytochrome c, it was reoxidized by potassium ferricyanide (1 mM) and the resulting extinction difference was plotted against the cytochrome c concentration. This plot was linear up to 200 µM ferrocytochrome c and from its slope the extinction coefficient at 510−535 nm ε Coul = 5.9 mM−1 cm−1 was determined.

Cytochrome Synthase (CS) and Succinate: Cytochrome C Reductase (Complex II & III): The activities of CS and succinate: cytochrome c reductase were determined at 30°C by standard methods (14).

Muscle Histology

Consecutive cryostat sections of muscle biopsy specimens (6 µm) were stained for hematoxylin and eosin, oil red O, periodic acid-Schiff, Gomori’s trichrome, NADH, myofibrillar adenosine triphosphatase (ATPase) at pH 4.2, 4.6, and 9.4, COX, and succinate dehydrogenase (SDH) as described (15). For quantification of the percentage of SDH-hyperreactive fibers, or COX-negative fibers, 6 randomly chosen, nonoverlapping areas from adjacent consecutive sections containing about 100 fibers were digitally acquired using an Olympus IX-70 inverse microscope connected to a CF 8/1 CCD camera (Kappa, Giechen, Germany). Each image was calibrated using 95% and 5% thresholds for white (equal to 100%) and black (equal to 0%), respectively. SDH-positive fibers reaching the 95% white threshold in the COX-reaction were assigned as COX-negative. Fibers reaching the 5% black threshold were counted as SDH-hyperreactive.

**Statistical Analysis**

Correlations between metric variables were obtained by calculation of the nonparametric Spearman correlation coefficients (r) and the corresponding two-tailed significance levels. When the observed correlation as measured by the Spearman method was significant, parametric Pearson’s coefficients (r) were chosen for graphical and statistical presentation of results. Comparison of means between groups was performed by the t-test. For all statistical analyses, significance level was set at p ≤ 0.05.

**RESULTS**

The clinical and genetic data of 20 patients with mitochondrial disorders (age range 28 to 60 years; median 42.1 years) are summarized in the Table. Genetic analysis confirmed the presence of large-scale deletions (2−7.5 kb) with a degree of heteroplasmy ranging from 16% to 78% in all patients.
Biochemical analysis showed decreased levels of complex I in 20 patients compared with 19 controls (0.37 ± 0.18 U/g vs 1.39 ± 0.47 U/g, respectively, p < 0.0001). Furthermore, significantly decreased levels of enzyme activities were found for complex II & III (0.55 ± 0.19 U/g; n = 16 vs 1.73 ± 0.70 U/g, n = 28 controls, p < 0.0001) as well as complex IV (2.91 ± 1.26 U/g, n = 20 vs 7.05 ± 3.47 U/g, n = 28 controls, p < 0.0001). In contrast, we found a significant increase of CS activities in 20 patients compared with 22 controls (16 ± 6.5 U/g vs 11 ± 2.2 U/g, p < 0.0001) reflecting the degree of adaptive mitochondrial proliferation in patients with large-scale mtDNA deletions.

To address the mosaic distribution of mitochondrial defects in skeletal muscle, we performed a histological analysis of about 600 fibers in each muscle biopsy. The typical histological pattern (patient 20 in the Table) is illustrated in consecutive cryostat sections stained for Gomori’s trichrome (Fig. 1a), succinate dehydrogenase (Fig. 1b), and cytochrome c oxidase (Fig. 1c). In each biopsy typical ragged red fibers were present (*), which were for succinate dehydrogenase hyperreactive and negative for cytochrome c oxidase (o). Additionally, fibers were present that showed an increased subsarcolemmal staining in Gomori’s trichrome as well as an increased subsarcolemmal SDH reactivity (Fig. 1a, b, +). In our quantitative analysis, both types of fibers (* and +) were evaluated as ragged red fibers or SDH-hyperreactive fibers, respectively. However, it is important to note that not all of these fibers were COX-negative (Fig. 1c). On the other hand, some COX-negative fibers (o) showed no abnormalities in Gomori’s trichrome and in succinate dehydrogenase staining. In all biopsies, the amount of ragged red fibers ranged from 0.2% to 31%, the amount of SDH-hyperreactive fibers from 0.7% to 32%, and the amount of COX-negative fibers from 1.1% to 38.2%.

**Correlations between Histological, Biochemical, and Genetic Findings**

As a first step, we correlated the percentage of ragged red fibers and SDH-hyperreactive fibers, both of which are classical morphological markers of mitochondrial proliferation. As shown in Figure 2a, a highly significant positive correlation between both parameters was demonstrated ($r_p = 0.926$, p < 0.0001).

To address the question of whether the degree of mitochondrial proliferation is also mirrored on the biochemical level (as indicated by our histological parameters), we correlated the percentage of ragged red fibers with the level of CS activity, which is a mitochondrial marker enzyme. As shown in Figure 2b, a highly significant positive correlation between both parameters was found ($r_p = 0.736$, p < 0.0001).

COX-negative fibers are morphological markers indicating respiratory chain enzyme (complex IV) deficiencies. On correlating the percentage of COX-negative fibers with the level of COX activities, no statistically significant correlation between both parameters was found ($r_p = -0.384$, p = 0.094). However, since respiratory chain

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**Table:**

Clinical and Genetic Data of 20 Patients with Large-Scale mtDNA Deletions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Degree of heteroplasmy</th>
<th>Deletion size</th>
<th>Muscle biopsy</th>
<th>Limb muscle weakness</th>
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<td>1</td>
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<td>+</td>
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<tr>
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</tr>
<tr>
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<tr>
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<td>M. biceps</td>
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</tr>
<tr>
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<tr>
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complex deficiencies can be masked by the individual degree of adaptive mitochondrial proliferation, we normalized complex IV activities by the corresponding CS activities. In doing so, we found a highly significant inverse correlation (Fig. 3a) between the percentage of COX-negative fibers and COX/CS ratios ($r_p = -0.627, p < 0.01$).

To examine whether mtDNA mutations equally effect the activities of mitochondrially encoded complexes, we correlated CS normalized complex activities with each other. Here, positive correlations between complex NADH:CoQ$_1$ oxidoreductase/CS and COX/CS ratios (Fig. 3b; $r_p = 0.483, p < 0.05$) as well as complex II & III/CS ratios ($p < 0.05$) were obtained. Furthermore, these CS-normalized activities of the respiratory chain complexes delineated patients from controls. The NADH:CoQ$_1$ oxidoreductase/CS ratios of 19 from the 20 patients (range 0.008 to 0.0909) were lower than the lowest ratio of controls (range 0.063 to 0.186). With the complex II & III/CS ratio all patients (range 0.011 to 0.053) could be delineated from controls (range 0.063 to 0.21). And, the COX/CS ratios of 18 from the 20 patients (range 0.05 to 0.55) were lower than the lowest ratio obtained in control biopsies (range 0.31 to 1.12).

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**Fig. 2.** a: Scatter plot between the percentage of SDH-hyperreactive and ragged red fibers ($r_p = 0.926, p < 0.0001$). b: Scatter plot between the percentage of ragged red fibers and levels of citrate synthase activity. ($r_p = 0.736, p < 0.0001$).

**Fig. 3.** a: Scatter plot between the percentage of COX-negative fibers and cytochrome c oxidase/citrate synthase ratios ($r_p = -0.627, p < 0.01$). b: Scatter plot between cytochrome c oxidase/citrate synthase and NADH:CoQ$_1$ oxidoreductase/citrate synthase ratios ($r_p = 0.483, p < 0.05$). Data point with error bars is the average of 19 normal control skeletal muscle samples.
heteroplasmy ranging from 16% to 78% in all patients. Correlation of genetic, morphological, and biochemical measures revealed clear-cut genotype phenotype relations in our series of patients.

Previous studies provided incongruent results with respect to genetic and histological relationships in mitochondrial disorders due to large-scale deletions. Our observation of a highly significant correlation between the percentage of COX-negative fibers and ragged red fibers with the degree of mtDNA heteroplasmy are in accordance with an earlier report of Goto et al in 22 patients (4). The studies of Yamamoto et al and Laforet et al suggested a correlation between mtDNA heteroplasmy and the numbers of COX-negative and SDH-hyperreactive fibers, but these findings failed to be statistically significant (5, 7). In contrast, 2 other studies failed to demonstrate a relationship between histological and genetic parameters (6, 8). Though the reasons for these discrepancies are not entirely clear, we believe that the number of fibers analysed in each muscle biopsy is of critical importance. In the study by Goto et al (4), as well as in our own, more than 500 fibers were analysed; in contrast, a much smaller number was evaluated in all other studies (5–8).

Applying improved biochemical methods we found statistically significant correlations between biochemical and histological as well as genetic findings. The percentage of ragged red fibers and SDH-hyperreactive fibers was positively correlated with the level of CS activity indicating that both histological parameters reflect the degree of adaptive mitochondrial proliferation in diseased muscle. In addition, we found a highly significant and inverse relationship between the percentage of COX-negative fibers and COX/CS ratios. Furthermore, a highly significant relationship between the degree of mtDNA heteroplasmy and CS normalized complex I and complex IV ratios was demonstrated. Thus, the degree of mtDNA heteroplasmy is mirrored on the histological as well as on the biochemical level.

These results clearly diverge from previous reports that failed to establish any correlation between biochemical and histological or genetic parameters (5–8). In our opinion these discrepancies are due to several methodological reasons: 1) Earlier reports used the standard NADH:cytochrome c reductase (complex I + III) assay for the determination of putative complex I deficiencies, which is known to have a rather large rotenone-insensitive part in tissue samples containing microsomal and plasma membrane NADH oxidases. As demonstrated by Estornell et al (13), this experimental error can be overcome by measuring rotenone-sensitive NADH:CoQ oxidoreductase, which has a higher complex I specificity. 2) In previous reports, COX activity was determined by modifications of the Wharton and Tzagoloff assay procedure (16). This test is based on the measurement of

**DISCUSSION**

Our genetic analysis confirmed the presence of single large-scale deletions in all 20 patients with a degree of

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**Fig. 4.** a: Scatter plot between the degree of mtDNA heteroplasmy and the percentage of ragged red fibers ($r_p = 0.784$, $p < 0.0001$). b: Scatter plot between the degree of heteroplasmy of mtDNA deletions and NADH:CoQ1 reductase/CS ratios. ($r_p = -0.648$, $p < 0.005$).
the decrease in ferrocytochrome c absorption in its \( \alpha \)-band at 550 nm. A crucial aspect of this assay is the high extinction coefficient of ferrocytochrome c, which allows the maximal use of 80 \( \mu \)M substrate. In contrast, we measured the cytochrome c oxidase activity in the \( \beta \)-band of ferrocytochrome c where 200 \( \mu \)M substrate can be used. At the latter concentration, the analysis of 28 normal human skeletal muscle homogenates revealed more than 2-fold higher cytochrome c oxidase activities compared with control values given by other groups that used the classical assay (3–5, 17). In other words, measurements well above the \( K_m \)-value of cytochrome c oxidase for ferrocytochrome c resulting in higher absolute activities are more sensitive in depicting COX abnormalities in patients with respiratory chain enzyme deficiencies.

Another key finding of our study was the observation that CS normalized complex I and complex II and III ratios correlated with those of complex IV ratios. This indicates similar enzyme deficiencies in complexes with different amounts of mitochondrially encoded subunits (complex I−7 subunits, complex III−1 subunit, complex IV−3 subunits). These results imply that large-scale deletions, all of which affect at least 1 tRNA gene, have similar effects on all mitochondrially-encoded proteins irrespective of the individual mutation size or localization. This can be explained by the finding that the steady-state levels of tRNAs in mitochondria are not in large excess, so a 50% reduction of 1 charged tRNA is sufficient to cause a translation defect (18).

Furthermore, we were able to demonstrate that even low degrees of heteroplasmy of mtDNA deletions result in biochemical abnormalities. This finding strongly argues against a well-defined mutation threshold in skeletal muscle. In contrast, clear-cut interdependencies between the degree of mtDNA heteroplasmy and mitochondrial respiratory chain defects were reported in studies using cybrids of mtDNA free cells and mitochondria containing deleted mtDNA (10, 11). In these studies, 50% to 55% deleted mtDNA was required to cause mitochondrial dysfunction. Our findings are, however, not in contradiction to the threshold concept (10, 11, 18, 19), which can be applied only to single fiber level or to a homogeneous cell population. Since skeletal muscles of CPEO, KSS, and MM patients consist of both fibers with mutated mtDNA above and below the threshold, it is obvious that due to this mosaicism threshold effects can be masked and phenotypic effects are averaged over the whole fiber population. Therefore, even rather low levels of mutated mtDNA in skeletal muscle may cause a pathological phenotype since mtDNA mutations affect individual muscle fibers.

In summary, our study revealed unequivocal genotype phenotype correlations in skeletal muscle specimens of 20 patients harbouring large-scale mtDNA deletions. For practical purposes, it is notable that the extent of historical abnormalities reflects the degree of biochemical abnormalities as well as the degree of heteroplasmy of mtDNA deletions. Furthermore, our data demonstrated that single large-scale mtDNA deletions equally affect the activity of all mitochondrially encoded respiratory chain complexes. However, the results clearly indicate the absence of any well-defined mtDNA deletion threshold in skeletal muscle.

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