

Phenotypic diversity associated with the *MT-TV* gene m.1644G>A mutation, a matter of quantity

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Abstract

We describe four patients from three independent families with the m.1644G>A in the *MT-TV* gene, previously reported without demonstration of its deleterious impact.

Very high mutation proportion co-segregated with cytochrome oxidase defect in single muscle fibers and respiratory defect and in cybrids as shown by spectrophotometric assays and polarography.

The mutation appeared to have a very steep threshold effect with asymptomatic life with proportions up to 70% mutation, progressive encephalopathy above 80% and severe Leigh-like syndrome above 95% mutation. One patient did not fit within that frame but presented with characteristics suggesting the presence of an additional disease.

Keywords : mitochondrial DNA, heteroplasmy, mitochondrial diseases, clinical diagnosis

1 Introduction

Genotype/phenotype relationship in mitochondrial diseases are reputed to be particularly complex. That situation is in part due to the huge diversity of the causal genetic alterations, involving numerous genes located on the nuclear genome or the mitochondrial DNA (mtDNA). The possible heteroplasmy of mtDNA mutations, i.e. their co-existence with wild type mtDNA molecules, is another major blurring factor of the genotype/phenotype relationship in mtDNA-related diseases (Lightowlers et al., 1997). The mutation proportion may significantly vary between organs (Ciafaloni et al., 1991, Shanske et al., 1990) thus implying that the mutation deleterious impact should be evaluated with respect to its proportion in each affected tissue. As a consequence most reported mtDNA mutations are considered potentially deleterious based on their absence in controls and the high phylogenic conservation of the modified nucleotide

position. They remain however with a “not confirmed” status unless they have been observed in a sufficient number of independent occurrences with similar phenotype (see Mitomap at www.mitomap.org).

The m.1644G>A mutation in the mtDNA tRNA^{Val} (*MT-TV*) gene fully exemplifies that situation. It has been reported twice in a heteroplasmic state associated with different presentations. In one family 75% mutation in blood were associated with deafness and hypertrophic cardiomyopathy while in one patient with 85% mutation in blood, with encephalopathy (Menotti et al., 2004). In an independent report it was found at 85% in the muscle of a patient with typical MELAS syndrome (Tanji et al., 2008). Although no biochemistry was provided, the mutation was associated with mitochondrial myopathy with scattered muscle fibers showing mitochondrial proliferation and/or cytochrome c oxidase defect.

We hereafter describe four patients from three different families carrying the same mtDNA *MT-TV* gene m.1644G>A point-mutation who presented with significant phenotypic variation with respect to age of onset, symptoms and disease progression. We demonstrated the deleterious potential of the mutation in cybrids with 100% mutation and in single muscle fibers analysis. These data in association with the genotype/phenotype analysis of the patients suggest a very steep threshold effect of the mutation with the highest proportions, reaching homoplasmy, segregating with a severe encephalopathy compatible with Leigh syndrome while proportions up to 70% are compatible with a normal life.

2 Patients, methods

2.1 Patients

Clinical and imaging data of the four patients are summarized in Table 1.

2.1.1 Patient 1

Family history was unremarkable with the exception of a cousin on the maternal side who was reported as “handicapped” without further specification. The patient first sought medical attention at 25 years of age because of bilateral progressive hearing loss. At 45 years old, he presented with complete bilateral hearing loss, chronic cephalalgias, and gait limitation due to both fatigability and muscle weakness. Mild cognitive impairment, cerebellar ataxia, proximal muscular weakness and lower limb amyotrophy were present. Electroneuromyography showed only myogenic changes in the quadriceps. Blood CPK were increased at 635 IU/L (Normal<250). Cerebellar and brain stem atrophy were seen on MRI (not shown). The patient died from a dissecting aortic aneurysm at 50 years of age.

2.1.2 Patient 2

The patient had two healthy brothers and a severely handicapped sister who had had congenital rubella. He attempted suicide at 18 years of age and was hospitalized in a psychiatric ward. At 22 years of age he was again hospitalized for fatigue and altered behavior with mutism, incoherent speech followed by somnolence, memory disturbance and disorientation. He progressively improved within several weeks and was discharged. He was readmitted few months later for recurrent altered behavior with confusion and delirium, which again regressed within 3 months. Neuropsychological tests revealed deficient episodic memory and a marked frontal syndrome with perseveration and de-inhibition. Brain MRI showed bilateral, asymmetrical signal abnormalities of the anterior parts of caudate and putamen, as well as global atrophy of the frontal, temporal and posterior parietal cortex (Figure 1A). Electroneuromyography was consistent with mild sensory axonal polyneuropathy. Echocardiography showed a grade 4 mitral insufficiency. The patient is currently 27 years old, and suffers from mutism and disorientation.

2.1.3 Patient 3 (Patient II-3 in Family 1 shown in Figure 2)

She was born after an uncomplicated pregnancy. Acquisition of walking and speech was normal. Psychomotor regression and axial dystonia were noticed after the age of three years and the patient thereafter progressed to severe disability with generalized dystonia, akinetorigid Parkinson syndrome and marked cognitive impairment. Insulin-dependent diabetes mellitus appeared at 18 years of age. L-Dopa therapy at 50 mg three times a day was without effect. The patient was referred to our clinic at 41 years of age. She was wheelchair-bound because of generalized dystonia associated with Parkinson syndrome and anarthria. She could only understand and act upon simple commands. Tendon reflexes were abolished but bilateral Babinski sign was present. Brain MRI showed biputaminal signal abnormalities consistent with necrosis (Figure 1C and D). Electroneuromyography showed axonal sensorimotor polyneuropathy of the lower extremities.

2.1.4 Patient 4 (Patient III-1 Family 1)

She was born from non-consanguineous parents after an uncomplicated pregnancy. She is the niece of Patient 3 (Supplemental Figure 1). Developmental delay was noted in the neonatal period. Partial seizures, strabism and cerebellar ataxia progressively occurred during her first year of life. At 4 and 6 years of age, physical examination disclosed profuse myoclonus involving trunk and extremities, truncal ataxia, dysmetria and mild dysmorphia including clinodactyly, high forehead and large ears. Moderate atrophy of the vermis and punctiform hyperintensities of the parieto-occipital white matter were seen on brain MRI (Figure 1E and F). Echocardiography was normal. Karyotypic analysis, FISH 15q11-q13, telomere analysis, search for *MECP2* mutations and Angelman syndrome were negative. Single nucleotide polymorphisms micro-array analysis disclosed 10 regions with loss of heterozygosity (LOH) greater than 1Mb that suggested a consanguinity by descent (Rehder et al., 2013).

2.2 Material and Methods

2.2.1 Samples

All analyses were performed after written informed consent from the patients according to the local institution rules. Rapidly frozen samples from muscle were kept at -80°C until use. Skin fibroblasts were cultured in 4.5 g/L glucose DMEM supplemented with 10% fetal bovine serum, 200 μM uridine and antibiotics. Patient 3's fibroblasts were used to create cybrids as described.(King and Attardi, 1989) Cells from buccal mucosa, urinary sediment and blood were freshly used for DNA extraction.

DNA samples were obtained from muscle using standard procedure with SDS and proteinase K digestion followed by phenol extraction and isopropanol precipitation. They were obtained from all other samples using QiaAmp DNA minikit (Qiagen, Courtaboeuf, France) and from single muscle fibers as described. (Bataillard et al., 2001)

2.2.2 *OXPHOS activities*

Spectrophotometric analyses were performed on homogenates from frozen muscle, fibroblasts and cybrids as described (Medja et al., 2009). Analyses of oxygen consumption were performed on permeabilized fibroblasts and cybrids as described (Auré et al., 2007).

2.2.3 *Genetic studies*

Large-scale rearrangements of the mitochondrial genome were screened by long range PCR while mtDNA amount was evaluated by real-time PCR amplification on an Abiprism 7900 (Applied Biosystems®) (Kim et al., 2008). Sequence of the whole mtDNA was obtained by direct sequencing for patients 2 and 4, and using Mitochip2 microarray technology (Affymetrix) for Patient 3 (van Eijsden et al., 2006). Screening of the 22 mitochondrial tRNA genes of Patients 1 muscle mtDNA was performed by Denaturing Gradient Gel Electrophoresis (DGGE) (Sternberg et al., 2001). Direct sequencing of mtDNA fragments was performed in a 48 capillaries 3730 sequencer (Applied Biosystems®) with Big Dye terminator v3.1 sequence kit and SeqScape2.5 software for sequence analysis. The patients' mtDNA haplotypes were

determined following the description of the main branching points of mtDNA lineages (www.mitomap.org).

The proportion of the m.1644G>A variant was evaluated by both DGGE and fluorescent Restriction Fragment Length Polymorphism (RFLP) analysis. The latter took advantage of the fact that mutation m.1644G>A creates a restriction site for ApoI. MtDNA fragments were amplified from nucleotide 1487 to 1691 using 20 bases long primers. To avoid fluorescent heteroduplex formation FAM-labelled forward primer was only added before the last PCR cycle. The amplified fluorescent mtDNA fragments were then digested with ApoI and run onto a 3730 DNA analyser (Applied Biosystems) giving 205 bp long undigested fragments and 155 bases long fluorescent digested fragments. The proportion of mutant mtDNA was obtained by the peak height of the digested fragments divided by the sum of the different peak heights. Analysis was performed with Gene Mapper® software.

2.2.4 Statistical methods

Comparisons between two groups were performed with the non-parametric Mann and Whitney test and significance threshold was set at 0.05.

3 Results

3.1 The four patients presented with a mitochondrial disease disclosed by high lactate levels, abnormal muscle histology, and/or defective enzymatic activity of the respiratory chain.

Lactate was increased in the blood of Patients 1 and 2 and in the CSF of Patient 3. Abnormal lactate peaks were observed in the basal ganglia of Patient 2 (Figure 1B). In contrast lactate metabolism appeared normal for Patient 4 whose blood lactate level was normal and brain spectroscopy did not find any lactate peak (Table 1).

Muscle biopsies of Patients 1, 2 and 3 disclosed scattered cytochrome c oxidase deficient muscle fibers. Cytochrome c oxidase activity was reported homogeneously low in Patient 4's

muscle. None of the patients' biopsies had typical ragged red fibers but diffuse mitochondrial proliferation with succinate dehydrogenase histochemical staining was reported for Patient 1 muscle (Supplemental Figure 1).

A combined defect of respiratory complexes, involving complexes I and IV, was observed in Patients 1, 2 and 3 muscle biopsies (Table 2). In Patient 1's muscle it was only observed after normalization to citrate synthase activity, which was increased together with complex II activities in accordance with the mitochondrial proliferation observed with muscle histology. All muscle respiratory chain activities appeared normal in Patient 4 muscle thus contradicting the low cytochrome c oxidase histochemical activity. However significant combined respiratory complexes defect was observed in the cultured skin fibroblasts from Patients 4 as well as in those from Patients 2 and 3 (Table 3). It comprised defect of respiratory complex I and IV, the former being shown by analysis of the oxygen consumption and the latter by spectrophotometric measurement.

3.2 Search for a mtDNA alteration causing combined OXPHOS defect disclosed the same m.1644G>A mutation in the four patients

Large scale mtDNA rearrangements, mtDNA depletion or any mtDNA tRNA mutations could be the cause of a combined defect of OXPHOS activities similar to that observed in the patients. Long range PCR and real time quantitative PCR excluded the presence of mtDNA rearrangements or depletion (data not shown). Screening of the 22 mtDNA tRNA genes (Patients 1) or total mtDNA analysis with Mitochip (Patient 3) or sequencing (patients 2 and 4) disclosed the presence of the m.1644G>A transition in the tRNA^{val} gene (*MT-TV*). It was confirmed by direct sequencing of the *MT-TV* gene. Determination of the patients' haplogroup showed that Patient 1 belonged to haplogroup I, Patient 2 to H2, and Patients 3 and 4, from the same family, to haplogroup H1. The m.1644G>A mutation has therefore occurred independently in several mtDNA haplogroups.

The mutation proportion was evaluated by fluorescent restriction and DGGE in all the tissues available from the patients and their family members (Table 4 and Figure 3). These results showed that the mutation was heteroplasmic, present in high proportion (>90%) in the muscle of Patient 1 and homoplasmic, or almost homoplasmic, in all the samples from Patients 2 and 3. In contrast, the mutation load was much lower in all Patient 4's samples (between 61 and 74%) falling in the range of mutation proportion observed in her older unaffected relatives (I-1, II-1, and II-5, see Figure 2). In each case, the mutation load appeared homogeneously distributed in the diverse tissues from a same individual (standard deviation of the mutation load in samples from the 5 subjects with multiple samples ranging from 0 to 7%) (Table 4).

3.3 The deleterious potential of the m.1644G>A mutation was demonstrated both in vivo with single muscle fibers analysis and ex vivo by its transfer into cybrid cells

Analysis of isolated muscle fibers from Patient 2 showed that COX defective single muscle fibers had higher mutation proportion ($92\pm 16\%$, $n=16$) than single muscle fibers with preserved COX activity ($73\pm 28\%$, $n=9$, with $p=0.048$).

The m.1644G>A mutation was then transferred into 143B rho0 cells and a clone homoplasmic for the 1644G>A mutation was further analyzed. Its cybrid nature was verified by polymorphic microsatellite markers that were those of parental 143B cells while the mtDNA originated from the patient (data not shown). Polarographic and spectrophotometric analyses of the cybrid clone showed a profound defect of respiration and ATP synthesis as well as low complex IV activity. The transfer of the mutation thus co-segregated with significant OXPHOS defect (Table 3).

DISCUSSION

In this paper we describe four patients from three unrelated families carrying the same m.1644G>A mutation in the *MT-TV* gene encoding the mitochondrial tRNA^{Val}. The mutation was demonstrated deleterious using stringent criteria for pathogenic mutations (Tuppen et al.,

2008). It modifies a highly conserved nucleotide in nearly all other species, extending back to yeasts and fungi, within a highly conserved 4-nucleotide group between the anticodon stem and T-stem of the variable loop of the tRNA^{Val} that is likely involved in the secondary structure of the tRNA (see Mamit-tRNA: Compilation of mammalian mitochondrial tRNA genes <http://mamit-tRNA.u-strasbg.fr>). It was absent in large databases of mtDNA polymorphisms (2700 control subjects (Ingman and Gyllensten, 2006) and Genbank sequencing data 18363 subjects as collected in www.mitomap.org /polymorphism coding). No additional mtDNA mutation was found in the other mtDNA tRNA genes. Very high proportions of the mutation were demonstrated as responsible for significant OXPHOS defect in cybrid cells and in single muscle fibers.

A steep threshold effect of the mutation was illustrated by the high mutation load ($73\pm 28\%$) associated with normal COX activity in Patient 2's single muscle fibers. The homogeneity of the mutation load in the diverse tissues obtained the patients and their relatives (see Table 4) allowed comparing mutation load and clinical phenotype. That analysis reinforced the steep threshold effect observed in single muscle fibers as 100% mutation (Patients 2 and 3) was associated with the most severe phenotype, compatible with Leigh syndrome, while 92% mutation (Patient 1) was observed with relatively late onset and slowly progressive myopathy and 60-80% mutation were found in asymptomatic relatives. Taking into account relative imprecision in percentage assessment, the literature data were also compatible with that threshold effect. In one report 85% mutation in blood were found in an adult patient with encephalopathy, deafness and heart involvement but 74% in the asymptomatic sibling (Menotti et al., 2004). In the second report 85% mutation were associated in a 37 years old patient with acute transient neurologic episodes, normal lactatemia but a small lactate peak in brain MR spectroscopy (Tanji et al., 2008).

Patient 4 clearly stood out of that frame of genotype/phenotype relationship because of her severe phenotype despite a mutation load at $66\pm 6\%$. Although that patient could have a much higher mutation load in the nervous system than in peripheral tissues, this is unlikely considering the relative homogeneity between tissues of the mutation proportion. In addition the normal level of lactate in CSF is against this hypothesis. Patient 4 presented with several atypical characteristics with respect to the other patients including very early age at onset, fixed psychomotor delay, mild dysmorphic features, and profuse myoclonias. She lacked several of the progressive characteristic clinical signs (deafness, dystonia, ataxia, diabetes mellitus) and/or paraclinical hallmarks (biputamin lesions, high levels of serum or CSF lactate, and histological muscle abnormalities) that were present in the other patients. In addition spectrophotometric assays of the respiratory chain were normal in her muscle thus contrasting with the constant combined defects observed in the other patients. They contradicted the reported low cytochrome c oxidase histochemical reaction, which in any case was a weak parameter in the absence of a mosaic pattern of the defect. Presence of another, yet unknown, aggravating or causing disease factor in Patient 4 was thus suggested. The consanguinity by descent shown by the single nucleotide polymorphisms micro-array suggested the presence of a recessive factor. However none of the 55 genes included in the regions with loss of heterozygosity had previously been involved in early onset encephalopathy or mitochondrial disease. Additional high throughput genetic investigations of Patient 4 nuclear DNA were not undertaken because they had little chance of success in the absence of an affected sibling. The diagnosis of the cause of the child disease was thus considered dubious and the genetic counsel provided accordingly.

In conclusion, our study demonstrated that the m.1644G>A mutation is deleterious. Putting aside Patient 4 whose genetic status remained controversial, the phenotype associated with the m.1644G>A mutation appeared tightly correlated with the levels of heteroplasmy with very

high proportions causing severe, Leigh-like, cerebral disease with high levels of lactate in blood or CSF, slightly lower mutation proportions, above 80%, also causing cerebral disease but without basal ganglia involvement or obvious lactate accumulation, and proportions lower than 70% being compatible with an asymptomatic life.

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FIGURES LEGENDS

Figure 1: Brain MRI of Patients 2, 3 and 4 with the m.1644G>A mutation.

Hypersignal of the head of the caudate nuclei (square) with high lactate doublet on spectroscopy (arrow) were noted in the brain of Patient 2 (panels A and B). Bilateral hyperintensities of putamina and hyposignal of pallidi consistent with necrosis (short arrows) were present in the brain of Patient 3, they were associated with mild cerebellar atrophy (long arrow) (panels C and D). Mild punctiform hyperintensities in the white matter (short arrow) and mild cerebellar atrophy (long arrow) were noted in the brain of Patient 4 (panels E and F). FLAIR sequences (A, C, D, E), T1 sequence (F) and spectroscopy (B).

Figure 2: Pedigree of Family 3

Affected patients are indicated with black symbols. The asymptomatic grandmother had numerous miscarriages of unknown cause. One of her daughter died during an accident at one year of age (II-6).

Figure 3: Denaturing gradient gel electrophoresis showing heteroplasmy of the m.1644G>A mutation

Pt 3= Patient 3, Pt 4= Patient 4, M= DNA extracted from muscle, U= DNA from cells of the urinary sediment, Bu= DNA from buccal cells, Na= DNA from nasal cells, Bl= DNA from blood (Bl); C= control DNA with the Cambridge reference mtDNA sequence; +C= mix of the DNA with control DNA prior to amplification. Heteroduplex molecules, indicated with the arrowhead on the right side, are generated during amplification of a heteroplasmic mtDNA population (samples from Pt 3 mother and from Pt 4) or during amplification of a mix of mutant and control mtDNA (samples +C). The homoduplex molecules are indicated with the black arrows on the right side of the figure. Wild type molecules, having a G instead of an A, are more stable and therefore migrate further than the mutant molecules.

Supplemental Figure 1: Cytochrome c oxidase defect with a mosaic pattern coexisted with variable mitochondrial proliferation in Patients 1, 2 and 3 muscle

SDH= succinate dehydrogenase histochemical activity; COX= cytochrome c oxidase

histochemical activity; bars=100 μ m.

Mitochondrial proliferation was marked in Patient 1's muscle but mild in Patient 2 or 3 muscle;

COX defect with a mosaic pattern was observed in the three patients, it was associated with

diffuse decreased COX staining in Patients 2 and 3 while scattered muscle fibers retained

normal COX activity in Patient 1 muscle.