HHS Public Access

Author manuscript

*Mitochondrion*. Author manuscript; available in PMC 2016 March 23.

Published in final edited form as:

*Mitochondrion*. 2013 November ; 13(6): 721–728. doi:10.1016/j.mito.2013.02.003.

# Mitochondrial DNA variations in Madras motor neuron disease

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## Abstract

Although the Madras Motor Neuron Disease (MMND) was found three decades ago, its genetic basis has not been elucidated, so far. The symptom at onset was impaired hearing, upper limb weakness and atrophy. Since some clinical features of MMND overlap with mitochondrial disorders, we analyzed the complete mitochondrial genome of 45 MMND patients and found 396 variations, including 13 disease-associated, 2 mt-tRNA and 33 non-synonymous (16 *MT-ND*, 10 *MT-CO,* 3 *MT-CYB* and 4 *MT-ATPase*). A rare variant (m.8302A>G) in *mt-tRNA*$^{Leu}$ was found in three patients. We predict that these variation(s) may influence the disease pathogenesis along with some unknown factor(s).

### Keywords

MMND; deafness; mitochondria; mtDNA; tRNA; haplogroup

## 1. Introduction

Madras Motor Neuron Disease (MMND) is a rare disorder, clinically characterized by childhood onset, sporadic occurrence, weakness and wasting of limbs, multiple lower cranial nerve palsies, pyramidal dysfunction and associated sensorineural deafness (Meenakshisundram et al., 1970; Nalini et al., 2006). Variant of this disorder (MMNDV) include the features of optic atrophy and mild cerebellar involvement (Gourie Devi and Nalini, 2003). Available literature indicates that the unique MMND phenotype is restricted

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**Disclosure of conflicts of interest**

The authors declare no conflicts of interest.

to India, particularly southern India. It was first reported from Madras (now called Chennai), Southern India and hence named as “Madras Motor Neuron Disease” (Meenakshisundram et al., 1970). Subsequently a few reports from Bengaluru and Vellore in South, Mumbai in West and Kolkata in East were reported (Nalini et al., 2008). Although the disease was first reported three decades ago, genetic basis behind the disease has not been elucidated so far.

MMND phenotypes overlap with Brown-Vialetto-Van Laere Syndrome (BVVL); however, screening for BVVL associated genes (*SLC52A1, SLC52A2, SLC52A3*; and *C9ORF72*) did not show any mutations (Nalini et al.). Clinical features of MMND also overlap with the symptoms of mitochondrial disorders. Indeed, it is evident that mitochondrial gene variants can lead to motor neuron disease. A 5-bp deletion (heteroplasmic) was found in the Cytochrome c Oxidase I (*MT-COI*) gene of the patient with motor neuron disease (MND) (Comi et al., 1998). The view that MMND might be a mitochondrial disorder was not consistent with initial analyses of serum biochemistry and muscle histopathology (Nalini et al., 2008). However, because normal muscle histology may be seen in cases of Chronic Progressive External Opthalmoplegia (CPEO) with well-defined mitochondrial DNA (mtDNA) mutations, it has been suggested that definitive testing for mitochondrial disorders requires sequencing of mtDNA (Sundaram et al., 2011). Because elements of the MMND are suggestive of mitochondrial disease, and some MMND families do not show a clear Mendelian inheritance pattern, we predicted a possible role of mtDNA variations in disease phenotype.

Mitochondrial haplogroups are defined by the common mtDNA polymorphisms and have been reported as modifiers of the phenotype for several diseases (Raule et al., 2007). For paradigm, the mtDNA haplogroup affects the clinical expression of the Leber hereditary optic neuropathy (LHON) and increases the penetrance of the m.11778A>G and m. 14484T>C mutations in “J” haplogroup background (Hudson et al., 2007; Ghelli et al., 2009). However, the incomplete penetrance has been attributed to the both genetic and environmental factors. Since, the MMND phenotype is restricted to southern India; and in order to check whether any particular haplogroup is seen predominately, we have undertaken whole mitochondrial genome sequencing and mtDNA haplogroup analysis in MMND.

## 2. Material and Methods

### 2.1 Subjects

Over the last 40 years (1971 to 2011), a total of 130 patients with MMND have been recorded and evaluated at the National Institute of Mental Health and Neurosciences, Bengaluru, a tertiary national referral center for neurological disorders. All the medical records coded for anterior horn cell disorders were scrutinized retrospectively back to 1970; those coded for motor neuron disease variants in the young were selected. Among 40 families, 45 affected members with either sporadic MMND or familial MMND were studied. The data including clinical features, electromyography, nerve conduction studies, audiological investigations, visual evoked potentials, CT scan and MRI brain findings were recorded and later transferred into SPSS 13 for analysis. After obtaining informed written consent, blood sample was drawn from the patients and their relatives. The Institutional

Ethical Committees (IECs) of both the participating institutes; National Institute of Mental Health and Neurosciences (NIMHANS), Bengaluru, India and the CSIR-Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India, approved this study.

### 2.2 Audiological assessment

All patients underwent hearing acuity testing at octave frequencies from 250 Hz to 8000 Hz for air conduction and 250 Hz to 4000 Hz for bone conduction. The degree of hearing impairment was classified based on the average of air conduction pure tone thresholds at all octave frequencies tested hereafter referred to as all frequency average (AFA). This method of averaging was adopted instead of traditional three-frequency puretone (500 Hz, 1 KHz and 2 KHz) average for a relatively better reflection of average hearing level. Based on AFA, the patients were grouped as per the three frequency average classification (Goodman, 1965) as normal hearing (10 to 25dB), mild (26 to 40dB), moderate (41 to 55dB), moderately severe (56 to70dB), severe (71 to 90dB) and profound (>90dB) hearing loss. Pure tone audiometric findings were analyzed in terms of percentage of ears affected, as many patients showed asymmetrical audiometric configurations.

### 2.3 Analysis of complete mitochondrial genome

DNA was extracted from the blood collected from patient and their available relatives using the standard protocol (Thangaraj et al., 2002). The complete mitochondrial genome was amplified using 24 sets of primers (Rieder et al., 1998) to generate overlapping fragments. The amplicons were directly sequenced using the ABI BigDye Terminator cycle sequencing kit, (Applied Biosystems, Foster City, CA, USA) and analyzed using ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Complete mtDNA sequences were aligned with the revised Cambridge reference sequence (*rCRS*) (NC_012920) (Anderson et al., 1981; Andrews et al., 1999) using Sequence Analysis and the AutoAssembler tools. Patients' mothers, sibs, maternal relatives (wherever available) and 300 ethnically matched control samples were also analyzed; adopting the above method. We also compared the data with 1,649 samples with various diseases that are associated with mitochondrial dysfunction. The observed mutations were compared with mitochondrial databases such as Mitomap (http://www.mitomap.org); mtDB (http://www.genpat.uu.se/mtDB) and HmtDB (http://www.hmtdb.uniba.it:8080/hmdb) for their significance. The mtDNA haplogroup have been assigned to each individuals based on the mutation, using available literatures (www.phylotree.org; mtDNA tree Build 15 (30 Sep 2012). All the data analyses were carried out using MEGA and CLUSTAL W software for checking the conservation of amino acid in mitochondrial encoded protein coding genes and *mt-rRNA*. In addition, we have used a reported system for determining evolutionary conservation of *mt-tRNA* point mutation (Yarham et al., 2012) and pathogenicity (Yarham et al., 2011).

### 2.4 PCR-RFLP

In order to evaluate the heteroplasmy at the nucleotide position 8302, we have performed PCR-RFLP. Since there is no restriction site at the nucleotide position 8302, we have designed a forward primer by modifying $7^{th}$ (C → G) and $19^{th}$ (G → C) base to destroy and create a restriction site for *XbaI*, respectively (5′-CCCCCTgTAGAGCCCACTcT - 3′: nt

8281- 8300; and a reverse primer (5′ – ATTTAGTTGGGGCATTTCACTG – 3′: nt 8358 – 8380). PCR was performed for 30 cycles (95 °C for 30s, 55 °C for 30s, 72 °C for 1min) on the GeneAmp 9700 Thermal Cycler (Applied Biosystems, Perkin-Elmer). PCR products were initially sequenced using the reverse primers to confirm the mismatches introduced. After confirming the mismatch by sequencing, PCR products were digested with *XbaI*. The digested amplicons were size fractionated using 10% non-denaturing acrylamide gel electrophoresis. The gel was stained with ethidium bromide and visualized under UV. Gel image was captured and the bands were evaluated subjected to densitometric analysis.

### 2.5 Screening for the A8302G mtDNA mutation

We have screened for the A8302G mutation in 10,844 samples, of which 2,245 shared the R haplogroup. These samples belonged to different social (Caste/Tribe/Religious) groups from different regions of India, Bangladesh and Nepal. The names of the populations and their geographical locations are given in Table S1.

### 2.6 Statistical analysis

Descriptive statistics mean ± standard deviation (range) for continuous variables and number (percentage) for categorical variables were used to express data. All the data analyses were carried out using SPSS Ver. 13.0.

## 3. Results

### 3.1 Clinical findings

All the 45 patients (from 40 families) of MMND were from Southern India, including 17 from Karnataka, 15 from Andhra Pradesh, 9 from Tamil Nadu and 4 from Kerala. Among these, 7 patients were familial MMND and 13 were familial MMNDV, 16 were sporadic MMND and 9 were sporadic MMNDV. Consanguinity was noted in 5 (11.1%) patients. Pedigree analysis revealed that in most families the disease was transmitted as an apparent autosomal recessive trait (Figure S1) and no autosomal dominant trait was observed. In two families (F5 and F8), a maternal inheritance pattern was suggested (Figure S1). There were 19 men and 26 women (42: 58) and the mean age (range) at onset was 14.2 ± 5.7 years (1–32) and at registration was 18.7 ± 6.4 (8–32) years. Mean duration of illness was 62.8 ± 47.5 (3–180 months). The major initial symptom at onset was impaired hearing in the majority (60%) of patients and upper limb weakness and atrophy in 15.6%. A significant number of patients had a slender habitus (Figure 1).

The neurological deficits of the patients are given in Table 1. The salient findings were bilateral sensori-neural hearing impairment in all and evidence of bulbar dysfunction in more than 75% of patients. Primary optic atrophy was noted in 18 (40%) and associated vision impairment in 12 patients. Atrophy and weakness of the distal muscles of the upper limbs were observed in 25 (55.6%) patients. Twelve patients demonstrated minimal cerebellar signs; gait ataxia was seen in all cases. Two also had cerebellar dysarthria. There were 7 deaths and the age at death ranged from 13 to 45 years.

### 3.2 Biochemical, histopathological, and imaging analysis

In all patients the following were normal: complete blood count, serum electrolytes, urine analysis, thyroid function, liver and renal function, and serum creatine kinase tests. In 6 cases, total serum hexoseaminidase A and B activity levels were also normal. Electromyography in 42 out of 45 patients revealed evidence of active denervation in 32 (76.1%), chronic denervation in 32 (76.1%) and reinnervation in 25 (59.5%). Motor and sensory conductions were normal with no evidence of conduction block. Left biceps muscle biopsy was performed in 17 cases, which showed evidence of neurogenic atrophy in 11 (64.7%) samples and remaining 6 (35.3%) was normal. There was no evidence of mitochondrial myopathy. Electron microscopy was normal in 6 cases. Visual evoked potentials performed in 10 cases revealed absent waveforms in 3 cases, prolonged P100 latencies in one patient and all these have MMNDV. CT scan of brain in 3 cases and MRI of brain in 10 cases were normal.

### 3.3 Audiological findings

Audiometry was performed in all 45 patients. Various degrees of hearing acuity (from normal hearing to profound hearing loss) were demonstrated amongst all. The results of the distortion product (DP) otoacoustic emissions (OAE) and auditory brain stem response (ABR) were available for 22 cases. The most significant finding was the presence of good OAEs in presence of impaired AFA (ranging from mild to profound degree of SN hearing loss). In all these ears the ABR was also abnormal, where the waveforms were either totally dys-synchronized or had delayed absolute as well as inter peak latencies.

### 3.4 Analysis of complete mitochondrial genome

Complete mtDNA sequencing of 45 patients and their available maternal relatives revealed a total of 396 variations across the genome. All the variants were analyzed using the mitochondrial databases, such as; Mitomap, mtDB and HmtDB to check for the significance of the mutations. Among the total variations, 13 were reported to be associated with various diseases (Table 2) and 35 private (33 non-synonymous and 2 in *mt-tRNA*) variations (Table 3).

The disease associated mutations includes; 3 in 12S rRNA (m.1243 T>C; m.1291T>C and m. 1453A>G) gene, 4 in *mt-tRNA* genes m.4454T>C (*mt-tRNA*$^{met}$), m.5843A>G (*mt-tRNA*$^{Tyr}$), m.12308G>A (*mt-tRNA*$^{Leu}$) and m.15924A>G (*mt-tRNA*$^{Thr}$), 3 in NADH dehydrogenase (*MT-ND*) genes of complex I (m.5460A>G in *MT-ND2*; m.10398A>G in *MT-ND3* and m.13708G>A in *MT-ND5*), one mutation in each complexes *MT-COI* gene (m. 6267G>A) of complex IV, *MT-ATPase6* (m.8701A>G) of complex V and *MT-CYB* (m. 15497G>A) of complex III (Table 2).

Most of the variants are well-known and none of them could be stated as “novel” (Bandelt et al., 2009). Hence, we have analyzed the private non-synonymous variants in each MMND families other than haplogroup-defined variations, which may influence the phenotype expression (Hudson et al., 2007; Ji et al., 2008). We observed 35 private variants (33 non-synonymous and 2 in *mt-tRNA* genes) (Table 3). These variations were noticed in 26 out of 45 MMND patients.

A total of 74 non-synonymous variants were observed in the MMND patients, of which 33 were found to be private non-synonymous variants (Table 3). A total of 16 out 33 variants were observed in *MT-ND* genes that includes 3 variants in *MT-ND1* (m.3565A>G; m. 3533A>G; m.4232T>C), 5 in *MT-ND2* (m.4501C>T; m.4674A>G; m.5046G>A; m. 5319A>G; m.5461C>T), 1 in *MT-ND3* (m.10084T>C), 2 in *MT-ND4* (m.11061C>T; m. 11321A>G), 1 in *MT-ND4L* (m.10610A>G) found in two families (F9 and F11), 2 in *MT-ND5* (m.12491C>T; m.14061C>T) and 2 in *MT-ND6* genes (m.14163C>T; m.14634T>C). Ten variations were observed in *MT-CO* genes that include 3 in *MT-COI* (m.6250C>T; m. 7149A>G; m.7229A>G), 4 in *MT-COII* (m.7664G>A; m.7830G>A; m.7854T>C; m. 8133C>T) and 3 in *MT-COIII* (m.9316T>C; m.9612G>A; m.9948G>A). Three variations in *MT-CYB* gene (m.14798T>C; m.15287T>C; m.15323G>A (F32 and F40). In addition, four variations were observed in complex V, of which 3 in *MT-ATPase6* (m.9095G>A; m. 9098T>C; m.9140C>T) and one variation in *MT-ATPase8* (m.8436C>T).

In tRNA genes, a total 13 variations were observed, of which 2 were private variants, m. 8302A>G (Figure 2A) in *mt-tRNA*$^{Lys}$, which was found in three families (F3, F28 and F36) and was not found in controls. Since this variant was observed in three families, we have used PCR-RFLP method to measure the ratio of wild type (A) vs mutant (G) at the nucleotide position 8302 in all the three available maternal family members. Although direct sequencing method did not find the wild type allele (A), the PCR-RFLP method helped in finding the low level (11.00% – 17.93%) of wild type allele (Figure 2Ba). However, there is no significant level of allelic difference observed between affected and unaffected individuals of the family studied (Figure 2Bb) and remaining one variant was in *mt-tRNA*$^{His}$ gene (m.12172A>G).

### 3.5 Phylogenetic analysis

The phylogenetic analysis of mtDNA-based haplogrouping of the MMND patients revealed that they fell in different haplogroups (Figure 3). The M derived haplogroup were observed in 43.9% of the individuals analyzed, that include M2a (3), M2b (1), M3a (1), M5a (4), M6a (1), M35a (1), M38a (1), M4″67 (1), M66 (2), M42b (1), M52a (1) and G2a (1). Haplogroup R was observed in 21.95% of the patients with its sub-haplogroups: R5a1a, R6a, R8, R8b1, R30b and R31b subgroups. The sub-haplogroup of U; U1a (2), U2 (5) [*i.e.* U2a (1), U2c (3)], U4b (1) and U5b (1) were present in 20.07% of the patients. The haplogroup W6 was observed in two patients. In addition, the haplogroups HV, HV2, H2a and H2b were also observed one each.

## 4. Discussion

Madras Motor Neuron Disease is a unique disorder, predominantly reported from Southern India. The underlying cause of the disease remains the biggest challenge for the researchers and clinicians. No causative MMND gene defect has been identified. Because some MMND pedigrees are consistent with maternal transmission of the MMND trait, and because elements of the MMND phenotype are similar to the clinical profile in mitochondrial disorders, we have explored the role of mitochondria in causing MMND. It is well established that many mitochondrial diseases result from mutations in mtDNA (Wallace et al., 1988; Holt et al., 1990). We have therefore sequenced the complete mtDNA of 45

MMND patients and their available maternal relatives to assess the inheritance pattern of MMND.

Complete mtDNA sequencing of 45 patients and their available maternal relatives revealed a total of 396 variations across the mitochondrial genome, including 13 disease-associated and 35 private variations that includes 33 non-synonymous, 2 in *mt-tRNA* genes. A total of 32 variations were observed in *mt-rRNA* genes, including three mutations in 12S rRNA gene [(m.1243 T>C (F1and F34); m.1291T>C (F29) and m. 1453A>G (F12)], which is reported to be associated with hearing impairment (Table 2) (Rydzanicz et al., 2009; Ballana et al., 2006). These mutations were found in four families (8.8%), thus indicating the involvement of *mt-rRNA* mutations in hearing impairment of these patients. In *mt-tRNA* genes, 4 variations reported to be associated with disease was observed in 10 patients/families. Of which, m.4454T>C in $mt\text{-}tRNA^{met}$ was observed in three families (F8, F23 and F33) with same haplogroup (M5a2a1) background (Table 3). Interestingly, this mutation was reported to be associated with hypertension in Han-Chinese, where the oxygen consumption rate of the cells found to be decreased (Zhu et al., 2009). Although this variant exist in low frequency among controls (1.6%), considering the fact that this mutation has been reported with phenotypic effect among Han-Chinese, we hypothesis that the MMND phenotype in the study samples might be due to its present in M5a2a1 haplogroup background (www.phylotree.org) (Table 3). The m.5843A>G in $mt\text{-}tRNA^{Tyr}$ was reported in atypical mitochondrial cytopathy presenting with focal segmental glomerulosclerosis in one patient (F15) (Scaglia et al., 2011).

Five diseases associated mutations were observed in protein coding genes that includes 3 in *MT-ND* genes of complex I (m.5460A>G in *MT-ND2*; m.10398A>G in *MT-ND3* and m. 13708G>A in *MT-ND5*). The m. 5460A>G (*MT-ND2*), is a haplogroups (L0, L1, L3, M, N, H, U, R) defining variant, which was reported earlier in association with Alzheimer's and Parkinson's diseases and also known to be polymorphic (Mitomap) have been detected in three families [F1 (W6), F34 (W6) and F39 (M52a)] in the present study. The m.10398A>G was the "N" haplogroup defining marker, however we observed this mutation with "M" haplogroup in about 40% MMND patients. This polymorphism has been reported to alter the cell pH, and associated with metabolic syndrome and breast cancer (Kazuno et al., 2006). The m.13708G>A (*MT-ND5*), which was previously reported with Leber's Hereditary Optic Neuropathy (LHON), was found in two families [F12 (M2b1b) and F27 (R5ala). This mutation was earlier reported as J haplogroup marker and associated with LHON in European population (Torroni et al., 1997; Howell et al., 1991). However, two patients in the present study with m.13708G>A mutation were from different haplogroup [F12 (M2b1b) and F27 (R5ala) background and showing different clinical features suggesting that this mutation may modulate the disease expressions, irrespective of the haplogroup. A study on LHON patients from China suggests that the secondary mutation, G13708A, shows variable penetrance with different mtDNA haplotype background. They further suggested that unknown nuclear gene and/or other factors contributing to different clinical penetrance of LHON (Wang et al., 2008).

One missense mutation (m.6267G>A) detected in *MT-COI* gene was reported earlier in prostate cancer was found in a patient (F20). The polymorphism m.8701A>G in *MT-*

*ATPase6* of complex V has been reported to alter mitochondrial matrix pH and intracellular calcium dynamics (Kazuno et al., 2006) was observed in 42.2% of MMND patients of the present study. The m.15497G>A mutation in *MT-CYB* changing amino acid from glycine to serine, reported elsewhere to cause exercise intolerance (Tarnopolsky et al., 2004), was observed in a single patient (F35). Out of 13 disease-associated mutations, 3 mutations (m. 1291A>G, m.5843A>G and m.15497G>A) were not found in controls (Table 2) and the remaining 10 mutations were observed in both controls and MMND patients with different haplogroup background, hence these mutations may also play a role along with nuclear/ unknown factor(s) in the disease phenotype.

Thirty-three out of 74 were private non-synonymous variations; of which 16 were in *MT-ND* genes, 10 were in *MT-CO,* 3 were in *MT-CYB* and 4 were in *MT-ATPase* genes. Although these non-synonymous variations were located widely across the mitochondrial genome, the highest frequency of mutations was observed in the *MT-ND* and *MT-CO* genes. Accumulation of mitochondrial point mutations in *MT-ND* genes (Complex I) leads to aging and oxidative damage (Beal, 2005). In *MT-ND* genes, we observed 123 variations, of which 16 were private non-synonymous and 3 were disease-associated. We note that mutations in complex I were reported to cause Parkinson's disease, mitochondrial encephalomyopathy lactic acidosis and stroke like episodes (MELAS), and Leigh's disease (Shanske et al., 2008; Malfatti et al., 2007; Smigrodzki et al., 2004). In the *MT-CO* gene we found 10 private non-synonymous and one disease-associated mutations. Cytochrome c oxidase deficiency in humans is associated with a wide range of clinical phenotypes (Pecnia et al., 2004). Cytochrome c oxidase mutations arise in genes encoding four nuclear-encoded assembly factors (Surf1p, Cox10p, Sco1p and Sco2p); (Bratton et al., 2003). Of particular interest in this study of MMND is the report that the heteroplasmic state of a 5-bp deletion in the *MT-COI* gene is associated with MND, which is unusual for mitochondrial disorders (Comi et al., 1998).

Mutations in tRNA genes affect the translation of mitochondrial proteins. We observed four disease-associated mutations (m.4454T>C; m.5483A>G; m.12308A>G and m.15924A>G) and two private mutations (m.8302A>G and m.12172A>G). The m.8302A>G in mt-$tRNA^{Lys}$ was observed in three families [F3 (R8b1), F28 (R*) and F36(R*)] with R haplogroup background. Since, this mutation was not seen in the reported data for the "R" haplogroup defining motifs, we checked this variant exclusively in 10,844 random individuals inhabited all over India, Bangladesh and Nepal (Figure S2) and compared with 1649 samples of various diseases associated with mitochondrial dysfunction (neuromuscular disorders-Vanniarajan et al., 2006; cardiomyopathy-Prasad et al., 2006; oligoasthenozoospermia-Rani et al., 2006; recurrent pregnancy loss-Vanniarajan et al., 2011; Noonan syndrome-Rani et al., 2010; chronic periodontitis- Govindaraj et al., 2011; and unpublished data). This mutation was also present in one patient showing clinical features of Leigh's disease (Unpublished data) but absent in remaining 10,844 random samples analyzed. Earlier study has shown that the m.8302A>T mutation was observed in patients with mitochondrial disorders and further they could not able to characterized the variant (Sternberg et al., 2001). In addition, the m.8302A>G variant was seen in one sample (JQ704923) (Behar et al., 2012) falling in the HV0 haplogroup; however, it is not a

haplogroup defining variant (www.phylotree.org). In the present study, we found m. 8302A>G variant in homoplasmic (Sanger sequencing) condition in three families with MMND (Figure 2A), however, PCR-RFLP analysis showed a low level (>18%) of wild type allele 'A' in all the patients, their mother and the sibs (Figure 2B). However, there is no significant difference between affected and unaffected individuals among the families. These three families (F3, F28, F36) belong to FMMNDV and the clinical features were common like optic atrophy, visual impairment and SNHL; and upper and lower muscle weakness were different among them (Table S2). Hence, m.8302A>G variant could be a rare polymorphic event exists in major haplogroup "R" background.

The inheritance pattern in most of the pedigrees suggested an autosomal recessive mode of inheritance; in two, F5 and F8, the pattern suggested maternal inheritance (Figure S1). In family F5, complete mtDNA sequencing failed to detect any pathogenic mutations. In family F8 there was one private non-synonymous homoplasmic variant (m.11321A>G) in *MT-ND4* gene replacing the amino acid from asparagine to aspartic acid was detected. The mother, maternal uncle and sister of the pedigree (F8), all of whom have this missense mutation, Because these individuals did not have the MMND phenotype, this mutation in not likely to have caused MMND, unless the mutation is relatively impenetrant or there is heteroplasmy with reduced copy numbers of mutant mtDNA in these individuals. Another interesting family (F18) shows three private non-synonymous variations (m.7854T>C; m. 8133C>T and m.14163C>T), which were not assigned to any particular haplogroup (Table 3). However, the patient is sporadic, which the three mutations were also observed in the mother, hence we could predict that mtDNA mutations along with some nuclear genes and or environmental factors may play a significant role in the pathogenesis of the patient.

In conclusion, our data revealed the presence of numerous mtDNA point mutations in individuals with MMND. Although we do not find either a single mtDNA mutation or mutations in a single mitochondrial gene in all cases, association of observed mtDNA mutations with MMND cannot be ignored. Our data are consistent with the alternative view that mtDNA variants may have an indirect role in the pathogenesis of MMND, perhaps interacting with a causal genetic defect and one or more environmental factors. It is likely that full genome or exome sequencing (now underway) will be an important next step in defining understanding of the genetic basis of MMND.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank all the sample donors who have participated in this study. KT was supported by the Department of Biotechnology (DBT), Government of India; and the Council of Scientific and Industrial Research (CSIR), Government of India. RHB received generous support from the ALS Therapy Alliance, Project ALS, the Angel Fund, the Pierre L. de Bourgknecht ALS Research Foundation, the Al-Athel ALS Research Foundation, and NIH/ NINDS grant 1R01NS050557.

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## Highlights

- Madras motor neuron disease is a neurological disease restricted to south India
- Major symptoms of this disease are deafness, upper limb weakness and atrophy
- Complete mtDNA analysis showed 13 disease-associated and 35 private variations
- A rare variant m.8302A>G was observed in *mt-tRNA*$^{Leu}$ in three patients

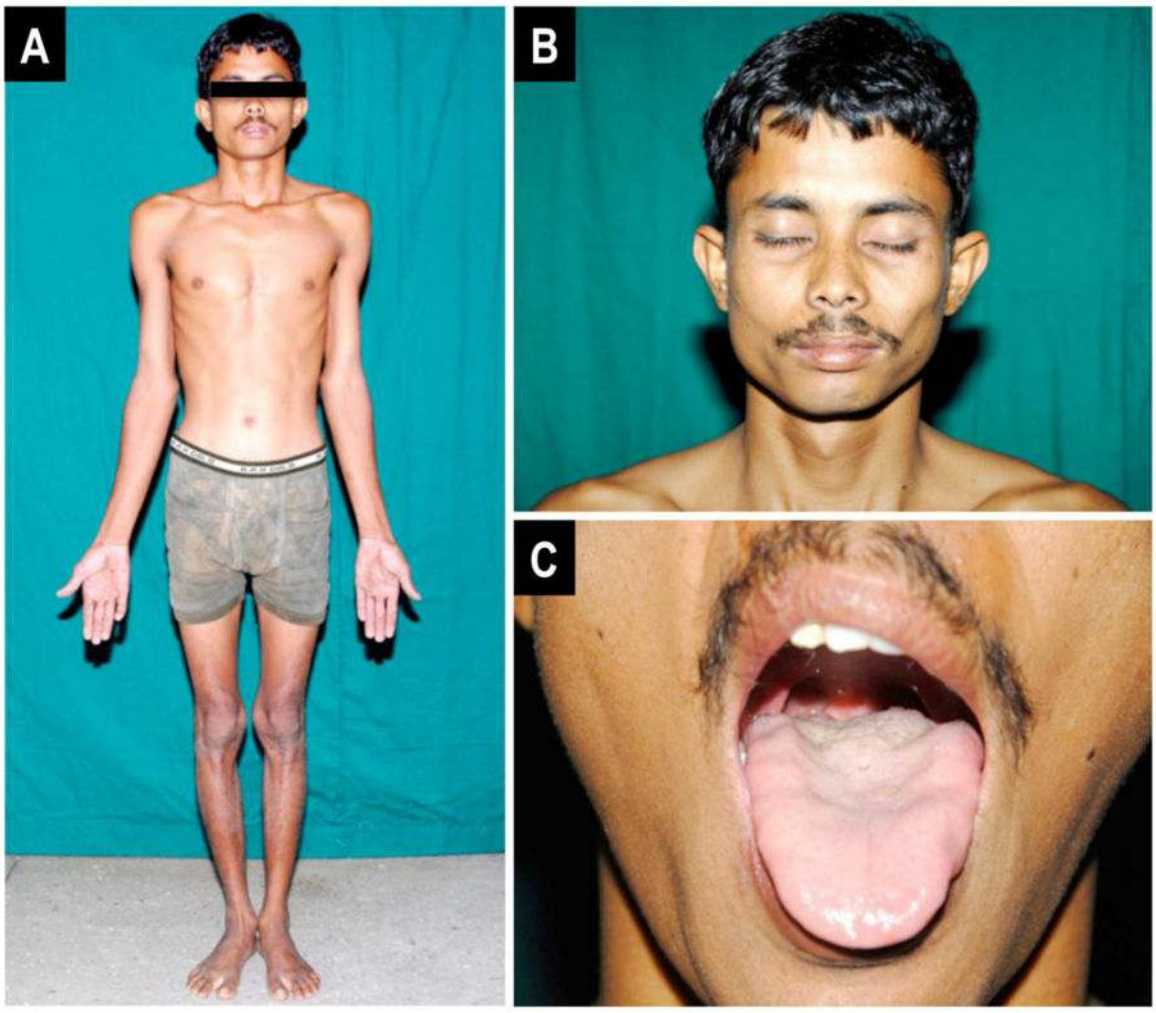


**Figure 1.**
Clinical features of MMND. A) Slender habitus of the patient with MMND. B) Lower motor neuron bifacial weakness. C) Atrophy of tongue.

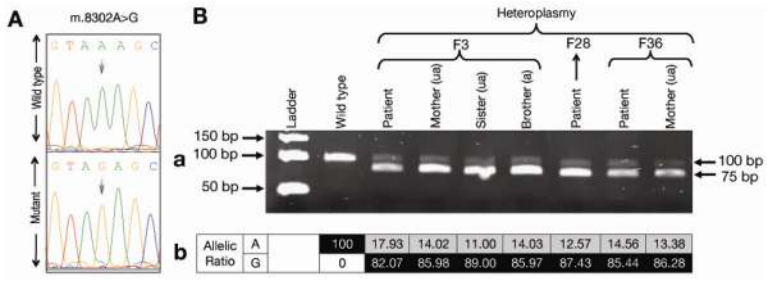


**Figure 2.**

**A)** The sequence electropherogram of m.8302A>G variation. The upper panel showing (arrow) the wild type nucleotide 'A' at the position 8302 in *mt-tRNA* lysine gene, while the lower panel showing the mutant allele 'G' which was observed in three families (F3, F28 and F36).

B) Detection of m. 8302A>G mutation by PCR-RFLP. a) The gel picture showing the wild type allele (A) (100bp) and mutant (G) (73bp) allele. b) The ratio between the wild type and mutant alleles are given for the three families (F3, F28 and F36).

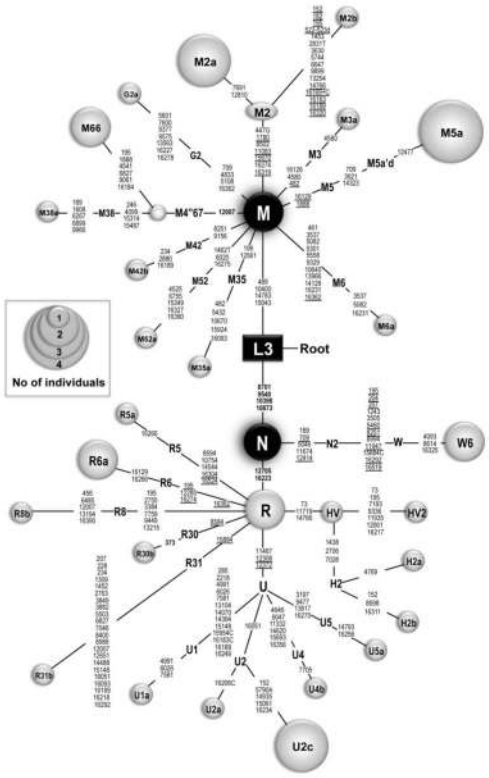


**Figure 3.**

Phylogenetic tree, constructed based on complete mtDNA sequence of MMND. Underlined nucleotide positions were recurrent mutations.

## Table 1

Clinical features of MMND observed in 45 patients.

| **Clinical characteristic** | **Value N= 45 (%)** |
|---|---|
| Age at presentation (mean ± SD) years | 18.8 ± 6.4 (range 8–32) |
| Age at onset (mean ± SD) years | 14.2 ± 5.7 (range 1–24) |
| Duration of illness (mean ± SD) months | 62.8 ± 47.5 (range 3–348) |
| M:F | 19:26 |
| Consanguinity | 5 (11.1) |
| Major symptom at onset | |
| Hearing impairment | 27 (60.0) |
| UL wasting/weakness | 7 (15.6) |
| Neurological deficits | |
| Bil optic atrophy | 18 (40.0) |
| Bil visual impairment | 10 (22.2) |
| Bifacial weakness | 24 (53.3) |
| Sensorineural deafness | 45 (100.0) |
| IX to XI cranial nerves | 36 (80.0) |
| Severe hypophonia | 35 (77.8) |
| XII cranial nerve | 36 (80.0) |
| Atrophy and weakness | |
| Upper limbs | 33 (73.3) |
| Lower limbs | 15 (33.3) |

**Table 2**

List of disease-associated mtDNA mutations were observed in MMND.

| NP | Gene | Amino acid change | Disease association (a) | Haplogroup Variant (hg) (b) | Patient | | Controls | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Haplogroup | N=45 (%) | Haplogroup | N=300 (%) |
| T1243C | 12S rRNA | - | Hearing Impairment | Yes (L0, C4c1, W) | W6 | 2 (4.4) | W | 4 (1.3) |
| T1291C | 12S rRNA | - | Hearing Impairment | Yes (L1c1b) | H2a2a | 1 (2.2) | Nil | 0 |
| A1453G | 12S rRNA | - | Possible DEAF risk factor | Yes (M2b) | M2b1b | 1 (2.2) | M2b | 2 (0.67) |
| T4454C | mt-tRNA Met | - | Mitochondrial dysfunction and Hypertension | Yes (J1c2b, H1an1a, M5a2, M7b3a) | M5a2a1 | 3 (6.7) | M5a | 5 (1.6) |
| G5460A | MT-ND2 | Ala → Thr | AD, PD | Yes (L0, L1, L3, M, N, H, U, R) | W6, M52a | 3 (6.7) | M, W, J, I | 14 (4.7) |
| A5843G | mt-tRNA Tyr | - | FSGS/Mitochondrial CM | Yes (Q) | R6a | 1 (2.2) | Nil | 0 |
| G6267A | MT-COI | Ala → Thr | Prostate Cancer | Yes (L1, M, H1) | M38a | 1 (2.2) | M38 | 3 (1.0) |
| A8701G | MT-ATPase6 | Thr → Ala | Mitochondrial matrix pH alteration | Yes (L, N, D, H, U) | M | 19 (42.2) | M | 143 (47.6) |
| A10398G | MT-ND3 | Thr → Ala | Altered cell pH/metabolic syndrome/ Breast cancer | Yes (L, N, R) | M | 18 (40) | M, R, J | 154 (51.3) |
| A12308G | mt-tRNA Leu | - | CPEO/Stroke/CM | Yes (U, T) | U | 7 (15.6) | U | 40 (13.3) |
| G13708A | MT-ND5 | - | LHON | Yes (L, M, N, D, R, J, U) | M2b1b, R5ala | 2 (4.4) | M5, J1b | 5 (1.6) |
| G15497A | MT-CYB | Gly → Ser | PIEI | Yes (G, H, U5, R7a) | M42b1 | 1 (2.2) | Nil | 0 |
| A15924G | mt-tRNA Thr | - | LIMM | Yes (L, M, D, N, U, R) | M35a2 | 1 (2.2) | M35a, U5a | 6 (2.0) |

[a]The nucleotide variants were listed in a format for web-based searches and

[b]The search was performed on phylotree (mtDNA tree Build 15 (30 Sep 2012).

AD- Alzheimer's disease; PD- Parkinson's disease; FSGS- Segmental glomerulosclerosis; CPEO- Chronic progressive external opthalmoplegia; LHON- Leber hereditary optic neuropathy; CM- Cardiomyopathy; PIEI-Paracrystalline inclusions with exercise intolerance; LIMM- Lethal infantile mitochondrial myopathy; hg- Haplogroup.

**Table 3**

Private non-synonymous, mt-tRNA variants observed in MMND families.

| Family | Nucleotide variant | Amino acid change | Gene | Reported Population context[a, b, c, d] | Haplogroup[e] | Haplogroup defining[e] | Reported disease context[a, c] | Conservation[f,g] |
|---|---|---|---|---|---|---|---|---|
| F1 | A12172G | - | mt-tRNA His | No | W6 | No | No | No |
| F3 | A8302G | - | mt-tRNA Lys | Yes | R8b1 | No | No | No |
| F4 | T4232C | I309T | MT-ND1 | Yes | U2c1 | Yes (L, R, J, U5a) | No | MC |
| | T9098C | I191T | MT-ATPase6 | Yes | | Yes (L3, M57, B2c) | No | No |
| F7 | C8436T | T24I | MT-ATPase8 | Yes | M3a2 | No | No | No |
| | C14061T | I575M | MT-ND5 | Yes | | No | No | No |
| F8 | A11321G | N188B | MT-ND4 | Yes | M5a2a | Yes (R14) | No | No |
| F9 | G9948A | V248I | MT-COIII | Yes | M66 | Yes (A10, U8b1a1) | No | MC |
| | A10610G | M47I | MT-ND4L | No | | No | No | No |
| F11 | A10610G | M47I | MT-ND4L | No | M66 | No | No | No |
| F13 | G7830A | R82H | MT-COII | Yes | G2a1 | Yes (N1, N9) | No | MC |
| F14 | C12491T | T52M | MT-ND5 | Yes | R* | No | No | MC |
| F15 | T10084C | I9T | MT-ND3 | Yes | R6a | Yes (C5a2, D2b2, D4b1a, R5ala, J1c8, U5, U7) | No | No |
| F16 | G7664A | A27T | MT-COII | Yes | U5a1d2b | Yes (B4b1a1a, L2a2b, M62) | No | No |
| F17 | A5319G | T284A | MT-ND2 | Yes | M6a | Yes (M1a1b1, M5c, H5a6, U5ala2) | No | No |
| | C11061T | S101P | MT-ND4 | Yes | | Yes (R11, Q2, L0d3) | No | No |
| F18 | T7854C | V90A | MT-COII | Yes | U2a | No | No | No |
| | C8133T | T183I | MT-COII | Yes | | No | No | MC |
| | C14163T | A171T | MT-ND6 | Yes | | No | Yes | No |
| F20 | A7149G | I416V | MT-COI | Yes | M38a | No | No | No |
| F22 | T9316C | P37S | MT-COIII | Yes | M2a1 | Yes (M22a, H3as) | No | No |
| F24 | T14634C | M14V | MT-ND6 | Yes | U1a3 | Yes (N3, L3d1b3) | No | No |
| F25 | A4674G | I69V | MT-ND2 | Yes | H2b | Yes (H1av1a) | No | No |
| F27 | A3565G | T87A | MT-ND1 | Yes | R5a1a | Yes (A2ae, R24) | No | No |
| | C9140T | A205V | MT- ATPase6 | Yes | | Yes (O) | No | No |

| Family | Nucleotide variant | Amino acid change | Gene | Reported Population context[a, b, c, d] | Haplogroup[e] | Haplogroup defining[e] | Reported disease context[a, c] | Conservation[f,g] |
|---|---|---|---|---|---|---|---|---|
| F28 | A8302G | - | mt-tRNA Lys | Yes | R* | No | No | No |
| F31 | G5046A | V193I | MT-ND2 | Yes | M4″67 | Yes (L, M, N) | No | No |
| | C6250T | A116V | MT-COI | Yes | | No | No | No |
| F32 | C3533T | T76I | MT-ND1 | Yes | R31b | No | No | No |
| | C5461T | A331V | MT-ND2 | Yes | | No | No | No |
| | G15323A | A193T | MT-CYB | Yes | | Yes (M3, G1, A2f3, H1m) | No | No |
| F34 | T15287C | P181L | MT-CYB | Yes | W6 | Yes (M5a1, I6a, H10c1) | No | MC |
| F35 | C4501T | S11P | MT-ND2 | Yes | | No | No | No |
| | A7229G | M466G | MT-COI | Yes | M52a | Yes (X2alb1, H1c4a, J1b1a1d) | No | MC |
| | G9612A | V136M | MT-COIII | Yes | | Yes (H49a2, W1b) | No | MC |
| | T14798C | P18L | MT-CYB | Yes | | Yes (A2ad1, K, J1c, T2g) | No | No |
| F36 | A8302G | - | mt-tRNA Lys | Yes | R* | No | No | No |
| F38 | G9055A | A177T | MT-ATPase 6 | Yes | U4b1a1 | Yes (M, Z, A2x, H, U8, U5, B4, J1) | No | No |
| F40 | G15323A | A193T | MT-CYB | Yes | HV | Yes (M3, G1, A2f3, H1m) | No | No |

[a,b,c,d]The nucleotide variants were listed are searched using Mitomap, mtDB, HmtDB and Google search, respectively.

[e]The search was performed on phylotree (mtDNA tree Build 15 (30 Sep 2012).

[f]The conservation analysis was performed by comparing human mtDNA sequence with 21 different species (rCRS NC_012920, Mus musculus NC_005089, Rattus norvegicus NC_001665, Bos taurus NC_006853, Danio rerio NC_002333, Macaca mulatta NC_005943, Canis lupus familiaris NC_002008, Felis catus NC_001700, Equus caballus NC_001640, Cavia porcellus NC_000884, Pan troglodytes NC_001643, Sus scrofa NC_000845, Oryctolagus cuniculus NC_001913, Capra hircus NC_005044, Camelus_dromedarius NC_009849, Panthera_tigris NC_010642, Elephas_maximus NC_005129, Cervus_elaphus NC_007704, Canis_lupus NC_008092, Giraffa_camelopardalis NC_012100, Bufo_gargarizans NC_008410, Delphinus_capensis NC_012061) for protein coding genes and

[g]conservation status for mt-tRNA point mutation were checked based on the reported literature (Yarham et al., 2012). MC represents moderately conserved.