RESEARCH PAPER

**Novel C12orf65 mutations in patients with axonal neuropathy and optic atrophy**

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**ABSTRACT**

**Objective** Charcot-Marie Tooth disease (CMT) forms a clinically and genetically heterogeneous group of disorders. Although a number of disease genes have been identified for CMT, the gene discovery for some complex form of CMT has lagged behind. The association of neuropathy and optic atrophy (also known as CMT type 6) has been described with autosomal-dominant, recessive and X-linked modes of inheritance. Mutations in Mitofusin 2 have been found to cause dominant forms of CMT6. Phosphoribosylpyrophosphate synthetase-I mutations cause X-linked CMT6, but until now, mutations in the recessive forms of disease have never been identified.

**Methods** We here describe a family with three affected individuals who inherited in an autosomal recessive fashion a childhood onset neuropathy and optic atrophy. Using homozygosity mapping in the family and exome sequencing in two affected individuals we identified a novel protein-truncating mutation in the C12orf65 gene, which encodes for a protein involved in mitochondrial translation. Using a variety of methods we investigated the possibility of mitochondrial impairment in the patients cell lines.

**Results** We described a large consanguineous family with neuropathy and optic atrophy carrying a loss of function mutation in the C12orf65 gene. We report mitochondrial impairment in the patients cell lines, followed by multiple lines of evidence which include decrease of complex V activity and stability (blue native gel assay), decrease in mitochondrial respiration rate and reduction of mitochondrial membrane potential.

**Conclusions** This work describes a mutation in the C12orf65 gene that causes recessive form of CMT6 and confirms the role of mitochondrial dysfunction in this complex axonal neuropathy.

**INTRODUCTION**

Inherited neuropathies are the most common genetic neurological disorders and affect ~1 in 2500 individuals.1 Charcot–Marie Tooth (CMT) includes a group of hereditary disorders in which motor and sensory neuropathy is the sole or primary part of the disease. CMT is traditionally classified into two types based on electrophysiological and neuropathological criteria, where CMT1 defined as ‘demyelinating’ and CMT2 as ‘axonal’.2 CMT can be associated with a variety of additional clinical features. The association between hereditary motor and sensory neuropathy and optic atrophy, also known as CMT6 (OMIM #601152), has been reported in families with different modes of inheritance, comprising over 50 cases.3–8 The clinical manifestations of CMT6 consist of distal muscle weakness and wasting starting in the lower limb with reduced reflexes and globe and Stocking sensory loss. There is progressive visual acuity loss due to optic atrophy, eventually leading to blindness. The age at onset is usually in childhood for the neuropathy and in the second decade for the optic atrophy.

Autosomal dominant CMT6 has previously been found to harbour mutations in the Mitofusin 2 (MFN2) gene.9 X-linked recessive mutations have been identified in the phosphoribosylpyrophosphate synthetase I (PRPS1) gene in CMT6 families.10 Conversely, no genetic cause for the autosomal-recessive forms of CMT6 has been identified until the work described here, where we identify a novel mutation in the C12orf65 gene and then characterise this mitochondrial protein in CMT6.

**METHODS**

**Samples**

DNA samples from multiple individuals in a large Indian family with CMT6 were collected at the Royal Free Hospital, London. An additional 93 probands affected by complex axonal neuropathy (axonal neuropathy plus one of the following: optic atrophy, retinitis pigmentosa, psychomotor retardation, cerebellar ataxia or pyramidal signs) were collected at the National Hospital for Neurology and Neurosurgery (NHNN), London. All patients were negative for known mutations in the following genes MFN2, MPZ, GJB1, BSCL2 and NEFL. Additional 90 samples with axonal neuropathy and evidence of mitochondrial impairment on muscle biopsy were collected from the neurometabolic unit at the NHNN. Genomic DNA was purified from peripheral blood cells using standard procedures.

**Nerve biopsy**

The nerve biopsy soon after the surgical removal was immersed in a fixative for overnight containing 3% buffered glutaraldehyde and 0.2M sodium...
cacodylate buffer. Then, specimens were cut with razor in 1–
2 mm thick pieces and osmicated in secondary fixative—
osmium tetroxide. After fixation, the specimens were impreg-
nated into epoxy resin from which semithin (~1 μm) sections or
ultrathin (~70 nm) sections were cut and put on glass slides or
grids, respectively. Semithin sections were stained either with
Methylene blue-Azure A and basic fuchsin or Toluidine blue
and examined by light microscopy on various magnifications.
Ultrathin (~70 nm) sections were stained with uranyl acetate/
lead citrate and examined by electron microscopy on various
magnifications.

**Single Nucleotide Polymorphism genotyping and
autozygosity mapping**

Genome-wide SNP genotyping was performed at National
Institute of Health (NIH) (Bethesda, USA). Each individual was
assayed on a Illumina HumanHap300 BeadChip, yielding
approximately 317,000 SNPs. Samples were processed, hybri-
dised and scanned following the instructions of the manufac-
turer. Clustering, normalisation and genotype calls were
performed using the GenomeStudio 2010.3 Genotyping Module (Illumina). Regions of shared homozygosity that segre-
gated with disease were visually identified using the Illumina
Genome Viewer tool within the BeadStudio suite.

Whole-exome sequencing was carried out at NIH (Bethesda,
USA). Nimblegen SeqCap EZ Exome (in solution capture) Kit
was used for the exome capture. Sequencing was performed on
a Genome Analyser IIx, according to the manufacturer’s instruc-
tion. Raw sequencing reads were aligned to the hg18 build of
the reference genome using the software Novoalign. Calling was
performed using Samtools V.0.18 and the resulting calls were
annotated using ANNOVAR.

**Mutation validation and screening in the additional cohorts**

Primers for PCR amplification were designed by Generunner
(http://www.generunner.net/). After PCR amplification, coding
regions and exon–intron junctions of C12orf65 gene were
sequenced by Sanger’s sequencing, using the Big Dye
Terminator Cycle Sequencing Kit V3 (Applied Biosystems,
Foster City, California, USA) and analysed by a 3130 Genetic
Analyser sequencing machine (Applied Biosystems). Sequences
were examined in silico for mutations by Sequencer software
4.9 (Gene Codes Corporation, Ann Arbor, Michigan, USA).

**Lymphoblast cell cultures**

Lymphoblast cells were obtained from Case 1 and two
unaffected relative carriers. Lymphoblastoid cell lines were
established by Epstein–Barr virus transformation of lymphocytes
isolated from peripheral blood. Cell lines were stored at the
European Collection of Cell Cultures. Informed consent was
obtained for all samples. Patient and control lymphoblasts were
thawed and maintained in culture in modified RPMI-1640
medium containing 300 mg/L L-Glutamine and HEPES
(Invitrogen) supplemented with 10% heat-inactivated Fetal
Bovine Serum (FBS) (Invitrogen) at 37°C and 5% CO2. Fresh
medium was added every 3 days and cultures were expanded
accordingly.

**Transcript analyses**

Purification of total RNA was performed using the Qiagen
miRNeasy Mini Kit, Hilden, Germany (catalogue #217004).
cDNA synthesis was performed using Qiagen Omniscript RT
Kit, Hilden, Germany (catalogue #205110). Multiplex quantita-
tive real-time PCR assays for the levels of C12orf65 and
RPL13A were performed using SYBR Green PCR Master Mix
Kit (Applied Biosystems) with a Corbett Rotor-Gene real-time
quantitative thermal cycler (Corbett Research/Qiagen). Thermal
cycling and gene-specific primers are available upon request.
Relative quantity of each C12orf65 level was normalised by
RPL13A.

**Expression profiling of C12orf65 in human Central Nervous
System tissue**

Expression data were generated from control human central
nervous system (CNS) tissue using Affymetrix Exon 1.0 STArrays.
CNS samples were collected by the Medical Research Council
Sudden Death Brain and Tissue Bank, Edinburgh, UK,11 and the
Sun Health Research Institute, an affiliate of Sun Health
Corporation, USA.12 A full description of the samples used and
the methods of RNA isolation and processing can be found in
Trabzuni et al.11 and Trabzuni et al.14 All arrays were preprocessed
with robust multiarray average quantile normalisation with GC
background correction13 and log2 transformation in Partek’s
Genomics Suite V6.6 (Partek, St. Louis, Missouri, USA). Regional
differences in gene-level expression were investigated with Partek’s
mixed-model ANOVA is the name for a statistical test, and gender
and batch effects (date of hybridisation and brain bank) were
included as cofactors.

**Blue native in-gel complex V assay**

The mitochondrial fraction was obtained from the lymphoblas-
toid cell pellets (7.5×10^7 cells) using two low-speed centrifuga-
tion steps (600 g×10 min) at 4°C, separated by a homogenisation
step. Then, the mitochondrial membranes were solubilised with a
750 mM amino hexanoic acid/50 mM Bis Tris buffer + 4% n-dodecyl β-D maltoside detergent. Samples were left on ice for
30 min and a further high spin (14 000 g×10 min) was used to
pellet insoluble material. An equal quantity of mitochondrial
protein was loaded from each sample. Blue Native (BN) gel was
run as previously described,16 using a 3–12% Bis–Tris gel
(Invitrogen) to ensure discrete separation of complex V. Complex
V activity was measured in a reverse direction. Complex V assay
was performed by incubating the gel overnight in stain contain-
ing 34 mM Tris, 270 mM glycine, 14 mM magnesium chloride,
6 mM lead (II) nitrate and 8 mM ATP.

**O2 consumption**

To measure mitochondrial respiration rate in intact cells,
approximately 1×10^7 cells were suspended in respiration
medium (HBSS with 10 mM D-glucose) in a Clark-type oxygen
electrode thermostatically maintained at 37°C. The oxygen
electrode was calibrated with air-saturated water, assuming
406 nmol O2 atoms/mL at 37°C. Oxygen consumption was
measured over 10 min with addition of oligomycin (final con-
centration 2 μg/mL) and FCCP (0.5 μM). All data were obtained
using an Oxigraph Plus System (Hansatech Instruments, UK)
with chart recording software.

For measurements of mitochondrial membrane potential (ΔΨm),
cells were loaded with 25 nM tetramethylrhodamine
methylster (TMRM) for 30 min at room temperature in HBSS
(156 mM NaCl, 3 mM KCl, 2 mM MgSO4, 1.25 mM KH2PO4,
2 mM CaCl2, 10 mM glucose and 10 mM HEPES, pH adjusted
to 7.35), and the dye was present during the experiment.
TMRR is used in the redistribution mode and therefore a
reduction in TMRM fluorescence represents ΔΨm depolarisa-
tion. Z-stack images were obtained for accurate analysis. The
values for wild-type (WT) were set to 100% and the other gen-
otypes were expressed relative to WT.17
RESULTS
Clinical details
Three members of a large consanguineous Indian family (figure 1A), affected by axonal neuropathy (table 1) and optic atrophy, were identified at the London Royal Free Hospital. Case 3 was first seen at the age of 35 years and then again at the age of 51 years. She is the first cousin of Cases 1 and 2 and presented as a child with delayed milestones, later at the age of 9 years she had lower limb and at 11 years upper limb weakness, along with static cognitive problems and visual difficulties. The examinations at the age of 35 and 51 years were remarkably similar with only mild deterioration of her clinical features. She had no dysmorphic features, but severe bilateral optic atrophy and static but significant cognitive problems. She had a brisk jaw jerk and a pout reflex. Marked distal symmetrical weakness and wasting affecting the limbs. In contrast to her cousins, tone in her upper and lower limbs was reduced with severe distal weakness in upper and lower limbs. Upper limb reflexes were normal. Knee and adductor reflexes were abnormally brisk. Ankle and plantar reflexes were absent. She had a moderately severe thoracic scoliosis. Sensation was impossible to assess reliably due to cognition but was likely to be abnormal. As a crude estimate of visual acuity, they were able to watch television and recognise faces across the room and she could do needlework.

Case 1 noted distal wasting and weakness at the age of 8, which slowly spread proximally. When he was examined (at the age of 34), he had severe muscle wasting of lower and upper limbs, bilateral optic atrophy and macular colloid bodies. Pyramidal signs were present in the upper limbs. Case 3 presented with a similar syndrome characterised by a very slowly progressing axonal neuropathy (onset in childhood), bilateral optic atrophy and pyramidal signs. This CMT6 family was originally reported as a case report in 1987 when the family members were in their middle 30s.14

Nerve biopsy
Light microscopy analysis of sural nerve biopsy from Case 1 showed a marked reduction in large myelinated fibres. The fascicles were populated by numerous small myelinated fibres, the majority of which were associated with regeneration clusters. Ultrastructural assessment confirmed the absence of both active and chronic demyelination and showed no evidence of ongoing axonal degeneration. The remaining myelinated fibres revealed normal thickness and morphology of the myelin. Loss of unmyelinated fibres was indirectly confirmed by increased amounts of endoneural collagen with the formation of collagen pockets among flattened Schwann cell profiles (figure 2).
Genetic analyses
Autozygosity mapping on two affected cousins (Case 1 and Case 3) identified five homozygous chromosomal segments (>1 Mb), concordant in both cousins encompassing 15.8 Mb and containing 226 genes (NCBI build 37.2). Whole-exome sequencing was used to perform a comprehensive search for pathogenic mutations in both cousins. The target region was sequenced at an average sequence depth of 26.6 for all samples. To identify potential causal variants, we selected all coding variants in the homozygous regions present in both samples. Then,
we filtered them by discarding (i) variants documented in the dbSNP and the 1000-Genomes Project and (ii) synonymous substitutions. Five variants passed these filters: two were missense, one was a non-frameshift deletion and one was a nonsense mutation. The latter is a 1 bp deletion in C12orf65 gene, resulting in a premature stop codon (NM_001143905: c.346delG: p.V116X) (figure 1B). As mutations in C12orf65 have been recently described to cause a severe encephalomyopathy, as well as spastic paraplegia and optic atrophy, we assessed segregation of this mutation in the family. The deletion was homoygous in the affected cases, and either absent or heterozygous in the unaffected relatives (figure 3). To further investigate the presence of the mutation in patients with complex neuropathy, we sequenced the coding exons of C12orf65 in an additional cohort of 183 patients. None of the patients harboured potentially pathogenic variants.

To determine whether the truncating mutation induced mRNA nonsense-mediated decay, we tested the level of expression of C12orf65. This analysis showed that level of the C12orf65 mRNA was not reduced in the patients’ cells versus controls (data not shown).

C12orf65 is a nuclear gene that encodes a mitochondrial matrix protein that appears to contribute to mitochondrial translation. Nonsense mutations in this have been found in patients with a mitochondrial disease associated with combined oxidative phosphorylation enzyme (OXPHOS) deficiency. Patients’ cells were analysed by BN-PAGE analysis to study mitochondrial respiratory chain protein complexes. This analysis showed a decreased activity of complex V as well as a defect in assembly and stability of complex V in the patient sample compared with controls (figure 4A).

In order to investigate the effect of mutations on mitochondrial respiration, we measured the rate of oxygen consumption in lymphocytes. The basal oxygen consumption in Case 3 cells was significantly reduced compared with control cells. Oligomycin (inhibitor of complex V) inhibited the respiration in control lymphocytes but to a significantly lesser extent in patients’ cells (p<0.001; figure 4B), confirming the decreased activity of complex V in Case 3 cells. Addition of 1μM of the uncoupler FCCP accelerated respiration to maximal levels in control lymphocytes. The basal oxygen consumption in Case 3 cells was significantly lesser degree in Case 3 cells, suggesting that the activity of the respiratory chain in these cells is limited.

Mitochondrial membrane potential (Δψm) is a major indicator of mitochondrial function and health. Δψm in Case 3 and control lymphoblasts was assessed by the fluorescent indicator tetramethylrhodamine methyl ester, TMRM. Case 3 cells were associated with a significant reduction in the TMRM signal (and hence in Δψm) compared with controls. There were no differences between cell groups in mitochondrial morphology (figure 4C).

**DISCUSSION**

The present study describes a novel homozygous mutation in the C12orf65 gene in patients with neuropathy and optic atrophy consistent with slowly progressive CMT6. The clinical phenotype is similar to the other CMT6 family members reported, but there are additional cognitive features and the disease is more slowly progressive with family members living a likely normal lifespan. Mutations in C12orf65 have been previously described in children with Leigh syndrome, optic atrophy and ophthalmoplegia, as well as in patients with SPG55, also characterised by neuropathy and optic atrophy, but as opposed to the cases presented here they had no cognitive impairment and a less severe phenotype.

These data suggest a genotype–phenotype correlation, where the mutation site correlates with disease severity. Indeed C12orf65 encodes for a 166 amino acid protein involved in protein translation termination and contains one RF-1 domain,

![Image of mitochondria impairment](image_url)

**Figure 4** Mitochondria impairment in the patient’s lymphoblasts. (A) BN-PAGE (in gel activity) shows reduced complex V activity in Case 3 compared with controls in lymphoblasts (demonstrated by reduced band density). V (F1-F0)=complex V holoenzyme; F1=catalytic site of complex V only. (B) Oxygen consumption in Case 3 and controls. Oxygen consumption was measured in immortalised lymphocytes using a Clark oxygen electrode. Respiration was inhibited by blocking ATP production using oligomycin (2 μg/ml) and maximised by adding the uncoupler FCCP (1 μM). Data are represented as mean±SEM. (C) and (D) Mitochondrial membrane potential. Mitochondrial membrane potential in control, carrier and Case 3 lymphoblasts was determined by tetramethylrhodamine methylester (TMRM) fluorescence. Control was taken as 100%. On the x-axes, C = controls; P = patient. ** indicates p<0.01 compared to wild-type values.
found also in peptide chain release factors (figure 1C). The patients with a severe infanthood encephalopathy and death as a child carry mutations in C12orf65 interrupting the RF-1 domain (p.L84X).\textsuperscript{17} Conversely, mutations sparing the RF-1 domain seem to cause a milder phenotype as reported in the cases presented here as well as in the SPG55 cases. Of note, the mutation site in our cases (p.V116X) would suggest a shorter protein as opposed to the SPG55 cases (p.R132X) and therefore a more severe phenotype.

We also investigated mitochondrial function in patient cell lines and showed that this was impaired at multiple levels of mitochondrial function. Mitochondrial membrane potential as well as mitochondrial respiration rate were reduced; BN gel analysis showed a decrease in complex V activity as well as a defect in the assembly of the complex.

These results highlight the importance of mitochondrial dysfunction in peripheral nerve disease and in optic atrophy. In peripheral nerve disease, the mitochondrial dysfunction is frequently associated with axonal CMT. Indeed Mitofusin 2, a mitochondrial membrane protein involved in mitochondrial fusion,\textsuperscript{19} and in the regulation of mitochondrial membrane potential and the OXPHOS system.\textsuperscript{20} Furthermore mutations in GDAPI, which encodes a mitochondrial membrane protein, and mutations in MT-ATP6, which encodes the ATP6 subunit of the mitochondrial ATP synthase (OXPHOS Complex V), as a cause of CMT further highlight the role of the mitochondria in the peripheral nerve axon.\textsuperscript{21} Similarly, the most common inherited optic neuropathies, Leber hereditary optic neuropathy (LHON) and autosomal dominant optic atrophy, are the result of mitochondrial dysfunction. LHON is caused by primary mitochondrial DNA (mtDNA) mutations affecting the respiratory chain complexes,\textsuperscript{22} while the majority of optic atrophy families have mutations in the OPA1 gene, which encodes for an inner mitochondrial protein important for mtDNA maintenance and oxidative phosphorylation.\textsuperscript{23, 24}

Although we could not assess the activities of the mitochondrial respiratory chain complex c, II–III and IV, the decrease in mitochondrial membrane potential rate and the reduction in mitochondrial respiration function to a global and uniform mitochondrial respiration dysfunction, consistent with other reports describing loss of function mutations in C12orf65 gene.\textsuperscript{17}

CMT6 is a combination of two clinical processes caused by defective mitochondrial function that leads to axonal neuropathy and optic atrophy. The three genes identified in CMT6 confirm this and the phenotype is variable, a frequent finding in mitochondrial disorders. Overall CMT highlights the link between the axon and mitochondrial dysfunction and suggested that this process is likely to be affected in other forms of acquired axonal dysfunction such as multiple sclerosis and idio-pathic neuropathy. From a genetic perspective, the combination of axonal neuropathy and CMT in any patient should alert the clinician to the possibility of a mitochondrial defect with a clear list of candidate genes to investigate.

DT contributed this study by acquisition of data and analysis of data. MR contributed this study by acquisition of data and analysis of data. ZI contributed this study by acquisition of data and analysis of data. MMR contributed this study by acquisition of data and revising the manuscript. SB contributed this study by acquisition of data and analysis of data. IH contributed this study by acquisition of data and analysis of data. JH contributed to this study by conceptualisation and supervision. AS contributed this study by acquisition of data and analysis of data. HH contributed this study by design, conceptualisation, acquisition of data, interpretation of data, supervision and drafting the manuscript. All the above-mentioned members approved the final version of this article to be published.

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