Mitochondrial protein alterations in a familial peripheral neuropathy caused by the V144D amino acid mutation in the sphingolipid protein, SPTLC1

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Abstract Axonal degeneration is the final common path in many neurological disorders. Subsets of neuropathies involving the sensory neuron are known as hereditary sensory neuropathies (HSNs). Hereditary sensory neuropathy type I (HSN-I) is the most common subtype of HSN with autosomal dominant inheritance. It is characterized by the progressive degeneration of the dorsal root ganglion (DRG) with clinical symptom onset between the second or third decade of life. Heterozygous mutations in the serine palmitoyltransferase (SPT) long chain subunit 1 (*SPTLC1*) gene were identified

as the pathogenic cause of HSN-I. Ultrastructural analysis of mitochondria from HSN-I patient cells has displayed unique morphological abnormalities that are clustered to the perinucleus where they are wrapped by the endoplasmic reticulum (ER). This investigation defines a small subset of proteins with major alterations in abundance in mitochondria harvested from HSN-I mutant *SPTLC1* cells. Using mitochondrial protein isolates from control and patient lymphoblasts, and a combination of 2D gel electrophoresis, immunoblotting and mass spectrometry, we have shown the increased abundance of ubiquinol-cytochrome c reductase core protein 1, an electron transport chain protein, as well as the immunoglobulin, Ig kappa chain C. The regulation of these proteins may provide a new route to understanding the cellular and molecular mechanisms underlying HSN-I.

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Introduction

Subsets of neuropathies involving the sensory neuron are known as hereditary sensory neuropathies (HSNs). HSNs are associated with a range of clinical presentations, pathologic alterations, electrophysiological abnormalities and increasingly specific biochemical or molecular genetic abnormalities [10].

Hereditary sensory neuropathy type I (HSN-I) is the most common subtype of the HSN [10]. With autosomal dominant inheritance, it is characterised by the progressive degeneration of the dorsal root ganglion (DRG) and an onset of clinical symptoms between the second or third decade of life [25]. HSN-I is rarely fatal but imposes lifelong disability with the disease initially manifesting with sensory loss in the feet,

followed by distal muscle wasting and weakness, and subsequent positive sensory phenomena such as lancinating or ‘shooting’ pains. Heterozygous mutations in the serine palmitoyltransferase (SPT) long chain subunit 1 (*SPTLC1*) were identified as the pathogenic cause of HSN-I [1, 6]. The associated mutations in this gene occur at single amino acids which are highly conserved throughout different species and are therefore likely to interfere with SPT functionality and structure [25].

SPT is a pyridoxal 5′-phosphate-dependent multimeric enzyme that catalyses the first step in the biosynthesis of sphingolipids, ceramide and sphingomyelin [14]; mutations in the SPT subunits thus result in potential dysfunction and perturbations in sphingolipid synthesis and metabolism linked to a variety of diseases, in particular HSN-I [26]. As the rate-determining enzyme in the de novo sphingolipid synthesis pathway, SPT is therefore a key enzyme in the regulation of cellular sphingolipid content by condensation of palmitoyl coenzyme A (CoA) with L-serine to form 3-ketodihydrosphingosine. SPT is composed of three known subunits: SPTLC1, SPTLC2 and SPTLC3 [12]. SPT is a type 1 integral membrane protein with a single highly hydrophobic domain in the amino-terminal region that anchors the enzyme to the endoplasmic reticulum (ER) membrane [18, 26, 29, 30].

Neurodegenerative diseases are a clinically heterogeneous group of chronic progressive illnesses with varying, but distinct, clinical manifestations. Many of the genetic causes of such disorders, including Huntington’s disease, some forms of familial amyotrophic lateral sclerosis (ALS), Charcot-Marie-Tooth syndrome type II (CMT II), Parkinson’s disease, Friedreich’s ataxia and Alzheimer’s disease, are well established; notably, despite obvious differences in underlying aetiologies, a role for mitochondrial dysfunction is evident in the pathogenesis of all these diseases [9, 17, 19].

Mitochondrial dysfunction in neurons can lead to a myriad of different effects such as apoptosis, oxidative stress, excitotoxicity and destructive rises in intracellular calcium levels that contribute to several pathologies of the nervous system [13]. Mitochondrial transport is also intimately dependent upon the functional state of the cell and of mitochondria themselves. Functioning mitochondria are essential for neuronal survival due to their long axonal processes and high demand for energy [17]. Mitochondrial membrane depolarisation and inhibition of ATP synthesis have been shown to alter movement of organelles; 80 % of slightly depolarised mitochondria in DRG neurons undergo retrograde movement, implying that unhealthy mitochondria are returned to the cell body for repair or removal, reducing the number of mitochondria that are transported in the anterograde direction [22].

Recently, it has been shown that mitochondria from HSN-I patient cells, expressing the V144D SPTLC1 mutant, have exceptionally electron-dense cristae [23]. Considering this finding, in this study, we have investigated altered protein expression changes from the mitochondria of HSN-I patient

cells using an integrated cell biology and proteomic methodology. This combined approach yields a detailed profile of mitochondrial proteins using coupled 2DE and mass spectrometric technologies. The resulting proteomic analyses identified, for the first time, a statistically significant increase in the abundance of ubiquinol-cytochrome c reductase core protein I and the immunoglobulin protein, Ig kappa chain C protein.

Materials

All cell culture stock solutions, including RPMI-1640, foetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), 4(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) (1 M) and phosphate-buffered saline (PBS), were purchased from Gibco Invitrogen (Australia). Cell culture consumables were purchased from BD Falcon (Greiner, USA). MTCO2, Tomm 22, ubiquinol-cytochrome c reductase core protein 1, Ig kappa chain C and GAPDH primary antibodies were purchased from Abcam (USA). Secondary HRP mouse antibodies and 4′, 6-diamidino-2-phenylindole (DAPI) stains were purchased from Sigma-Aldrich (Australia).

Methods

EBV-transformed lymphoblasts

Epstein-Barr virus (EBV)-transformed control and V144D HSN-I patient lymphoblasts were kindly provided by Prof. Garth Nicholson (Molecular Medicine Laboratory, Anzac Research Institute, Sydney) [7].

Lymphoblast cultures

Lymphoblasts were cultured in RPMI-1640 media (Gibco), supplemented with FBS (10 % v/v), penicillin (1 U/mL), streptomycin (1 µg/mL), L-glutamine (2 mM) and HEPES (1 M) at 37 °C in a humidified atmosphere of 5 % CO₂, using T75 cm² culture flasks (Greiner, Interpath). Prior to use in biochemical assays, lymphoblasts were collected by centrifugation at 1500×g (5 min at RT) and washed in PBS. Cell counts were obtained using the Countess Automated Cell Counter (Invitrogen, Australia).

Isolation of mitochondrial proteins

Briefly, mitochondria were isolated using a sucrose density gradient [2, 24]. Lymphoblasts were first centrifuged at 1500×g for 5 min, and the cells were then washed in 10 mL of ice-cold 1× PBS prior to suspension in 10 mL ice-cold CaSRB buffer (10 mM NaCl, 1.5 mM CaCl₂,

10 mM Tris–HCl, pH 7.5) and left on ice for 10 min. Cells were homogenised using a Dounce homogenizer (Kimble-Chase, USA), and 7 mL of 2.5× MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM Tris–HCl, pH 7.6) was added to restore isotonicity. Homogenate was centrifuged at 700×*g* for 5 min to remove nuclei and unbroken cells. The resulting supernatant was centrifuged at 15,000×*g* for 10 min to pellet the crude mitochondria. Sucrose gradients were made in 4-mL high-speed centrifuge tubes (Beckman Coulter, USA) by adding 1 mL of 1.7 M sucrose buffer (1.7 M sucrose, 10 mM Tris-base, 0.1 mM EDTA, pH 7.6) overlaid with 1.6 mL of 1.0 M sucrose buffer (1.0 M sucrose, 10 mM Tris-base, 0.1 mM EDTA, pH 7.6). The mitochondrial pellet was resuspended in 1.6 mL of 1× MS buffer and overlaid on top of the sucrose gradient and centrifuged at 40,000×*g* for 30 min. The mitochondrial band, in the middle of the gradient, was gently removed using a 20-G needle, transferred to a 1.5-mL tube and centrifuged at 16,000×*g* for 15 min. The resulting pellet was resuspended in 2D solubilisation buffer containing 8 M urea, 2 M thiourea, 4 % (w/v) CHAPS and a cocktail of protease inhibitors.

Protein concentration

Determination of total cellular protein was performed using the EZQ Protein Estimation Assay (Invitrogen, Australia) as previously described by Churchward et al. [4].

2D gel electrophoresis

Protein concentration estimations (EZQ assay) were performed on patient and control mitochondrial protein fractions; a total of 100 µg protein was used for each 2DE analysis. 2DE was carried out as previously described [3, 11, 27]; briefly, mitochondrial proteins were reduced and alkylated in solutions containing total protein extraction buffer (containing 8 M urea, 2 M thiourea and 4 % CHAPS without ampholytes), total extraction buffer with 2 % ampholytes, TBP/dithiothreitol (DTT) disulphide reduction buffer (2.3 mM tributyl phosphine and 45 mM DTT) and alkylation buffer (230 mM acrylamide monomer).

After incubation, the treated samples were added to 7-cm non-linear pH 3–10 immobilised pH gradient (IPG) strips (Bio-Rad ReadyStrip) and left to rehydrate for 16 h at RT. Isoelectric focusing was then carried out at 20 °C using the Protean IEF Cell (Bio-Rad, USA); the initial 15 min at 250 V followed by linear ramping to 4,000 V at 50 µA/gel for a further 2 h. After 2 h, isoelectric focusing was continued at 4000 V (constant) for a total of 37,500 Vh.

After IEF, the IPG strips were incubated in IEF equilibration buffer with 2 % DTT and 350 mM acrylamide monomer

to ensure complete reduction and alkylation. The equilibrated IPG strips were then resolved in the second dimension using the MiniProtean II (Bio-Rad).

A 12.5 % T, 2.6 % C polyacrylamide gel was buffered with 375 mM Tris buffer (pH 8.8), 0.1 % (w/v) sodium dodecyl sulphate and polymerised with 0.05 % (w/v) ammonium persulphate and 0.05 % (v/v) tetramethylethylenediamine (TEMED). A stacking gel containing a 5 % T, 2.6 % C polyacrylamide buffered with 375 mM Tris buffer (pH 8.8), 0.1 % (w/v) sodium dodecyl sulphate (SDS) and included 0.1 % bromophenol blue was added to the resolving gel. The IPG strips were placed onto the stacking gel and overlaid with 0.5 % (w/v) low melting agarose dissolved in 375 mM Tris (pH 8.8), with 0.1 % (w/v) SDS. Electrophoresis was carried out at 4 °C using pre-chilled Tris-glycine-SDS electrode buffer; 150 V was initially used to rapidly drive proteins out of the IPG strips and into the stacking gel for 5–10 min, and the voltage reduced to 90 V for 2–3 h. The gels were fixed with 10 % methanol and 7 % acetic acid for 1 h. The gels were washed three times with distilled water for 20 min.

The gels were stained with colloidal Coomassie Blue (0.1 % (w/v) CCB G-250, 2 % (v/v) phosphoric acid, 10 % (w/v) ammonium sulphate, 20 % (v/v) methanol) for 20 h, with constant shaking at RT [11] and subsequently de-stained five times with 0.5 M NaCl, 15 min each. Imaging of CBB-stained gels on the FLA-9000 imager (FUJIFILM, Tokyo, Japan) was carried out at 685/750 excitation/emission with a photomultiplier tube (PMT) setting of 600 V and pixel resolution set to 100 µm [11]. Analysis of 2D gel images was performed using Delta 2D software with automated spot detection (local background region, 96; average spot size, 32; and sensitivity in percentage, 20.0) (version 4.0.8; DECODON GmbH, Gerfswald, Germany).

SDS-PAGE and immunoblotting

Control and patient mitochondrial protein fractions (25 µg total protein) were subjected to SDS-PAGE on 12.5 % resolving gels and transferred to PVDF membrane. The membranes were blocked with 5 % skim milk in TBS buffer containing 0.1 % Tween-20. Whole membranes were blocked and incubated with anti-Tomm22, anti-ubiquinol-cytochrome c reductase core protein 1, anti-Ig kappa chain C, anti-GAPDH, and anti-MTCO2 at 1:1,000, for 16 h at 4 °C. The membrane was then incubated with secondary horse radish peroxidase antibody (1:2,000 dilution) for 1 h at RT. Blots were developed using an enhanced chemiluminescence (ECL) detection kit (Pierce Thermo Scientific, USA). The membrane was developed on CL-Xposure Film (Thermo Fisher Scientific, USA) using an AGFA X-ray developer.

Mass spectrometry

2D gels were analysed for uniquely present or absent protein spots in control versus V144D mutant (i.e. all-or-none changes). The protein spots of interest were excised from gels and de-stained overnight with 1:1 absolute acetonitrile and 25 mM ammonium bicarbonate. The gel pieces were then reduced and alkylated in 10 mM dithiothreitol (DTT) and 15 mM iodoacetic acid (IAA) and subsequently incubated with trypsin solution (10 ng/ μ L, pH 7.4) for 16 h at 37 °C. The digested peptides were concentrated in a speedy vac and resuspended in 0.1 % formic acid for subsequent analysis. LC-MS/MS analysis was carried out on a nanoAquity UPLC (Waters Corp., Milford, MA, USA) linked to a Xevo QToF mass spectrometer from Waters (Micromass, UK). Three microlitres of digested peptides was loaded onto a nanoAquity C18 BEH130 column (1.7 μ m, 75 μ m $\times$ 150 mm) and then resolved and eluted from the column using a binary gradient program at a flow rate of 0.4 μ L/min: mobile phase A was 0.1 % formic acid in water, and mobile phase B was 0.1 % formic acid in acetonitrile. The nano-UPLC gradient was as follows: 0 min, 99:1 A/B; 1 min, 99:1 A/B; 31 min, 50:50 A/B; 33 min, 15:85 A/B; 36 min, 15:85 A/B; and 37 min, 99:1 A/B. The mass spectrometer was operated in positive ESI mode with capillary voltage of 2.3 kV, cone voltage of 25 V and source temperature of 80 °C. Targeted MS/MS data or data-dependent acquisition (DDA) was acquired with collision energy ramping from 30 to 40 eV on the eight most intense peaks of MS mode with mass ranges of 350–1,500 Da. The data were acquired using Masslynx software (version 4.1, Micromass, UK).

The acquired DDA data from Masslynx were converted to PKL files by Protein Lynx Global Server (Waters, UK). The MS/MS data files were searched against SwissProt database with semi-trypsin as the enzyme. The following parameters were used in Mascot for identification of the peptides: maximum missed cleavage of 2; positive peptide charge of 2, 3 and 4; peptide mass tolerant of 0.5 Da in MS and MS/MS data base; fixed modification, carbamidomethyl (C); and variable modifications, oxidation (M).

Immunofluorescence

Lymphoblasts (1×10^6 cells) were suspended in 1 mL of warm PBS. After centrifugation at 1,000 $\times$ g for 5 min, at RT, the cell pellet was resuspended in 4 % paraformaldehyde for 15 min. Cells were then placed in 0.5 % TritonX-100 and incubated at 37 °C for 30 min. The cells were then centrifuged and blocked in 5 % BSA solution at 37 °C for 30 min. After washing in PBS, the cells were resuspended in primary antibody, MTCO2 (Abcam, 1:50), ubiquinol-cytochrome c reductase core protein 1 and Ig kappa chain C (Abcam, 1:100) and incubated for 1 h at RT. The cells were subsequently washed and resuspended in secondary antibody, anti-mouse rhodamine (Millipore,

1:200), and incubated for 1 h at RT. DAPI (1 μ g/ μ L) was added to the cell suspension, and after 2 min, the cells were centrifuged and washed two times with PBS. Aliquots (300 μ L) were added to six-well culture plates containing coverslips coated in Histogrip (Invitrogen, USA) and centrifuged at 500 $\times$ g for 10 min. The coverslips were washed in warm PBS, left overnight to dry and mounted onto glass slides prior to confocal imaging. The LSM 5 confocal microscope comprising the LSM 5 exciter laser scanning microscope with Axiovert 200 M inverted optical microscope (Carl Zeiss, Jena, Germany) was used for the acquisition of immunofluorescence images. For all acquisitions, a plan-Apochromat 63 $\times$ /1.40 oil DIC objective was used with an excitation wavelength of 405 and 543 nm. Fluorescence was detected in a bandwidth of 460–560 nm. Data was analysed using the Carl Zeiss Zen 2009 Software.

Flow cytometry

For flow cytometry analyses, lymphoblasts were isolated as above; the cells were then suspended in 1 mL of 4 % paraformaldehyde in PBS and incubated for 15 min at RT. Thereafter, the cell suspension was centrifuged at 1,000 $\times$ g for 5 min at RT and then resuspended in 0.3 % Triton X-100 for 15 min at 37 °C. Cells were incubated in primary antibody for 1 h at RT. After incubation, the cell suspension was centrifuged at 1,000 $\times$ g for 5 min and the pellet resuspended in secondary antibody, anti-mouse FITC (Millipore, 1:200), for 1 h at RT. The cell suspension was washed two times in PBS and analysed using the MACSQuant flow cytometer (Milenyi Biotech). Live cells were gated, and the mean of fluorescence was obtained per 10,000 cellular events with an excitation wavelength of 488 nm and emission filter of 525/50 nm. Data was analysed with MACSQuant software.

Results

Expression of GAPDH and mitochondrial markers in HSN-I patient-derived lymphoblasts

Quantitative immunoblotting was used to determine whether both Tomm 22 (translocase of the outer mitochondrial membrane) and MTCO2 (cytochrome c oxidase subunit II) were expressed in protein lysates from isolated mitochondrial fractions and in the total cell extracts from control and V144D mutant HSN-I patient-derived lymphoblasts (V144D cells) (Fig. 1a, b). Analyses indicated that there was no statistically significant change in expression of these proteins in either protein sample (Fig. 1d), with values of 1.74 and 4.24 for control and V144D Tomm 22 mitochondrial fractions, and 5.6 and 11.30, and 1.46 and 2.06 from control and V144D

Fig. 1 Immunoblot analysis of Tomm 22, MTCO2 and GAPDH. Expression of GAPDH and mitochondrial markers in HSN-I patient-derived lymphoblasts. **a** Immunoblot of Tomm 22; **b** immunoblot of MTCO2; **c** immunoblot of GAPDH. *Lanes 1* and *2* represent control mitochondrial proteins; *lanes 3* and *4* represent control total proteins; *lanes 5* and *6* represent V144D mitochondrial proteins; and *lanes 7* and *8* represent V144D total proteins. Representative graphs showing no statistically significant ($p>0.05$) difference between mitochondrial control and patient lymphoblast lysates ($n=3$) of Tomm 22 (blue) and MTCO2 (red) (**d**) and GAPDH (**e**) are shown. All blots were normalised to GAPDH. Error bars depict SE of means

mitochondrial and total fractions respectively. As a comparative housekeeping protein for quantitation, as well as a protein loading control, analysis of GAPDH was carried out in order to establish relative protein expression levels (Fig. 1c). Despite some variability, 3,167.06 and 1,973.15 for control and V144D mitochondrial fractions and 7,766.75 and 7,091.37 for control and V144D total fractions, there were no statistically significant changes in the expression of GAPDH in either the controls or V144D cells (Fig. 1e).

2D gel images of mitochondrial proteins from control and patient-derived lymphoblasts

Total isolated mitochondrial proteins were resolved and quantitatively assessed using a refined 2DE protocol (Fig. 2) [3, 11, 27]. Mitochondrial proteins from control and V144D cells were resolved using mini gel format; image analysis indicated

583±7 and 571±6 detectable proteins in the control and V144D cell mitochondria, respectively. Further analysis of the total mitochondrial protein profiles from control and V144D cells revealed three consistent ‘all-or-none’ protein changes in the V144D cells relative to control lymphoblasts. These protein species were located at pI/MW (kDa) coordinates of 5.7/55, 6.6/24 and 8.3/24 (Table 1). Subsequent LC/MS analysis identified these proteins to be ubiquinol-cytochrome c reductase core protein 1 and Ig kappa chain C.

Expression of ubiquinol-cytochrome c and Ig kappa from HSN-I patient-derived lymphoblasts

Immunoblot analysis was performed on isolated mitochondria and total cell lysates from control and V144D cells in order to further quantitatively assess changes in protein abundance. Blots were normalised to GAPDH as shown in Fig. 1c. These

◀ **Fig. 2** Representative images of 2D gels and regions of mitochondrial proteins from control and patient-derived lymphoblasts. **a** Control and V144D mitochondrial proteins; **b** resolved protein species having a vastly altered abundance as indicated (*red arrow*). The molecular weights are in kilodaltons (kDa), and the IEF dimension is in pH units

data showed a concomitant increase in the amount of ubiquinol-cytochrome c (5.01 compared to 2.59) in the protein samples from V144D cells compared to the control samples ($p < 0.05$; Fig. 3a, c). In contrast, immunoblotting confirmed a significant decrease in the amount of Ig kappa protein in the total lysates of V144D cells compared to controls (1.47 compared to 2.71) ($p < 0.05$; Fig. 3b, d).

SPTLC1 mutations cause no change to intracellular localisation

In order to establish the intracellular localisation and abundance of ubiquinol-cytochrome c, immunofluorescence studies were performed on control and V144D cells. There were no detectable changes in intracellular localisation of MTCO2 or ubiquinol-cytochrome c protein in control and V144D cells, whereby both proteins were peripherally localised. When the intracellular localisation of Ig kappa protein was assessed, there was also no detectable change between the control or V144D cells and the localisation of the protein was also unchanged in the periphery (Fig. 4).

Relative quantification of MTCO2, ubiquinol-cytochrome c and Ig kappa in V144D cells

Immunostained MTCO2, ubiquinol-cytochrome c and Ig kappa control and V144D cells were analysed using fluorescence-assisted cell sorting (FACS) to determine the total fluorescence per cell (Fig. 5a, b). There was no overall shift or increase in the fluorescence histograms from control and V144D mutant cell populations with respect to the MTCO2 protein. However, there was a marked increase in the relative fluorescence intensity of ubiquinol-cytochrome c in the V144D cells compared to that of control lymphoblasts with an increase in relative fluorescence of 80 ± 1.5 OD (a 2.2-fold

increase) respectively, relative to the stained controls (Fig. 5c). There was an increase in Ig kappa peak intensity in the V144D cells (an ~ 1.5 -fold increase) with an observed peak width increase in the control cells.

Discussion

HSN-I is an autosomal dominant sensory neuropathy resulting in the dying back of the peripheral sensory neurons and a progressive degeneration of the dorsal root ganglia [21]. HSN-I is caused by missense mutations in the *SPTLC1* gene, but the actual cellular and molecular mechanisms underlying the disease remain poorly understood. A recent study has shown mitochondrial ultrastructural changes to be linked with ER stress in HSN-I cells [23]. Using an integrated proteomic and cell biology approach, we have identified a significant increase in the amount of ubiquinol-cytochrome c reductase core protein 1 in the mitochondria from HSN-I (V144D) patient-derived lymphoblasts relative to control lymphoblasts. Of further interest, there is a decreased amount of the immunoglobulin protein, Ig kappa chain C, in the V144D cells as well as a change in the pI of this protein.

Mitochondria are the intracellular energy producing organelles where substrates are metabolised to fuel oxidative phosphorylation through the electron transport chain within their inner membrane [31]. The electron transport chain consists of four multimeric enzyme complexes. These complexes facilitate the flow of electrons from the reducing substrates to oxygen to build a proton gradient required for ATP generation [5]. Ubiquinol-cytochrome c reductase core protein 1 (also known as cytochrome b-c1 complex subunit I) is a central component of the electron transport chain, catalysing the oxidation of ubiquinol (ubihydroquinone) and reduction of cytochrome c [5].

In order to test whether there were protein changes due to the HSN-I SPTLC1 mutation, we assessed the proteomes of mitochondria isolated from control and V144D cells using high-resolution 2DE, to enable quantitative assessments (Fig. 2a). Tomm 22 and MTCO2,

Table 1 Mass spectrometry

Spot number	Protein identified	Accession number	Number of unique peptides matched	Sequence coverage	Mascot protein score	Predicted pI	Predicted MW (kDa)	Mascot pI	Mascot MW (kDa)
I	Ubiquinol-cytochrome c reductase core protein 1	P31930	4	18 %	208	5.7	55.00	5.9	53.3
II	Ig kappa chain C	P01834	14	88 %	908	6.6	22.00	5.5	11.7
III	Ig kappa chain C	P01834	12	86 %	1280	8.3	22.00	5.5	11.7

Summary table of mascot protein identification. LC-MS/MS and Mascot Database searching identified ubiquinol-cytochrome c reductase core protein 1 and Ig kappa chain C from V144D patient-derived lymphoblast and Ig kappa chain C from control lymphoblasts isolated mitochondria

Fig. 3 Immunoblot blot analysis of ubiquinol-cytochrome c and Ig kappa chain C. Expression of ubiquinol-cytochrome c and Ig kappa chain C from HSN-I patient-derived lymphoblasts. **a** Immunoblot detection of ubiquinol-cytochrome c. **b** Immunoblot detection of Ig kappa chain C. *Lanes 1 and 2* represent control mitochondrial proteins; *lanes 3 and 4* represent control total proteins; *lanes 5 and 6* represent V144D mitochondrial proteins; and *lanes 7 and 8* represent V144D total proteins. **c**, **d** Representative graphs showing statistically significant ($*p<0.05$) difference between control patient lymphoblasts and the mutant V144D lymphoblasts of ubiquinol-cytochrome c and Ig kappa chain C respectively ($n=3$). Blots were normalised to GAPDH (Fig. 1c). Error bars depict SE of means

Fig. 4 Immunofluorescence of ubiquinol-cytochrome c, MTCO2 and Ig kappa chain C. *SPTLC1* mutations cause no change to the intracellular localisation of ubiquinol-cytochrome c, MTCO2 and Ig kappa chain C. Representative confocal micrographs showing ubiquinol-cytochrome c, MTCO2 and Ig kappa chain C stained lymphoblasts (red) and DAPI nuclear stain (blue). Scale bar= 5 μ m

Fig. 5 Flow cytometry analysis of ubiquinol-cytochrome c, MTCO2 and Ig kappa chain C. Relative quantification of ubiquinol-cytochrome c, MTCO2 and Ig kappa chain C in HSN-I patient-derived lymphoblasts expressing the V144D mutant SPTLC1 genes. Flow cytometry analysis

of the relative fluorescence intensity of ubiquinol-cytochrome c (**a**), MTCO2 (**b**) and Ig kappa chain C (**c**) in control and V144D patient-derived lymphoblasts. *Red histogram* represents the V144D patient lymphoblasts, and *blue histogram* represents control lymphoblasts ($n=3$)

mitochondrial marker proteins, confirmed the quality of the isolated mitochondrial fraction used for analysis. There were no statistically significant changes in expression of these mitochondrial markers, suggesting that these proteins turn over at a constant rate in both the control and V144D cells. Analysis of GAPDH from control and V144D cells also indicated no significant changes in this protein (Fig. 1e). There was a protein selectively detected in the V144D cells at pI 5.7 and molecular weight 55 kDa; this protein was undetectable in the control protein profile (Fig. 2b). This protein proved to be ubiquinol-cytochrome c reductase core protein 1. Quantitative immune-blotting confirmed a significant (i.e. 2-fold) increase in the amount of ubiquinol-cytochrome c

reductase core protein 1 in V144D cells relative to control cells.

Ubiquinol-cytochrome c reductase core protein 1 functions to ensure that the electron transfer rate is optimal and that fast dissociation of electrons occurs following transfer [8]. These processes are essential to maintain the electron flow and to prevent any potential electron leaks or break down of the respiratory chain [8]. Ubiquinol-cytochrome c reductase core protein 1 is also involved in free radical generation, producing reactive oxygen species (ROS) within mitochondria. ROS production can disrupt the homeostasis and interactions within the mitochondrial matrix resulting in the loss of the oxidative phosphorylation, along with the disruption of mitochondrial functions and physiology leading to cell death [5].

Further analysis was performed using immunostaining (Fig. 4) and FACS (Fig. 5) to determine cellular localisation and expression of ubiquinol-cytochrome c reductase core protein 1 and MTCO2. Immunostaining revealed no distinct difference between the localisation of ubiquinol-cytochrome c reductase core protein 1 in the control versus V144D cells. FACS analysis correlated with the previous expression data, exhibiting a concomitant 2.2-fold increase in fluorescence intensity of ubiquinol-cytochrome c reductase core protein 1 in the V144D cells relative to controls. In contrast, the MTCO2 protein displayed no increase in fluorescence intensity in control versus patient (V144D) lymphoblasts.

Furthermore, in the comparison of the control and V144D cell proteomes, we identified two other marked protein changes. Both of these proteins were located in the 24-kDa molecular weight region but were located in different pI (6.6 and 8.3 respectively) regions. Both proteins were identified as Ig kappa chain C. The cause of the apparent shift in pI is yet to be determined; however, it seems likely due to an as yet unidentified posttranslational modification (potentially a glycosylation, phosphorylation or methylation, or any combination of the three). This is the first study to identify a change in an immunoglobulin due to the SPTLC1 mutation causing HSN-I. The Ig kappa light-chain constant region undergoes little to no variation in human immunoglobulins and forms part of the five immunoglobulin classes produced in mature B cells [16]. While B cells inherently produce Ig kappa, we have determined a statistically significant decrease in the amount of Ig kappa chain C in the total cell lysate of V144D cells relative to control lymphoblasts. Immunoglobulin light chains have been implicated in and are biomarkers of diseases such as multiple myeloma and primary systemic or amyloid light-chain (AL) amyloidosis [28]. In these diseases, it has been shown that the free Ig kappa chain associates with sphingomyelin on the plasma membrane of the myeloma cells forming aggregates that are required for intercalation with membranes [15]. This further suggests the important role sphingolipids play in these disease processes.

The novel findings in this study thus suggest a link to oxidative phosphorylation, via ubiquinol-cytochrome c reductase core protein 1 (perhaps through regulation of ROS production), and that ensuing interference with energy production ultimately leads to axonal retraction that is the hallmark characteristic of hereditary sensory neuropathy. Clearly, if ROS production increases with the increased levels of ubiquinol-cytochrome c reductase core protein 1, the ensuing potential cellular damage would only further contribute to a progressing avalanche of damage that could well characterize axonal retraction at both the molecular and cellular levels. In that regard, it is notable that our proteomic analyses did not identify marked alterations in the levels of known antioxidant, chaperone or other repair proteins; do the mutations stymie such responses? The data also indicate for the first time that

there is also a potential immunological component to this neurodegenerative disorder, characterised by significantly decreased amounts of the immunoglobulin protein, Ig kappa chain C. The exact role of this protein and its relationship to HSN-I will be the aim of further investigations. Are reduced levels of this protein responsible for apparent reductions in cellular repair responses? Clearly, far more work is required to fully elucidate the mechanisms underlying peripheral neuropathies, but the novel findings arising from this first coupled, quantitative proteomic cell biological analysis provide critical new directions not even previously hypothesised. The findings in this study, coupled with the findings of Marshall et al. [20] and Myers et al. [23], suggest that there may well be underlying molecular alterations broadly common to neurodegenerations as a whole, linked to both mitochondria and lipids.

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