ORIGINAL ARTICLE

A founder mutation in Vps37A causes autosomal recessive complex hereditary spastic paraparesis

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ABSTRACT

Background Members of two seemingly unrelated kindreds of Arab Moslem origin presented with pronounced early onset spastic paraparesis of upper and lower limbs, mild intellectual disability, kyphosis, pectus carinatum and hypertrichosis.

Methods The authors performed neurological and developmental examinations on the affected individuals. The authors conducted whole genome linkage and haplotype analyses, followed by sequencing of candidate genes; RNA and protein expression studies; and finally proof of principle investigations on knockdown morpholino oligonucleotide injected zebrafish.

Results The authors characterise a novel form of autosomal recessive complex hereditary spastic paraparesis (CHSP). MRI studies of brain and spinal cord were normal. Within a single significantly linked locus the authors ultimately identified a homozygous missense mutation c.1146A>T (p.K382N) in the vacuolar protein sorting 37A (Vps37A) gene, fully penetrant and segregating with the disease in both families. Mobility was significantly reduced in Vps37A knockdown morpholino oligonucleotide injected zebrafish, supporting the causal relationship between mutations in this gene and the phenotype described in the patients of this study.

Conclusions The authors provide evidence for the involvement of Vps37A, a member of the endosomal sorting complex required for transport (ESCRT) system, in upper motor neuron disease. The ESCRT system has been shown to play a central role in intracellular trafficking, in the maturation of multivesicular bodies and the sorting of ubiquitinated membrane proteins into internal luminal vesicles. Further investigation of mechanisms by which dysfunction of this gene causes CHSP will contribute to the understanding of intracellular trafficking of vesicles by the ESCRT machinery and its relevance to CHSP.

INTRODUCTION

First described in 1880,1 hereditary spastic paraplegia (HSP) comprises a heterogeneous group of neurodegenerative disorders characterised by progressive lower limb spasticity, retrograde degeneration of the crossed cortico-spinal tracts and thinning of the posterior columns in the spinal cord.2 Complicated forms, also known as ‘complex hereditary spastic paraparesis (CHSP)’, are characterised by the addition of such neurological features as spastic quadriaparesis, seizures, dementia, amyotrophy, extrapyramidal disturbance, cerebral or cerebellar atrophy, optic atrophy, and peripheral neuropathy, as well as by extra neurological manifestations such as dysmorphism, albinism, retinitis pigmentosa, deafness, dementia, amyotrophy and ichthyosis.2–4 CHSP forms are generally inherited as autosomal recessive traits. In Mediterranean countries, CHSP forms are more common, due to the increased frequency of consanguinity and its association with autosomal recessive pathologies.4–5

Currently, more than 40 HSP loci and 21 causative genes for pure and HSP forms have been identified.5 With some genes shown to cause phenotypes associating with both forms, the historical classification of HSP types by pure and complex has become blurred.5 Alternatively, HSP types are classified by the biological function of the proteins encoded by the causative genes. Accordingly, a large group of HSP associated proteins have been found to be involved in membrane trafficking and protein sorting pathways, including microtubule-based transport.4 Some of these proteins are involved with the endosomal sorting complex required for transport (ESCRT) system, comprising ESCRT-0, -I, -II and -III. The ESCRT machinery is involved in the maturation of multivesicular bodies (MVB) and sorting of ubiquitinated membrane proteins into internal luminal vesicles.6 Spastin and spartin, which are mutated in two common forms of HSP (SPG4 and SPG20), have been previously shown to bind the ESCRT-III proteins CHMP1B and Ist1, respectively; and spartin plays a role in cytokinesis.7,8 For a comprehensive discussion of the role of ESCRTs in neural functions in general, and in HSP in particular, see this recent review.9

We present two Arab Moslem consanguineous kindreds with multiple affected individuals presenting a unique phenotype of CHSP. We determined the causative mutation in the gene vacuolar protein sorting 57A (Vps37A), encoding for a subunit of the ESCRT-I complex.

METHODS

The ethics committee of Western Galilee Hospital, Nahariya, and the supreme Helsinki committee of the Israeli Ministry of Health approved the study.


New disease loci

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For numbered affiliations see end of article.

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All study participants and parents of minors signed informed consent.

**Clinical examinations**

Complete physical, neurological and developmental examinations were performed on available and consenting individuals with CHSP from the two kindreds. Family history, laboratory test results, metabolic measurements, operations and medical procedures, including brain MRI, EEG, EMG, and muscle biopsy were accessed.

**Genotyping**

To localise the mutated gene we performed linkage analysis using a genome-wide scan with 2000 microsatellite markers distributed throughout the genome, at average intervals of 2 cM (DeCode, Iceland), as previously described. Additional markers on suspected regions were selected from the UCSC Human (Homo sapiens) Genome Browser Gateway and the ‘Linkage Mapping Set v2.5 MDS and MD10’ kits (Applied Biosystems, Carlsbad, California, USA). Data were analysed using the Superlink Online software under an autosomal recessive mode of inheritance, with 99% penetrance and a disease allele frequency of 0.001, assuming uniform allele frequency distribution.

**Candidate gene selection and mutation analysis**

Information about the 24 genes and ORFs found in the genomic segment tightly linked to the disease (supplementary table 1) was gathered from the public databases (Ensemble Genome Browser, UCSC Genome Browser, UCSC Human Gene Sorter, GeneCards and OMIM), and their relevance to HSP was assessed. Relevant criteria included:

- Genes already known to be involved in HSP or with pathways involved in HSP
- Genes expressed in the central or peripheral nervous system, in neurons, glia and Schwann cells
- Genes which have homology to known participants in HSP pathology
- Genes known to be involved in other neurodegenerative diseases
- Genes which have orthologues known to be involved in neurodegenerative diseases in model animals
- Genes involved in the development and differentiation of neurons and dendritic cells, in particular paroxysmal axons of the motor and sensory systems.

Coding regions and at least 50 bp of flanking intron regions of eight genes, encompassing the top candidate genes in the linked interval, were sequenced and analysed using the ABI Prism 3100 automated sequencer according to the manufacturer’s standard protocol (Applied Biosystems) and nucleotide blast (NCBI). Known single nucleotide polymorphisms (SNPs) with no link to disease (supplementary table 1) were accessed.

**Disease mutation cosegregation**

The presence of Vps37A c.1146A>T mutation was tested in all participating members of both kindreds, in 50 healthy controls from the same village and in 214 ethnically matched healthy controls (Arab Muslims, residents of the northern region of Israel), via restriction analysis with Hinf1. Genomic DNA was amplified by PCR, using primers flanking Vps37A exon 11: 5'-ATTTTCTAGTTTGGCACCCTG-3' forward primer and 5'-TTCTACTATTAGACCTAATG-3' reverse primer, followed by enzymatic digestion with Hinf1 (New England Biolabs, Ipswich, Massachusetts, USA). PCR products were separated on 8% acrylamide gel. The wild type allele presents two bands of 250 bp and 111 bp; and the mutated, three bands of 280 bp, 72 bp and 39 bp.

**RNA and cDNA analyses**

We examined the nature of Vps37A expression in different tissues from both healthy and affected individuals. Vps37A mRNA transcript (Human Multiple Tissue cDNA Panel, BD Biosciences, San Jose, California, USA) was studied in eight normal tissue types: heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas.

To compare the level of transcription of Vps37A in patients and healthy controls, we purified total RNA from whole peripheral blood using the RiboPure-Blood Kit (Ambion, Life Technologies, Carlsbad, California, USA), and extracted total RNA from fibroblasts using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, Missouri, USA). RNA samples were reverse transcribed to cDNA and PCR amplified to detect Vps37A and β-actin. PCR conditions for Vps37A and β-actin were 30 s at 94°C, 50 s at 55°C and 10 s at 72°C for 34 cycles. Primer sequences for Vps37A were: 5'-CCTCTGCAAGGAAATCTGC-3' and 5'-CCCTCCAAGAAATCTTCTGC-3'. Primer sequences for β-actin were: 5'-GGCACATGATGAGCTCCCG-3' and 5'-GCTGGAA-GTGGACACCGA-3'.

**Western blotting**

We compared the level of Vps37A protein expression in blood lymphocytes and skin fibroblasts from affected individuals and healthy control fibroblasts (Cascade Biologics, Portland, Oregon, USA). Actin was used as a protein loading control.

**Coimmunoprecipitation assay**

Since Vps37A interacts with other ESCRT-I proteins, such as Vps28 and Tsg101/vps23, we investigated whether Vps37A (K382N) is capable of interacting with members of the ESCRT-I protein complex. Protein extracts were made from control (Cascade Biologics) and mutant fibroblasts. Wild type and patient fibroblasts were washed with PBS and lysed with 1% Nonidet P40, 50 mM TRIS pH 8, 150 mM NaCl, and a protease inhibitor mix (Roche Diagnostics, Indianapolis, Indiana, USA). Rabbit polyclonal Vps37A (Proteintech Group Inc, Chicago, Illinois, USA) was coupled to M-280 magnetic dynabeads (Invitrogen, Life Technologies, Carlsbad, California, USA) and a coimmunoprecipitation assay followed by western blotting was performed. Equivalent amounts of total protein cell extracts, as determined by BCA assay (Pierce Biotechnology, Rockford, Illinois, USA), were mixed with the Vps37A coupled dynabeads, or dynabeads only (negative control), and incubated overnight at 4°C in lysis buffer. The beads were boiled in Laemmli buffer (Biorad, Hercules, California, USA) and eluates were loaded onto a 4%–12% tris-glycine gel (Invitrogen, Life...
Figure 1  Pedigrees of kindreds 1 (A) and 2 (B) showing haplotypes across the complex hereditary spastic paraparesis linked locus. Ten microsatellite markers spanning 6.8 cM on chromosome 8 were used for haplotype reconstruction. Roman capitals indicate generations. The code numbers of all family members appear below symbols. Filled symbols indicate affected members. Black dots indicate obligate carriers. Circles, female subjects; squares, male subjects; slant, deceased. The haplotype assumed to carry the disease allele is framed in square.
Table 1  Clinical phenotypes of patients with CHSP

<table>
<thead>
<tr>
<th>Family/patient</th>
<th>Sex</th>
<th>AO</th>
<th>Muscles tone and tendon reflexes lower extremities</th>
<th>Muscle tone and tendon reflexes upper extremities</th>
<th>Deep sensation vibration and position</th>
<th>GMFCS (level)</th>
<th>Cognitive and language developmental delay</th>
<th>MRI/CT of brain</th>
<th>Kyphosis/pectus carinatum</th>
<th>Hypertrichosis</th>
<th>Small joint hyper-flexibility</th>
<th>Miscellaneous</th>
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</thead>
<tbody>
<tr>
<td>1/VI</td>
<td>F</td>
<td>15 m</td>
<td>+ ++ Clonus</td>
<td>Normal muscle tone, increased tendon reflexes</td>
<td>Normal</td>
<td>III Uses walker*</td>
<td>Mild</td>
<td>Normal MRI of brain and spine at 3 Y</td>
<td>++/--</td>
<td>--</td>
<td>+</td>
<td>Normal hearing and vision</td>
</tr>
<tr>
<td>1/VI4</td>
<td>F</td>
<td>36 m</td>
<td>+ ++ Clonus</td>
<td>Increased muscle tone and tendon reflexes</td>
<td>Diminished vibration sensation</td>
<td>III Uses walker*</td>
<td>Mild, speech delay</td>
<td>Normal CT at 3 Y, normal MRI at 9 Y, mild ventriculomegaly</td>
<td>+/-</td>
<td>++</td>
<td>+</td>
<td>Normal hearing and vision</td>
</tr>
<tr>
<td>1/VI5</td>
<td>F</td>
<td>15 m</td>
<td>+ ++ Clonus</td>
<td>Normal muscle tone, increased tendon reflexes</td>
<td>Normal</td>
<td>III Uses walker</td>
<td>Normal intelligence, mild speech difficulties</td>
<td>Normal MRI at 7 Y</td>
<td>+/+</td>
<td>--</td>
<td>+</td>
<td>Normal hearing and vision</td>
</tr>
<tr>
<td>1/VI6</td>
<td>M</td>
<td>10 m</td>
<td>+ + Spontaneous clonus</td>
<td>Increased muscle tone and tendon reflexes</td>
<td>Loss of vibration sensation</td>
<td>IV Uses wheelchair and walker*</td>
<td>Mild, speech delay</td>
<td>At 18 m mild white matter changes—left, non-specific</td>
<td>++/+</td>
<td>--</td>
<td>+</td>
<td>Micro-meatus, normal hearing and vision</td>
</tr>
<tr>
<td>1/VI7</td>
<td>M</td>
<td>12 m</td>
<td>+ +++ Severe clonus</td>
<td>Increased muscle tone and tendon reflexes</td>
<td>Abnormal position sensation</td>
<td>IV Uses walker</td>
<td>Moderate</td>
<td>NA</td>
<td>++/+</td>
<td>+</td>
<td>+</td>
<td>Normal hearing and vision</td>
</tr>
<tr>
<td>1/VI8</td>
<td>F</td>
<td>12 m</td>
<td>++</td>
<td>Increased muscle tone, increased tendon reflexes—severe</td>
<td>Normal</td>
<td>III Uses walker*</td>
<td>Severe, no speech</td>
<td>Suspected cortical dysplasia, insular left, otherwise normal MRI at 4 Y</td>
<td>++/+</td>
<td>--</td>
<td>+</td>
<td>Severe hearing impairment, far sighted needs glasses</td>
</tr>
<tr>
<td>1/VI9</td>
<td>M</td>
<td>12 m</td>
<td>++</td>
<td>Normal muscle tone, increased tendon reflexes—mild</td>
<td>Normal</td>
<td>III Uses wheelchair and walker*</td>
<td>Mild, delayed speech</td>
<td>NA</td>
<td>++/+</td>
<td>+</td>
<td>+</td>
<td>Severe hearing impairment</td>
</tr>
<tr>
<td>2/III</td>
<td>F</td>
<td>7 m</td>
<td>+++ Clonus, dystonia</td>
<td>Increased muscle tone and tendon reflexes, dystonia</td>
<td>Normal</td>
<td>IV Uses wheelchair and walker</td>
<td>Mild, delayed speech</td>
<td>Normal CT at 3 Y</td>
<td>++/+</td>
<td>--</td>
<td>+</td>
<td>Far sighted needs glasses</td>
</tr>
<tr>
<td>2/III12</td>
<td>F</td>
<td>8 m</td>
<td>+++ Dystonia</td>
<td>Increased muscle tone and tendon reflexes, dystonia</td>
<td>Normal</td>
<td>V</td>
<td>Moderate; delayed speech</td>
<td>Normal brain and spine MRI at 6 Y</td>
<td>++/+</td>
<td>NA</td>
<td>NA</td>
<td>FTT microcephaly normal hearing and vision</td>
</tr>
</tbody>
</table>

*Botulinum injections.

AO, age at onset; CHSP, complex hereditary spastic paraparesis; FTT, failure to thrive; GMFCS, Gross Motor Function Classification System; m, months; NA, not available; Y, years.

+ and -- indicated clinical manifestation. – = none; + = weak; ++ = moderate; +++ = severe; NA = not available.
Table 2  Fine mapping of kindred I, chromosome 8 linked region

<table>
<thead>
<tr>
<th>Marker information</th>
<th>Position</th>
<th>Genetic* (cM)</th>
<th>Physical † (Mb)</th>
<th>Recombination fraction</th>
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<td></td>
<td></td>
<td>0</td>
<td>0.01</td>
<td>0.05</td>
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<td>DBS550</td>
<td>21.33</td>
<td>10919926</td>
<td></td>
<td></td>
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<tr>
<td>DBS552</td>
<td>26.43</td>
<td>12762448</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBS1754</td>
<td>27.4</td>
<td>12999960</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBS1790</td>
<td>27.4</td>
<td>13076501</td>
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<td></td>
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<td>DBS1827</td>
<td>30.49</td>
<td>14828720</td>
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<tr>
<td>DBS549</td>
<td>31.73</td>
<td>15659945</td>
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<tr>
<td>DBS484</td>
<td>34.76</td>
<td>16075550</td>
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<tr>
<td>DBS261</td>
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<td>17836463</td>
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<td>DBS258</td>
<td>41.55</td>
<td>20377440</td>
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<td></td>
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<td>DBS280</td>
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<td>20437063</td>
<td></td>
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<td>DBS1053</td>
<td>42.85</td>
<td>21291741</td>
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<tr>
<td>DBS1116</td>
<td>42.85</td>
<td>21440568</td>
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<tr>
<td>DBS282</td>
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<td>22542515</td>
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<td>DBS405</td>
<td>45.41</td>
<td>22900470</td>
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<td>DBS1733</td>
<td>46.26</td>
<td>22690059</td>
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<td>DBS1734</td>
<td>46.26</td>
<td>22817140</td>
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<tr>
<td>DBS131</td>
<td>27395748</td>
<td>0.6</td>
<td>0.6</td>
<td>0.5</td>
</tr>
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</table>

Logarithm of odds scores for markers located across the region on chromosome 8p linked to complex hereditary spastic paraparesis in kindred 1. The area flanked by markers DBS550 and DBS131 produced a peak score of 6.7 at marker DBS261 (italic) at 0. Boldface numbers indicate scores ≥3.

*Sex average marker locations according to Marshfield genetics map. cM, centiMorgan.
†Physical marker locations obtained from UCSC Human Genome Browser Gateway. Mb, megabase.

Technologies, Carlsbad, California, USA). Equivalent amounts of total protein, as determined by BCA assay (Pierce Biotechnology), from fibroblasts, lymphocytes or IP assays were loaded onto 4%–12% tris-glycine gels. After blotting, the nitrocellulose membranes (Invitrogen, Life Technologies, Carlsbad, California, USA) were probed with primary antibodies against Vps57A, Vps101 and Vps28. HRP-labelled antimouse antibody or antirabbit antibody (Amersham Biosciences, Piscataway, New Jersey, USA) was used as a secondary antibody. The antigen–antibody complexes were detected with an ECL kit (Amersham Biosciences). Antibodies used for western blotting were: rabbit polyclonal anti-Vps57 (Proteintech Group Inc Novus Biologicals, Littleton, Colorado, USA), Tsg101 and Vps28 (Santa Cruz Biotechnology Inc, Santa Cruz, California, USA).

Zebrafish and morpholino injection

To investigate the in vivo function of Vps37A in vertebrates and to further establish the causative relationship between Vps37A and the CHSP phenotype, we created a vps37a knockdown morpholino oligonucleotide (MO) zebra fish. Wild type zebrafish (AB x T up LF) were staged and housed as previously described. Groups of 25–50 stage-matched embryos were collected at 96 hpf and postfertilisation (hpf). For vps37a knockdown, antisense MO (GeneTools, Philomath, Oregon, USA) were designed against the vps37a gene (Accession number: NM 001145152), 25 base pairs upstream of the transcript start codon (vps37a 5'ATG: 5'-GTCTGTATAGTTACCTCAGGCCACAT-3') and the intron 1-exon 2 splice site (vps37a Sp: 5'-CTCTGTTTTTTCATGCA-CAAGAAAA-3'). Embryos were injected with MO (1 ng/embryo) at the 1- to 2-cell stage and allowed to develop at 28.5°C to desired stages. To evaluate functional consequences of Vps37A knockdown, and to detect reduced larval mobility, we applied a touch response test at 96 hpf as published previously. For rescue experiments, full length human VSP37A was amplified by PCR from a human cDNA clone (IMAGE clone: 5275060, Accession: BI550753.1, Open Biosystems) and cloned into pCS2+ (Invitrogen, Life Technologies, Carlsbad, California, USA).

Mutagenesis was undertaken using the QuickChange site directed mutagenesis kit (Stratagene, Agilent Technologies, Santa Clara, California, USA) according to the manufacturer’s instructions. The missense mutation was introduced for VPS37A (Evdo) using the primer sets (Forward: 5'-gagcagagaagagacttcatgagggctag-3'; Reverse: 5'-catctgcctgctgctggctg-3'). VPS37A plasmids were linearised with Not1 and mRNA synthesised using Ambion Message Machine SP6 kit. Either wild type or mutagenised mRNA (75 pg) were injected into the cytosol of one cell stage embryos with vps37a MO.

RESULTS

Family and patients

Medical history and physical examinations of seven individuals from one kindred (figure 1A) and two from a second (figure 1B) revealed common features (table 1), including normal pregnancy and delivery, developmental and motor delay from the first or second year of life, followed by unsteadiness in standing, and difficulties in walking. All affected children presented with spasticity in the lower limbs that progressed to the upper extremities, requiring recurrent physiotherapy and ligament lengthening operations. Several patients were treated with botulinum injections with moderate improvement, regaining the ability to walk with the aid of a walker. All patients presented mild to moderate delays in cognition and speech. Marked kyphosis was noted in all patients. However, patients II3 and III13, from kindred 2 (figure 1B), presented a more severe phenotype (table 1). When assessed, laboratory test results, metabolic measurements, EEG, EMG, muscle biopsy and oxidative phosphorylation studies were normal (data not shown). Patients were not dysmorphic. Brain and spinal cord MRIs were essentially normal with non-specific findings in some patients. Seven additional members (IV7, V9.14, figure 1A) of the first kindred and one additional of the second (II9, figure 1B) were suspected of being affected with an identical phenotype, but were not available for examination or genetic analysis.
Figure 2  (A) Genomic DNA sequence analysis of Vps37A reveals a homozygous A>T transition at position c.1146 (indicated by arrow) in exon 11, resulting in a lysine to asparagine substitution at position 382 of the Vps37A protein (p.K382N). The samples: wild type: unrelated control; healthy heterozygote: kindred 2, II10; affected homozygote: kindred 2, III12. (B) ConSeq results for PFAM multiple alignment of mod(r) domain demonstrate that within this conserved domain, the mutated lysine (marked by red square) is highly conserved, and predicted to be solvent exposed and to have a functional role. (C) Evolutionary conservation of the Vps37A C-terminal part. NCBI-blastp protein sequence alignment of the Vps37A C-terminal encompassing the mutation site reveals high conservation of lysine at position 382 (in red) among various species.
Figure 3  (A) Expression of Vps37A in multiple tissue samples. Human adult multiple tissue cDNA panel was amplified with the sequences at coding regions of Vps37A and G3PDH as primers. Vps37A was expressed in all human adult tissues tested. It was expressed at a high level in pancreas, placenta and heart, and at a lower level in skeletal muscle and brain (semiquantitative). (B) Vps37A expression in healthy and affected pancreas, placenta and heart, and at a lower level in skeletal muscle and in all human adult tissues tested. It was expressed at a high level in coding regions of Vps37A and G3PDH as primers. Vps37A was expressed adult multiple tissue cDNA panel was amplified with the sequences at (lane 4) fibroblasts did not show binding with Vps37A, Vps28 or Tsg101. CHSP (lane 2) fibroblasts. Western blot was probed for Vps37A, Vps28 with an anti-Vps37A antibody on protein extracts from control (lane 1) and (D) Interaction of Vps37A with members of the ESCRT-I complex. Co-IP with an anti-Vps37A antibody on protein extracts from control (lane 1) and CHSP (lane 2) fibroblasts. Western blot was probed for Vps37A, Vps28 and Tsg101. Both Vps37A (wild type) and Vps37A (K382N) showed interaction with Vps28 and Tsg101 (lanes 1 and 2). The negative control, beads only co-IP on protein lysates of wild type (lane 3) and mutant (lane 4) fibroblasts did not show binding with Vps37A, Vps28 or Tsg101.

Mode of inheritance
The healthy consanguineous parents of all affected individuals in the first kindred are descended from a single ancestor, indicating autosomal recessive inheritance and suggesting a founder mutation. The pedigree of the second kindred is also consistent with autosomal recessive inheritance.

Whole genome screen and linkage analysis
Genome wide screening of nine members of kindred 1 (figure 1A), followed by fine mapping using 30 additional DNA samples from members of this kindred, supported sole linkage to a 12 Mb (25 cM) region spanning markers D8S250 to D8S1754, with a maximum LOD score of 6.7, on marker D8S261 (table 2). Linkage to all other chromosomal regions was excluded by negative LOD scores.

Haplotype reconstruction
Haplotype reconstruction for kindred 1 revealed a uniform homozygote region shared by all affected members, and cosegregating with the disease, flanked by markers D8S261 and D8S405. Detection of the identical homozygous region between markers D8S280 and D8S405 in healthy individual VI-18 narrowed the CHSP locus to the region between markers D8S254 and D8S280, an interval of 3.8Mb (6.8 cM, position according to the March 2006 release of the human genome assembly, hg18) (figure 1A).

Haplotype reconstruction of kindred 2 using a slightly different repertoire of markers within the same linked region, dictated by informatively of DNA markers, revealed a common haplotype cosegregating with the disease in affected individual II6 (figure 1B). However, affected individual III1 inherited the disease linked allele from her father, and a slightly different allele from her mother (figure 1B), possibly due to expansion of the repeat number in marker D8S261 that occurred in one of the founders of this family.

Candidate gene selection and mutation analysis
The common genomic segment encompasses 24 genes and predicted transcripts (supplementary table 1). The relevance of these genes to HSP was assessed according to the criteria detailed in the Methods section.

Out of the 24, eight genes were defined as good candidates to have the disease-causing mutation: FGF20, NAT1, PFD3, LPL, SLC18A1, ATP6V1B2, LZTS1 and VPS37A. All eight candidate genes were sequenced, and 16 polymorphisms were found. Out of these, 15 were previously reported SNPs with no link to the pathology. VPS37A c.1146A>T was the only novel sequence variant found.

We identified a novel A>T point substitution in VPS37A (OMIM 609927), in the coding region of exon 11, rendering a lysine to aspargine inframe substitution at position 382-p. K382N (figure 2A). Cross-species alignment of the amino acid sequences for VPS37A showed lysine in position 382 to be highly conserved throughout evolution (figure 2B,C).

No SNP in this location was previously reported SNPs with no link to the pathological. VPS37A c.1146A>T was the only novel sequence variant found.

We identified a novel A>T point substitution in VPS37A (OMIM 609927), in the coding region of exon 11, rendering a lysine to aspargine inframe substitution at position 382-p. K382N (figure 2A). Cross-species alignment of the amino acid sequences for VPS37A showed lysine in position 382 to be highly conserved throughout evolution (figure 2B,C).

Analysis of the impact of the mutation on protein function was predicted to be deleterious using Polyphen (‘Probably Damaging’ with a score of 1.000) and Panther (‘Deleterious’ with 88.4% probability), while SIFT tool predicted the mutation to be ‘Tolerated’ with a score of 0.22. PredictProtein analysis of the normal and mutant proteins gave similar results with no indication of change.
Lysine 382 is located at the C’ terminus of the protein, in an unstructured region. Analysis of the tertiary structure predicted that no structural change would be caused by the substitution. Prediction of the impact of the mutation on the protein stability, using the MuPro tool, was inconclusive, with some of the methods used showing increased stabilisation while others showing it to decrease.

Previously published causative mutations for HSP in Palestinians, in exon 8 and 13 of the KIF1A gene,12 13 were examined and ruled out.

**Disease mutation cosegregation**

Using direct sequencing and restriction analysis we showed complete segregation and thus full penetrance of the mutation Vps37A c.1146A>T with the disease phenotype; all affected individuals were found homozygous to the change, obligate carriers and some siblings of the affected were heterozygous, and no healthy members of kindreds 1 and 2 were homozygotes. The absence of the identified point substitution in 428 chromosomes of health ethnically matched control samples refuted the possibility of a unique ethnic polymorphism. Interestingly, random analysis of DNA samples from 50 healthy individuals, residents of the same village, but not related to our patients, revealed three carriers for the point substitution, none of them homozygous, pointing indeed towards a founder mutation in this genetic isolate.

**Vps37A mRNA expression**

Vps37A mRNA transcript (Human Multiple Tissue cDNA Panel, BD Biosciences) was observed in the eight normal tissue types examined: heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas, with lower levels in the brain and skeletal muscle (figure 3A). Expression levels of Vps37A mRNA derived from lymphocytes and fibroblasts of affected individuals were found equivalent to those of healthy individuals (figure 3B).

**Western blotting and coimmunoprecipitation assay**

Equal amounts of protein lysates, as determined by BCA assay from both affected and control fibroblasts and lymphocytes, showed equivalent amounts of Vps37A protein on western blot probed with a Vps37A-specific antibody (Santa Cruz Biotechnology) (figure 3C), indicating that the novel missense mutation does not cause destabilisation of the Vps37A protein. We found that both Vps28 and Tsg101 coimmunoprecipitated with Vps37A (wild type) and Vps37A (K382N) in equal amounts (figure 3D, lanes 1 and 2). The beads-only co-IP, which served as...
a negative control, did not show a specific immunoprecipitation with the Vps37A, Vps28 or Tsg101 protein (figure 3D, lanes 3 and 4).

Zebrafish and morpholino injection
Zebrafish embryos injected with 1 ng of vps37a MO showed no obvious dysmorphology at 96 hpf (figure 4A,B). The Vps37A MO-injected zebrafish at 96 hpf showed striking and significant loss of motility in comparison with standard MO-injected controls (figure 4C), providing evidence for Vps37A involvement in CHSP. Rescue with wild type but not with mutated VPS37A proved specificity of this morpholino approach (figure 4C).

DISCUSSION
A mutation in Vps37A causes a novel form of autosomal recessive CHSP
We characterised a novel form of autosomal recessive CHSP in two Arab Moslem kindreds. We showed a homozygous missense mutation in Vps37A, c.1146A>T (p.K382N), to be the cause of this pathology. The causative relation between this mutation and autosomal recessive CHSP is substantiated by several findings: The disease and the region on chromosome 8p22 are linked with statistical significance and a common haplotype is inherited with full segregation. Mutation c.1146A>T of Vps37A, which is in full segregation with the disease in both families, is not a known SNP according to 1000 Genomes Project and dbsNP and was not detected in any of 428 control chromosomes from the same ethnic origin. The haplotype fully corresponds with the mutation in affected individuals and is absent in healthy family members. The two kindreds reside in the same small genetically isolated village and are highly consanguineous. The amino acid K382 is located in the mod(r) motif in Vps37A and is highly if not completely conserved among species. Further, our demonstration of significantly reduced mobility in Vps37A MO-injected zebrafish compared with standard MO-injected controls with specific rescue experiments supports Vps37A involvement in CHSP. A number of unique mutations have been identified in similar isolated populations residing in villages in the same area.16-19

Vps37A and the ESCRT-I complex
Vps37A encodes a subunit of the ESCRT-I complex. The lysine in position 382 is highly conserved throughout evolution, appearing in all examined organisms. The impact of the mutation on protein function was predicted to be deleterious using PolyPhen and Panther, while SIFT tool predicted the mutation to be ‘Tolerated’. PredictProtein analysis of the normal and mutant proteins gave similar results with no indication of change. Analysis of the tertiary structure of VPS37A predicted that no structural change would be caused by the substitution, and we show that mutant Vps37A protein expression does not destabilise the ESCRT-I complex. The observation that Vps37A (p.K382N) is still able to interact with members of the ESCRT-I complex Vps28 and Tsg101 suggests that the conserved K382 is not involved in ESCRT-I assembly, but rather affects normal muscle tone and strength via a different biological pathway. Thus, this position may be related to interactions with other proteins or other forms of regulation of the protein which are currently unknown.

Bache and coworkers demonstrated that in mammalian cells the ESCRT-I complex, namely Vps57, Tsg101, Vps28 and MVB12, is required for downregulation of the epidermal growth factor receptor via degradation.20 While the tissue and cell-specific role of the ESCRT machinery is not well characterised in humans, ESCRT-I is known to play a role in HIV budding and infection,21 as well as in melanosome biogenesis in epidermal melanocytes.22 Bache et al20 identified hVps57A as the human counterpart of Vps57 in yeast, and the equivalent of the protein hepatocellular carcinoma related protein 1. Severe consequences of downregulation of expression of other ESCRT-I members or failure of formation of the ESCRT-I have been reported. Homozygous Tsg101-/-mouse embryos have been shown to fail to develop past day 6.5 of embryogenesis;23 interference of Tsg101 expression has been shown to lead to neoplastic transformation.24 Loss of TSG101 function in endo-lysosomal trafficking plays a significant role in the pathogenesis of spongiform neurodegeneration in Mgrn1 null mutant mice.25

Expression and function of Vps37A
Our observation of Vps37A expression in cDNA of eight normal human tissues, including the brain, supports previous evidence that Vps37A is expressed in most human tissues, with the highest expression in the liver.26

While the functional role of K382 is still unknown, K382 in the mod(r) domain is thought to be involved in regulation of protein expression through its binding to ubiquitin. This binding signals protein degradation, both by the proteasome and lysosome.27 Interaction between Vps37A and ubiquitin remains speculative despite the similarity between the C-terminal of Vps57A and the ubiquitin E2 variant, located in the protein Tsg101.20 27 Another possibility is that K382N impairs lysine acetylation. Lysine acetylation is not limited to modification of histones but occurs in other proteins as well, and participates in cellular regulatory processes such as silencing and protein stability.28

Web resources
- Ensemble Genome Browser (http://www.ensemble.org/index.html)
- GeneCard (http://www.genecards.org/index.shtml)
- Marshfield genetic Map (http://research.marshfieldclinic.org/genetics/home/index.asp)
- NCBI-MapView (http://research.marshfieldclinic.org/genetics/home/index.asp)
- PedTool (http://bioinfo.cs.technion.ac.il/pedtool/)
- UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway)
- NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/)
- UniProt (Universal Protein) Resource (http://www.expasy.org/sprot)
- ConSeq website (http://conseq.tau.ac.il)
- Superlink online (http://bioinfo.cs.technion.ac.il/superlink-online)
- 1000 Genome project (http://www.1000genomes.org/)
- RCSB PDB (http://www.rcsb.org/pdb/home/home.do)
- UCSC Chimera (http://www.cgl.ucsf.edu/chimera)
- PolyPhen tool (http://genetics.bwh.harvard.edu/pph2/index.shtml)
- SIFT tool (http://sift.jcvi.org/)
- PANTHER tool (http://www.pantherdb.org/)
- PredictProtein package (www.predictprotein.org/)
- MUpro tool (http://www.ics.uci.edu/~baldig/mutation.html)
It is possible that Vps37A-p.K382N causes CHSP by impairing a mechanism that is not related to endosomal protein sorting. ESCRT subunits are known to be involved in functions not related to membrane trafficking in a wide range of disease pathologies.29 30

Defects in membrane trafficking and neurodegenerative disease

The current study shows for the first time the involvement of the ESCRT-I member, Vps37A, in CHSP. Spastin, atlastin, REEP1 and spartin are other proteins involved in ESCRT complexes that are encoded by HSP causative genes.31–39 Other defects in membrane trafficking have also been associated with HSP. Infantile-onset ascending hereditary spastic paralysis16 and ALS230 are two recently documented examples. Furthermore, we note that defects in ESCRT compounds have been implicated with neurodegenerative diseases other than HSP, such as frontotemporal dementia and amyotrophic lateral sclerosis.29 40 The current findings enable accurate genetic counselling to patients and their families, carrier testing and early prenatal diagnosis in a large consanguineous population in northern Israel, and possibly in other CHSP families worldwide. The molecular mechanisms underlying the phenotype are not yet known, but are possibly related to vesicular trafficking or abnormal ubiquitination.

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Contributors

YEZ as part of her PhD thesis performed DNA linkage analyses and sequencing expression studies. WW performed the biochemical and cell biology experiments. NK participated in ascertaining the patients and families, and provided genetic counselling to the families. DS, AB and RR performed the neurological and clinical investigation on the patients. VS performed sequencing studies and bioinformatics analyses. MK participated in ascertaining the patients and families, and in performing the molecular and linkage analyses. BG and WS performed the neurological, clinical and laboratory investigation on the patients and families. HC and VS performed immunohistochemistry studies and participated in the cellular biology studies. TF ascertainment of the patients, performed clinical analyses and follow-up. BG participated in the statistical analyses for the linkage studies. AFV participated in the clinical evaluation of the patients and performed botulinum treatments. YA participated in the biochemical analyses and the design of experiments. AMW performed the zebrafish studies. RK participated in the zebrafish studies, linkage analyses and designing of the experiments. TCF2 is the principal investigator of this project. She designed the research, recruited and examined patients, performed and supervised research, analysed clinical, molecular and biochemical data. All authors helped in writing and reviewing the manuscript.

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Competing interests

None.

Patient consent

All study participants and parents of minors signed locally approved and appropriate informed consent forms, delivered by board certified geneticists/genetic counselors able to communicate in the patients’ local language, following local and international Helsinki committees’ directives. The anonymity of patients is kept.

Ethics approval

The ethics approval was provided by the Ethics committee of Western Galilee Hospital, Nahariya, Israel and by the supreme Ethics committee of the Israeli Ministry of Health.

Provenance and peer review

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