Methods

Immunohistochemistry

Tissue material available for analysis from the proband included a full-thickness biopsy of the ileum. Similar specimens were obtained from stored surgical and autopsy material from a deceased sibling. All tissue specimens were fixed in formalin and embedded in paraffin. Then, 4-μm sections were stained with hematoxylin and eosin, and near-consecutive sections were cut for immunohistological analysis. Immunohistochemistry for smooth muscle actin (SMA) applied a monoclonal antibody from mouse host, Clone 1A4 (Dako, Glostrup, Denmark), using 1:200 dilution for the primary antibody, in a PT Link-FLEX system at high pH according to the manufacturer’s instructions (Dako). The supplementary antibodies to smooth muscle myosin heavy chain (SMMHC) (monoclonal from mouse host, clone SMMS-1; Dako), and desmin (monoclonal from mouse host, clone D33, Dako), as well as S–100 (polyclonal from rabbit host, Dako) were also used according to the manufacturer’s instructions. Microscopic evaluation was done using Nikon Eclipse Ni microscope (Nikon Corp., Tokyo, Japan), Nikon Digital Sight DS-Fi2 camera, and Nikon NIS-Elements F 4.00.00 software.

DNA isolation and exome sequencing

Genomic DNA was extracted from EDTA-treated blood on a QIAGEN EZ1 advanced XL (Qiagen, Hilden, Germany) and mechanically fragmented on a Covaris S220 (Covaris inc., Woburn, Massachusetts, USA). TrueSeq DNA sample preparation and exome enrichment (62 Mb) were carried out according to the manufacturer’s instructions (Illumina Inc., San Diego, California, USA). The samples were sequenced 100 bp paired-end using an Illumina HiScan SQ. The presence or absence of the pathogenic variant was verified by Sanger sequencing on an ABI3130XL (Life Technologies Ltd, Paisley, UK) using standard protocols. CLC Main Workbench (CLC bio, Aarhus, Denmark) was used for primer design and Sanger sequence analysis.

DNA sequence analysis and bioinformatic filtering

Bioinformatic analysis consisted of a standard protocol including image analysis and base calling by Illumina RTA 1.12.4.2, demultiplexing by CASAVA 1.8 (Illumina) and alignment of sequence reads to the reference genome GRCh37/hg19 by BWA [10]. Picard was used for removing PCR duplicates [11]. The Genome Analysis Toolkit (GATK) was applied for base quality score recalibration, insertion and deletion (INDEL) realignment, and single-nucleotide polymorphism (SNP) and INDEL discovery [12,13]. Annotation of sequence variants was performed using Annovar [14]. First, a candidate gene approach was attempted. The Human Genome Mutation Database (HGMD) [15] and Online Mendelian Inheritance in Man (OMIM) [16] were searched for genes associated with visceral neuropathy, pseudo-obstruction or pseudo-obstruction. The patient was examined for potential deleterious mutations in the genes DCHS2, EDN3, EDNRB, FLNA, MTBP51, POLG, RET, SOX10, TCAP, TKT, and TYPY (also known as TP or ECGF1). The chromosomal regions 3p21, 4q31-q32, 9q31, 19q12, 16q23, Xq28 were also searched, but no probable pathogenic variant was discovered. Subsequently, bioinformatic filtering of whole exome data was performed using Filtus [17] software. Integrative Genomics Viewer (IGV) was used for visualization of NGS data [18]. It was assumed that the pathogenic mutation would change an amino acid or splice site (see Table e1). Furthermore, any mutations causing intestinal pseudo-obstruction are expected to be rare, and consequently variants with prevalence above 0.1% in online databases containing normal genetic variation (Exome Variant Server and 1000 Genomes project) were removed. Also, all variants present in 100 in-house exomes were removed. It was assumed that the pathogenic mutation would be heterozygous in the proband and absent in the healthy mother. This resulted in a list of variants in 157 genes, and a search for these genes in the Human Genome Mutation Database revealed approximately 200 associated phenotypes/disorders [19]. All phenotypes were evaluated manually, and “Visceral myopathy, familial” was the only one considered interesting. The gene associated with familial visceral myopathy was Enteric smooth muscle actin γ2 (ACTG2) [20]. The proband harbored three variants in ACTG2, but only one, c.442C>A:p.Arg148Ser (NM001615) was not present in the healthy mother. It was apparently heterozygous and this was confirmed by Sanger sequencing. The mutation was recently reported in a family segregating with autosomal dominant familial visceral myopathy [20].

References

13 McKenna A, Hanna M, Banks E et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010; 20: 1297–1303
16 Online Mendelian Inheritance in Man, OMIM. Available from: http://omim.org/
Fig. e2  Intraoperative photograph of the abdomen. Two lateral mini-laparotomy incisions were made.

Fig. e5  Postoperative photograph of the abdomen 4 years after the last operation.

Table e1  Criteria and filter settings used for in silico filtering of genetic variants. The number of genes containing filtered variants are shown for the proband and her healthy mother, as well as the number of genes fitting autosomal dominant inheritance.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Filter</th>
<th>Genes in proband, n</th>
<th>Genes in healthy mother, n</th>
<th>Genes fitting autosomal dominant inheritance, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Located in/near coding regions</td>
<td>Exonic or splice site</td>
<td>10191</td>
<td>10400</td>
<td>3313</td>
</tr>
<tr>
<td>Change amino acid sequence of protein</td>
<td>Not synonymous SNV</td>
<td>6762</td>
<td>6970</td>
<td>2131</td>
</tr>
<tr>
<td>Rare in online variant databases</td>
<td>ESP65001 and 1000G2&lt;0.001</td>
<td>1052</td>
<td>1095</td>
<td>548</td>
</tr>
<tr>
<td>Minimum technical quality (depth)</td>
<td>Depth &gt; 5x</td>
<td>967</td>
<td>1049</td>
<td>463</td>
</tr>
<tr>
<td>Rare in Norwegian population</td>
<td>Not in in-house variant database</td>
<td>179</td>
<td>218</td>
<td>157</td>
</tr>
</tbody>
</table>

SNV, single nucleotide variation.

1 1000 Genomes Project, http://www.1000genomes.org/

2 ESP6500 Exome Sequencing Project (6500 individuals), http://evs.gs.washington.edu/EVS/