HHS Public Access

Author manuscript

J Clin Immunol. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:

J Clin Immunol. 2016 January ; 36(1): 19–27. doi:10.1007/s10875-015-0225-6.

A Multiplex Kindred with Hennekam Syndrome due to Homozygosity for a *CCBE1* Mutation that does not Prevent Protein Expression

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Abstract

Collagen and calcium-binding EGF domain-containing protein 1 (*CCBE1*) bi-allelic mutations have been associated with syndromes of widespread congenital lymphatic dysplasia, including Hennekam Syndrome (HS). HS is characterized by lymphedema, lymphangiectasia, and intellectual disability. *CCBE1* encodes a putative extracellular matrix protein but the HS-causing mutations have not been studied biochemically. We report two HS siblings, born to consanguineous parents of Turkish ancestry, whose clinical phenotype also includes protein losing enteropathy, painful relapsing chylous ascites, and hypogammaglobulinemia. We identified by whole exome and Sanger sequencing the homozygous CCBE1 C174Y mutation in both siblings. This mutation had been previously reported in another HS kindred from the Netherlands. In over-expression studies, we found increased intracellular expression of all forms (monomers, dimers,

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Compliance with Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Conflict of Interest

The authors declare they have no competing financial interests.

trimers) of the CCBE1 C174Y mutant protein, by Western blot, despite mutant mRNA levels similar to wild-type (WT). In addition, we detected increased secretion of the mutant CCBE1 protein by ELISA. We further found the mutant and WT proteins to be evenly distributed in the cytoplasm, by immunofluorescence and confocal microscopy. Finally, we found a strong decrease of lymphatic vessels, with a corresponding diminished expression of CCBE1, by immunohistochemistry of the patients' intestinal biopsies. In contrast, mucosal blood vessels and muscularis mucosae showed normal CCBE1 staining. Our findings show that the mutant CCBE1 C174Y protein is not loss-of-function by loss-of-expression.

Keywords

Hennekam Syndrome; lymphedema; lymphangiectasia; *CCBE1*

Introduction

Hennekam Syndrome (HS) (OMIM entry #235510) was first defined in 1989 by Raoul C. Hennekam and colleagues, describing autosomal recessive (AR) intestinal lymphangiectasia and lymphedema associated with intellectual disability, in two pairs of siblings (two males and two females) from separate nuclear families within the same consanguineous kindred [1]. Subsequent publications described other kindreds, better delineating the heterogeneous clinical manifestations of this syndrome including lymphedema of varying severity and locations, lymphangiectasia of different organs, intellectual disability of varying severity, some with and without facial dysmorphy, and a few other features (seizure disorders, hypothyroidism, and congenital skull, skeletal, cardiac, and renal anomalies) [2–10]. However, it was not until the era of next generation sequencing that the genetic etiology of this disease started to be deciphered.

Connell et al. and Alders et al. first reported patients with bi-allelic *CCBE1* mutations (OMIM entry # 612753) [4, 11, 12]. HS is a genetically heterogeneous disease as up to 45 patients with HS have been reported in the literature to date, including only 13 patients (29 %) with bi-allelic *CCBE1* coding mutations (Fig. 1) [4, 11, 12]. Regulatory mutations have not been excluded in other patients. However, more recently, 9 HS patients with bi-allelic *FAT4* mutations were described [13]. Interestingly, HS is allelic at the *FAT4* locus to Van Maldergem Syndrome, which shares some features with HS, such as intellectual disability and facial dysmorphy, but not features of lymphatic dysplasia including lymphedema and lymphangiectasia [13]. The genetic etiology of about half of HS patients remains unknown.

The collagen and calcium-binding EGF domain-containing protein 1 (*CCBE1*) gene encodes a putative extracellular matrix protein crucial for lymphangiogenesis. CCBE1 is secreted into extracellular matrix and interacts with key connective tissue components [11, 14, 15]. Functional analysis of *CCBE1* mutations demonstrated that mutant zebrafish lacked lymphatic vessels, developed edema, and lacked thoracic ducts [11, 16]. Furthermore, *Ccbe1* mutant mice also lack lymphatic vessels and develop edema [14]. Hence, *CCBE1* has been shown to be a master gene across species for the development of lymphatic vessels,

accounting for human *CCBE1* mutations being associated with widespread lymphatic dysplasia.

Interestingly, other bi-allelic *CCBE1* mutations have been described in one case of Aagenaes syndrome, a rare AR disorder, characterized by severe chronic lymphedema and neonatal intrahepatic cholestasis, without lymphangiectasia or mental retardation [17]. Fetal hydrops has also been documented in unrelated children, each with a sibling suffering from HS or Aagenaes syndrome [4, 17]. The *CCBE1* mutations causing any of the three associated conditions have not been studied experimentally. Currently, the evidence supporting the disease-causing nature of these mutations relies on the rarity of the mutant alleles, their in silico prediction to be deleterious, their segregation with phenotype in a model of AR inheritance, their occasional recurrence in unrelated kindreds, and the significant proportion of kindreds carrying mutations.

The pathogenesis of the three related conditions caused by CCBE1 mutations also remains elusive. Mutations in genes other than *CCBE1* can underlie these and other, related conditions. Indeed, mutations of genes along the VEGFR3 signaling pathway, including *FLT4, GCJ2,* and *PTPN14* have been implicated in isolated primary lymphedema, whereas mutations in *FOXC2, SOX18, GATA2, IKBKG, FAT4,* and *CCBE1* have been implicated in syndromic primary lymphedema [18–20]. In contrast, there are no genetic etiologies for patients with isolated or syndromic primary intestinal lymphangiectasia [21, 22]. In this context, we studied two siblings with HS and attempted to characterize the *CCBE1* mutations found.

Materials and Methods

Case Report

We report two siblings, P1, a 22 year-old sister and P2, a 21 year-old brother, born to consanguineous parents of Turkish ancestry living in Germany. Both siblings since infancy have developed a distinctive clinical phenotype marked by limb lymphedema, relapsing chylous ascites (Fig. 2), inguinal hernias, enteric protein loss, hypoproteinemia, and hypogammaglobulinemia. Both siblings had a history of mild development delay requiring extra scholarly assistance to pass primary school, compared to their healthy unaffected siblings. Furthermore, both siblings had speech delays up until age 4 years with gain of full age-appropriate speech capabilities by age 7 years. They were subsequently able to complete all educational requirements. Of note, P1 and P2 do not display any overt facial dysmorphy (patients refused publication of photographs or further genetic subspecialty consultations). In adulthood, both P1 and P2 developed hypothyroidism treated with levothyroxine.

P1 was born full-term with normal weight for gestational age. At 3 weeks of age, she developed failure to thrive, protein losing enteropathy, and massive generalized edema. At that time, her laboratory evaluations were notable for low total serum protein of 3.6 gm/dL (normal range 4.7–6.7 gm/dL), low IgG level of 58.6 mg/dL (normal range 200–800 mg/dL), and high levels of alpha-1 antitrypsin of 20 mg/gm (normal range <1 mg/gm) of stool. Her complete blood count, electrolyte, and liver function profiles were all normal. Subsequent radiologic imaging showed enlarged plicae circulares indicating intestinal

lymphangiectasia. Bilateral inguinal hernia required surgical correction by 5 months of age. She was treated with albumin and immunoglobulin replacements (750 mg/kg every 2 weeks), aggressive diuresis (furosemide and aldactone), and modified diet (20 % protein, 50 % carbohydrate, 30% fat of which 50% are composed of medium-chain triglycerides) with significant improvement in edema, protein losing enteropathy, and hypogammaglobulinemia, and hence doubling of birth weight by age 6 months. Despite these improvements, P1's childhood and adulthood has been complicated by relapsing painful chylous ascites, frequent peritoneal drainage, and hospital admissions, with missed days from school and work. Octreotide therapy was started at age 21 years, with moderate improvement of symptoms and therapy was truncated after 6 months due to side effects (abdominal pain and diarrhea). At the age of 21 years, diet was optimized with restriction of dietary fat but enhanced medium-chain triglycerides intake. On this optimized diet, peripheral lymphedema regressed with normalization of serum protein (7.8 gm/dL) and immunoglobulin (1400 mg/dL) levels, without necessitating albumin or immunoglobulin replacement for 15 months. Nevertheless, she is still suffering from chylous ascites, but the frequency of abdominal paracentesis has significantly decreased.

P2 was born premature at 32 weeks of gestational age with weight appropriate for gestational age. Five minutes after birth, his neonatal course was complicated by severe respiratory distress requiring intubation and mechanical ventilation. He had no cardiac anomalies. At birth, P2 already had profound ascites and eyelid edema; limb edema developed at 3 weeks of age. Pre-treatment laboratory evaluations from P2's childhood are not available; however he had hypoproteinemia and hypogammaglobulinemia. Subsequent radiologic imaging of the small intestine revealed enlarged Kerckring's circular folds in the jejunum and ileum indicating intestinal lymphangiectasia. In addition, he had inguinal and umbilical hernia requiring surgical correction by 4 months of age. At 11 weeks of age he had hypoproteinemia of 4.6 g/dL (normal range 4.7–6.7 gm/dL) and hypoalbuminemia of 2.7 gm/dL (normal range 3.6–5.0 gm/dL). While immunoglobulin levels (363 mg/dL) were still within normal range (200–550 mg/dL) corrected for age of 11 months, he developed progressive hypogammaglobulinemia.

P2 was also treated with albumin and immunoglobulin replacements, diuretics, and modified diet (20 % protein, 50 % carbohydrate, 30 % fat of which 50 % are composed of medium-chain triglycerides) with improvement in edema, protein losing enteropathy, and hypogammaglobulinemia. However, he continued to have growth delay until puberty, after which both height and weight normalized for age. P2, like his sister, had his childhood and adulthood complicated by relapsing painful chylous ascites, frequent peritoneal drainage, and hospital admissions, with missed days from school and work. At age 20 years, intermittent octreotide therapy over several months provided moderate improvement and was stopped due to side effects (diarrhea and abdominal pain). Potentially due to his lower compliance to dietary fat intake with enriched medium-chain triglycerides intake, he continues to have moderate edema of bilateral lower extremities, hypoproteinemia (5 gm/dL), and hypogammaglobulinemia (615 mg/dL) requiring intravenous immunoglobulin substitution (30 gm every 3 to 4 weeks).

Clinically, the history of these patients suggests that a dietary treatment with substitution of lipids by medium-chain triglycerides may have an important influence on the course of illness and the development of symptoms in patients with CCBE1-caused lymphatic dysplasias. Most recent peripheral blood immunologic and peritoneal fluid laboratory evaluations for P1 and P2 are shown in Tables 1 and 2, respectively. CD4+ and CD8+ T cell were normal; whereas B cells were slightly elevated. NK-cells were initially decreased in both P1 and P2 (Table 1), however later spontaneously normalized in P1 at follow up (Table 1). Serum inflammatory markers were elevated during periods of ascites exacerbation (Table 1), with chylous ascitic fluid high in TNF-α and interleukin-6 (Tables 2 and 3). Levels of vitamins B12, C, E and A were within normal range with vitamin supplementation, but both P1 and P2 had persistent vitamin D deficiency (not shown).

Sequencing

The consanguinity of the patients' parents suggests an autosomal recessive mode of inheritance. Whole exome sequencing (WES) performed at New York Genome Center [23, 24] was completed using sequencing platform Illumina HiSeq2500 with exome kit Agile SureSelectXT Human All Exon V4+ UTRs. Variant analysis of WES data filtered for shared homozygous variants between P1 and P2 identified the homozygous CCBE1 C174Y mutation in both siblings.

The *CCBE1* mutation in both P1 and P2, and familial segregation was confirmed by Sanger sequencing using forward (CCAGATATTGATGAGTGTGCC) and reverse (GGCACTTGGAAACTTTAGAC) primers.

Transfection Assays

Site-directed mutagenesis using forward (GAAGATGATGGGAAGACATATACCAGGGGAGACAAATATCC) and reverse (GGATATTTGTCTCCCCTGGTATATGTCTTCCCATCATCTTC) primers was performed on complete open reading frame wild-type (WT) CCBE1 sequence in pCMV6 entry vector with C-terminal Myc-DDK tag (OriGene Technologies). Overexpression of the WT and mutant CCBE1 C174Y protein were accomplished by 24–48 h transfection of constructs into HEK293T cells using X-tremeGENE 9 (Roche).

RNA Isolation and Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was isolated from transfected HEK293T cells using RNeasy Mini kit (Qiagen). The concentration and purity of the RNA were calculated using NanoDrop 1000 spectrophotometer. cDNA was synthesized by reverse transcription using 2 μg of total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). We quantified the induction of CCBE1 WT and mutant mRNA expression in transfected HEK293T cells by qRT-PCR using CCBE1 probe (Life Technologies, TaqMan Gene Expression Assays), normalized to GAPDH (Life Technologies, TaqMan Gene Expression Assays). The reactions were performed in triplicates on a 7500 Fast Real-Time PCR System (Applied Biosystems).

Western Blotting

HEK293T cell pellets were washed in phosphate-buffered saline (PBS), lysed in sample buffer supplemented with protease inhibitors, and concentration determined by assay. Reduced (by addition of β-mercaptoethanol) and non-reduced protein was then loaded into the wells of a gel, for SDS-PAGE and western blotting. Proteins were stained using antibodies rabbit polyclonal anti-CCBE1 IgG at 1:2,000 dilution (HPA041374, Sigma-Aldrich), donkey polyclonal anti-rabbit IgG HRP at 1:10,000 dilution (GE Healthcare), mouse monoclonal anti-FLAG IgG HRP at 1:5,000 dilution (Sigma-Aldrich), and rabbit polyclonal anti-GAPDH HRP IgG at 1:1,000 dilution (Santa Cruz). The bands were detected by enhanced chemiluminescence (ECL). Western blotting was performed in triplicates.

Immunofluorescence

Overexpression of the WT and mutant CCBE1 C174Y protein were accomplished by 24–48 h transfection of constructs into HEK293T cells using X-tremeGENE 9 (Roche). The cells were fixed with 4 % paraformaldehyde, permeabilized with 0.1 % Triton X-100, incubated with primary antibody rabbit polyclonal anti-CCBE1 IgG at 1:2,000 dilution (HPA041374, Atlas Antibody, Sigma-Aldrich) in 4 % goat serum, washed with PBS, and incubated in secondary antibody Alexa Flour 488 goat anti-rabbit IgG at 1:800 dilution (Invitrogen). Cells were counterstained with nuclear stain DAPI at 1:5000 dilution (Invitrogen), washed, and mounted with ProLong Diamond (Life Technologies). Cells were visualized by Inverted TCS SP8 laser scanning confocal microscope and Leica Application Suite X.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections from duodenal biopsies were stained on a BenchMark ULTRA automated instrument (Ventana Medical Systems, Inc.). Heat-induced epitope retrieval was performed using CC1 at 95 °C for 64 min followed by antibody incubation at 36 °C for 16 min. The rabbit polyclonal anti-CCBE1 antibody was used at 1:500 dilution (HPA041374, Atlas Antibody, Sigma-Aldrich). The lymphatic specific mouse monoclonal D2–40 antibody was used at a dilution of 1:50 (Zytomed). D2–40, in normal mesenchymal tissue of the gastrointestinal tract, is highly specific for lymphatic endothelial cells and does not cross react with any other tissue type. Binding of anti-CCBE1 or D2–40 primary antibodies to the antigen was visualized using ultraView Universal DAB Detection Kit, an indirect, biotin-free detection system (Ventana). Subsequently sections were counterstained with Haematoxylin and Bluing Reagent (Ventana).

Enzyme-Linked Immunosorbent Assay (ELISA)

Cultured media from transfected HEK293T cells were concentrated by using Amicon Ultra-2 Centrifugal Filter Unit with Ultracel-10 membrane (EMD Millipore Corporation). All concentrations were measured using Bradford assay followed by a standard curve. A sandwich ELISA of concentrated supernatant (170 μg of total protein) was performed using 2.5 μg/mL of rabbit polyclonal anti-human CCBE1 capture antibody (Atlas Antibodies, HPA041374), and 1:20, 000 monoclonal anti-Flag HRP detection antibody (Sigma-Aldrich, A8592). A sensitive colorimetric substrate for ELISA HRP detection using the 3,3′,5,5′-Tetramethylbenzidine (TMB) Microwell Peroxidase Substrate System (Kirkegaard & Perry

Laboratories, Inc.) was utilized. Absorbance data were read by VICTOR X4 (PerkinElmer, Inc.). Statistical analysis with unpaired *t* test with 95 % confidence interval was performed using GraphPad PRISM (version 5). All experiments were done in triplicates.

Results

Homozygous CCBE1 C174Y Mutation in P1 and P2

Whole exome sequencing (WES) identified a shared homozygous cytosine to thymine mutation on chromosome 18 position 57,134,003 (reference genome GRCh37/hg19) in P1 and P2, resulting in a cysteine to tyrosine missense mutation at amino acid residue 174 (C174Y). This non conservative mutation is located in the calcium binding EGF domain of the protein [25] (Fig. 1). The cysteine is conserved across species including vertebrates (NCBI HomoloGene). This variation has never been reported in public databases of about 60,000 individuals (Exome Variant Server ESP6500 data release, 1000 Genomes phase 3 release, ExAC data version 0.2) or in our own in-house database of over 3,000 exomes. This amino acid residue is highly conserved (NCBI HomoloGene) and the missense mutation is predicted to be possibly damaging by Polyphen-2 and deleterious by Sift, with an intermediate CADD score of 14.61 [26–28]. The *CCBE1* gene has a GDI score of 2.75, predicted to be “medium” damage for all disease types [29]. The same homozygous mutation was previously reported in a patient with HS from the Netherlands [12]. Sanger sequencing confirmed AR segregation; both unaffected siblings and parents are heterozygous for this mutation, whereas the two patients are homozygous (Fig. 3). WES did not identify mutations in *FAT4*, in both P1 and P2. These data, together with the previously reported mutations in CCBE1, including C174Y itself (Fig. 1), strongly suggested that the mutation is disease-causing.

In Vivo and Ex Vivo Expression Profile of Mutant C174Y CCBE1

We next attempted to study the molecular mechanism by which these mutations are disease-causing. Whereas the genotype-phenotype association has been previously documented, the molecular mechanism has not been reported for the nine disease-causing mutations in the literature, including C174Y (Fig. 1) [4, 11, 12]. Western blotting of transfected HEK293T cells with overexpression of WT and C174Y mutant plasmids shows moderately increased intracellular expression of mutant CCBE1 C174Y protein compared with WT, in both reduced and non-reduced conditions (Fig. 4a). This difference in protein expression is not seen at the mRNA level as qPCR showed similar mRNA transcript levels of the mutant CCBE1 C174Y compared with WT (Fig. 4b). Immunofluorescence and confocal microscopy identified both WT and mutant CCBE1 C174Y to be expressed in the cytoplasm, and not the nucleus (Fig. 5). In the same experimental setting, ELISA of cultured supernatants of transfected HEK293Tcells shows increased CCBE1 C174Y protein compared with WT (Fig. 6). Finally, immunohistochemistry of both patients’ duodenal biopsy with a lymphatic vessel-specific antibody D2–40 demonstrated significant paucity of lymphatic structure compared with normal control (Fig. 7, P2 not shown). The corresponding areas were not marked with a CCBE1-specific antibody, contrasting with mucosal blood vessels and muscularis mucosae that showed normal CCBE1 staining (Fig. 7).

Discussion

We report two siblings with HS from a consanguineous kindred of Turkish ancestry living in Germany. They are homozygous for the CCBE1 C174Y mutation, which was discovered by next generation sequencing [30, 31]. We further show that this mutation does not impair protein expression; it even results in moderately enhanced cellular expression of all forms of the protein (monomer, dimer, trimer) *in vitro*. Although the collagen domains of CCBE1 have been demonstrated to be more critical in lymphangiogenic activity *in vivo*, compared with the calcium-binding EGF domain [32], not all HS-causing variations of mutant CCBE1 proteins have been characterized in previous publications [4, 11, 12, 17]. The WT CCBE1 protein can be secreted [11, 14, 15]. Our results show somewhat increased secretion of mutant C174Yprotein compared to WT *in vitro*.

The interaction of secreted CCBE1 with key extracellular matrix connective tissue components may be necessary for the structural integrity of lymphatic cells [14]. Hence, we hypothesize that although CCBE1 C174Y molecules may be secreted, it fails to adopt a conformation suitable for their biological activity, and increased secretion of mutant protein may be a compensatory mechanism for a non-functional CCBE1 protein. Alternatively, the mutant protein may be rapidly degraded in physiological conditions *in vivo* explaining diminished CCBE1 staining in P1's immunohistochemistry (Fig. 7), which is not reflected by experimental conditions *in vitro*. Furthermore, CCBE1 has been shown to interact and enhance the activity of metalloproteinase ADAMTS3, which in turn cleaves the N-terminal part of pro-VEGFC (inactive form) to make VEGF-C (active form) [15]. However, there was no difference in VEGF-C levels in the serum of P1 and P2 compared to healthy controls (data not shown). In any case, further studies are needed to explore in more depth both the immunological, molecular and cellular basis of this disease.

Homozygosity for the CCBE1 C174Y mutation had been previously reported in a 2-year-old girl from the Netherlands with HS (P3) (Fig. 1) [12]. Similar to P1 and P2, P3 was also affected by lymphedema, lymphangiectasia, intellectual disability, growth and developmental delay (Table 1) [12]. However, unlike P1 and P2, P3 had facial dysmorphy, metopic craniosynostosis, and adrenal neuroblastoma, and had not chylous ascites or inguinal hernias (Table 1) [12]. HS with normal face has been rarely reported and only prior to the identification of the first CCBE1 mutations [7]. Our results therefore extend the phenotype associated with the very same CCBE1 bi-allelic mutation. Previous studies have suggested that while the "core" phenotype (lymphedema, lymphangiectasia) of HS is fully penetrant globally, although with variable expressivity, other phenotypes are incomplete in penetrance and may or may not be associated with HS [4, 12]. We report the first occurrence of the same CCBE1 C174Y mutation in two kindreds. Our findings suggest that phenotypic variability is not necessarily a consequence of allelic heterogeneity at the *CCBE1* locus.

Instead, our findings suggest that genetic modifiers or environmental factors may be responsible for the heterogeneity of the phenotypes seen with incomplete penetrance in patients with mutant *CCBE1*-caused lymphatic dysplasias. Moreover, Shah et al. reported homozygous CCBE1 L133P mutation in 2 siblings, one with lymphedema-cholestasis (Aagenaes syndrome), and the other with fetal hydrops[20]. Furthermore, Connell et al.

reported homozygous CCBE1 C75S mutation in a kindred with three affected siblings, all of whom presented *in utero* with fetal hydrops, but two died and the third sibling survived and displayed HS [4]. While lymphatic dysplasia may be commonly associated with *CCBE1* mutations, *CCBE1* mutations are not a common cause of fetal hydrops [17, 33]. The fact that the same mutation can give rise to different phenotypes implies that environmental forces and/or loci other than CCBE1 are involved.

To conclude, known patients with CCBE1-caused HS share the same core clinical phenotype of lymphedema and lymphatic dysplasia, which displays complete penetrance. Other phenotypes in CCBE1-mutated HS patients (i.e. intellectual disability) are not fully penetrant. Alternatively, when taken as a whole, HS can be seen as displaying variable expressivity with complete penetrance of the core phenotype. Allelic heterogeneity at the *CCBE1* locus and genetic modifiers, at loci other than *CCBE1*, may contribute to phenotypic heterogeneity, together with somatic processes or environmental influences. The CCBE1 C174Y mutation confers increased intracellular protein expression and secretion *in vitro* concurrent with a paucity of CCBE1 protein *in vivo* by immunohistochemistry, suggesting that a lack of functional normal protein may underlie the pathogenesis of HS, at least in the 3 patients from the two kindreds homozygous for this mutation. The other HS-causing mutations reported in the literature have not been biochemically studied [4, 12]. Further studies are needed to decipher the molecular and cellular mechanisms by which CCBE1 C174Y and other mutants cause HS. For example, biochemical studies of other CCBE1 mutations (Fig. 1) [4, 12], including secretion and degradation studies, are necessary. Our study shows that at least the C174Y CCBE1 mutation is not loss-of-expression. It could be loss-of-function by misfolding and impaired functional activity.

Acknowledgments

We warmly thank the patients for their participation. This work was supported by the National Center for Research Resources and the National Center for Advancing Sciences (NCATS) of the National Institutes of Health (NIH) (grant 8UL1TR000043), the Rockefeller University, INSERM, Paris Descartes University, and the St. Giles Foundation.

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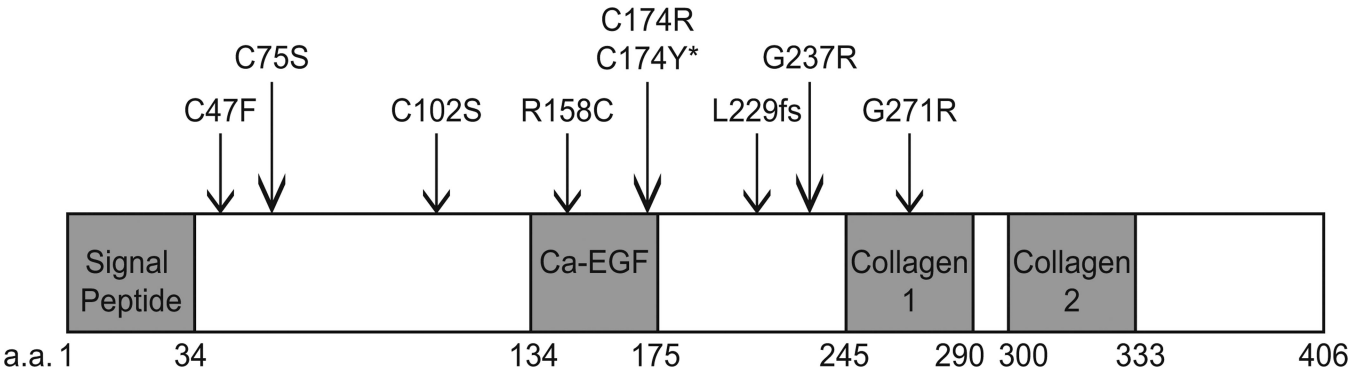

Fig. 1.

CCBE1 protein domains [25], and reported human mutant alleles [4, 12]. *Homozygous in patients P1, P2, and previously reported P3 [12]

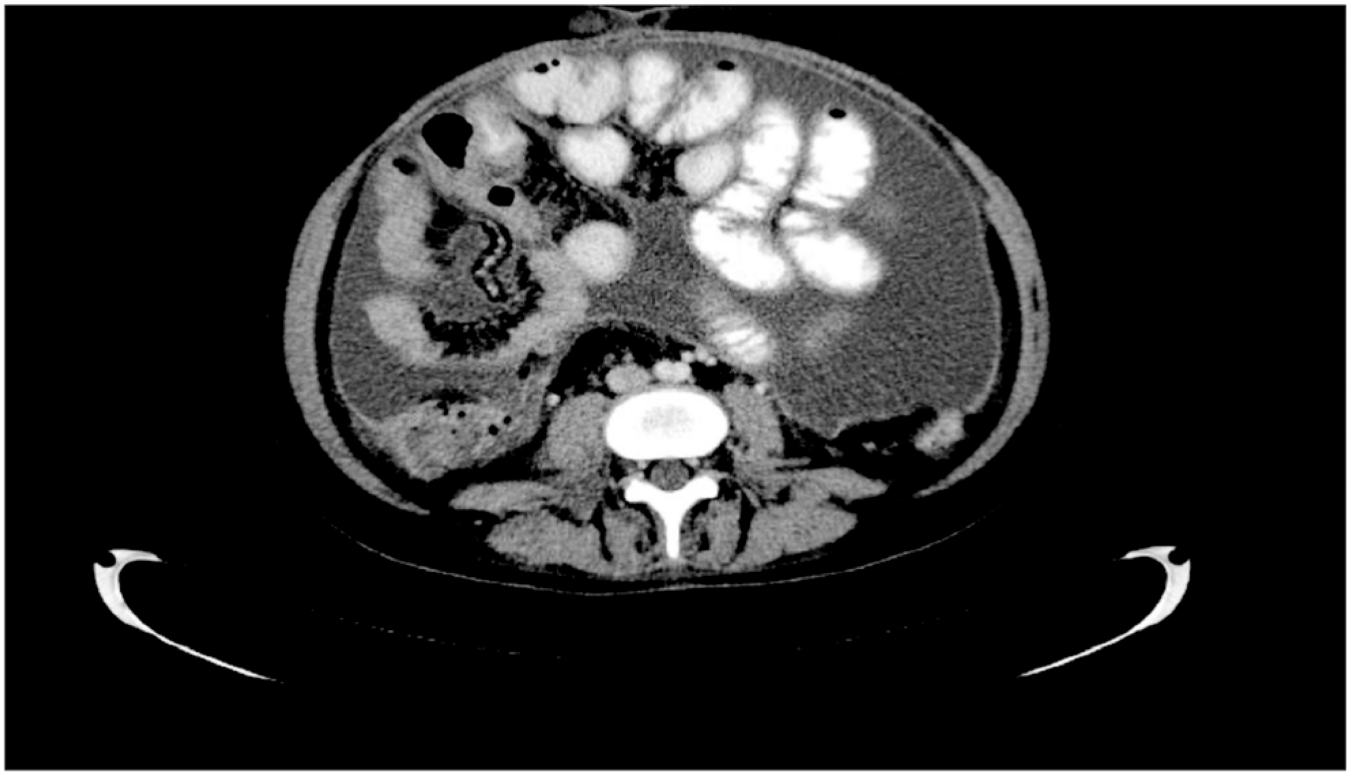

Fig. 2.
Patient P1, massive chylous ascites (CT scan)

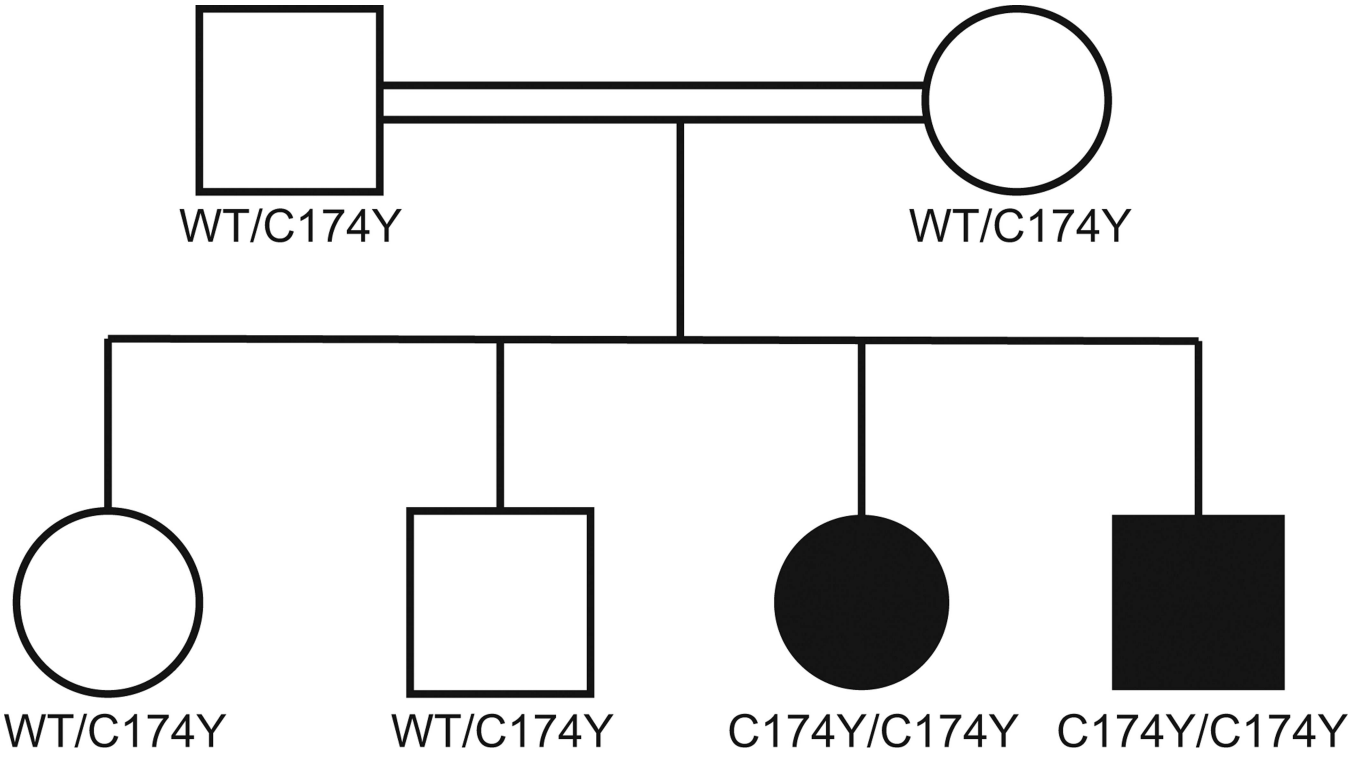

Fig. 3.
Familial segregation of CCBE1 C174Y mutation

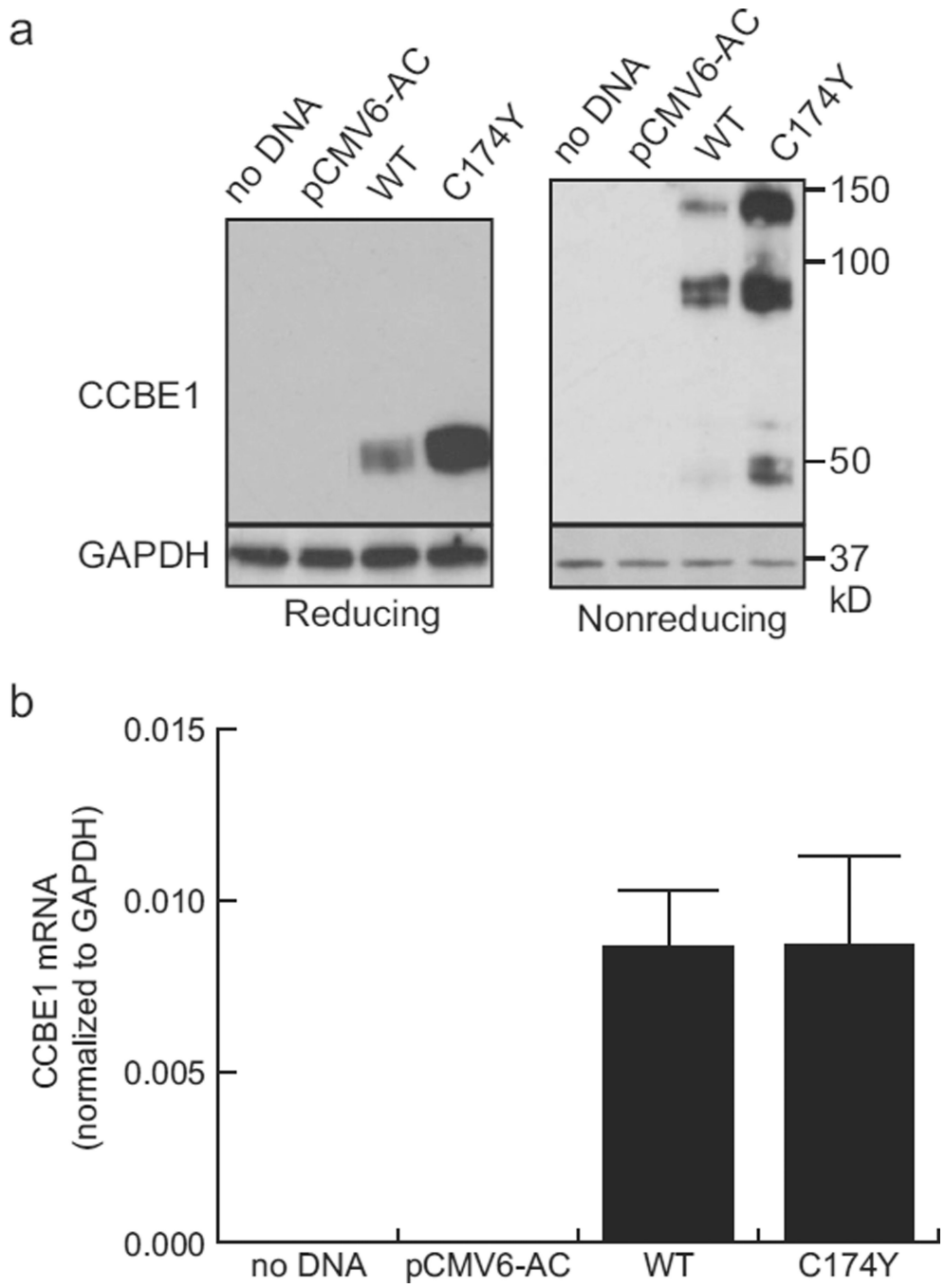

Fig. 4.

a Increased CCBE1 C174Y mutant expression of all forms (monomer, dimer, trimer) compared to wild-type, in transfected HEK293T cells, performed in triplicates (not shown) **b** No difference in mRNA level between the CCBE1 C174Y mutant and wild-type, in transfected HEK293T cells, performed in triplicates (not shown)

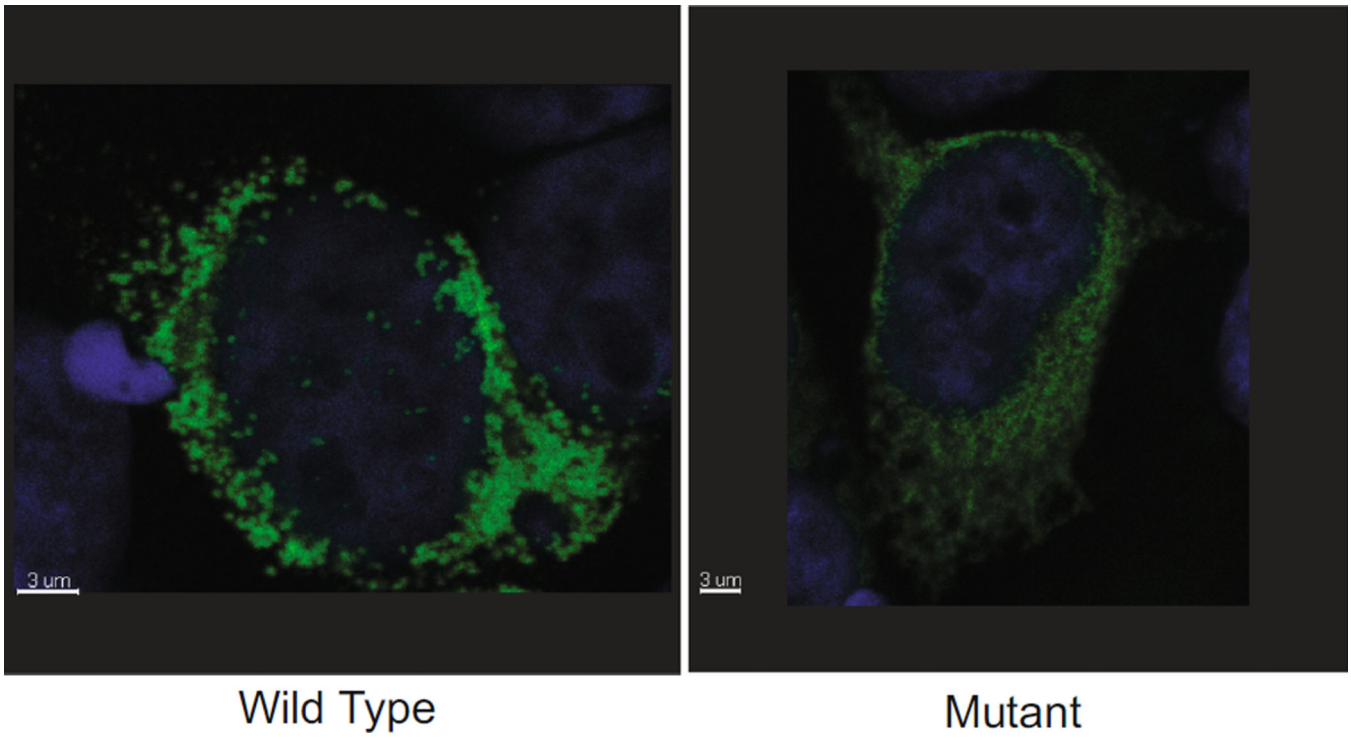

Fig. 5.
Confocal microscopy, CCBE1 in *green*, DAPI in *blue*

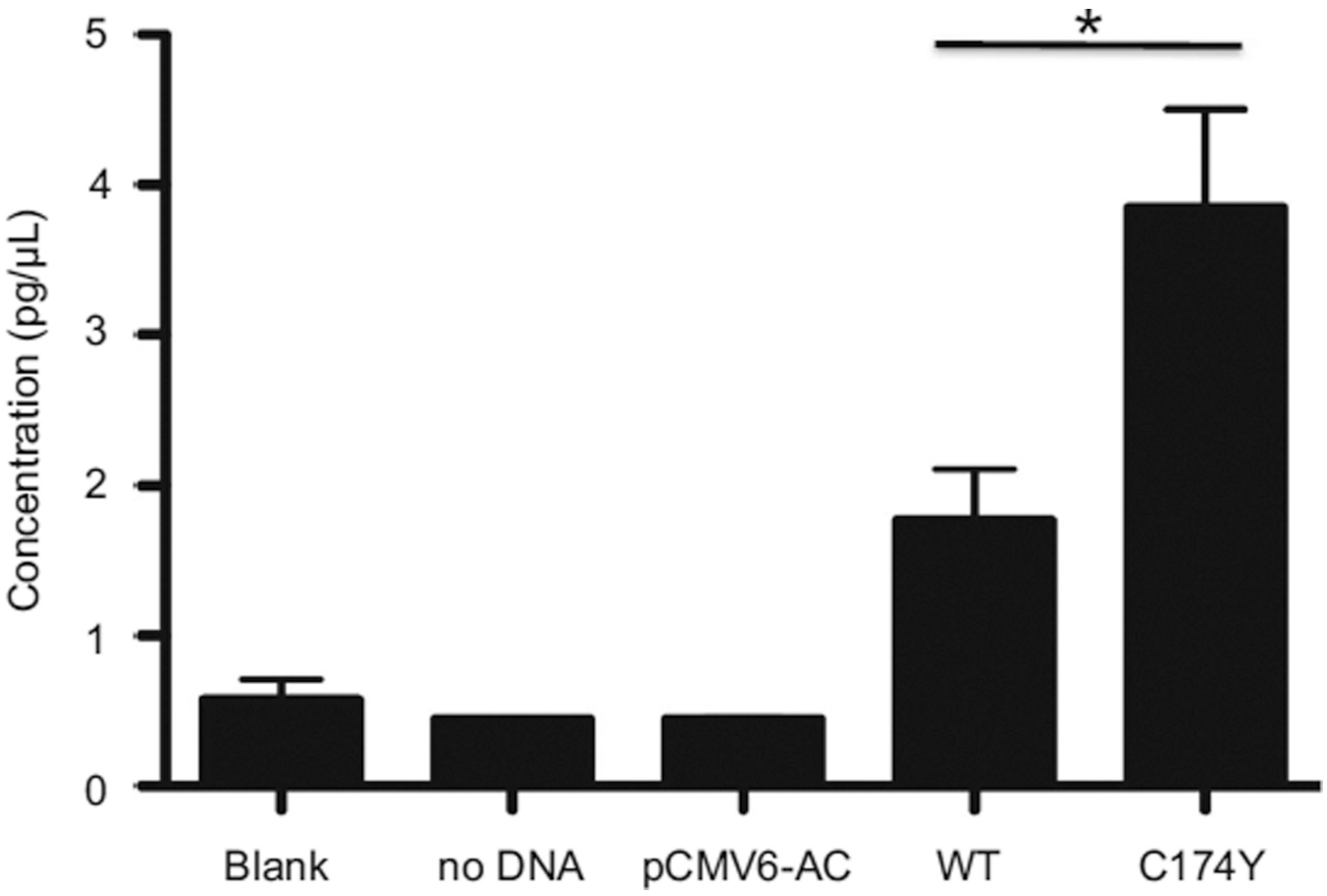

Fig. 6.

Increased CCBE1 C174Y mutant secretion compared to wild-type, in cultured supernatants of transfected HEK293T cells, performed in triplicates (not shown). * $p = 0.048$, unpaired t test

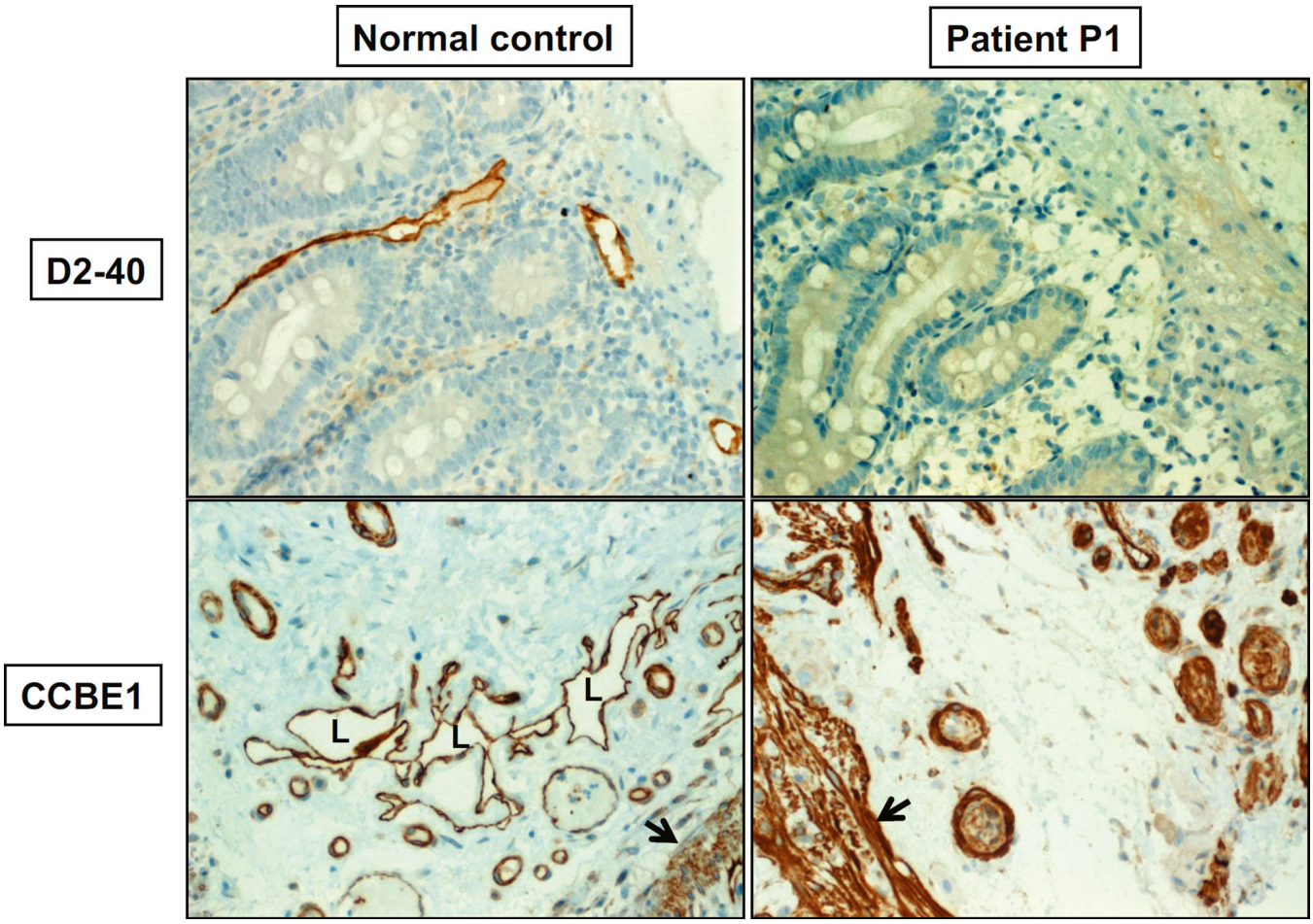

Fig. 7.

Intestinal (duodenum) lymphatic CCBE1 and D2–40 staining in age-matched normal control compared to patient P1, at 400× magnification. Patient P1 lacks of intestinal lymphatic structures. *L* denotes lymphatic structures. *Arrow* denotes muscularis mucosa layer

Table 1

Laboratory parameters of P1 and P2 in serum

Specimen type	P1	P2	Normal range
Hemoglobin	12.5	13.7	12–16 gm/dL
Platelets	147	274	140–400 × 10^3/μL
Leukocytes	5.3	9.87	4–10 × 10^3/μL
Lymphocytes (%)	**21.9**	**19.7**	25–40 %
CD3+ T cells (Absolute)	1255	1463	600–2,000/μL
CD4+ T cells (Absolute)	816	855	430–1,300/μL
CD8+ T cells (Absolute)	426	494	200–700 /μL
B cells (Absolute)	**373**	**380**	60–300/μL
CD16+ NK cells (Absolute)	125 *1*	**57**	120–350/μL
Immunoglobulin G	1400	615	751–1,560 mg/dL
Immunoglobulin A	178	104	82–453 mg/dL
Immunoglobulin M	**422**	136	46–304 mg/dL
Immunoglobulin E	23.7	54.3	0–100 U/mL
Immunoglobulin G 1	**934**	437	280–800 mg/dL
Immunoglobulin G 2	397	164	115–570 mg/dL
Immunoglobulin G 3	**185**	83.2	24–125 mg/dL
Immunoglobulin G 4	<7	13.4	5.2–125 mg/dL
C Reactive Protein	1.7–**31.2**	3.6–**94.2**	0–8 mg/L
TNF-α	5.6–**12.3**	7.1–10.3	0–8.1 pg/mL
Interleukin-6	2.2–**18.8**	**22.6–88.1**	0.1–15 pg/mL
IFN-γ	0.1–**8.3**	2.5–**9.5**	<1 pg/mL
IFN-α	ND	0.1–**46.4**	<1 pg/mL

Shown are representative laboratory values obtained at different time points of disease (intestinal lymphangiectasia) exacerbation within a 15-month period. Abnormal values are indicated in bold. ND, not done.

*1*P1 had low (94/ μL) CD16+ NK cells 1 year prior to laboratory value shown

Table 2

Laboratory parameters of P1 and P2 in peritoneal fluid

Specimen type	P1	P2	Normal range*
Triglycerides	**852**	67 [1] / **975** [2]	<200 mg/dL
Cholesterol	200	96 [1] / 118 [2]	<200 mg/dL
Total protein	**46.3**	**31.7** [1] /**3.1** [2]	66–83 mg/dL
Leukocytes	**265**	**200** [1] / **600** [2]	4–10 × 10^3/μL
Lymphocytes (%)	32.9	**18.7**	25–40 %
Immunoglobulin G	**ND**	**528**	751–1,560 mg/dL
C Reactive Protein	0.5	ND	0–8 mg/L
TNF-α	**15**	**39.4**	0–8.1 pg/mL
Interleukin-6	**706**	**>1000**	0.1–15 pg/mL
IFN-γ	**4.4**	ND	<1 pg/mL
IFN-α	**124**	ND	<1 pg/mL
Microbiological cultures	sterile	sterile	Not applicable

Patient P2 has different time points shown:

[1] Same time point analyses as the inflammatory markers,

[2]: Representative laboratory values obtained at different time points of disease (intestinal lymphangiectasia) exacerbation within a 15-month period.

* No normal ranges available for periotoneal fluids; serum normal range values are shown

Table 3

Phenotypic comparison of three patients with CCBE1 *C174Y* homozygous mutation

	P1	P2	P3*
Gender	F	M	F
Consanguinity	+	+	+
Growth delay	+	+	+
Developmental delay	+	+	+
Facial dysmorphy	−	−	+
Lymphedema	+	+	+
Lymphangiectasia (Intestinal)	+	+	+
Chylous ascites	+	+	−
Metopic craniosynostosis	−	−	+
Adrenal neuroblastoma	−	−	+
Inguinal hernias	+	+	−

* P3 was previously reported [12]