

Evaluation of Clinical Manifestations in Patients with Severe Lymphedema with and without *CCBE1* Mutations

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Key Words

Autosomal recessive · *CCBE1* · Genotype-phenotype · Hennekam syndrome · Lymphangiectasia · Lymphatic dysplasia · Lymphedema

Abstract

The lymphedema-lymphangiectasia-intellectual disability (Hennekam) syndrome (HS) is characterised by a widespread congenital lymph vessel dysplasia manifesting as congenital

lymphedema of the limbs and intestinal lymphangiectasia, accompanied by unusual facial morphology, variable intellectual disabilities and infrequently malformations. The syndrome is heterogeneous as mutations in the gene *CCBE1* have been found responsible for the syndrome in only a subset of patients. We investigated whether it would be possible to predict the presence of a *CCBE1* mutation based on phenotype by collecting clinical data of patients diagnosed with HS, with or without a *CCBE1* mutation. We report here the results of 13 *CCBE1* positive patients, 16 *CCBE1* negative pa-

tients, who were clinically found to have classical HS, and 8 patients in whom the diagnosis was considered possible, but not certain, and in whom no *CCBE1* mutation was identified. We found no statistically significant phenotypic differences between the 2 groups with the clinical HS phenotype, although the degree of lymphatic dysplasia tended to be more pronounced in the mutation positive group. We also screened 158 patients with less widespread and less pronounced forms of lymphatic dysplasia for *CCBE1* mutations, and no mutation was detected in this group. Our results suggest that (1) *CCBE1* mutations are present only in patients with a likely clinical diagnosis of HS, and not in patients with less marked forms of lymphatic dysplasia, and (2) that there are no major phenotypic differences between HS patients with or without *CCBE1* mutations.

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Lymphedema is in most patients a secondary phenomenon and caused by external factors such as infection, trauma, surgery, or radiation. In at least 10% of patients, lymphedema is a primary and heritable condition caused by defects in the design or development of lymph vessels. To date, only a limited number of genes are known to be involved in heritable lymphedema syndromes. Congenital isolated lymphedema can be caused by mutations in the lymphatic specific growth factor VEGFC receptor *VEGFR3* [Irrthum et al., 2000; Karkkainen et al., 2000], and sometimes the lymphedema in patients with mutations in *GJC2* is congenital [Ferrell et al., 2010; Ostergaard et al., 2011]. Congenital lymphedema is frequently syndromic, and causative genes have been found for the lymphedema-distichiasis syndrome (*FOXC2*), hypotrichosis-lymphedema-telangiectasia syndrome (*SOX18*), incontinentia pigmenti (*IKBK*), Noonan syndrome (*PTPN11* and other RAS-MAPK pathway genes), congenital disorder of glycosylation type 1a (*PMM2*), and the choanal atresia and lymphedema syndrome (*PTPN14*) [Van Schaftingen and Jaeken, 1995; Fang et al., 2000; Döffinger et al., 2001; Tartaglia et al., 2001; Irrthum et al., 2003; Au et al., 2010]. Mutations in *VEGFR3* have also been found in a patient with congenital lymphedema, unusual facial morphology and distal limb defects [Ghalamkarpour et al., 2009], which may indicate that some patients designated to have isolated lymphedema may in fact have a syndromic form. Many syndromic forms are known for which the causative gene(s) have not been identified yet [Hennekam et al., 2010].

Recently, mutations in *CCBE1* were shown to cause a generalised form of syndromic lymphatic dysplasia [Alders et al., 2009; Connell et al., 2010]. This syndrome was

first described in 1989 [Hennekam et al., 1989] in a highly inbred family with 3 affected children who had generalised lymphedema predominantly in limbs, intestinal lymphangiectasia, distinctive facial features, and variable intellectual disabilities. Since then about 30 patients with this lymphedema-lymphangiectasia-intellectual disability or Hennekam syndrome (HS) have been reported [Van Balkom et al., 2002].

The *CCBE1* gene is a putative extracellular matrix protein and crucial for lymphatic development in zebrafish [Hogan et al., 2009]. Mutations in *CCBE1* were detected in only a subset (23%) of patients clinically diagnosed with HS suggesting genetic heterogeneity [Alders et al., 2009]. Here we report on the results of molecular analysis in a series of HS patients and a series of patients with clinically less marked lymphatic dysplasia, and the analysis of the phenotype of HS patients with and without *CCBE1* mutations, to investigate whether a correlation between clinical phenotype and presence of *CCBE1* mutation can be established.

Methods

Between 01.01.2000 and 01.01.2011, 51 patients were referred to the diagnostic lab of the Academic Medical Center in Amsterdam for molecular studies. A questionnaire asking detailed information about the clinical phenotype was sent to all referring physicians. In all patients, except one (patient 12), clinical photographs were available which were evaluated along with the questionnaire data by a single observer (R.C.H.). Statistical analysis of the data was done using the two-tailed Fisher's exact test. p values for comparison of developmental delay in the various subgroups were also calculated keeping the different subcategories (normal, B, DD, sDD, and GMD). p values <0.05 are considered clinically significant.

In addition, samples of 158 patients with less marked forms of lymphatic dysplasia were screened for mutations in *CCBE1*. These patients had been referred for molecular analysis of *VEGFR3*, *FOXC2* and *SOX18*, and none had mutations in these genes.

Mutation analysis of *CCBE1* was performed by PCR amplification of all coding exons followed by bidirectional sequencing using the BigDye kit v1.1 (Applied Biosystems) and an ABI3700 genetic analyzer (Applied Biosystems), or by high resolution melting (HRM)-based screening (Light Cycler, Roche) combined with bidirectional sequencing on CEQ2000 capillary sequencer (Beckman Coulter). Sequence results were analyzed using CodonCode aligner (CodonCode Corporation) or Sequencher™ 4.5 software.

Results

In total, 34 (67%) of the questionnaires were returned. The major clinical data are summarised in table 1 and illustrated in figure 1. Of 2 mutation positive patients (4 and 5), no questionnaire was returned, and clinical data



Fig. 1. a Phenotype of patient 10 with a *CCBE1* mutation at age 2 years showing hair whorl in anterior hairline, hypertelorism, thick lateral eyebrows, epicanthi, depressed nasal bridge, and (pitting) lymphedema of distal limbs. **b** Changes in facial phenotype and lower limbs lymphedema in patient 11 with a *CCBE1* mutation at age 3 months, 2 years, 8 years and 15 years. Note similarities with patient in figure 1a, and variation in facial lymphedema. **c** Lower limb lymphedema in patient 11 at age 3 months, 2 years and 8 years.

from these patients were obtained from the earlier publication [Alders et al., 2009]. The single patient with a *CCBE1* mutation published by another group [Connell et al., 2010] was also added to table 1 as patient 13 to provide a complete literature overview; this patient has also been clinically investigated by one of us (R.C.H.). Clinical data were not available from 16 patients without a *CCBE1* mutation.

We divided the group of patients into 3 groups: Group 1: those with a mutation in *CCBE1*; Group 2: those without a mutation in *CCBE1* but in whom the diagnosis HS was considered to be likely and Group 3: patients without a *CCBE1* mutation and in whom the clinical diagnosis of HS was considered to be possible but uncertain, or even unlikely, but the referring physician wanted to exclude *CCBE1* as the causative gene. In 2 patients, (patient 19 and

Table 1. Groups according to diagnosis and mutations**a** Group 1: diagnosis likely, *CCBE1* mutation positive

Patient	1	2	3	4 [±]	5 [±]	6	7	8	9	10	11	12	13 ^s	Total
Gender	m	m	m	f	m	f	m	m	f	m	f	f	f	7m/6f
Consanguinity	–	+	+	+	–	+	+	+	+	–	–	–	–	7/13
Family history positive	–	+	–	–	–	+	–	+	–	–	–	–	+	4/13
Mutation <i>CCBE1</i> allele 1	R158C	C102S	C75S	C75S	C174R	G237R	C75S	C102S	C174Y	L229fs	C174R	C47F	C75S	13/13
allele 2	L229fs	C102S	C75S	C75S	L229fs	G237R	C75S	C102S	C174Y	G271R	L229fs	L229fs	C75S	
Age at last investigation	20 y (d22 y)	17 y	8 y	3 y	1 y	5 y (d6 y)	29 y	1 y	2 y	2 y	19 y	20 y	6 y	
Developmental delay	DD	B	B	–	GMD	B	–	DD	B	–	B	B	–	9/13
Growth <P3	–	+	+	–	–	+	+	+	+	–	+	+	+	9/13
OFC <P3	–	+	–	–	+	+	–	–	+	–	+	+	–	6/13
Unusual face	+	+	+	+	+	+	+	+	+	+	+	+	+	13/13
Lymphedema limbs	+	+	+	+	+	+	+	+	+	+	+	+	+	13/13
Lymphangiectasia														
Gut	+ ^b	+	+	+	+	+	+	+ ^b	+	+	+	+	+ ^b	13/13
Lung	+	?	+	+	+	+	+	+	–	–	+	–	+	9/12
Pericardial	+	+	–	+	–	+	–	+	–	–	–	–	–	5/13
Kidney	+	?	–	–	?	–	–	–	–	–	–	–	–	1/11
Genital	+	+	–	–	+	+	+	+	–	?	–	–	+	7/12
Hydrops	–	–	–	–	+	–	–	–	–	–	+	–	+	3/13
Heart malformation	–	–	–	VSD	–	–	–	–	–	–	–	ASD	–	2/13
Blood vessel abnormality	–	–	–	H	–	+	–	–	–	–	–	TR, AR	–	3/13
Other	1		2, 3		3		2, 4	5		6, 7	8		3	

b Group 2: diagnosis likely, mutation negative

Patient	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	Total
Gender	m	m	f	m	f	m	f	f	m	m	f	m	m	m	f	m	10m/6f
Consanguinity	+	+	+	+	–	–	+	–	–	–	?	+	+	+	+	–	9/15
Family history positive	+	–	–	–	–	–	–	–	–	–	–	+	+	+	+	–	5/16
Mutation <i>CCBE1</i>	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/16
Age at last investigation	12 y	23 y	1 y	9 y	8 y	18 y	4 y	17 y	5 mo (d5 mo)	1 y	35 y	1 y	40 y	26 y (d28 y)	36 y (d36 y)	29 y	
Developmental delay	–	B	DD	B	B	sDD	DD	sDD	DD	B	–	DD	B	B	sDD	B	14/16
Growth <P3	+	–	+	+	–	+	–	+	+	–	–	+	+	+	–	+	10/16
OFC <P3	?	–	?	?	–	–	–	–	+	–	?	–	–	–	+	?	2/11
Unusual face	+	a	+	+	+	a	+	+	+	+	+	+	+	+	+	+	14/16
Lymphedema limbs	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	15/16
Lymphangiectasia																	
Gut	+	–	?	+	+	+	–	+	+	+	+	–	+	+	+	+	12/15
Lung	–	–	?	+	–	–	–	+	?	+	–	+	+	+	+	+	8/14
Pericardial	–	+	?	+	–	–	–	?	–	–	–	–	–	–	–	+	3/14
Kidney	–	–	?	?	–	–	–	?	–	–	–	–	–	–	–	–	0/13
Genital	–	–	?	+	+	+	–	?	+	+	–	+	+	+	+	+	10/14
Hydrops	–	–	–	–	–	–	–	–	+ ^c	–	–	–	–	–	–	–	1/16
Heart malformation	–	–	–	+	–	–	–	+	PDA	PDA	–	–	–	–	–	–	4/16
Blood vessel abnormality	–	–	H	–	–	VP	–	–	–	–	dup v.c.i.	VP	–	–	–	–	4/16
Other	9, 10			10, 11	10	12, 13	12, 14	10, 12, 15	16, 17	6, 10		18	17, 20			10	

36) another diagnosis was confirmed cytogenetically (patient 19: del(22)(q13.3)) or molecularly (patient 36: mutation in *PTPN11* confirming Noonan syndrome). Patient 19 was diagnosed as likely HS, and the deletion of chromosome 22q13.3 was identified only after *CCBE1* mutation analysis. The patient with Noonan syndrome was known to carry a *PTPN11* mutation prior to *CCBE1* mutation analysis, but the lymphedema was so marked that it was thought useful to exclude heterozygosity or homo-

zygosity for *CCBE1* as an explanation for the lymphedema.

We found no *CCBE1* mutation in the 158 patients with less marked lymphatic dysplasia, and also no mutation in the 8 patients in whom the clinical diagnosis was considered possible but uncertain. Mutations in *CCBE1* were present in a total of 13 patients. Mutations were mostly missense mutations clustered in the collagen domain, the Ca-binding EGF domain or the cysteine rich domain up-

Table 1 (continued)

c Group 3: diagnosis uncertain or unlikely, mutation negative

Patient	30	31	32	33	34	35	36	37	Total
Gender	f	m	f	f	f	f	m	f	2m/6f
Consanguinity	–	+	–	–	–	–	–	+	2/8
Family history positive	–	–	–	–	–	–	–	–	0/8
Mutation <i>CCBE1</i>	–	–	–	–	–	–	–	–	0/8
Age at last investigation	10 mo (d10 mo)	16 y	4.5 y	16 y	6 y	8 y	6 mo (d6 mo)	6 y	
Developmental delay	sDD	–	DD	B	–	DD	DD	B	6/8
Growth <P3	+	+	+	+	–	+	+	–	6/8
OFC <P3	+	+	+	+	–	+	–	–	5/8
Unusual face	+	+	+	+	–	+	+	+	7/8
Lymphedema limbs	+	+	+	mild	+	+	+	+	8/8
Lymphangiectasia									
Gut	+	+	–	+	+	+	–	+ ^b	6/8
Lung	+	+	+	–	–	+	–	+	5/8
Pericardial	+	–	–	–	–	+	–	–	2/8
Kidney	+	–	–	–	–	–	–	–	1/8
Genital	–	+	–	–	–	–	–	–	1/8
Hydrops	–	–	–	–	–	–	–	+	1/8
Heart malformation	hypoplastic left heart	–	PDA, PFO	ASD	–	–	ASD	aortic stenosis	5/8
Blood vessel abnormality	–	–	–	–	–	–	–	–	0/8
Other	19	20, 23	3, 9	21	22	6	24		

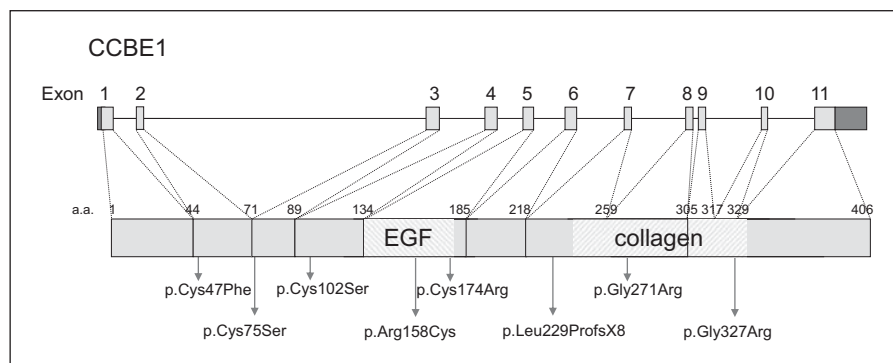
+ = Present; – = absent; mo = month; y = year; (d...) = in case a patient died, age of death; DD = developmental delay; sDD = severe developmental delay; B = borderline developmental delay; GMD = gross motor delay; PDA = persistent ductus arteriosus; PFO = patent foramen ovale; ASD = atrial septal defect; VSD = ventricular septal defect; a = unusual face, but not typical HS; H = haemangioma; AR = accessory renal artery; TR = tortuosity retinal vessels; VP = difficult to access blood vessels; dup v.c.i. = duplication vena cava inferior.

Other: 1 = Glaucoma; 2 = growth hormone deficiency; 3 = hypothyroidism; 4 = testis carcinoma; 5 = diaphragmatic hernia; 6 = metopic craniosynostosis; 7 = adrenal neuroblastoma; 8 = delayed puberty; 9 = hearing loss, campodactyly with partial cutaneous syndactyly; 10 = seizures; 11 = abnormal abdominal

situs; 12 = macrocephaly; 13 = deletion 22q13.3; 14 = brain atrophy, especially vermis atrophy, overgrowth; 15 = agenesis callosal body; 16 = retroperitoneal lymphangioma caudal of the pancreas; 17 = gingival hypertrophy; 18 = low CD3 and CD4, slightly decreased NK cell function; 19 = severe vertebral osteoporosis; 20 = scoliosis; 21 = acute lymphatic leukaemia, myopia; 22 = brain heterotopia, aplasia cutis with underneath skull defect; 23 = pectus carinatum; 24 = molecularly confirmed Noonan syndrome.

^a Patients described in Alders et al. [2009]. ^s Patients described in Connell et al. [2010] and also investigated in person by one of us (R.C.H.). ^b Including chylous ascites. ^c Hydrops fetalis, improved prenatally.

Fig. 2. Overview of the mutations described in *CCBE1*. The upper panel represents the 11 exons of the *CCBE1* gene; the lower panel represents the *CCBE1* protein. Functional domains in the protein (according to Uniprot) and the location of the mutations are indicated.



stream of the EGF domain (fig. 2). The mutations in the collagen domain change conserved glycines in the repeating Gly-X-Y motif of the collagen domain and probably affect the stability of the triple helix conformation of the domain. All mutations in the EGF domain and upstream of this domain involve cysteines (cysteines are either mutated or introduced by the mutations), which are crucial amino acids for proper folding of the protein. One recur-

rent mutation introducing a frameshift and encoding a truncated protein lacking the collagen domain was found.

We performed statistical analysis on the clinical findings listed in table 1 in the *CCBE1* positive and negative patients. No statistically significant differences were present between the patient groups with a clinical diagnosis of HS with and without a *CCBE1* mutation. All patients carrying *CCBE1* mutations had lymphedema of the limbs,

unusual face and lymphangiectasia in the gut, representing the most distinctive features of the syndrome. Development varied from normal to severe delay within the group of patients thought to have HS, and indeed this variability is recognised even within a single family [Hennekam et al., 1989]. No difference existed between the patients with a *CCBE1* mutation and those without. Growth parameters were disturbed in a similar way in those with and without a *CCBE1* mutation, but microcephaly was more common in those with a *CCBE1* mutation. Lymphedema and lymphangiectasia were universal in *CCBE1* positive patients but less so in those without a mutation. The lymphedema was sufficiently marked at birth to diagnose hydrops foetalis in 3 of the *CCBE1* positive cases, in 1 of the *CCBE1* negative HS cases and in 1 of the cases thought to have another entity (patient 37). Two *CCBE1* positive cases needed respiratory support at night, and 2 other *CCBE1* positive cases died in childhood or early adulthood due to respiratory problems. Recurrent chylous ascites was present in 3 molecularly confirmed patients, in none of the unconfirmed patients and in patient 37 (Noonan syndrome). In general there is a tendency for the lymphatic dysplasia to be more marked in the *CCBE1* positive patients than in the *CCBE1* negative patients.

Seizures did not occur in the group of molecularly confirmed HS patients, but were found in 6 of the HS cases without molecular confirmation. Cardiac anomalies were less common in *CCBE1* positive cases than in those without a mutation or with another form of generalised lymphatic dysplasia, and blood vessel anomalies were also more common in *CCBE1* negative HS cases. Unusual clinical manifestations in *CCBE1* positive patients included metopic craniosynostosis, diaphragmatic hernia, growth hormone deficiency (each occurring in one case), and hypothyroidism ($n = 3$). Metopic craniosynostosis was also present in one of the HS cases without a *CCBE1* mutation and in a patient from the group with possible HS. One *CCBE1* positive case developed a testicular carcinoma in early puberty, and another was found to have an adrenal neuroblastoma in early infancy. One of the patients in the possible HS group had acute lymphatic leukaemia.

Discussion

In this study, we compared the clinical features of patients diagnosed with HS with and without a mutation in the *CCBE1* gene. In addition, a group with less marked lymphatic dysplasia was screened for mutations in *CCBE1*.

Our results show that *CCBE1* mutations are present only in patients with the clinical diagnosis of HS. None of the patients with a possible but less likely diagnosis had mutations in this gene. Two patients had a molecular diagnosis other than HS (22q13.3 deletion; Noonan syndrome). In both patients, the lymphatic dysplasia was very severe, but no additional *CCBE1* mutation could be found to explain the severe lymphatic abnormalities in these cases.

In the group of patients with the clinical diagnosis of HS, about 40% of the patients had *CCBE1* mutations. No significant differences were observed between the mutation positive and mutation negative patients with clinically diagnosed HS. All mutation positive patients had lymphedema of the limbs, lymphangiectasia in the gut and the unusual facial morphology considered at least in part being secondary to the intra-uterine facial lymphedema, indicating that absence of these features may correlate with absence of a *CCBE1* mutation. The severity of the *CCBE1* related lymphedema also is apparent in the need for respiratory support at night in 2 patients and early death due to respiratory insufficiency in 2 other patients. Also, the presence of seizures may correlate with absence of a *CCBE1* mutation, as none of the *CCBE1* positive patients had seizures whilst several of the *CCBE1* negative cases did. Otherwise the patients with and without mutation were remarkably similar, suggesting that other gene(s) involved in HS may well act within the same pathway as *CCBE1*.

The function of *CCBE1* is not completely understood yet. Unlike other lymphangiogenesis associated genes, *CCBE1* is not expressed in endothelial cells, but is spatially and temporally expressed along the migration routes of lymphatic endothelial cells and may be a guidance molecule involved in lymphangioblast budding and migration [Hogan et al., 2009]. *CCBE1* contains a Ca-binding EGF domain. These domains are usually involved in protein-protein interactions, and one might speculate that mutated genes encoding proteins that interact with *CCBE1* may also cause HS.

We conclude that *CCBE1* mutations cause a widespread and severe form of lymphatic dysplasia, associated with variable intellectual disabilities, unusual facial morphology and infrequently malformations. This indicates it causes a truly syndromic entity. There is no marked clinical difference in patients with and without a *CCBE1* mutation, although there is a tendency for the lymphatic dysplasia to be less marked in those without *CCBE1* mutations. Further research for the identification of possible other genes causing HS is in progress and will take into account that other causative genes may well act in the same pathway as *CCBE1*.

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