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Mutations in the VEGFR3 Signaling Pathway Explain 36% of Familial Lymphedema

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Functional pathway · Genetic · Mutation · Phenotype

Abstract
Lymphedema is caused by dysfunction of lymphatic vessels, leading to disabling swelling that occurs mostly on the extremities. Lymphedema can be either primary (congenital) or secondary (acquired). Familial primary lymphedema commonly segregates in an autosomal dominant or recessive manner. It can also occur in combination with other clinical features. Nine mutated genes have been identified in different isolated or syndromic forms of lymphedema. However, the prevalence of primary lymphedema that can be explained by these genetic alterations is unknown. In this study, we investigated 7 of these putative genes. We screened 78 index patients from families with inherited lymphedema for mutations in *FLT4, GJC2, FOXC2, SOX18, GATA2, CCBE1*, and *PTPN14*. Altogether, we discovered 28 mutations explaining 36% of the cases. Additionally, 149 patients with sporadic primary lymphedema were screened for *FLT4, FOXC2, SOX18, CCBE1*, and *PTPN14*. Twelve mutations were found that explain 8% of the cases. Still unidentified is the genetic cause of primary lymphedema in 64% of patients with a family history and 92% of sporadic cases. Identification of those genes is important for understanding of etiopathogenesis, stratification of treatments and generation of disease models. Interestingly, most of the proteins that are encoded by the genes mutated in primary lymphedema seem to act in a single functional pathway involving VEGFR3 signaling. This underscores the important role this pathway plays in lymphatic development and function and suggests that the unknown genes also have a role.

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The lymphatic system is a blind-ended endothelium-lined network [Butler et al., 2009] that collects and transports extravasated fluid, plasma proteins and immune cells back to the blood circulation [Karkkainen et al., 2001]. Developmental defects or dysfunction of the lymphatic system cause lymphedema [Brouillard and Vikkula, 2007; Boon and Vikkula, 2012], a disorder characterized by abnormal swelling of one or more extremities due to impaired transport of the lymph [Irrthum et al., 2000]. Lymphedema can be primary (congenital) or secondary (acquired). Hereditary lymphedema is more easily recognized when it occurs as an autosomal dominant trait, even if there is reduced penetrance and variable expressivity [Irrthum et al., 2000]. Parental consanguinity suggests recessive inheritance [Ghalamkarpour et al., 2009b]. Lymphedema can occur in association with other clinical features or as part of a syndrome [Irrthum et al., 2003]. Currently, 9 genes have been linked to different types of primary lymphedema: *FLT4* (VEGFR3), *GJC2, FOXC2, SOX18, GATA2, CCBE1, PTPN14, KIF11*, and the lastly identified *VEGFC*.

FLT4 and *GJC2* (fms-related tyrosine kinase 4, encoding VEGFR3, and gap junction protein gamma-2, encoding connexin-47, CX47, respectively) code for proteins localized on the cellular membrane. *FLT4* is mutated in Nonne-Milroy disease (OMIM 153100), characterized by congenital bilateral lower limb lymphedema. All reported mutations are localized within the 2 intracellular tyrosine kinase domains of the encoded VEGFR3 receptor [Butler et al., 2007, 2009]. Cells expressing mutant VEGFR3 show inhibited autophosphorylation of the receptor, indicating downregulation of VEGFR3 signaling [Irrthum et al., 2000; Karkkainen and Petrova, 2000; Ghalamkarpour et al., 2009b]. Nonsynonymous mutations in *GJC2* were discovered in a few families with late-onset autosomal dominant lymphedema (OMIM 613480) affecting all 4 extremities ('four-limb lymphedema') and sometimes associated with saphenous vein insufficiency, blepharoptosis, and involvement of the face or genitalia. Some families showed reduced penetrance [Ferrell et al., 2010; Ostergaard et al., 2011a]. The GJC2 amino acid substitutions likely cause gain-of-function, as loss-of-function mutations in GJC2 are found in patients with inherited autosomal recessive Pelizaeus-Merzbacher-like disease (PMLD, OMIM 608804), a hypomyelinating disorder of the central nervous system [Uhlenberg et al., 2004].

The second group of lymphedema-causing genes encodes 3 transcription factors *(FOXC2*, *SOX18* and *GATA2). FOXC2* (forkhead box C2) is mutated in puberty or late-onset primary lymphedema associated with distichiasis (LDS, OMIM 153400). FOXC2 regulates the expression of genes involved in cell growth, proliferation, differentiation, and longevity [Fang et al., 2000]. The majority of the *FOXC2* mutations are insertions, deletions or nonsense mutations, leading to mRNA decay or truncated loss-of-function proteins [Dagenais et al., 2004; Ghalamkarpour et al., 2009a; van Steensel et al., 2009]. FOXC2 suppresses PDGFB production [Shimoda et al., 2011], and loss of its activity leads to accumulation of vascular smooth muscle cells in collecting lymphatics of knock-out mice and also in patients [Petrova et al., 2004; Norrmen et al., 2009].

Recessive and dominant mutations in *SOX18* (SRY-box 18) cause the hypotrichosis-lymphedema-telangiectasia syndrome (HLTS, OMIM 607823), characterized by congenital lymphedema, reduced body hair including the absence of eyelashes and eyebrows, and localized cutaneous telangiectasias. This transcription factor plays an important role in early blood vessel modeling [Downes et al., 2009] as well as in the differentiation of lymphatic endothelial progenitor cells from venous precursors [Francois et al., 2008]. The 3 published *SOX18* mutations are localized in the DNA-binding domain (recessive) or in the transactivation domain (truncating/dominant) [Irrthum et al., 2003]. The $Sox18^{-/-}$ mice exhibit only a minor coat defect [Pennisi et al., 2000a]. In contrast, the spontaneous *ragged* mutant of *Sox18* has defective lymphatic and cardiovascular tissues and hair follicle defects [Pennisi et al., 2000b].

Mutations in *GATA2* (GATA-binding protein 2) have been linked to a predisposition to myelodysplastic syndrome (MDS, OMIM 614286) and to acute myeloid leukemia (AML, OMIM 601626). Subsequently, mutations were identified in patients with primary lymphedema with myelodysplasia (also known as Emberger syndrome, OMIM 614038) [Hahn et al., 2011; Ostergaard et al., 2011b] and the monocytopenia with mycobacterial infection syndrome (MonoMAC, OMIM 614172), associated with dendritic cell, monocyte, B lymphocyte and natural killer lymphocyte deficiency (DCML) [Kazenwadel et al., 2012]. The phenotypes are not exclusive, underscored by a Japanese patient with a *GATA2* mutation with MonoMAC and Emberger syndromes [Ishida et al., 2012]. There are no obvious genotype-to-phenotype correlations [Hyde and Liu, 2011; Holme et al., 2012].

The gene coding for the extracellular protein CCBE1 (collagen and calcium-binding EGF domain-1) that enhances the lymphangiogenic effects of VEGFC in vivo [Bos et al., 2011] is essential for fetal liver erythropoiesis [Zou et al., 2013]. CCBE1 was identified to be important

Table 1. 227 samples were screened for 7 lymphedema genes and 40 (17%) mutations were found

Gene	Families		Sporadic and unknown inheritance	
	samples tested	mutations	samples tested	mutations
FLT4	71	16	101	7
GJC2	55	1	–	–
FOXC2	64	5	135	4
SOX18	22	3	63	1
GATA2	45	1	–	–
CCBE1	55	2	86	0
PTPN14	37	0	118	0
Total	78	28 (35.9%)	149	12 (8%)

for lymphatic development by genetic knock-down screening in zebrafish [Hogan et al., 2009]. In human patients, *CCBE1* homozygous and compound heterozygous mutations cause the Hennekam lymphangiectasia-lymphedema syndrome (OMIM 235510), characterized by severe peripheral lymphedema associated with intestinal lymphangiectasias, characteristic facial features, growth and mental retardation, and hydrops fetalis [Hennekam et al., 1989; Alders et al., 2009, 2013; Connell et al., 2010].

An intracellular phosphatase (PTPN14) was shown to interact with the VEGFC-receptor VEGFR3 by co-immunoprecipitation upon activation by VEGFC. An intragenic deletion encompassing both sides of exon 7 of *PTPN14* (protein tyrosine phosphatase, non-receptor type 14) was identified in a consanguineous family with autosomal recessive choanal atresia and lymphedema (OMIM 613611) [Au et al., 2010]. This mutation causes a frameshift p.Ser194Argfs*19.

Whole exome sequencing successfully revealed 2 additional genes, mutations of which cause primary lymphedema. Firstly, *KIF11* mutations (kinesin family member 11, a DNA-interacting protein) were discovered to cause MLCRD (microcephaly, lymphedema, chorioretinal dysplasia) and CDMMR (chorioretinal dysplasia, microcephaly and mental retardation), 2 allelic syndromes that have now been regrouped as MCLMR (microcephaly with or without chorioretinopathy, lymphedema, or mental retardation, OMIM 152950) [Ostergaard et al., 2012]. MCLMR can be sporadic or inherited as an autosomal dominant trait, and the mutations are predicted to result in loss-of-function of EG5, the encoded homotetrameric kinesin motor protein playing a role in mitotic spindle assembly and function [Ostergaard et al., 2012]. The second gene, *VEGFC* (vascular endothelial growth factor C), is the major ligand of VEGFR3. A frameshift mutation was identified in 1 family, resulting in loss-of-function of the mutant allele [Gordon et al., 2013].

All the gene identifications reported to date have been performed using specific and limited numbers of families; thus, it is impossible to determine the proportion of all inherited primary lymphedema that can be explained by mutations in these genes. Therefore, we screened a total of 78 familial index patients for 7 of the genes that were known at the beginning of this study. We screened another 149 samples with presumably sporadic primary lymphedema or lymphedema of unknown mode of inheritance for 5 of the genes.

Materials and Methods

Sample Collection

DNA or whole blood samples were collected from patients seen in collaborating clinics. Informed consent was obtained from each participant, as approved by the ethical committee of the Medical Faculty at the Université catholique de Louvain (Brussels, Belgium) and the respective local committees. DNA was extracted manually from whole blood using a DNA purification kit (Gentra) or Wizard genomic DNA purification kit (Promega).

Screening Method

A series of 78 index patients from families with primary hereditary lymphedema was screened for mutations in *FLT4, GJC2, FOXC2, SOX18, GATA2, CCBE1*, and *PTPN14*. The screenings were performed sequentially. The new samples were included in screens of the remaining genes (table 1). Once a mutation was found, the sample was not tested for the remaining genes. In some cases, patients exhibiting distinct signs/symptoms associated with a disease were prioritized for specific genes (e.g. hypotrichosis or alopecia for *SOX18*) (online suppl. table 1, www.

Table 2. Mutations found in familial cases of lymphedema ordered according to amino acid position

Gene Sample ID	Nucleotide change	Amino acid change	Protein effect	dbSNP135	Cosegregation[a]	Mutation reported by
FLT4						
Family-1	c.2554G>T	p.Gly852Cys	probably damaging	not reported	NA	–
Family-2	c.2554G>T	p.Gly852Cys	probably damaging	not reported	NA	–
Family-3[b]	c.2563G>A[c]	p.Ala855Thr[c]	probably damaging	rs121909657[d]	♀♂	Ghalamkarpour et al., 2009b[e]
Family-4	c.2569G>C	p.Gly857Arg	probably damaging	not reported	NA	Karkkainen et al., 2000
Family-5	c.2585C>T	p.Ala862Val	probably damaging	not reported	♂	–
Family-6	c.2626G>T	p.Val876Leu	probably damaging	not reported	♀	–
Family-7	c.2632G>A	p.Val878Met	probably damaging	rs121909654[d]	♂	Ghalamkarpour et al., 2006[e]
Family-8	c.2647+2T>C	–	splice-site	not reported	NA	–
Family-9	c.2797G>C	p.Gly933Arg	probably damaging	not reported	♀	Evans et al., 2003
Family-10	c.3104A>G	p.His1035Arg	probably damaging	rs121909653[d]	♂	Irrthum et al., 2000[e]
Family-11	c.3121C>T	p.Arg1041Trp	probably damaging	rs121909650[d]	♂	Evans et al., 2003
Family-12	c.3121C>T	p.Arg1041Trp	probably damaging	rs121909650[d]	NA	Evans et al., 2003
Family-13	c.3121C>G	p.Arg1041Gly	probably damaging	not reported	NA	–
Family-14	c.3257T>C	p.Ile1086Thr	probably damaging	rs121909655[d]	♂	Ghalamkarpour et al., 2006[e]
Family-15[b]	c.3316G>A	p.Glu1106Lys	possibly damaging	rs121909656[d]	♀	Spiegel et al., 2006[e]
Family-16	c.3323_3325del	p.Phe1108del	in-frame deletion	not reported	♂	Evans et al., 2003; Ghalamkarpour et al., 2006[e]
GJC2						
Family-17	c.143C>T	p.Ser48Leu	probably damaging	not reported	NA	Ferrel et al., 2010
FOXC2						
Family-18[b]	c.595dup[c]	p.His199Profs*264[c]	frameshift	not reported	♀	Finegold et al., 2001
Family-19	c.902_923dup	p.Pro309Glyfs*161	frameshift	not reported	NA	–
Family-20	c.982_983del	p.Gly328Leufs*134	frameshift	not reported	♂	–
Family-21	c.983dup	p.Tyr329Leufs*134	frameshift	not reported	♀	–
Family-22	c.1315_1316dup	p.Phe440Argfs*33	frameshift	not reported	NA	–
SOX18						
Family-23[b]	c.283T>A[c]	p.Trp95Arg[c]	probably damaging	rs28936693[d]	♀♂	Irrthum et al., 2003[e]
Family-24[b]	c.310G>C[c]	p.Ala104Pro[c]	probably damaging	rs28936692[d]	♀♂	Irrthum et al., 2003[e]
Family-25	c.720C>A	p.Cys240*	premature stop codon	rs74315430[d]	present in sibling	Irrthum et al., 2003[e]
GATA2						
Family-26	c.130G>T	p.Glu44*	premature stop codon	not reported	present in sibling	–
CCBE1						
Family-27[b]	c.223T>A[c]	p.Cys75Ser[c]	probably damaging	rs121908250[d]	♀♂	Alders et al., 2009[e]
Family-28[b]	c.520T>C[c]	p.Cys174Arg[c]	probably damaging	rs121908254[d]	♀♂	Alders et al., 2009
PTPN14						
No familial mutation found.						

[a] NA = Family members not available; ♀ = mutation maternally inherited and present in other family members; ♂ = mutation paternally inherited and present in other family members; ♀♂ = consanguineous inheritance. [b] Consanguineous family. [c] Homozygous mutation. [d] Reported by dbSNP135 as probably pathogenic allele. [e] Patient published by our group.

karger.com/doi/10.1159/000354097). In addition to the familial samples, we screened 5 genes *(FLT4, FOXC2, SOX18, CCBE1*, and *PTPN14)* in a cohort of 149 patients in which the primary lymphedema was presumably sporadic or the mode of inheritance was unknown (table 1). The recently discovered *KIF11* and *VEGFC* genes were not included in the study.

The coding regions of the genes (exons and splice-sites) were screened either by denaturing high performance liquid chromatography (DHPLC, Wave System 3500A, Transgenomic), high resolution melting (HRM) with a LightCycler® 480 (Roche Applied) or direct sequencing (CEQ2000 capillary sequencer (Beckman Coulter) or a 3130*xl* Genetic Analyzer (Life Technologies)). For the *FLT4* receptor, we screened only the kinase domains (encoded by exons 17–25), for which all the known mutations have been reported. Amplicons with an abnormal DHPLC or HRM profile were re-amplified and sequenced. Sequences were analyzed with Sequencher® 4.5 (Gene Codes Inc.) or with CLC Main Workbench 6© (CLC Bio) software.

Table 3. Mutations found in sporadic cases and samples with unknown mode of inheritance ordered according to amino acid position

Gene Sample ID	Nucleotide change	Amino acid change	Protein effect	dbSNP135	Cosegregation[a]	Paternity attested	Mutation reported by
FLT4							
Patient-1	c.2587T>C	p.Ser863Pro	probably damaging	not reported	de novo	yes	–
Patient-2	c.2677C>G	p.Leu893Val	probably damaging	not reported	de novo	yes	Ghalamkarpour et al., 2009a[d]
Patient-3[b]	c.2819G>C	p.Arg940Pro	probably damaging	not reported	NA	–	–
Patient-4	c.3105C>G	p.His1035Gln	probably damaging	not reported	de novo	yes	Ghalamkarpour et al., 2006[d]
Patient-5	c.3341C>T	p.Pro1114Leu	probably damaging	rs121909652[c]	de novo	yes	Karkkainen et al., 2000
Patient-6	c.3391G>A	p.Gly1131Ser	probably damaging	not reported	de novo	yes	–
Patient-7	c.3410C>T	p.Pro1137Leu	probably damaging	not reported	NA	–	Evans et al., 2003; Ghalamkarpour et al., 2009a[d]
FOXC2							
Patient-8	c.493_501del	p.Arg165_Arg167del	in-frame deletion	not reported	incomplete penetrance	yes	–
Patient-9	c.914_921del	p.Tyr305Cysfs*155	frameshift	not reported	NA	–	Bell et al., 2001
Patient-10	c.930_936dup	p.Tyr313Argfs*152	frameshift	not reported	de novo	yes	Finegold et al., 2001
Patient-11	c.943C>T	p.Gln315*	premature stop codon	not reported	de novo	yes	Ghalamkarpour et al., 2009a[d]
SOX18							
Patient-12		p.Cys240*	premature stop codon	rs74315430[c]	de novo	yes	Irrthum et al., 2003[d]
CCBE1							
No mutation found.							
PTPN14							
No mutation found.							

[a] NA = Family members not available. [b] Unknown mode of inheritance. [c] Reported by dbSNP135 as probably pathogenic allele. [d] Patient published by our group.

Variant Analysis

All variants were searched against dbSNP135 (http://www.ncbi.nlm.nih.gov/projects/SNP/) [Sherry et al., 2001]. The effects of amino acid changes on proteins were predicted using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) [Sunyaev et al., 1999; Ramensky et al., 2002; Adzhubei et al., 2010]. MutationTaster (http://www.mutationtaster.org/) [Schwarz et al., 2010] was also used, in particular to predict the effects of splice-site changes. The nomenclature of the mutations follows the guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/) and was verified with Mutalyzer (https://mutalyzer.nl/) [Wildeman et al., 2008]. When DNAs of other family members were available, cosegregation or de novo nature was checked. For the sporadic cases, paternity tests were performed using the PowerPlex16 HS system (Promega) on a 3130*xl* Genetic Analyzer (Life Technologies). All mutations have been submitted to the Vascular Anomalies Mutation Database (www.icp.ucl.ac.be/vikkula/VAdb/).

Results

We found an *FLT4* (VEGFR3) mutation in 16 families (table 1; 6 mutations published previously) [Irrthum et al., 2000, 2003; Ghalamkarpour et al., 2006, 2009a, b]. These mutations included 12 distinct nonsynonymous changes (two of which were found in 2 families), 1 in-frame deletion (p.Phe1108del) and 1 intronic change in a consensus splice site (c.2647+2T>C) (table 2). Of the 12 substitutions, 11 were predicted to alter the function of the receptor, whereas the last one (p.Glu1106Lys) is 'possibly damaging'. According to MutationTaster, the intronic splice-site change is predicted to be 'disease-causing'. Some of these substitutions are listed in dbSNP135 as 'probably pathogenic' alleles (table 2).

Only 1 mutation was found in *GJC2* (p.Ser48Leu) (table 2). The same change has been reported in 2 other families [Ferrell et al., 2010; Ostergaard et al., 2011a]. It is predicted to be 'probably damaging' for the protein function.

In *FOXC2*, we found 4 nucleotide insertions and 1 deletion, all leading to reading frameshifts, expected to produce a truncated protein (table 2). Among the samples screened for *SOX18* (table 1), we identified 3 families with a mutation: 2 with amino acid substitutions (p.Trp95Arg and p.Ala104Pro) and 1 with a nonsense change (p.Cys240*) (table 2). The substitutions were homozygous mutations in consanguineous families, where-

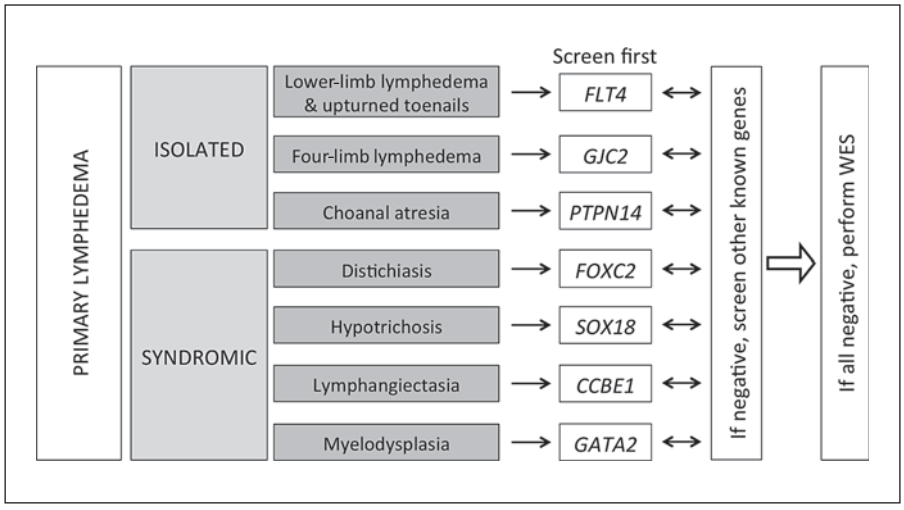

Fig. 1. Scheme for the genetic analysis of lymphedema patients. WES = Whole exome sequencing.

as the nonsense mutation was dominant [Irrthum et al., 2003]. All are reported as 'pathogenic' in dbSNP135. In the third transcription factor gene, *GATA2*, we identified only 1 mutation, which leads to a premature termination codon (p.Glu44*).

Within the 55 families tested for *CCBE1* (table 1), we found 2 homozygous substitutions: p.Cys75Ser and p.Cys174Arg. Both were predicted to be 'probably damaging' and referred to as a 'probably pathogenic' allele in dbSNP135 (table 2). No pathogenic change was identified in *PTPN14* (table 2).

We also screened a series of 149 samples of index patients presumed to have sporadic lymphedema or with an unknown family history (table 1). We identified 7 patients with nonsynonymous VEGFR3 mutations, predicted to be 'probably damaging' (table 3). Among these, only p.Pro1114Leu was reported in dbSNP135 as a 'probably pathogenic' allele. We also found 4 patients with a *FOXC2* mutation (table 3). These included 1 deletion (p.Tyr305Cysfs*155), 1 duplication (p.Tyr313Argfs*152), 1 in-frame deletion (p.Arg165_Arg167del) and 1 premature stop codon (p.Gln315*). In *SOX18*, we identified 1 premature stop codon (p.Cys240*), which had previously been found in a familial case. No mutation was detected in *CCBE1* or *PTPN14* (tables 1, 3) in this non-familial screen. In all, 7 mutations appeared de novo, while 1 was inherited from an unaffected parent, therefore showing incomplete penetrance (Patient-8). The inheritance of 3 others could not be assessed (table 3).

Discussion

We screened 78 families with primary hereditary lymphedema for 7 genes reported to be mutated in some families. We discovered mutations in *FLT4* (16 patients mutated/71 samples tested), *GJC2* (1/55), *FOXC2* (5/64), *SOX18* (3/22), *GATA2* (1/45), and *CCBE1* (2/55). Overall, a mutation was found in one-third (n = 28) of the familial cases (table 1). These numbers are lower when the mode of inheritance is sporadic or unknown. Yet, in 149 such samples tested, a mutation was found in 12 (8%), and all but 3 were conclusively de novo. These included 7 mutations in *FLT4* (6.9%, 7/101), 4 in *FOXC2* (2.9%, 4/135) and 1 in *SOX18* (1.6%, 1/63) (table 1). In aggregate, only about 17% of the primary lymphedema samples can be explained by a change in the 7 genes tested. Thus, when evaluating a patient with primary lymphedema, family history is an important guide to genetic screening. Associated symptoms direct to the most likely gene(s) (fig. 1). For a large proportion of the families, the mutated gene remains unknown.

We also evaluated possible genotype-phenotype correlations and protein function. The majority (56%) of the lymphedema-causing mutations were discovered in the transmembrane receptor VEGFR3, encoded by *FLT4*. Most of the mutations are amino acid substitutions that alter conserved residues located in the 2 intracellular tyrosine kinase domains. They can occur either in familial or sporadic cases. The only exceptions were a recessive mutation in the ATP-binding domain (p.Ala855Tyr) for which a homozygous state is required to decrease receptor activity, as well as 2 less common types of *FLT4*

Table 4. Phenotypes associated with mutations in the known lymphedema genes

Gene	Disease name (OMIM No.)	Type of lymphedema	Associated symptoms
FLT4	Nonne-Milroy disease (153100)	congenital or early-onset lower limb lymphedema, predominantly at the dorsum of the feet	hydrocele, prominent veins, infection, upturned toenails and hydrops fetalis
GJC2	hereditary lymphedema (613480)	postpubertal lower or upper limb lymphedema	vein insufficiency, blepharoptosis, involvement of face and genitalia
FOXC2	lymphedema-distichiasis syndrome (153400)	pubertal or late-onset lower limb lymphedema	double row of eyelashes, cardiovascular anomalies, cleft palate, blepharoptosis, hydrops fetalis, cystic hygroma and bone defects
SOX18	hypotrichosis-lymphedema-telangiectasia syndrome (607823)	lower limb lymphedema	hypotrichosis, telangiectasia and blepharoptosis
GATA2	primary lymphedema with myelodysplasia (Emberger syndrome) (614038)	lower limb primary lymphedema	hydrocele, predisposition to myelodysplastic syndrome and acute myeloid leukemia, deafness and warts
CCBE1	Hennekam syndrome (235510)	upper and lower limb lymphedema	mental retardation and lymphangiectasia
PTPN14	choanal atresia and lymphedema (613611)	lower limb lymphedema	choanal atresia

changes: an in-frame deletion (p.Phe1108del) found in 3 distinct families [Evans et al., 2003; Ghalamkarpour et al., 2006; Connell et al., 2009], and an intronic splice-site change (c.2647+2T>C). By cloning and RT-PCR, it has been demonstrated that a deletion in a similar position of another splice-site (c.2542+2delT, formerly IVS17+2delT) generates an abnormal splicing product: intronic insertion and exon 17 skipping [Futatani et al., 2008]. The phenotype caused by the identified VEGFR3 mutations is as described for Nonne-Milroy disease in earlier reports [Brice et al., 2005; Ghalamkarpour et al., 2006]. Most patients had bilateral congenital lower-limb lymphedema, predominantly on the dorsal feet. In some older patients it progressed higher on the leg. Familial history is not obligatory [Ghalamkarpour et al., 2006], as de novo mutations were identified in 7 patients. Several patients had associated features: hydrocele (n = 5), upturned toenails (n = 4) and saphenous insufficiency (n = 1) (table 4).

CCBE1 mutations were identified in 2 families, accounting for 4.8% of the mutated patients. In zebrafish, the expression of *ccbe1* overlaps with that of *vegfc* (the VEGFR3 ligand) in the somatic mesoderm [Hogan et al., 2009], and in mice, CCBE1 enhances the lymphatic responses to VEGFC [Bos et al., 2011]. All *CCBE1*-mutated patients clearly had Hennekam syndrome [Alders et al., 2009], and no change in this gene was found among the sporadic primary lymphedema patients. Thus, future screens of CCBE1 could be restricted to patients with generalized lymphatic dysplasia, with or without facial anomalies, with a recessive mode of inheritance [Alders et al., 2009; Connell et al., 2010] (table 4).

Mutations in the 3 transcription factor genes *(FOXC2, SOX18* and *GATA2)* account for 35% of the mutations identified. The majority result in premature truncation of the protein (tables 2, 3) and likely loss-of-function or dominant-negative effect, assuming that the shortened protein is expressed and stable, and can bind to its target sequences or dimerize with the wild-type protein. Mutations in transcription factors commonly have pleiotropic effects. Accordingly, all the patients exhibited syndromic phenotypes (table 4). *FOXC2* patients had lymphedema and distichiasis [Fang et al., 2000; Bell et al., 2001], and patients with mutations in *SOX18* had hypotrichosis with lymphedema and telangiectasia [Irrthum et al., 2003]. One patient with *GATA2* mutation did not have Emberger syndrome [Hahn et al., 2011; Ostergaard et al., 2011b] (table 4). Patients with a mutation in one of these 3 genes had characteristic features of their respective syndrome; however, the contrary was not always the case. Some individuals with clear signs of these phenotypes were not found to have a change in one of these genes, suggesting technical limitations in detecting all mutations, or more likely, locus heterogeneity.

GJC2 (encoding connexin-47) is mutated in patients with various phenotypes. It causes autosomal dominant, often late-onset lymphedema of lower and upper extremities. Associated symptoms include blepharoptosis, recurrent cellulitis, venous insufficiency, and facial and genital involvement (table 4) [Ferrell et al., 2010; Ostergaard et al., 2011a]. In our sequential series, a *GJC2* mutation accounted for only 2.4% of mutated patients (a single family with a mutation). The index case had a late-onset (age 30) bilateral lymphedema, localized to the ankles.

No *PTPN14* mutation was found in our series. PTPN14 has a dynamic subcellular localization in vitro. It is nuclear in proliferating cells, but concentrated at intercellular

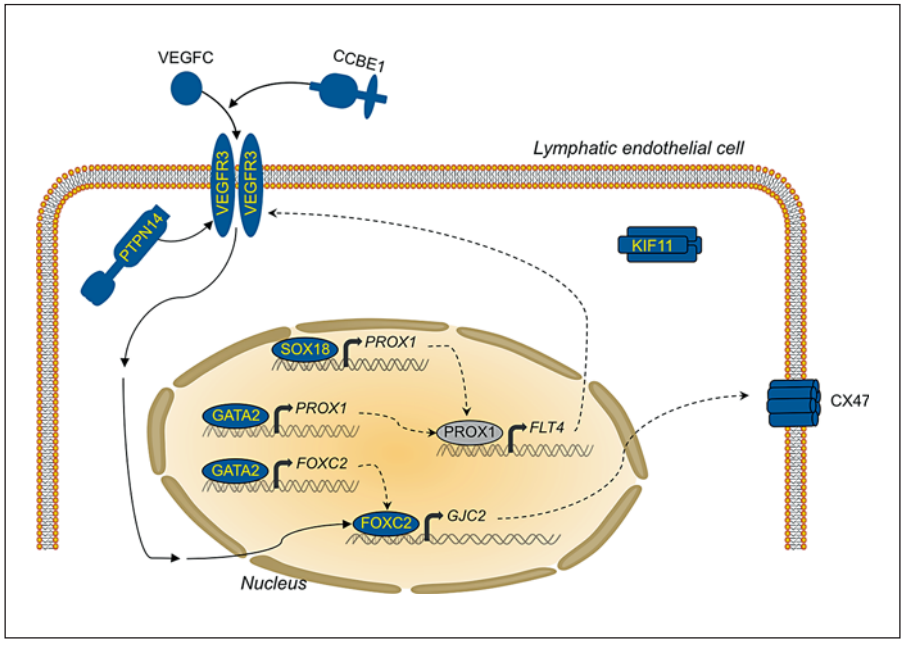

Fig. 2. The functions of the proteins encoded by the genes mutated in primary lymphedema cluster around a central molecular pathway: the VEGFR3 signaling.

junctions in confluent cells [Wadham et al., 2000; Benzinou et al., 2012]. In vivo, there is an overlap in expression of PTPN14 and VEGFC, and it has been shown that VEGFC stimulation enhances recruitment of PTPN14 to a complex including VEGFR3 (fig. 2). The only mutation reported so far in this gene is a homozygous deletion of exon 7, resulting in loss-of-function in a family with juvenile-onset lymphedema, choanal atresia, developmental delay, and pericardial effusion in some affected individuals [Au et al., 2010]. It may be that this is a very rare private mutation; *PTPN14* mutations probably explain only a very small proportion of primary hereditary lymphedema.

VEGFR3 signaling is a theme common to the functions of the proteins encoded by the 9 genes known to be mutated in patients with primary lymphedema (fig. 2). The functional link varies. It can be at the level of the ligand/receptor (VEGFR3, CCBE1), modification of receptor activity (PTPN14), production of the receptor (SOX18 and GATA2 via PROX1), (in)direct control of regulation of transcription (FOXC2, also via GATA2), or a downstream target gene (*GJC2*, and *FLT4* itself). Genetic interactions between the different molecules have been demonstrated in combined heterozygous mice or double knock-down zebrafish [Bos et al., 2011; Cermenati et al., 2013; Hagerling et al., 2013]. The link is less obvious between *KIF11*, which participates in mitotic spindle assembly, and mutations of which cause MCLMR [Ostergaard et al., 2012]. A comprehensive view suggests that regulation of the VEGFR3 signaling pathway is central to the molecular mechanism of primary lymphedema. This is underscored by the loss-of-function mutation discovered in *VEGFC* in a family with autosomal dominant Milroy-like primary lymphedema [Gordon et al., 2013].

Conclusion

This study shows that mutations in 7 lymphedema-causing genes explain about one-third of all familial primary lymphedemas. Although this proportion is increased due to the newly discovered genes *KIF11* and *VEGFC*, more than half of hereditary primary lymphedemas remain unexplained. Based on our analyses, the proteins encoded by mutated genes cluster around a central molecular pathway: VEGFR3 signaling (fig. 2). The detailed functional roles among all the players are as yet unclear. It is likely that the genes to be identified will also encode proteins that interact with this central pathway. This concept should help guide discovery of more genes, and their identification will divulge the precise regulation and function of VEGFR3 signaling.

It is clear that primary (hereditary) lymphedema exhibits high locus heterogeneity due to numerous private mutations. The emerging notion that some patients with secondary lymphedema also harbor germline changes in

genes that are mutated in primary lymphedema extends this pathogenetic view to explain the more common forms of lymphedema [Finegold et al., 2012]. Whole exome sequencing should help to overcome this heterogeneity and aid in the hunt for the missing genes responsible for primary lymphedema. Someday, pinpointed screening of a complete lymphedema gene panel may become a useful strategy to prevent primary, and secondary, lymphedema.

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