Genetics of lymphatic anomalies

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Lymphatic anomalies include a variety of developmental and/or functional defects affecting the lymphatic vessels: sporadic and familial forms of primary lymphedema, secondary lymphedema, chylothorax and chyloous ascites, lymphatic malformations, and overgrowth syndromes with a lymphatic component. Germline mutations have been identified in at least 20 genes that encode proteins acting around VEGFR-3 signaling but also downstream of other tyrosine kinase receptors. These mutations exert their effects via the RAS/MAPK and the PI3K/AKT pathways and explain more than a quarter of the incidence of primary lymphedema, mostly of inherited forms. More common forms may also result from multigenic effects or post-zygotic mutations. Most of the corresponding murine knockouts are homozygous lethal, while heterozygotes are healthy, which suggests differences in human and murine physiology and the influence of other factors.

Introduction
The lymphatic system plays a crucial role in tissue homeostasis. Blunt-ended lymphatic capillaries collect extravasated fluid, transport it via collecting lymphatic vessels and the thoracic duct, and return it to the blood circulation at the level of the left subclavian vein. The lymph travels through lymph nodes for immune surveillance. Larger lymphatic vessels contain valves to orient the passive flow, driven by neighboring arterial pulsations and muscular contractions. Lymphatic anomalies result from defects in the development, maturation, or function of this system. These include lymphatic malformations (LMs), lymphedema, chylothorax and chyloous ascites, and overgrowth syndromes with a lymphatic component, including the Klippel-Trenaunay-Weber, proteus, PTEN hamartoma tumor syndrome, and CLOVES (congenital lipomatous overgrowth, vascular malformations, epidermal nevi, and skeletal/spinal abnormalities) syndromes (1). Genetic studies and the generation of genetically modified mice have started to shed light on the etiopathogenic mechanisms underlying these diseases.

Lymphedema
The most frequent lymphatic anomaly is lymphedema. It is characterized by abnormal accumulation of interstitial fluid due to inefficient uptake and reduced flow, leading to swelling and disability, mostly in the extremities. Treatment is limited to pressotherapies, lymphatic drainage, and elastic compression and is never curative. Lymphedema can be primary (often congenital) or secondary (acquired, for example, after an infection or as a consequence of surgical breast cancer treatment).

Familial primary lymphedema commonly segregates in an autosomal dominant or recessive manner. It has been classified based on the age at onset into congenital (such as Nonne-Milroy lymphedema or type I lymphedema), peripubertal (hereditary lymphedema II, also known as Meige disease or lymphedema praecox), and late-onset lymphedema (onset after 35 years of age; also referred to as lymphedema tarda). Lymphedema can also occur in association with other clinical signs, as part of a syndrome. Nine-teen genes have been identified as being mutated in different isolated or syndromic forms of lymphedema (Table 1). On the basis of these data, a complex algorithm for clinical diagnosis has been proposed (2); however, the reduced penetrance of several of the signs and symptoms of the syndromes complicate its use. Interestingly, most of the proteins encoded by these genes seem to involve the VEGF-C/VEGFR-3 axis (3).

VEGF-C/VEGFR-3 signaling axis
The first mutations were discovered in FLT4, which encodes VEGFR-3 (Figure 1). Missense mutations in the tyrosine-kinase domain of the receptor cause primary congenital lymphedema (Nonne-Milroy lymphedema; OMIM 153100). This is usually present at birth, is bilateral, and affects the feet up to the knees (4, 5). Patients sometimes present with prenatal pleural effusion or in utero hydrops (6, 7). Nonne-Milroy lymphedema is usually an autosomal dominant disorder, yet de novo mutations are not infrequent (7–10). Thus, a family history of lymphedema is not required for diagnosis. Moreover, a family with a particular recessive VEGFR3 mutation has been reported (9). To date, mutations have been discovered in more than 100 families (Table 1 and refs. 3, 11).

Dominant VEGFR3 mutations inhibit receptor phosphorylation and prevent downstream signaling. The recessive mutation has a weaker effect, resulting in reduced ATP binding. In lymphoscintigraphy, hypoplasia or aplasia of the lymphatic system is observed (12). A similar phenotype is seen in the Chy mouse, which carries a spontaneous heterozygous point mutation in Vgfrc and is a model to study the pathophysiology and to perform preclinical trials of Nonne-Milroy lymphedema. The homozygous Vgfrc knockout mice die around E9.5 due to irregular vessels with defective lumens (13, 14).

A mutation in the VEGFR-3 ligand, VEGFC, was reported in one family (Figure 1 and ref. 15). Clinically, these patients are indistinguishable from those with a FLT4 mutation. The predicted mutant protein was expressed in zebrafish but lacked detectable activity. Based on the position of the mutation (a small deletion in the fourth exon), the mutant allele likely undergoes nonsense-mediated mRNA decay in human beings, resulting in negligible expression of the mutant protein. This would result in a loss of VEG-C function. Similarly, spontaneous heterozygous Chy-3 mice, which develop chylous ascites and lymphedema, have a deletion of the whole VEGFC gene (16). Homozygotes lack all lymphatic vasculature and heterozygotes have lymphatic hypoplasia (17). Haploinsufficiency is therefore the most probable pathogenic mechanism for mutations in VEGFC.
Collagen and calcium-binding EGF domain–containing protein 1 (CCBE1) binds to the extracellular matrix and potentiates the effects of VEGF-C on VEGFR-3 (Figure 1 and ref. 18). It is mutated in the zebrafish mutant full of fluid (fof), where it was shown that CCBE1 is required for lymphangioblast budding and angiogenic sprouting from venous endothelium (19). In humans, homozygous or compound heterozygous mutations that abolish CCBE1 function cause highly penetrant, generalized lymphatic anomalies, including lymphedema and visceral lymphangiectasias (Table 1). These are associated with typical facial features and mental retardation, components of the Hennekam lymphangiectasia-lymphedema syndrome (OMIM 235510) (20–22).

PTPN14 is a protein tyrosine-phosphatase that is recruited to the VEGFR-3 receptor upon VEGF-C stimulation (Figure 1 and ref. 23). A homozygous deletion of exon 7 was found in a single consanguineous family with lymphedema and choanal atresia (OMIM 608911). The mutation results in a shift in the reading frame and the appearance of a premature termination codon (23), probably leading to nonsense-mediated mRNA decay and loss of function. Ptpn14-deficient mice mimic the human phenotype, as they develop lymphedema postnatally due to hyperplastic vessels (23). Thus, hyperactive VEGFR-3 signaling due to loss of the phosphatase also perturbs normal lymphatic development.

Transcription factors
Several transcription factors act downstream of VEGFR-3 (Figure 1). As they have pleiotropic effects through several target genes, their mutations cause syndromic forms of lymphedema. Truncating and missense mutations in FOXC2 are found in patients with late-onset lymphedema (hereditary lymphedema II; OMIM 153200), often associated with distichiasis (double row of eyelashes) and sometimes ptosis (OMIM 153400) and/or yellow nails (OMIM 153300) (Table 1 and refs. 24–30). Distichiasis has a high penetrance, yet not all patients with this feature carry a mutation in FOXC2. Foxc2−/− mice have abnormal lymphatic patterning and arrested lymphatic valve development. In heterozygotes, increased recruitment of pericytes hampers the function of collecting lymphatics, reminiscent of the human phenotype (31).
VEGFR-3 expression is controlled by PROX1, a crucial transcription factor for initiation of lymphangiogenesis. PROX1 is under the control of another transcription factor, SOX18 (Figure 1). The latter is mutated in the rare hypotrichosis-lymphedema-telangiectasia syndrome (OMIM 607823), which is characterized by variable-onset lymphedema associated with sparse hair and cutaneous telangiectasias. A dominant nonsense mutation is located in the transactivation domain of SOX18 and two recessive substitutions in the DNA-binding domain (32). The former may compete for DNA binding without transcriptional activation of target genes, whereas the latter likely have less affinity to their promoter binding motifs. **Ragged** mice, which have Sox18 mutations, are phenotypically similar (32, 33).

**Table 1**

<table>
<thead>
<tr>
<th>Lymphatic anomalies/additional signs</th>
<th>Gene (protein)</th>
<th>Cases</th>
<th>Penetrance</th>
<th>Mutation type</th>
<th>Inheritance</th>
<th>Animal models</th>
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<tbody>
<tr>
<td>Isolated lymphedema</td>
<td></td>
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<tr>
<td>Primary congenital lymphedema/</td>
<td>FLT4 (VEGFR-3)</td>
<td>&gt;100</td>
<td>High</td>
<td>Inactivating</td>
<td>AD, AR,</td>
<td>Chy, Flt4&lt;sup&gt;+/−&lt;/sup&gt;</td>
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<tr>
<td>Nonne-Milroy lymphedema</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>de novo</td>
<td></td>
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<td>Milroy-like disease</td>
<td>VEGFC</td>
<td>1</td>
<td>High</td>
<td>LOF</td>
<td>AD</td>
<td>Chy-3, Vegfc&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>Syndromic lymphedema</td>
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<td>Hennekam lymphangiectasia-lymphedema syndrome/mental retardation</td>
<td>CCBE1</td>
<td>13</td>
<td>High</td>
<td>LOF</td>
<td>AR</td>
<td>fofofofofofofofofofofofofofofofofofofofo</td>
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<td>Lymphedema-distichiasis and yellow nail syndromes/ptosis</td>
<td>FOXC2</td>
<td>&gt;85</td>
<td>High</td>
<td>LOF</td>
<td>AD</td>
<td>Foxc2&lt;sup&gt;+/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hereditary lymphedema II (Meige disease)</td>
<td>GJC2 (CX47)</td>
<td>7</td>
<td>High</td>
<td>Missense</td>
<td>AD</td>
<td>(Gjc2&lt;sup&gt;+/−&lt;/sup&gt;)</td>
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<tr>
<td>Oculodentodigital dysplasia/lymphedema</td>
<td>GJA1 (CX43)</td>
<td>1</td>
<td>High</td>
<td>Missense</td>
<td>AD</td>
<td>(Gja1&lt;sup&gt;+/−&lt;/sup&gt;)</td>
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<td>Choroidal atresia/lymphedema</td>
<td>PTPN14</td>
<td>1</td>
<td>High</td>
<td>LOF</td>
<td>AR</td>
<td></td>
</tr>
<tr>
<td>Hypotrichosis-lymphedema-telangiectasia syndrome</td>
<td>SOX18</td>
<td>3</td>
<td>High</td>
<td>LOF</td>
<td>AR, AD, de novo</td>
<td>Ragged, (Sox18&lt;sup&gt;−/−&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Lymphedema-lymphangiectasia</td>
<td>HGF</td>
<td>4</td>
<td>Medium</td>
<td>LOF?</td>
<td>D-N</td>
<td>AD?</td>
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<td>MCLMR</td>
<td>Klf11</td>
<td>14</td>
<td>Low</td>
<td>LOF</td>
<td>AD</td>
<td>(Klf11&lt;sup&gt;−/−&lt;/sup&gt;)</td>
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<tr>
<td>Noonan syndrome 1 (54% with lymphedema)</td>
<td>PTPN11 (SHP2)</td>
<td>&gt;100</td>
<td>Medium</td>
<td>GOF</td>
<td>AD</td>
<td>Shp2&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Noonan syndrome 1 (65% with lymphedema)</td>
<td>SOS1</td>
<td>Few with lymphedema</td>
<td>Medium</td>
<td>GOF</td>
<td>AD</td>
<td>–</td>
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<tr>
<td>Primary lymphedema, myelodysplasia (Emberger syndrome)</td>
<td>GATA2</td>
<td>13</td>
<td>Low</td>
<td>LOF</td>
<td>AD</td>
<td>(Gata2&lt;sup&gt;−/−&lt;/sup&gt;)</td>
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<tr>
<td>OLEDAID</td>
<td>IKBKG (NEMO)</td>
<td>5</td>
<td>Low</td>
<td>Hypomorphic</td>
<td>X-linked</td>
<td>(Ikbkg&lt;sup&gt;−/−&lt;/sup&gt;)</td>
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<tr>
<td>CM-AVM/lymphedema</td>
<td>RASA1</td>
<td>Few with lymphedema</td>
<td>Low</td>
<td>LOF</td>
<td>AD</td>
<td>(Rasa1&lt;sup&gt;−/−&lt;/sup&gt;)</td>
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<tr>
<td>Cholestatics-lymphedema syndrome (Agenaes syndrome)</td>
<td>locus in 15q</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>AR</td>
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<tr>
<td>Syndromic chylothorax/chylous ascites, lymphangiectasia</td>
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<tr>
<td>Fetal chylothorax</td>
<td>ITGA9</td>
<td>6</td>
<td>High</td>
<td>Missense</td>
<td>AR, de novo</td>
<td>Ilga9&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>Noonan syndrome, cardiofaciocutaneous syndrome/chylothorax</td>
<td>KRAS</td>
<td>Few with chylothorax</td>
<td>Low</td>
<td>GOF</td>
<td>AD</td>
<td>Kras&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Noonan syndrome 1/lymphangiectasia</td>
<td>RAF1</td>
<td>Few with lymphangiectasia</td>
<td>Low</td>
<td>GOF</td>
<td>AD</td>
<td>Raf1-KI</td>
</tr>
<tr>
<td>Costello syndrome/chylosis ascites, chylothorax</td>
<td>HRAS</td>
<td>Few with chylosis ascites/chylothorax</td>
<td>Low</td>
<td>GOF</td>
<td>AD</td>
<td>Hras&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>Syndromes with LMs</td>
<td></td>
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<tr>
<td>Turner syndrome/nuchal translucency</td>
<td>Monosomy X</td>
<td>&gt;100</td>
<td>Medium</td>
<td>–</td>
<td>Sex-linked</td>
<td>(X0 mice)</td>
</tr>
<tr>
<td>Proteus syndrome, Pten hamartoma tumor syndrome</td>
<td>PTEN</td>
<td>–10</td>
<td>Medium</td>
<td>LOF</td>
<td>AD, de novo</td>
<td>(Pten&lt;sup&gt;−/−&lt;/sup&gt;)</td>
</tr>
<tr>
<td>CLOVES, Klippel-Trenaunay-Weber syndrome</td>
<td>PIK3CA</td>
<td>3</td>
<td>N/A</td>
<td>GOF</td>
<td>Somatic</td>
<td>p&lt;sub&gt;100-KI&lt;/sub&gt;</td>
</tr>
<tr>
<td>Proteus syndrome</td>
<td>AKT1</td>
<td>40</td>
<td>N/A</td>
<td>GOF</td>
<td>Somatic</td>
<td>Akt1&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<sup>a</sup>Number of index cases reported with a mutation.  <sup>b</sup>Penetrance of lymphatic anomalies when carrying a mutation.  <sup>c</sup>Germine mutants only. Parentheses indicate the absence of lymphatic anomaly, and bolded text indicates that the model partially mimics the human phenotype.  <sup>1</sup>Inactivation in adults. AD, autosomal dominant; AR, autosomal recessive; D-N, dominant negative; GOF, gain of function; KI, knockin; LOF, loss of function; –, not applicable. Question marks indicate that mutation type and/or inheritance is unclear.
GATA2 is a transcription factor that controls PROX1 and FOXC2 expression (Figure 1). Most GATA2 mutations are associated with dendritic cell, monocyte, B-lymphocyte, and natural killer lymphocyte deficiency (OMIM 614172), with susceptibility to myelodysplastic syndrome (OMIM 614286) and acute myeloid leukemia (OMIM 601626). However, some GATA2 mutations have been discovered in patients with primary lymphedema with myelodysplasia (Emberger syndrome; OMIM 614038), which is characterized by lymphedema and myelodysplasia (36, 37). There is no clear phenotype-genotype correlation, suggesting that modifiers play a role.

Besides cross-regulation, these transcription factors modify expression levels of several other proteins, some of which are involved in lymphangiogenesis (Figure 1). PROX1 regulates FLT4, and FOXC2 controls proteins essential for lymphatic valves, such as connexins. Thus, adequate spatiotemporal control of their activity is essential for proper lymphatic vessel development and function.

Nuclear and transcription factor regulators
Mutations in IKBKG and KIF11, which modulate the activity of transcription factors or affect nuclear dynamics, cause complex lymphedema syndromes. IKBKG (also known as NEMO) is an NF-kB modulator. It is mutated in patients with the rare X-linked syndrome anhidrotic ectodermal dysplasia with immunodeficiency, osteopetrosis, and lymphedema (OLEADA; OMIM 300301), associated with incontinentia pigmenti in the mother (OMIM 308300) (Figure 1). Five cases of OLEADA with an IKBKG mutation have been reported (Table 1 and refs. 38–41). Loss-of-function mutations cause incontinentia pigmenti, which is embryonic lethal in males (42). The OLEADA-causing mutations are hypomorphs, which diminish but do not abolish the ability of IKBKG to activate NF-kB (41). NF-kB upregulates PROX1 and cooperates with it to induce VEGFR-3 expression (Figure 1 and ref. 43).

KIF11 encodes EG5, which acts as a homotetrameric kinesin motor. Members of this protein family are involved in establishing a bipolar spindle during mitosis for chromosome positioning and centrosome separation. Heterozygous KIF11 mutations cause a specific syndrome consisting of lower limb lymphedema of variable expressivity associated with microcephaly with or without chorioretinopathy, lymphedema, or mental retardation (MCLMR; OMIM 152950) (44). The EG5 mutations causing MCLMR may have dominant-negative effects, since Kif11 mutant mice are phenotypically normal and Kif11−/− mice die prior to implantation (45). A link to the VEGF-C/VEGFR-3 pathway has not been established, but inhibition of EG5 activates the PI3K/AKT pathway (Figure 1 and ref. 46).

Connexins
The transcription factors involved in VEGF-C/VEGFR-3 signaling regulate expression of genes essential for lymphatic (and venous) valve development and maintenance. These include the gap junction proteins, connexins (Figure 1 and ref. 47). Mutations in two connexins have been identified in a few lymphedema patients (Table 1). They consist of amino acid substitutions that alter but do not abolish connexin function.

Substitutions of highly conserved amino acids in connexin 47 (CX47), encoded by GJC2, cause lymphedema in all four extremities (48, 49). In contrast, loss-of-function mutations, primarily premature stop codons, cause hypomyelinating leukodystrophy 2 (OMIM 608804), in which lymphedema does not occur. CX47 is expressed in lymphatic endothelial cells, on the upstream side of lymphatic valves (Figure 1 and ref. 47). The amino acid substitutions may have gain-of-function effects, as Cx47 homozygous knockouts have no lymphatic defect (Table 1 and ref. 50).

An amino acid substitution in another connexin, CX43 (GJA1), can also cause lymphedema, as part of the oculodentodigital dysplasia (OMIM 164200), which affects the eyes, face, teeth, and digits (51). CX43 is also highly enriched on the upstream side of lymphatic valves (Figure 1), where it forms hemi- and intercellular channels (47). The only GJA1 mutation reported so far to cause lymphedema likely specifically alters channel properties leading to valve dysfunction. Homozygous inactivation of Cx43 in mice is lethal at birth because of cardiac malformation (52).

Rasopathies and lymphedema
Lymphedema, chylothorax, or chylous ascites are also variably seen in patients with mutations in genes of the RAS signaling pathway, commonly referred to as rasopathies. Noonan syndrome 1 (OMIM 163950) is caused by germline mutations in PTPN11 encoding SHP2 (53), a mediator of tyrosine kinase receptor signaling (Figure 1), in 50% of patients. In another 13% of patients, the mutation is in SOS1 (53). Lymphedema is present in half of the patients with mutations in PTPN11, and in over 60% of those with a SOS1 mutation (Table 1 and ref. 54).

In some patients, activating mutations associated with lymphedema or lymphangiectasia are found in KRAS (55) or RAF1 (56). KRAS usually causes the cardiofaciocutaneous syndrome (57), and mutations in HRAS are associated with Costello syndrome (58, 59). In both disorders, chylothorax and chylous ascites can be seen. Such features have also been observed in a small number of patients with a RASA1 mutation causing capillary malformation-artériovenous malformation (CM-AVM) syndrome (60, 61). RASA1 encodes the RAS GTPase p120RASGAP (Figure 1), and inactivation of this gene in adult mice results in alteration of the lymphatic system (62). All these anomalies lead to increased RAS pathway activity.

Additional genes
In addition to the above-mentioned genes that are known to carry mutations in subgroups of patients with variable penetrance of lymphedema (Table 1), several other genes may be involved in lymphatic anomalies. A recurrent integron 69 (ITGA9) missense mutation seems to associate with severe chylothorax in human fetuses (63–65). ITGA9 expression is controlled by PROX1 (66). ITGA9 binds to fibronectin and is essential for the development of lymphatic valves (Figure 1) (67). Inactivation in mice results in fatal bilateral chylothorax (68).

Alterations in hepatocyte growth factor (HGF) and its receptor (MET) were reported in a few families with lymphedema (69). However, bioinformatic re-analysis of the MET changes predicted that these mutations are benign. The four HGF mutations, two of which are premature stop codons, are probably damaging. HGF is an interesting predisposing factor, as it stimulates lymphangiogenesis in vitro and in vivo (70). Confirmatory genetic data are needed.

Cholestasis-lymphedema syndrome (OMIM 214900), also known as Aagenaes syndrome, has been linked to chromosome 15 on the basis of haplotype sharing (71). The causative gene is unknown. A CCBE1 mutation was also found in one patient with cholesta-sis-lymphedema syndrome, suggesting locus heterogeneity (72).

Overall, genetic mutations in a subset of eight genes explain of lymphatic valves (Figure 1 and ref. 47). The amino acid substitutions may have gain-of-function effects, as Cx47 homozygous knockouts have no lymphatic defect (Table 1 and ref. 50).

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Overall, genetic mutations in a subset of eight genes explain
This comprises less than 25% of patients with lymphedema. Some of the remaining heritability could be due to mutations in the other 11 known genes, but such mutations are likely rare, since the syndromes caused by mutations in those genes are particular and well characterized and/or the penetrance of lymphedema is low. Although genetic screens are rarely, if ever, exhaustive, additional genes that are mutated in primary lymphedema are highly likely to exist.

**Secondary lymphedema**

Secondary lymphedema is induced by external stimuli, such as infection, surgery, or radiotherapy. It may be influenced by genetic predisposition (73). Predisposing changes were reported in the *MET* gene (69) and in the *GJC2* gene (74). Changes in the latter were found to increase risk for developing secondary lymphedema following breast cancer treatment.

Secondary lymphedema often develops years after an invasive treatment. Dissection of its genetic predisposition therefore requires long-term prospective studies. Lymphangiogenesis is under the control of a tightly orchestrated equilibrium between numerous players. Predisposition to secondary lymphedema may be based on weak changes in a number of these genes instead of strong, mutation-like changes. Their identification would help develop novel, targeted therapeutic modalities.

**LMs**

LMs are localized lesions that consist of dilated lymphatic channels filled with lymph but disconnected from the normal lymphatic system (75). LMs are congenital and enlarge when infected. They are sporadic, usually unifocal, and their etiopathogenesis is unknown. The lack of familial forms and the unifocality of the lesions suggest that the cause could be a somatic mutation, restricted to cells of the lesion, and that would be too damaging (i.e., lethal) if occurring germline. Such a scenario held true for sporadic venous malformations, which we have demonstrated to result from strongly activating somatic mutations in *TIE2* (76, 77). Other developmental disorders have since been shown to be due to similar post-zygotic mosaic mutations (78, 79).

LMs can be part of a syndrome, such as Turner syndrome (due to monosomy X), or overgrowth syndromes, such as proteus syndrome (OMIM 176920), Klippel-Trenaunay-Weber syndrome (capillary-lymphatico-venous malformation; OMIM 149000), and CLOVES syndrome (OMIM 612918) due to mutations in the PI3K/AKT pathway (Figure 1 and Table 1). *PTEN* mutations are found in a subset of patients with PTEN hamartoma tumor syndrome; some are inherited (80). In contrast, a recurrent somatic activating mutation was discovered in *AKT1* in patients with proteus syndrome (78). *Akt1*−/− mice have reduced lymphatic capillaries and valve numbers, underscoring an important function in lymphangiogenesis (81). Somatic mutations that activate *PI3KCA* were identified in patients with CLOVES and some with Klippel-Trenaunay-Weber syndrome (79). All of these proteins are core elements of the PI3K/AKT/mTOR pathway (Figure 1), suggesting that other overgrowth syndromes as well as sporadic LMs may be caused by somatic changes in the components of this pathway.

**Current methods and future directions**

Mutations in the VEGF-C/VEGFR-3 axis are a central mechanism in the etiopathogenesis of inherited lymphedema (Figure 1). Many of the mutated genes encode proteins involved in the downstream regulation of target gene expression or proteins of the RAS/MAPK pathway that regulate VEGFR-3 expression. Yet the cause of primary lymphedema remains unexplained for many patients. Some syndromes with localized lymphatic dysplasia are caused by somatic mutations that tend to cluster around PI3K/AKT signaling. There are connections between the two pathways (Figure 1), suggesting that the etiopathogenic causes of primary (and secondary) lymphedema may cluster around these intracellular functions.

For many years linkage analysis and positional cloning have been used to identify disease-causing genetic mutations, as was the case for VEGFR3 and FOXC2. In some instances, for example *SOX18* and *CBE1*, this approach was helped by the identification of spontaneous mutant animal models that guided the human genetic studies. In other cases, researchers utilized autozygosity mapping in large consanguineous families (*PTPN14*). More recently, the advent of next-generation sequencing has allowed analysis of large candidate regions or the whole exome (whole-exome sequencing [WES]). Using WES on several patients with a similar phenotype led to the identification of mutations in novel genes, such as *KIF11* and VEGFC. In the latter, restricting the analysis to candidate genes proved successful.

The unexplained lymphatic anomalies might also result from a combination of mildly mutated germline alleles that are more difficult to identify and mutations in regulatory regions and/or epigenetic changes. The latter two are not detected by WES. Epigenome and whole-exome sequencing may thus be indicated in the future. However, within primary lymphedema, in which locus heterogeneity is high and sporadic cases are not rare, targeted high-throughput sequencing of a panel of genes has become the method of choice for diagnostic screens.

It is difficult to treat lymphedema, whether primary or secondary. Genetic insights point toward VEGF-C/VEGFR-3 signaling as a target. Autologous grafts are currently being tested in combination with adenosyl viral expression of VEGF-C in preclinical studies of secondary lymphedema (82). This seems to increase lymphangiogenesis around the implanted lymph nodes, helping them to connect with the lymphatic system. Another candidate is HGF. Injection of a plasmid expressing HGF in rat tail (83) or in a mouse model of upper limb lymphedema that simulates breast cancer-related lymphedema (84) prevented overt development of lymphedema and stimulated development of new lymphatic vessels in the long term. Such growth factors may potentially be useful in inducing the development functional lymphatics.

The RAS/MAPK pathway is activated in some forms of syndromic lymphedema (Noonan syndrome) and CM-AVM syndrome. Many inhibitors of this pathway are being developed in laboratories and tested in preclinical trials for cancer therapy. Some of them may be applicable as therapies for syndromic lymphedema.

In regard to syndromes with germline or somatic mutations activating the PI3K/AKT signaling pathway, small-molecule inhibitors may become useful as treatment. Many such inhibitors exist, and some are in clinical use for cancer, including the mTOR inhibitor rapamycin. Preliminary results on PTEN lesions and lymphatic anomalies are encouraging (85, 86).

The role of genetics in lymphatic anomalies is complex, as illustrated by the 23 mutated human genes reported thus far (Table 1). Their respective prevalence has not been studied in detail on large series of patients, and the lymphatic defect is sometimes a rare or minor feature of the syndromic phenotype. With the increased availability of high-throughput sequencing on large sets of
patients with lymphatic defects, several additional genes will likely be revealed. The clarification of the defective signaling pathways will also allow the generation of better animal models to test new molecules as targeted therapies to treat lymphatic anomalies.

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