REPORT

GJC2 Missense Mutations Cause Human Lymphedema

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Lymphedema is the clinical manifestation of defects in lymphatic structure or function. Mutations identified in genes regulating lymphatic development result in inherited lymphedema. No mutations have yet been identified in genes mediating lymphatic function that result in inherited lymphedema. Survey microarray studies comparing lymphatic and blood endothelial cells identified expression of several connexins in lymphatic endothelial cells. Additionally, gap junctions are implicated in maintaining lymphatic flow. By sequencing *GJA1*, *GJA4*, and *GJC2* in a group of families with dominantly inherited lymphedema, we identified six probands with unique missense mutations in *GJC2* (encoding connexin [Cx] 47). Two larger families cosegregate lymphedema and *GJC2* mutation (LOD score = 6.5). We hypothesize that missense mutations in *GJC2* alter gap junction function and disrupt lymphatic flow. Until now, *GJC2* mutations were only thought to cause dysmyelination, with primary expression of Cx47 limited to the central nervous system. The identification of *GJC2* mutations as a cause of primary lymphedema raises the possibility of novel gap-junction-modifying agents as potential therapy for some forms of lymphedema.

Lymphedema is the abnormal accumulation of lymphatic fluid in interstitial space. Patients with lymphedema suffer from recurrent local infections, physical impairment, and cosmetic and psychosocial stigmatization and may be at increased risk for developing lymphangiosarcoma.[1] The population prevalence of lymphedema is estimated in the range of 1.3–1.4 per 1000.[2] Primary (inherited) lymphedema is less common than secondary lymphedema, which is associated with conditions such as filariasis, trauma, and cancer therapy. Recent studies in families with inherited forms of lymphedema have identified six genes, *FLT4*[3,4] (encoding VEGFR3) (MIM 153100), *FOXC2*[5,6] (MIM 153400), *SOX18*[7] (MIM 607823), *HGF*[8] (MIM 142409), *MET*[8] (MIM 164860), and *CCBE1*[9,10] (MIM 235510), causing lymphedema.

To identify other causal genes for lymphedema, we reviewed differential gene expression in lymphatic endothelial cells (LECs) versus blood endothelial cells (BECs) and noted that *GJA1* (encoding connexin [Cx] 43) (MIM 121014) is expressed in BECs and LECs whereas *GJC2* (encoding Cx47) (MIM 608803) is expressed only in LECs.[11] Gap junctions are intercellular channels formed by hexamers of connexin proteins on adjoining cells that facilitate the electrical and metabolic coupling of cells within a tissue via a variety of mechanisms. Rhodin first suggested a role for gap junctions on lymphatic vessels,[12] but there has been limited characterization of gap junction intercellular communication (GJIC) in lymphatic vessels or LECs.[13,14]

We investigated the connexins as potential genes for causal lymphedema mutations in the families ascertained through the University of Pittsburgh Lymphedema Family Study (UPLFS). This study was approved by the Institutional Review Board of the University of Pittsburgh, and informed consent was obtained from all subjects. Initially, families were ascertained by a physician's diagnosis of lymphedema in the proband (confirmed by medical records) and a lymphedema occurrence in a first-degree relative. We screened 150 probands from the UPLFS for mutations in *GJA1* (chromosome 6q22-q23), *GJA4* (chromosome 1p35.1) (MIM 121012), and *GJC2* (chromosome 1q41-q42). Sequences were aligned and curated with Sequencher v4.7 (Gene Codes Corp.). Mutations in *FLT4*, *FOXC2*, and *SOX18*, known lymphedema genes, were previously excluded in these probands by bidirectional sequence analysis. The sequences of *GJA4* (NM002060), *GJA1* (NM000165), and *GJC2* (NM020435) were downloaded from Entrez Nucleotide. Unique sequence amplification and sequencing primers (see Table S1 available online) were designed to amplify genes in overlapping fragments. These fragments were then sequenced in both directions with ABI BigDye v3.1 chemistry, and the products were resolved on an ABI 3730 DNA sequencer in the Genomics and Proteomics Core Laboratory of the University of Pittsburgh. Six lymphedema families of mixed European ancestry were identified with heterozygous dominant causal *GJC2* mutations (Table 1).

We identified two *GJC2* mutations in families suitable for linkage analysis: one cosegregating lymphedema and a C>T transition at nucleotide 143 leading to an S48L (family 135) substitution in extracellular loop 1 of Cx47, and another cosegregating lymphedema and a C>T transition at nucleotide 778 resulting in an R260C (family 168) substitution in extracellular loop 2 (Figure 1). Linkage analysis in these two families yielded a LOD score of 6.5 under

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Table 1. *GJC2* Mutations Observed in Primary Lymphedema Families

Family	Sequence Substitution	Amino Acid Change	Predicted Domain
337	AGATCCACAACC(A>C)CTCCACCTTCGT	H19P	N-terminal
135	GAGGCCATCTACT(C>T)GGCGGAGCAGGCC	S48L	Extracellular loop 1
251	CCACGCCGCGCGCCCC(G>A)GCGCACCTGCCG	R125Q	Intracellular loop
104	GAGGAGCCCATGCTG(G>A)GCCTGGGCGAGGAG	G149S	Intracellular loop
168	TGCTTCGTGTCG(C>T)GCCCTACTGAAAAG	R260C	Extracellular loop 2
151	CCCGCGCCGCCCC(C>T)G CCCTGCGCCTTC	P316L	C-terminal

a model of disease frequency = 0.0001, penetrance = 0.9, phenocopy rate = 0.0, assuming no recombination. The R260C mutation is located within the conserved SRPTEK motif, important for connexon docking. This motif is a target of peptide mimetic inhibitors of GJIC for Cx43 and Cx32.[15,16] Four additional unique *GJC2* mutations were observed in other, smaller families: H19P in the N-terminal domain, R125Q in the intracellular loop, G149S in the intracellular loop, and P316L in the C-terminal domain were transmitted from an affected parent to an affected child. Samples were not available from other family members, and these cases are consistent with, but not informative for, linkage. (Figure 2; Table 1).

GJC2 mutations occur only in affected or at-risk individuals, cause a change in a conserved amino acid of Cx47, and were not present in 250 sequenced, ethnically matched controls (0 of 500 alleles). These missense mutations affect amino acids highly conserved in mammalian evolution, showing only one variation of glycine to alanine in the case of the G149S mutation (Figure 3). Non-lymphedema-associated sequence variants were also identified (Table S2).

The current age or age at death, genotype with respect to *GJC2*, age at onset of lymphedema of the leg and/or hand, and other phenotypic features in the families demonstrating linkage are shown in Figure 1. Uncomplicated lymphedema of the leg or hand was the only constant feature reported in the affected individuals. Individual IV-20, family 135, was reported to have a nuchal fold at birth but was nonpenetrant for lymphedema. Many affected individuals had onset of lymphedema in childhood or adolescence. Individuals IV-4, family 168, and III-18, IV-19, and IV-20, family 135, were nonpenetrant males, showing reduced penetrance of *GJC2* mutations in these families. Generally, males showed a later age at onset than females. Other features reported in some lymphedema pedigrees (ptosis, cellulitis, venous insufficiency, etc.) appeared sporadically in these families. Four individuals in family 135 reported recurrent skin infections. In the four smaller families with mutations, the clinical phenotypes were similar to the families demonstrating linkage, including a later age at onset.

Of note, two additional rare mutations, one leading to a truncated Cx47 protein (E44ter) and a 22 bp deletion leading to a truncation of the *GJC2* protein at residue 30, were identified. These changes were not present in 500 control alleles but failed to segregate with disease in pedigrees. These early nonsense changes are predicted to code for a prematurely truncated polypeptide, leading to a null allele. The carriers of these truncation mutations showed no discernable phenotype, consistent with the Cx47-deficient mouse, in which heterozygous or homozygous null animals have no gross phenotype and no Cx47-specific developmental or functional abnormality.[17,18]

We show here that mutations in *GJC2* cause primary lymphedema, through linkage in two families and significant genetic evidence from four independent families. We hypothesize that coordinated gap junction function is needed to optimize the conduction of lymph from the periphery to the thoracic duct and is compromised in individuals with *GJC2* missense mutations. In vivo evidence in rat mesenteric lymphatics shows significant impairment of contraction propagation upon treatment with nonspecific gap junction inhibitors.[13,14] The *GJC2* mutations are notable because they support an abnormality in lymphatic function rather than the previously identified mutations in genes causing abnormal lymphatic development. Such functional abnormalities could potentially benefit from the current development of gap-junction-modifying drugs,[19,20] offering a novel medical treatment for lymphedema.

The role of *GJC2*/Cx47 in lymphatic function is unexpected because it has a demonstrated primary role in the central nervous system (CNS), with expression reportedly limited to oligodendrocytes.[17,21] Homozygous loss-of-function mutations in *GJC2* cause Pelizaeus-Merzbacher-like disease (PMLD; MIM 608804), characterized by severe CNS dysmyelination.[22,23] Neither individuals affected with PMLD nor their obligate heterozygous carriers of *GJC2* mutations are reported to have a lymphatic phenotype, although the clinical phenotype of lymphedema is often subtle. Likewise, the clinical information available on our lymphedema patients and families would be insensitive to a mild clinical neurological abnormality. We observed no mutations in the transmembrane domains where many of the PMLD mutations are found.[23]

The *GJC2* lymphedema mutations are distributed throughout the protein, with no geographical clustering.

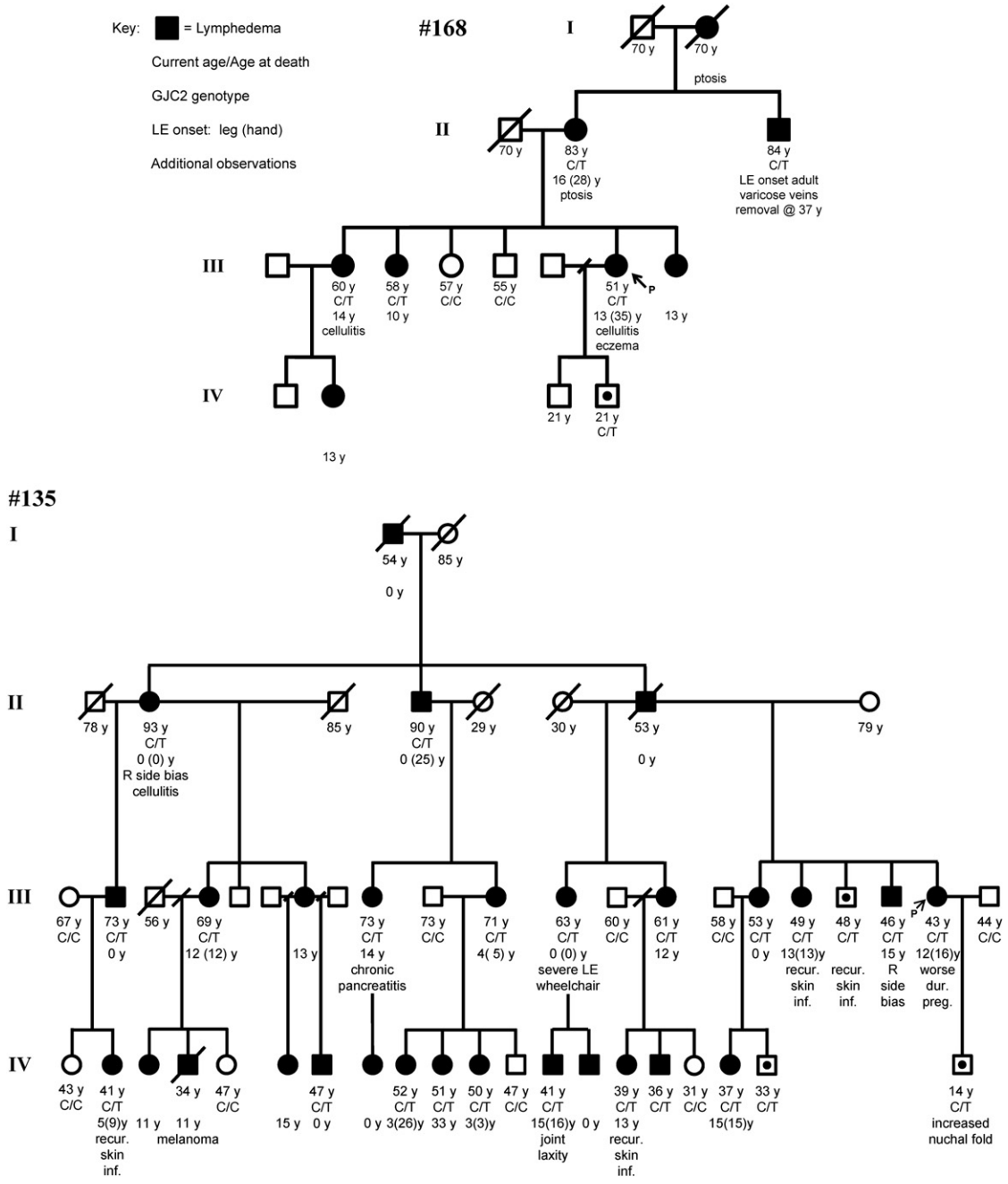

Figure 1. Pedigrees of the Two Linked Families
Pedigrees of the two linked families showing current age or age at death, cosegregation of *GJC2* missense mutation with lymphedema, age at onset of lymphedema of the leg and/or hand, and other phenotypic features. Family 168, R260C, and family 135, S48L, are shown. Filled shapes indicate affected individuals with lymphedema. LOD = 6.5. Arrows indicates the probands.

However, the two mutations located in the extracellular loop domains (i.e., S48L and R260C) are predicted to interfere with connexon (i.e., hemichannel) assembly into functional channels. The linked R260C mutation is located in a conserved SRPTEK motif important for connexon docking; the importance of this motif is further underscored by a homologous autosomal-dominant *GJA1* mutation (R202H) identified in families with oculodentodigital dysplasia (MIM 164200), with functional characteristics of poor plaque formation and impaired dye transfer and electrical coupling.[24,25] Similarly, we expect these two extracellular mutations to result in impaired channel activity and propose that this might result in impaired coordination of pulsatile lymphatic flow.[14] The mechanism through which the identified intracellular mutations mediate their effects is not clear, especially in light of the more recent recognition that connexin function is not limited only to their well-recognized channel activity but may involve hemichannel function or changes in cell adhesion or motility.[26–29] Further characterization of the

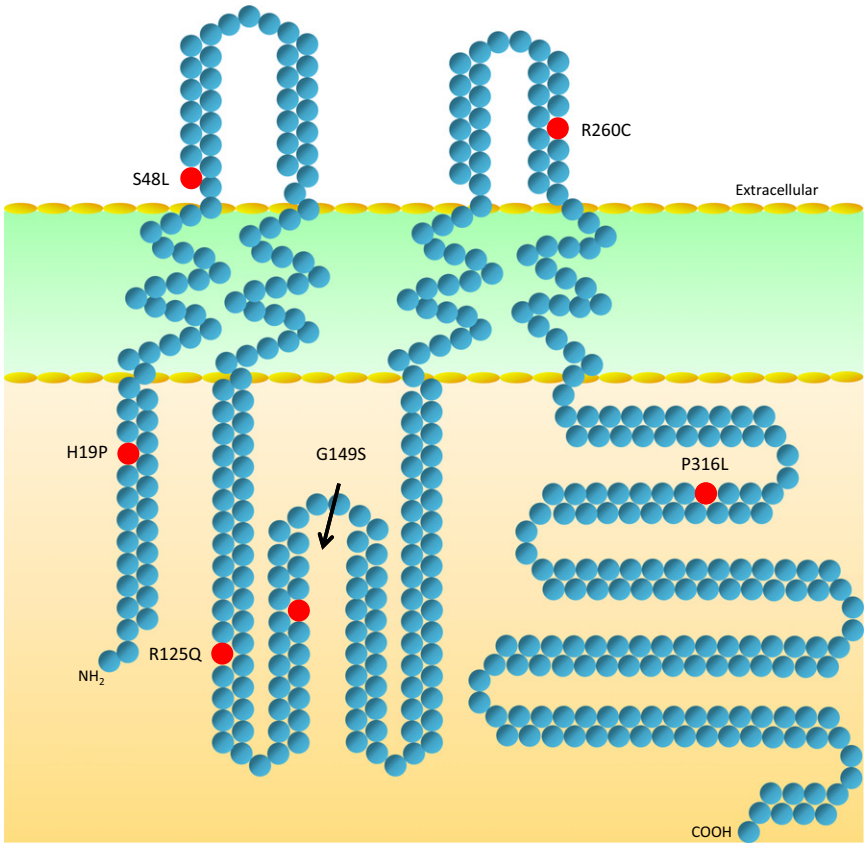

Figure 2. Schematic Drawing of Connexin 47 Protein Locating Lymphedema-Associated Amino Acid Substitutions
Domain break points and sequence are as described by Uhlenberg et al.[22] and http://www.uniprot.org/uniprot/Q5T442.

mutations reported here, especially with regard to their predicted dominant-negative effect with wild-type Cx47 or transdominant effect with other endogenous connexins expressed in LECs, will contribute to our understanding of the role of connexins in lymphatic function.

Although we are approaching the 400th anniversary of the first description of the lymphatic system in 1627,[30] lymphatics remain a poorly understood and largely neglected biological system. Despite an essential and active role in the maintenance of fluid homeostasis, trafficking of antigen-presenting and other immune cells, and pathological processes like chronic inflammation and cancer, the lymphatic vascular system is often viewed as a passive conduit rather than an interactive participant in these processes. The identification of mutations in the Cx gene family in primary lymphedema justifies further investigation into the manipulation of gap-junction-coordinated lymphatic flow and function as a possible therapeutic option for primary lymphedema.

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Human   MTNMSWSFLTRLLEEIHNHSTFVGKVWLTVLVVFRIVLTAVGGEAIYSDEQAKFTCNTRQPGCDNVCYDAFAPLSHVRFWVFQIVVISTPSVMYLGYAVHRLARASEQERRRALRRRPGP 120
Chimp   MTNMSWSFLTRLLEEIHNHSTFVGKVWLTVLVVFRIVLTAVGGEAIYSDEQAKFTCNTRQPGCDNVCYDAFAPLSHVRFWVFQIVVISTPSVMYLGYAVHRLARASEQERRRALRRRPGP 120
Cow     MTNMSWSFLTRLLEEIHNHSTFVGKVWLTVLVVFRIVLTAVGGESIYSDEQTKFTCNTRQPGCDNVCYDAFAPLSHVRFWVFQIVVISTPSVMYLGYAVHRLARASQDERRRASRRRP-S 119
Mouse   MTNMSWSFLTRLLEEIHNHSTFVGKVWLTVLVVFRIVLTAVGGESIYSDEQSKFTCNTRQPGCDNVCYDAFAPLSHVRFWVFQIVVISTPSVMYLGYAVHRLARASEQERRRALRRRPGT 120
Rat     MTNMSWSFLTRLLEEIHNHSTFVGKVWLTVLVVFRIVLTAVGGESIYSDEQSKFTCNTRQPGCDNVCYDAFAPLSHVRFWVFQIVVISTPSVMYLGYAVHRLARASEQERRRALRRRPGP 120
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Human   RRAPRAHLP---PPHAGWPEPADLGEEEPMLGLGEEEEEEETGAAEGAGEEAEEAGAEEACTKAVGADGKAAGTPGPTGQHDGRRRIQREGLMRVYVAQLVARAAFEVAFLVGQYLLYGF 237
Chimp   RRAPRAHLP---PPHAGWPEPADLGEEEPMLGLGEEEEEEETGAAEGAGEEAEEAGAEEACTKGVGADGKAAGTPGPTGQHDGRRRIQREGLMRVYVAQLVARAAFEVAFLVGQYLLYGF 237
Cow     RRAPRPPLPLPPPPHPGWPEPADLGEEEPMLGLGEEDED--PGVAEGLGE--DEEAEDTGAAKGAGGDTKVAGVPGPAGQHDGRRRIQREGLMRVYVAQLVARAAFEVAFLVGQYLLYGF 235
Mouse   RRLPRAQLP---PPPPGWPDTTDLGEAEPILAL-EEDEDEEPGAPEGPGEDTEEERAEDVAAKGGGGDGKTVVTPGPAGQHDGRRRIQREGLMRVYVAQVVVRAAFEVAFLVGQYLLYGF 236
Rat     RRLPRAQLP---PPPPGWPDTTDLGEAEPILAL-EEDEDEEPGAPEGPGEDTEEERTEDVAAKGGGGDGKTVVTPGPAGQHDGRRRIQREGLMRVYVAQLVVRAAFEVAFLVGQYLLYGF 236
        ** **. **   ** .***:.:**** **:*.* **:*:  .*..** **  :*   :  .:*. *.* *.. .***:*********************:*.*****************

Human   EVRPFFPCSRQPCPHVVDCFVSRPTEKTVFLLVMYVVSCLCLLLNLCEMAHLGLGSAQDAVRGRRGPPASAPAPAPRPPPCAFPAAAAGLACPPDYSLVVRAAERARAHDQNLANLALQA 357
Chimp   EVRPFFPCSRQPCPHVVDCFVSRPTEKTVFLLVMYVVSCLCLLLNLCEMAHLGLGSAQDAVRGRRGPPASAPAPPPRPPPCAFPAAAAGLACPPDYSLVVRAAERARAHDQNLANLALQA 357
Cow     EVRPFFACSRQPCPHVVDCFVSRPTEKTVFLLVMYVVSCLCLLLNLCEMAHLGLGNAQDAVRGRRPLPAS-PGPMPRPPPCALPAAPSGLACPPDYSLVVRTAEHARAQDQELASLALQA 354
Mouse   EVPPFFACSRQPCPHVVDCFVSRPTEKTVFLLVMYVVSCLCLLLNLCEMAHLGLGSAQDAVRGRRGASAAGPGPTPRPPPCAFPAAAAGLACPPDYSLVVRAAERARAHDQNLANLALQA 356
Rat     EVPPFFACSRQPCPHVVDCFVSRPTEKTVFLLVMYVVSCLCLLLNLCEMAHLGLGSAQDAVRGRRGASAAGPGPAPRPPPCAFPAAAAGLACPPDYSLVVRAAERARAHDQNLANLALQA 356
        ** ***.**************************************************.*********  .*: *.* *******:***.:**************:**:***:**:**.*****

Human   LRDGAAAGDR--DRDSSPCVGLPAASRGPPRAGAPASRTGSATSAGTVGEQGRPGTHERPGAKPRAGSEKGS-ASSRDGKTTVWI 439
Chimp   LRDGAAAGDR--DRDSSPCVGLPAASRGPPRAGAPASRTGSATSAGTVGEQGRPGTHERPGAKPRAGSEKGS-ASSRDGKTTVWI 439
Cow     LQDRRALGDL----DSPPGPGLPANARGPPKPGAPASGSGSATSGGTVGGQGRQG------IKPRMGSEKGSGSSSREGKTTVWI 429
Mouse   LRDGAAVAAVSADRDSPPCAGLNATSRGARRVGGLASGTGSATSGGTVGEQSRPGAQEQLATKPRAGSEKGS-TGSRDGKATVWI 440
Rat     LRDGAAVAAVSADRDSPPCSGLNATSRGPPRAGGPASGTGSATSGGTVGEQGRSGAQEQLATKPRVGSEKGS-TGSRDGKATVWI 440
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Figure 3. Amino Acid Alignment of Cx47 from Different Species
Tan indicates intracellular domains; green indicates transmembrane domains; white indicates extracellular domains. Red dots represent the positions of amino acids altered in lymphedema families.

Supplemental Data

Supplemental Data include two tables and can be found with this article online at http://www.cell.com/AJHG.

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Web Resources

The URLs for data presented herein are as follows:

University of Pittsburgh Lymphedema Family Study, http://www.hgen.pitt.edu/projects/lymph/
Entrez Nucleotide, http://www.ncbi.nlm.nih.gov/nuccore/
Genomics and Proteomics Core Laboratory of the University of Pittsburgh, http://www.genetics.pitt.edu/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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