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Clinical Report

A Novel *VEGFR3* Mutation Causes Milroy Disease

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Milroy disease, also known as primary congenital lymphedema, is a hereditary form of lymphedema with autosomal dominant inheritance. Individuals with Milroy disease are typically characterized by congenital onset of lymphedema of the lower limbs due to hypoplasia of the lymphatic vessels. The genetic basis of most cases of Milroy disease has not been established, although mutations in the vascular endothelial growth factor receptor *VEGFR3* (*FLT-4*) are responsible for some cases with 17 mutations described to date. In this report, we describe a novel *VEGFR3* mutation in exon 22 in a four-generation family in which congenital lymphedema segregates in an autosomal dominant manner. In addition to lymphedema, affected family members had other clinical manifestations associated with Milroy disease including hydrocele, ski jump toenails, large caliber veins, and subcutaneous thickening. We screened *VEGFR3* for mutations which revealed a novel 3059A > T transversion in exon 22 resulting in Q1020L missense mutation in the second tyrosine kinase domain of VEGFR3. This mutant allele segregated with lymphedema among affected individuals with incomplete penetrance. This is the first report of an exon 22 mutation in Milroy disease. © 2007 Wiley-Liss, Inc.

Key words: lymphedema; Milroy disease; VEGFR3

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INTRODUCTION

Lymphedema is defined as chronic tissue swelling of soft tissues due to abnormal drainage of lymph by lymphatic vessels [Mortimer, 1995]. Primary lymphedema can be genetic in origin and the most common form of hereditary primary lymphedema is Milroy disease, also known as primary congenital lymphedema (PCL; OMIM #153100). As with most forms of primary lymphedema, Milroy disease has an autosomal dominant mode of inheritance with incomplete penetrance. Milroy disease is usually characterized by bilateral lymphedema of the lower limbs, although unilateral lymphedema has also been reported [Brice et al., 2005]. Other clinical manifestations associated with Milroy disease include hydrocele, large caliber veins, upslanting of the toenail plates known as "ski jump" toenails, and papillomatosis of the toes [Brice et al., 2005].

The only known molecular cause of Milroy disease is mutation in the *VEGFR3* gene (formerly *FLT-4*) [Ferrell et al., 1998; Irrthum et al., 2000; Karkkainen et al., 2000; Evans et al., 2003; Daniel-Spiegel et al., 2005; Mizuno et al., 2005; Ghalamkarpour et al., 2006; Spiegel et al., 2006]. Linkage analysis on Milroy disease families suggests genetic heterogeneity and possibly complex inheritance for Milroy disease although some families clearly have linkage to the *VEGFR3* locus on 5q35.3 [Ferrell et al., 1998; Evans et al., 1999; Holberg et al., 2001]. *VEGFR3* encodes a receptor tyrosine kinase that is expressed by lymphatic endothelial cells (LECs) [Kaipainen et al., 1995; Partanen et al., 2000] and is activated by the vascular endothelial growth factors VEGF-C and VEGF-D to mediate lymphangiogenesis [Veikkola et al., 2001]. The *VEGFR3* gene is composed of 31 exons transcribed as two alternatively spliced transcripts encoding a protein with 7 immunoglobulin-like repeat domains and 2 tyrosine kinase

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domains [Iljin et al., 2001]. Only 17 *VEGFR3* mutations have been identified, and all mutations described localize to one of the two tyrosine kinase domains, illustrating the necessity of these domains in the signal transduction function of VEGFR3 [Ferrell et al., 1998; Irrthum et al., 2000; Karkkainen et al., 2000; Evans et al., 2003; Daniel-Spiegel et al., 2005; Mizuno et al., 2005; Ghalamkarpour et al., 2006; Spiegel et al., 2006].

In this report, we describe a large family that presented with a form of hereditary lymphedema consistent with Milroy disease. We screened all available family members for mutations in both *VEGFR3* tyrosine kinase domains. Our screen identified a novel 3059A > T transversion in exon 22 resulting in a Q1020L missense mutation located in the second tyrosine kinase domain. The mutation segregated with lymphedema among the affected individuals of the family in an autosomal dominant manner with incomplete penetrance.

MATERIALS AND METHODS

Ascertainment

The proband was self-referred to the Stanford Center for Lymphatic and Venous Disorders for evaluation of severe, congenital, bilateral lower extremity lymphedema and moderately severe hydrocele. A family history of lymphedema in the proband's family suggested the presence of a hereditary form of lymphedema. Therefore, informed consent for examination and DNA testing was obtained from all subjects in accordance with the University of Michigan Medical School Institutional Review Board. The family members were identified by the proband and all agreed to participate in the evaluation.

Phenotypic Analysis

All participating family members had a thorough assessment of their lower extremity anatomy by one of the authors (SGR), with qualitative assessment of the presence of pitting edema, hyperkeratosis, subcutaneous thickening, and the presence or absence of Stemmer sign. The morphology of the nail plates was assessed, along with documentation of the appearance of the superficial venous vasculature. Genital examination was performed to identify or exclude the presence of hydrocele in all male family members.

DNA Isolation

Genomic DNA was extracted from 5 or 10 ml blood samples using a guanidine HCl method for DNA extraction from blood [Ciulla et al., 1988].

VEGFR3 Sequencing

Each of the exons (exons 18–26) that encode the tyrosine kinase domains of *VEGFR3* were amplified using the GC Rich PCR System (Roche, Indianapolis, IN) with following primer sets (Table I). Exons 23 and 25 were amplified as previously described [Evans et al., 2003]. The resulting PCR products were treated with shrimp alkaline phosphatase (Roche) and exonuclease I (USB Biologicals, Cleveland, OH) and then submitted to the University of Michigan DNA Sequencing Core (http://seqcore.brcf.med.umich.edu/) for sequence analysis.

FOXC2 Mutation Screening

FOXC2 mutation screening was performed as previously described [Fang et al., 2000].

Multiple Sequence Alignment

VEGFR3 protein sequences were retrieved from Genbank. The following Genbank sequences were aligned using ClustalW [Thompson et al., 1994]. (http://www.ebi.ac.uk/clustalw/): *Homo sapiens* (NP_891555.1), *Canis familiaris* (XP_538585.2), *Mus musculus* (NP_032055.1), *Rattus norvegicus* (XP_579569.1), and *Gallus gallus* (XP_414600.1). The conservation of the Q1020 was compared to other receptor tyrosine kinases including: EGFR (BAD92679), FGFR1 (AAH18128), MET (EAL24359), PDGFRB (AAH32224), RET (AAH04257).

RESULTS

The proband was evaluated for severe bilateral lower extremity lymphedema as described in Materi-

TABLE I. *VEGFR3* Primers

Exon	Forward primer (5′-3′)	Reverse primer (5′-3′)
17[a]	GGAGGAGCAATGCGAATAC	AGACAAGGAGAGGTGGACAG
18	CTGTCCACCTCTCCTTGTCT	GTAGAATCTCGGATGAGACTG
19[a], 20, 21	ATTCACATCGGCAACCAC	AGGCACTAGGAAAAGGGAAG
22	TGACATGCTTGTTAGCTGTTCC	AACAGACTCTGCTGCTGACC
24	AGAACAGCAGCAGGGAGATAC	TTCAGAACGACCTGGCACAC
26	TGAAGGAGGAACGAGGGGTAG	AAAGATGCAAGGGTGTGGG

[a]Indicates exons that are incompletely amplified by primers.

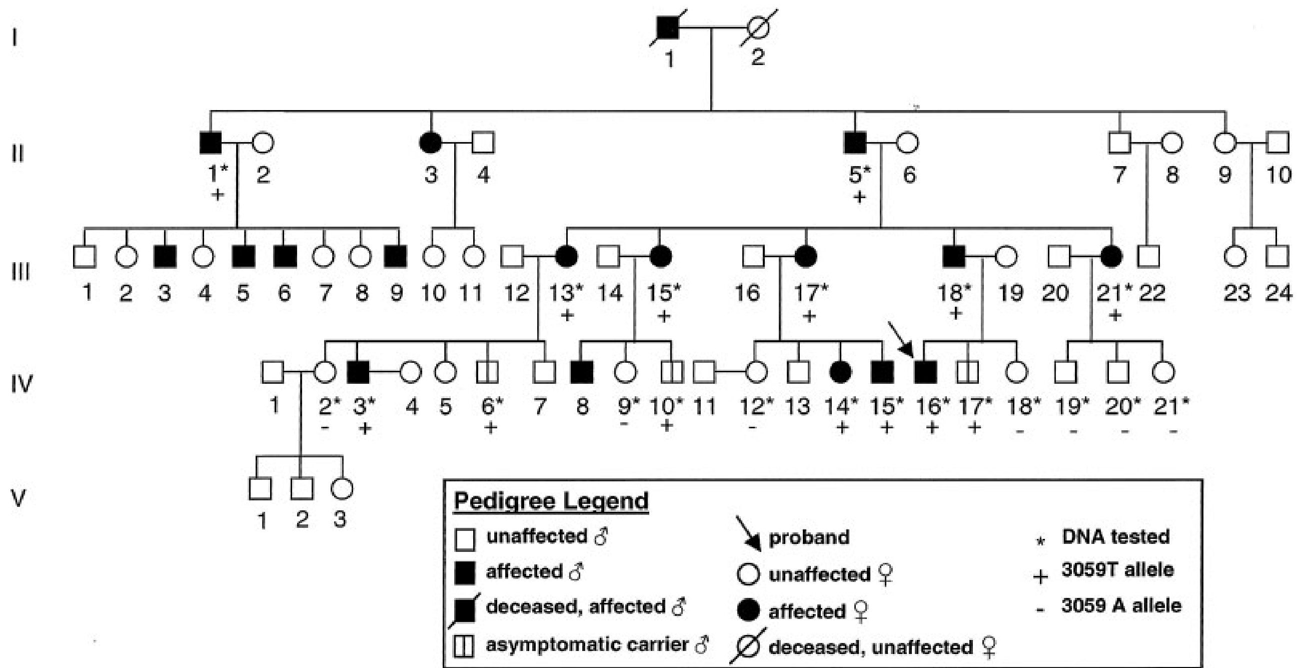


Fig. 1. Pedigree of large family with inherited, congenital lymphedema. (+) Refers to individuals that are heterozygous for 3059T allele. (−) Refers to individuals that are homozygous for 3059A allele.

als and Methods. Upon evaluation, the presence of lymphedema was confirmed, and the proband was also noted to have marked cutaneous fibrotic changes and hydrocele. The interview with the proband indicated a significant family history of lymphedema; therefore, a pedigree was ascertained (Fig. 1) and blood samples were collected from 20 related individuals, representing four generations of the family. Clinical evaluations of each of the related individuals are summarized in Table II. Lower limb lymphedema was primarily bilateral (10/11) and ranged from moderate to severe. All of the affected individuals had congenital onset of lymphedema, with the exception of individual II-5 who developed lymphedema at the age of 21. In addition to lymphedema, some affected individuals displayed hyperkeratosis, ski jump toenails, varicose veins, and hydrocele (Table II). For Milroy cases that have been rigidly defined both clinically and molecularly, each of these phenotypes has been previously reported in association with Milroy disease [Brice et al., 2005]. Possible distichiasis was noted in some of the individuals but was not verified by an ophthalmologist. Distichiasis is associated with lymphedema-distichiasis syndrome (LD), another form of inherited primary lymphedema (OMIM #153400). LD is caused by mutations in the forkhead transcription factor *FOXC2* [Fang et al., 2000]. Mutational screening did not detect mutations or polymorphisms in the *FOXC2* single coding exon. Further evaluation of the pedigree demonstrated that the possible distichiasis did not segregate with lymphedema and was also reported in family members without lymphedema and thus was excluded from consideration.

Since *VEGFR3* mutations are known to cause some cases of Milroy disease [Karkkainen et al., 2000], we screened *VEGFR3* for mutations. All previously

TABLE II. Clinical Evaluation of Affected Individuals

Individual	Lymphedema	Stemmer	Ski jump toenails	Associations
II-5	B Mild	+	−	Hydrocele
III-13	B Moderate	+	−	Varicose veins
III-15	B Severe	+	+	None
III-17	B Moderate	+	+	None
III-18	B Mild	+	+	Varicose veins
III-21	U′ Mild	+	−	Varicose veins
IV-3	B Mild	+	−	Hydrocele
IV-14	B Mild	−	−	None
IV-15	B Moderate	−	+	None
IV-16	B Severe	+	−	Hydrocele, v.v.

B, bilateral; U′, unilateral; v.v., varicose veins.

reported *VEGFR3* mutations localize to one of the two tyrosine kinase domains in the cytoplasmic portion of the VEGFR3 receptor (Table III) [Irrthum et al., 2000; Karkkainen et al., 2000; Evans et al., 2003; Brice et al., 2005; Daniel-Spiegel et al., 2005; Mizuno et al., 2005; Ghalamkarpour et al., 2006; Spiegel et al., 2006]. Therefore, we amplified and sequenced *VEGFR3* exons 18 through 26, which encompass both of the tyrosine kinase domains of VEGFR3. DNA sequencing revealed a novel 3059A > T transversion in exon 22 (Fig. 2A). Each of the affected individuals (I-1, I-5, III-13, III-15, III-17, III-18, III-21, IV-3, IV-14, IV-15, IV-16) are heterozygous for the 3059T allele (Fig. 1). With the exception of individuals IV-6 (age 19), IV-10 (age 17), and IV-17 (age 22), each of the unaffected individuals did not inherit the 3059T allele. The 3059T allele makes a non-conservative missense mutation changing an evolutionarily conserved glutamine residue to leucine at amino acid position 1020 of VEGFR3 (Fig. 2B,C).

DISCUSSION

We have identified a novel *VEGFR3* mutation that likely causes Milroy disease in a large family. It is the first mutation reported in exon 22 of the *VEGFR3* gene. Without functional data, the possibility remains that the identified change encodes a coding polymorphism. However, previous inquires by Iljin et al. [2001] and Evans et al. [2003] identified six and four polymorphisms, respectively, in the *VEGFR3* coding region, none of which encoded the Q1020L missense mutation. Additionally, twelve other coding SNPs in the *VEGFR3* gene (rs307821, rs307826, rs448012, rs744282, rs1049076, rs1049077, rs1049080, rs1130378, rs1130379, rs2270519, rs3736061, and rs3736062) are reported in the SNP database (NCBI) which also do not encode the Q1020L mutation. Based upon this previous work and the available data in the SNP database, the Q1020L amino acid change does not appear to be a polymorphism.

In addition to the available variation data, segregation of the 3059T allele among all affected family members argues strongly against it being a coding region polymorphism. Although three unaffected individuals (IV-6, IV-10, and IV-17) did inherit the 3059T allele but did not develop lymphedema or any of the other clinical associations, previous clinical studies of families with Milroy disease has demonstrated incomplete penetrance. In a study of ten families, Brice et al. [2005] reported ten percent of individuals with Milroy disease mutations in *VEGFR3* do not develop lymphedema which correlates with our 21 percent (3/14).

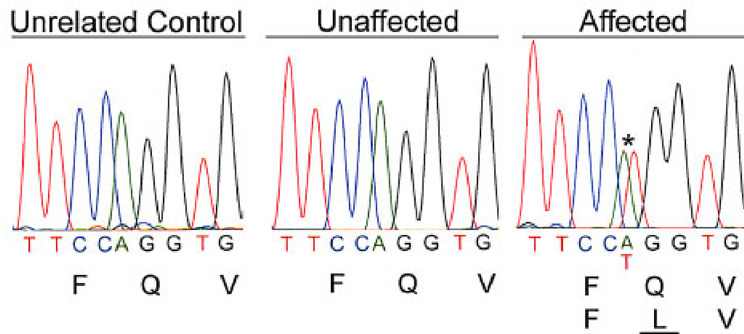
The 3059A > T mutation changes a conserved glutamine residue to leucine in the second tyrosine kinase domain causing a predicted loss or attenuation of VEGFR3 function. The replacement of glutamine with leucine introduces a nonpolar amino acid in place of a polar uncharged amino acid. Although the charge remains the same and the relative structure of the side chain is similar, the change in potential hydrogen bonding resulting from the presence of glutamine could disrupt the function of the second tyrosine kinase domain or alter the stability of the overall structure of VEGFR3. All known VEGFR3 mutations (Table III) are located in tyrosine kinase domains. Therefore, this mutation will likely result in defective VEGF-C and VEGF-D signaling due to dominant negative inhibition of VEGFR3 autophosphorylation as established by Karkkainen et al. [2000].

Our case illustrates a novel mutation in exon 22 of *VEGFR3* in the second tyrosine kinase domain. All known *VEGFR3* mutations in Milroy disease have been found in the tyrosine kinase domains. It remains unclear to date whether Milroy disease or

TABLE III. Known VEGFR3 Mutations

Mutation	Domain	Exon	Nucleotide change	Reference by first author
G854S	TK I	18	2560G > A	Evans et al. [2003]
G857R	TK I	18	2569G > A	Karkkainen et al. [2000], Mizuno et al. [2005]
V878M	TK I	18	2632G > A	Ghalamkarpour et al. [2006]
A915P	TK I	19	2743G > C	Evans et al. [2003]
C916W	TK I	19	2748C > G	Evans et al. [2003]
G933R	TK I	20	2797G > C	Evans et al. [2003]
Q1020L	TK II	22	3059A > T	This report
H1035R	TK II	23	3104A > G	Irrthum et al. [2000]
H1035Q	TK II	23	3105C > G	Ghalamkarpour et al. [2006]
R1041P	TK II	23	3122G > C	Karkkainen et al. [2000]
R1041W	TK II	23	3121C > T	Evans et al. [2003]
R1041Q	TK II	23	3122G > A	Evans et al. [2003]
L1044P	TK II	23	3131T > C	Karkkainen et al. [2000]
I1086T	TK II	24	3257T > C	Ghalamkarpour et al. [2006]
E1106K	TK II	24	3316G > A	Daniel-Spiegel et al. [2005], Spiegel et al. [2006]
ΔF1108	TK II	24	del3323-3325TCT	Evans et al. [2003]
P1114L	TK II	25	3341C > T	Karkkainen et al. [2000]
P1137L	TK II	25	3410C > T	Evans et al. [2003]

A VEGFR3 Exon 22 Sequencing Chromatograms



B Evolutionary Conservation of Q1020 Residue

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        1005          1020          1035
Human: WLSPLTMEDLVCYSFQVARGMEFLASRKCIH
  Dog: WLSPLTMEDLVCYSFQVARGMEFLASRKCIH
Mouse: WLSPLTMEDLVCYSFQVARGMEFLASRKCIH
  Rat: WLSPLTMEDLVCYSFQVARGMEFLASRKCIH
Chick: WQSPLTMEDLICYSFQVARGMEFLASRKCIH
       * ********:*********************
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C Conservation of Q1020 Among RTKs

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                       1020
VEGFR3: WLSPLTMEDLVCYSFQVARGMEFLASRKCIH
PDGFRB: ESPVLSYMDLVGFSYQVANGMEFLASKNCVH
   RET: -ERALTMGDLISFAWQISQGMQYLAEMKLVH
  EGFR: ----IGSQYLLNWCVQIAKGMNYLEDRRLVH
   KIT: -----TVKDLIGFGLQVAKGMKYLASKKFVH
 FGFR1: --EQLSSKDLVSCAYQVARGMEYLASKKCIH
                *:    *:: **::*     :*
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Fig. 2. **A**: DNA sequencing analysis of *VEGFR3* exon 22. A chromatogram from an individual with lymphedema (*affected*) is compared to an unaffected family member (*unaffected*) as well as an unrelated control (*unrelated*). The 3059A > T mutation is indicated by the asterisk (*). The amino acids encoded by the nucleotides depicted are shown below the nucleotide sequence with the mutant amino acid shown in red. **B**: Multiple sequence alignment of the VEGFR3 protein. VEGFR3 sequences from various species (indicated to the left of the polypeptide sequences) demonstrate the conservation of the TK II domain and the Q1020 residue highlighted in red. **C**: Multiple sequence alignment of VEGFR3 with other human RTKs. Alignment with other RTKs shows that Q1020 is conserved in general among RTKs. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

other variations of congenital lymphedema are caused by mutations in other domains of VEGFR3. The possibility exists that mutations that disrupt the ligand-binding function are more severe and incompatible with life. Therefore, mutational testing of the tyrosine kinase domains of VEGFR3 is a logical first step to address the molecular cause of Milroy disease.

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REFERENCES

Brice G, Child AH, Evans A, Bell R, Mansour S, Burnand K, Sarfarazi M, Jeffery S, Mortimer P. 2005. Milroy disease and the VEGFR-3 mutation phenotype. J Med Genet 42:98–102.

Ciulla TA, Sklar RM, Hauser SL. 1988. A simple method for DNA purification from peripheral blood. Anal Biochem 174:485–488.

Daniel-Spiegel E, Ghalamkarpour A, Spiegel R, Weiner E, Vikkula M, Shalev E, Shalev SA. 2005. Hydrops fetalis: An unusual prenatal presentation of hereditary congenital lymphedema. Prenat Diagn 25:1015–1018.

Evans AL, Brice G, Sotirova V, Mortimer P, Beninson J, Burnand K, Rosbotham J, Child A, Sarfarazi M. 1999. Mapping of primary congenital lymphedema to the 5q35.3 region. Am J Hum Genet 64:547–555.

Evans AL, Bell R, Brice G, Comeglio P, Lipede C, Jeffrey S, Mortimer P, Safarazi M, Child AH. 2003. Identification of eight novel *VEGFR-3* mutations in families with primary congenital lymphodema. JMG 40:697–703.

Fang J, Dagenais SL, Erickson RP, Arlt MF, Glynn MW, Gorski JL, Seaver LH, Glover TW. 2000. Mutations in FOXC2 (MFH-1), a

American Journal of Medical Genetics Part A: DOI 10.1002/ajmg.a

forkhead family transcription factor, are responsible for the hereditary lymphedema-distichiasis syndrome. Am J Hum Genet 67:1382–1388.

Ferrell RE, Levinson KL, Esman JH, Kimak KA, Lawrence EC, Barmada MM, Finegold DN. 1998. Hereditary lymphedema: Evidence for linkage and genetic heterogeneity. Hum Mol Genet 7:2073–2078.

Ghalamkarpour A, Morlot S, Raas-Rothschild A, Utkus A, Mulliken JB, Boon LM, Vikkula M. 2006. Hereditary lymphedema type I associated with VEGFR3 mutation: The first de novo case and atypical presentations. Clin Genet 70:330–335.

Holberg JH, Erickson RP, Bernas MJ, Witte MH, Fultz KE, Andrade M, Witte CL. 2001. Segregation analysis and a genome wide linkage search confirm genetic heterogeneity and suggest oliogogenic inheritance in some Milroy congenital primary lymphedema families. Am J Med Genet 98:303–312.

Iljin K, Karkkainen MJ, Lawrence EC, Kimak MA, Uutela M, Taipale J, Pajusola K, Alhonen L, Halmekyto M, Finegold DN, Ferrell RE, Alitalo K. 2001. VEGFR3 gene structure, regulatory region, and sequence polymorphisms. FASEB J 15:1028–1036.

Irrthum A, Karkkainen MJ, Devriendt K, Alitalo K, Vikkula M. 2000. Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. Am J Hum Genet 67:295–301.

Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K. 1995. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. PNAS 92:3566–3570.

Karkkainen MJ, Ferrell RE, Lawrence EC, Kimak MA, Levinson KL, McTigue MA, Alitalo K, Finegold DN. 2000. Missense mutations interfere with VEGFR-3 signaling in primary lymphoedema. Nat Genet 25:153–159.

Mizuno S, Yamada Y, Yamada K, Nomura N, Wakamatsu N. 2005. Clinical variability in a Japanese hereditary lymphedema type I family with an FLT4 mutation. Congenit Anom 4: 59–61.

Mortimer PS. 1995. Managing lymphedema. Clin Dermatol 13: 499–505.

Partanen TA, Arola J, Saaristo A, Jussila L, Ora A, Miettinen M, Stacker SA, Achen MG, Alitalo K. 2000. VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. FASEB J 14:2087–2096.

Spiegel R, Ghalamkarpour A, Daniel-Spiegel E, Vikkula M, Shalev SA. 2006. Wide clinical spectrum in a family with hereditary lymphedema type I due to a novel missense mutation in VEG FR3. J Hum Genet 51:846–850.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.

Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, Kubo H, Thurston G, McDonald DM, Achen MG, Stacker SA, Alitalo K. 2001. Signaling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. EMBO J 20:1223–1231.