

Original Article

A novel mutation in vascular endothelial growth factor receptor 3 causes primary lymphedema in a Chinese family

Ting Dai^{1*}, Honggang Wang^{2*}, Tao Yu^{2*}, Jian Qi², Guo Fu², Liwei Yan², Zhihao Liao³, Liqiang Gu², Xiaolin Liu², Ping Li², Xiang Zhou²

¹Department of Biotechnology, College of Life Sciences, Guangzhou Medical University, Guangzhou 511436, China; ²Department of Microsurgery, Trauma and Hand Surgery, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, China; ³Department of Microsurgery and Hand Surgery, The Third Affiliated Hospital of Guangzhou University of Traditional Chinese, Guangzhou 510080, China. *Equal contributors.

Received June 21, 2016; Accepted August 19, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Milroy disease, also known as primary congenital lymphedema, is a hereditary form of lymphedema with an autosomal dominant inheritance pattern. Individuals with Milroy disease are typically characterized by the congenital onset of lymphedema of the lower limbs as a result of the hypoplasia of the lymphatic vessels. A three-generation family in which Milroy disease segregates in an autosomal dominant manner was investigated. However, the affected family members did not suffer from the other clinical manifestations included hydrocele, ski jump toenails, and large caliber veins. Mutation analysis and functional studies were carried out in this pedigree. We screened VEGFR3 for mutations and identified a novel 3130 C>A transversion in exon 22, resulting in an L1044M missense mutation in the second tyrosine kinase domain of VEGFR3. This mutant allele resulted in the development of lymphedema due to its segregation among each of the affected individuals. Functional studies demonstrated that compared with a previously reported mutation, c. 3059 A>T, the mutation in our pedigree appears to cause only a slight reduction in VEGFR3 downstream signaling. This may be the reason for the milder phenotype noted in this family. We identified a novel mutation that caused Milroy disease in a Chinese family. This is the first report describing the functional changes induced by the c. 3130 C>A mutation. This finding has expanded our knowledge of the VEGFR3 gene's function and has advanced our understanding of hereditary lymphedema.

Keywords: Lymphedema, vascular endothelial growth factor receptor-3, point mutation

Introduction

Primary lymphedema is a disorder caused by the dysfunction of the lymphatic system and is characterized by peripheral edema that primarily affects the lower extremities. Three types of hereditary lymphedema are classified by their ages of onset, as follows: Milroy disease (MIM 153100) is characterized by congenital onset; Meige disease or lymphedema praecox (MIM 153200) typically occurs peripubertally or in association with pregnancy, and lymphedema tarda develops after 35 years of age [1, 2]. Milroy disease (hereditary lymphedema type I) was first described in 1892. It is characterized by the congenital onset of lower limb lymphedema and is inherited in an autosomal dominant manner [3]. In 1985, Dale estimated that

the prevalence of primary lymphedema was 1/6000 based on a single UK clinical study [4]. The prevalence of primary lymphedema in North America has been estimated as 1.15 children per 100,000 children by Smeltzer et al [5]. More recently, chronic lymphedema (primary and secondary causes) was estimated to affect as many as 1.33/1000 in the UK [6]. Figures such as those may vary; therefore, further study regarding the epidemiology and mechanism of lymphedema is required to develop a better understanding of and treatment for human hereditary lymphedema.

In the 1990s, several groups independently mapped MD to chromosome 5q35 [7-9]. VEGFR3 was shown to be the causative reason for some cases of MD [10-12]. VEGFR3 is a tyro-

sine kinase receptor for vascular endothelial growth factor-C and growth factor-D [13]. It plays an important role in the development and growth of lymphatic vessels [14, 15]. Functional studies of those mutations have shown that it is restricted to the tyrosine kinase domains of the receptor and may reduce tyrosine kinase activity, thereby affecting lymphatic development [11]. In the same paper, it was noted that the mutant receptor remained on the cell surface longer than did the wild type (WT) receptor, which presumably resulted in decreased kinase activity. In mice, VEGFR3 homozygous knockout animals die in utero due to the underdevelopment of major blood vessels [16]. However, heterozygous knockout mice are phenotypically normal. The Chy mutant mouse has a heterozygous kinase inactivating VEGFR3 mutation that results in the swelling of each of the limbs due to a lack of dermal lymphatic vessels, as is the case with patients with Milroy disease [17].

In this study, we describe a three-generation family with 7 individuals affected by congenital hereditary lymphedema as a result of an L1044M substitution in a highly conserved residue in the second tyrosine kinase domain of the VEGFR3 receptor. However, the affected family members did not suffer from the other clinical manifestations of Milroy disease, manifestations that include hydrocele, ski jump toenails, large caliber veins, and subcutaneous thickening. Luciferase assays indicate that this mutation appears to reduce VEGFR3 downstream signaling to a lesser extent than does a previously described mutation.

Material and methods

Patients

The proband was self-referred to our hospital for Lymphatic and Venous Disorders for the evaluation of mild, congenital, bilateral lower extremity lymphedema. Following a detailed examination, a family history of lymphedema in the proband's family suggested the presence of a hereditary form of lymphedema. Venous blood samples were collected from both affected and unaffected individuals in this family. An additional 141 healthy individuals were used as controls. Genomic DNA was isolated from the venous blood samples using standard methods. This experiments were per-

formed with each participant's informed consent and with the approval of the local ethics committee, which were in accordance with the with the Helsinki Declaration.

Molecular analysis

A molecular analysis was performed using a slightly modified method similar to that used previously by Evans et al [18]. All known VEGFR3 mutations causing Milroy disease are located within functional domains [19]. It was unclear whether Milroy disease or other types of congenital lymphedema are caused by mutations in other areas of VEGFR3. Therefore, a mutation analysis of the tyrosine kinase domain of VEGFR3 was a logical first step in uncovering the molecular basis of this disease [20].

Exons 17 through 26 of VEGFR3 were examined. A genetic analysis of the sequencing products was performed using an ABI 3100 Genetic Analyzer. All sequences were run in both forward and reverse. Each detected mutation was checked in 141 healthy individuals via sequencing. Primers for VEGFC were produced as previously described [18].

Phenotypic analysis

Each of the family members consented to and underwent a complete assessment by one of the authors (xz) to search for abnormalities of their lower limb anatomy, which included a qualitative assessment for keratosis, ski jump toenails, large caliber veins, and subcutaneous thickening [20]. In the male family members, a genital examination was conducted to confirm the presence of a hydrocele.

Multiple sequence alignment

ClustalW was used to align the VEGFR3 protein sequences of the following different species: Homo sapiens (NP_891555.1), Pan troglodytes (XP_518160.4) Canis familiaris (XP_538585.2), Gallus gallus (XP_414600.1), Mus musculus (NP_032055.1), Rattus norvegicus (XP_57956-9.1), Coturnix coturnix (CAA58267.1) and Danio rerio (NP_571020.1). Additionally, the mutation locus was compared with the following additional receptor tyrosine kinases found in Homo sapiens: PDGFRB (AAH32224), FGFR1 (AAH18128), RET (AAH04257), ALK (NP_004-295.2), EGFR (BAD92679) and EPH (AAI302-92.1).

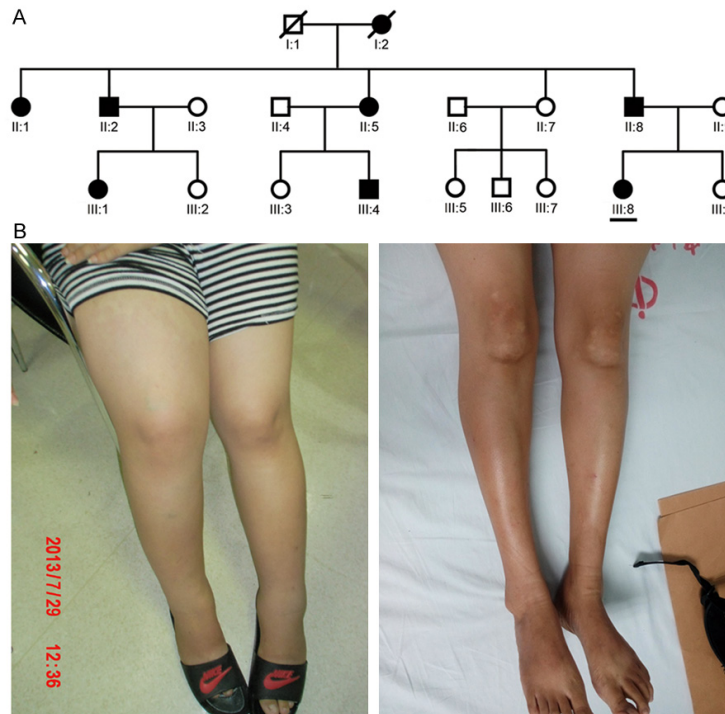


Figure 1. A Chinese family with inherited, congenital lymphedema. A: Family pedigree of a three generation Chinese family. Squares are males and circles are females. Blackened symbols indicated affected individuals, open symbols are unaffected individuals. The proband (III:8) is underlined. B: Photographs of individuals, III:8 and II:9. Photographs of foot of the proband showing lymphedema in both leg. No abnormalities were observed in his mother (right panel).

Expression vector construction

Human VEGFR3 open reading frame (ORF) cDNA was obtained from GeneChem (Guangzhou, Guangdong, China). The ORF was amplified by PCR and cloned into HindIII- and BamHI-digested pcDNA3.1/Z(+) (Invitrogen, Carlsbad, CA, USA) to create the expression plasmid, pcDNA3.1-VEGFR3. We generated the VEGFR3 mutants using the G QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), using the oligonucleotides, 5'-TGGTGGCCAGAGGGATGGAGT-3', for the Q10-20L (A>T) mutation, and 5'-ATGCTGTCTCGGAAAGCGACGTG-3', for the L1044M (C>A) mutation. The presence of the desired base changes was verified via DNA sequencing.

Analysis of downstream transcription factor activation via a luciferase assay

This analysis was performed based on a previously utilized method, although our approach was modified [21]. 293T cells were cultured in

Iscove's Modified Dulbecco's Medium with 10% fetal bovine serum, 100 mg/mL penicillin and 100 mg/mL streptomycin. Using the Mercury Pathway Profiling System (Clontech, Tokyo, Japan), we analyzed the effect of the VEGFR3 mutation on the ability of NF- κ B transcription factor complexes to activate luciferase in transiently transfected 293T cells. Cells were seeded into 24-well tissue culture plates 24 h prior to transfection at approximately 60% confluence. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Both wild-type and mutant VEGFR3 expression plasmids were cotransfected with NF κ B dependent Photinus pyralis luciferase reporter plasmids. The vector containing the herpes simplex virus thymidine kinase promoter upstream of the Renilla luciferase (PRL-TK, Promega) was selected as an internal control. Following an overnight

incubation with calcium phosphate precipitation, the cells were washed, lysed and assayed for firefly and Renilla luciferase expression using the Dual Luciferase Reporter Assay System (Promega). The values represent the means of three independent experiments performed in triplicate, and the bars in the figures denote the S.D. Student's t test was used to determine the statistical significance of the differences between the means of the unpaired samples.

Results

Clinical report

We investigated a Han Chinese family with distinctive congenital lymphedema (**Figure 1**). There were 7 affected individual among the 3 generations of the family under study. The index case involved an 11-year-old boy. The presence of lymphedema was confirmed upon his evaluation. Other clinical manifestations associated with Milroy disease include hydro-

Novel VEGFR3 mutation causes primary lymphedema

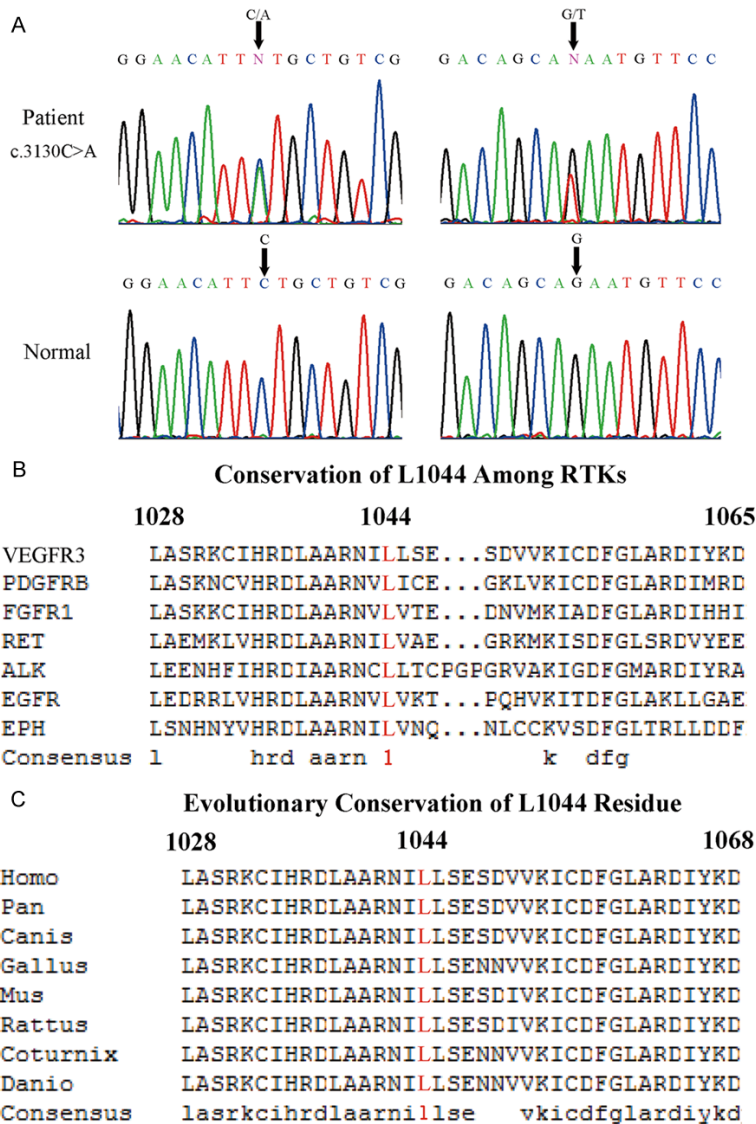


Figure 2. A novel missense mutation in VEGFR3 causes Milroy disease. A: The Milroy disease is associated with a point mutation at position 3130 of the VEGFR3 coding sequence that causes a L1044M substitution. Genomic DNA sequencing demonstrating the presence and consequence of the missense mutation c. 3130 C>A (p.Leu1044Met) in the proband. B: ClustalX alignment of a portion of the HOXD13 protein in 13 different species showing the evolutionary conservation of the glycine residue in position 220 (highlighted in red). C: Alignment among other receptor tyrosine kinases in Homo sapiens also shows conservation. Accession numbers of the proteins (in the same order as they appear in the figure) are: Homo sapiens (NP_891555.1), Pan troglodytes (XP_518160.4), Canis familiaris (XP_538585.2), Gallus gallus (XP_414600.1), Mus musculus (NP_032055.1), Rattus norvegicus (XP_579569.1), Coturnix coturnix (CAA58267.1), Danio rerio (NP_571020.1), PDGFRB (AAH32224), FGFR1 (AAH18128), RET (AAH04257), ALK (NP_004295.2), EGFR (BAD92679) and EPH (AAI30292.1).

jects. For the other family members, apart from congenital lymphedema, no other abnormalities were noted.

Genetic analysis

VEGFR3 mutations are known to cause Milroy disease. Previous studies have indicated that most VEGFR3 mutations that caused congenital lymphedema were localized to the tyrosine kinase domains of the VEGFR3 receptor. Therefore, we amplified and sequenced VEGFR3 exons 17 through 26, which encompass both of the tyrosine kinase domains of VEGFR3. Sequencing revealed a heterozygous C-to-A transition in exon 23, at position 3031 of the coding sequence, in the proband. This base change converts amino acid 1044 from leucine to methionine. The same base change was confirmed via reverse sequencing of the same patient; each of the affected individuals carried this mutation. However, it was not found in any of the unaffected family members or in the 141 unrelated and unaffected control subjects (**Figure 2A**). An alignment of VEGFR3 protein sequences showed that this position is highly conserved among many different species. Additionally, a cross alignment study involving other tyrosine receptor types indicates that it is conserved (**Figure 2B, 2C**). Therefore, this amino acid appears to play an important role in both the structure and the function of the VEGFR3 protein.

cele, hyperkeratosis, ski jump toenails and papillomatosis, although none of these was noticed during his examination. Further investigation of this family revealed 6 additional affected sub-

Functional analysis

According to previous reports, VEGFR3 exerts its function primarily via the activation of gene

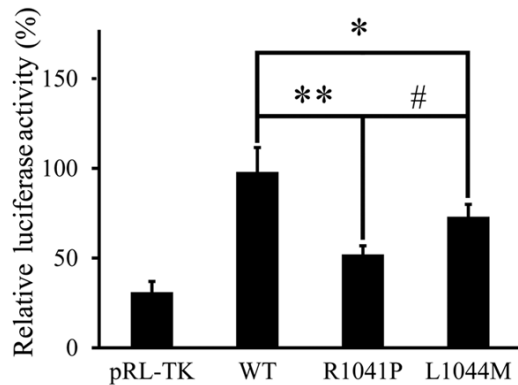


Figure 3. The mutation L1044M affects the transcription regulating functions of VEGFR3. Luciferase activity assayed in cell extracts of 293T cells. The significance of differences was calculated using the independent-samples T test. *, $P < 0.05$, **, $P < 0.01$ versus wild type. #, $P < 0.05$; versus R1041P.

expression via the NF κ B transcription factor complexes. Luciferase assays were conducted to determine whether the mutation affected the capability of VEGFR3 to facilitate downstream signaling. Luciferase reporter constructs such as pGL4.32 (luc2P/NF- κ B-RE/Hygro) were used. This vector contains five copies of an NF- κ B response element (NF- κ B-RE), which drives the transcription of the luciferase reporter gene. Upon activation, it activated the luciferase reporter gene. Additionally, we introduced the following internal control vector: pRL-TK, which contains the Herpes simplex virus thymidine kinase promoter region, located upstream of the Renilla luciferase. We co-transfected wild-type and mutant VEGFR3 expression plasmids and luciferase reporter plasmids into 293T cells. Luciferase activity was analyzed following stimulation with VEGF-C. The results are shown in **Figure 3**. Wild-type HOXD13 enhanced the activities of the reporters. Additionally, the c. 3059 A>T (p.Gln1020-Leu) and c. 3130 C>A (p.Leu1044Met) mutants exhibited reduced expression compared with the wild-type sequences (0.53-fold, $P < 0.01$ and 0.74, $P < 0.05$, respectively). The c. 3130 C>A (p.Leu1044Met) mutant also demonstrated significantly higher expression compared with the c. 3059 A>T (p.Ile314Leu) mutant (1.47-fold, $P < 0.05$). Therefore, our results indicate that the c. 3130 C>A (p.Leu1044Met) mutation affected VEGFR3's capacity to participate in downstream signaling.

Discussion

Our study has described a novel mutation in exon 23 of VEGFR3, which causes the substitution of methionine for the evolutionary conserved leucine in the tyrosine kinase domain of the receptor. Each of the affected individuals in the family that was tested carried the mutation. The same mutation was not found in any unaffected family members, nor was it found among 141 healthy controls.

A batch of studies has demonstrated the relationship between the VEGFR3 mutation and primary lymphedema [10-12]. Using in situ hybridization, Kukk et al found that mouse VEGF-C mRNA expression was detected primarily in the regions in which the lymphatic vessels undergo sprouting from embryonic veins, such as the perimetanepric, axillary and jugular regions. Previous reports indicate that VEGFR3 is expressed in almost all endothelial cells during early development [22]. In his study, Karkkainen et al established that VEGFR-3 plays an important role in the function of lymphatic vessels and that a mutation located within a critical domain of VEGFR-3 may interfere with normal VEGFR-3 signal transduction and cause primary lymphedema [11].

The mutation identified in this family caused a c. 3130 C>A transition in exon 23 of VEGFR3, resulting in a p.Leu1044Met substitution. The L1044M mutation represents the replacement of a non-sulfur-containing amino acid with a sulfur-containing amino acid (methionine). The introduction of a sulfur-containing amino acid represents a change that is likely to produce structural alterations. The MutationTaster program predicted a score of 0.041, and the PolyPhen program [23, 24] predicted that damage was likely as a result of this mutation, with a score of 1.000 (sensitivity: 0.00; specificity: 1.00). Additionally, this residue is highly conserved among different species and different RTKs (**Figure 1**). The high conservation of this glycine residue indicates that it may play an important structural role within a functional domain of the VEGFR3 protein.

Gordon et al offered the most current and complete description of Milroy disease and also provided a comprehensive overview of the genetic basis for primary lymphedema [19].

The edema of Milroy disease is usually painless and chronic. A typical Milroy lymphedema patient often presents at birth with bilateral swelling of the feet [18]. Swelling may extend beyond the ankle and progress upward to the knee [19]. Deep skin creases may be observed on the toes, and the skin often has a brawny texture [18]. Typically, patients develop enlargement of the diameter of the saphenous vein, which is indicative of an associated venous phenotypic abnormality. Other features include small dysplastic, up slanting toe nails, papillomatosis of the toes, recurrent cellulitis, and hydrocoeles in male patients [19]. However, in this family, the only clinical symptom noted was primary lymphedema. Therefore, we used luciferase assays to determine the reason for the discrepancy. Our results indicated that the c. 3130 C>A (p.Leu1044Met) mutant demonstrated only mildly reduced transcription activation ability compared with the c. 3059 A>T (p.Ile314Leu) mutant, which may partially explain the mild phenotype observed in this family. Additionally, in our data, the c. 3059 A>T (p.Ile314Leu) mutant demonstrated a 22% reduction in its transcription activation ability compared with the wild type sequence, which was consistent with this results noted in a previous report [21]. This result suggested that our assay was valid.

In this study, we illustrated a novel mutation in exon 22 of VEGFR3, within the second tyrosine kinase domain. A clinical analysis demonstrated that a patient in this family not only had swelling of his feet but also possessed a venous valvular abnormality. Our results confirmed the causative role of the VEGFR-3 gene in the pathogenesis of this disease. Compared with previously identified mutations, this mutation appears to result in only the slight impairment of the function of VEGFR-3. Therefore, this may be the reason for milder phenotype noted in this family. The knowledge of this more favorable disease course, as well as the various clinical presentations and the genetic basis of this disease, will contribute to improvements in prenatal counseling and in the management of affected families.

Acknowledgements

The authors would like to thank profession Qingtang Zhu for helpful discussions.

Disclosure of conflict of interest

None.

Address correspondence to: Ping Li and Xiang Zhou, Department of Microsurgery, Trauma and Hand Surgery, The First Affiliated Hospital of Sun Yat-sen University, 58 Zhongshan Er Road, Guangzhou 510080, China. Tel: 86 13600481606; Fax: 86 020-87750632; E-mail: lee-ping@21cn.com (PL); Tel: 86 15913113047; Fax: 86 020-87750632; E-mail: 110337622@qq.com (XZ)

References

- [1] Lewis JM and Wald ER. Lymphedema praecox. *J Pediatr* 1984; 104: 641-648.
- [2] Wheeler ES, Chan V, Wassman R, Rimoin DL, Lesavoy MA. Lesavoy, Familial lymphedema praecox: Meige's disease. *Plast Reconstr Surg* 1981; 67: 362-364.
- [3] Brice G, Child AH, Evans A, Bell R, Mansour S, Burnand K, Sarfarazi M, Jeffery S, Mortimer P. Mortimer, Milroy disease and the VEGFR-3 mutation phenotype. *J Med Genet* 2005; 42: 98-102.
- [4] Dale RF. The inheritance of primary lymphoedema. *J Med Genet* 1985; 22: 274-8.
- [5] Smeltzer DM, Stickler GB, Schirger A. Primary lymphedema in children and adolescents: a follow-up study and review. *Pediatrics* 1985; 76: 206-218.
- [6] Moffatt CJ, Franks PJ, Doherty DC, Williams AF, Badger C, Jeffs E, Bosanquet N, Mortimer PS. Lymphoedema: an underestimated health problem. *QJM* 2003; 96: 731-738.
- [7] Ferrell RE, Levinson KL, Esman JH, Kimak MA, Lawrence EC, Barmada MM, Finegold DN. Finegold, Hereditary lymphedema: evidence for linkage and genetic heterogeneity. *Hum Mol Genet* 1998; 7: 2073-8.
- [8] Witte MH, Erickson R, Bernas M, Andrade M, Reiser F, Conlon W, Hoyme HE, Witte CL. Phenotypic and genotypic heterogeneity in familial Milroy lymphedema. *Lymphology* 1998; 31: 145-5.
- [9] Evans AL, Brice G, Sotirova V, Mortimer P, Beninson J, Burnand K, Rosbotham J, Child A, Sarfarazi M. Mapping of primary congenital lymphedema to the 5q35.3 region. *Am J Hum Genet* 1999; 64: 547-5.
- [10] Irrthum A, Karkkainen MJ, Devriendt K, Alitalo K, Vikkula M. Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. *Am J Hum Genet* 2000; 67: 295-301.
- [11] Karkkainen MJ, Ferrell RE, Lawrence EC, Kimak MA, Levinson KL, McTigue MA, Alitalo K,

Novel VEGFR3 mutation causes primary lymphedema

- Finegold DN. Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. *Nat Genet* 2000; 25: 153-9.
- [12] Futatani T, Nii E, Obata M, Ichida F, Okabe Y, Kanegane H, Miyawaki T. Molecular characterization of two novel VEGFR3 mutations in Japanese families with Milroy's disease. *Pediatr Int* 2008; 50: 116-8.
- [13] Saaristo A, Partanen TA, Arola J, Jussila L, Hytönen M, Mäkitie A, Vento S, Kaipainen A, Malmberg H, Alitalo K. Vascular endothelial growth factor-C and its receptor VEGFR-3 in the nasal mucosa and in nasopharyngeal tumors. *Am J Pathol* 2000; 157: 7-14.
- [14] Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *Embo J* 1996; 15: 1751.
- [15] Yamada Y, Nezu J, Shimane M, Hirata Y. Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. *Genomics* 1997; 42: 483-8.
- [16] Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K, Breitman M, Alitalo K. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* 1998; 282: 946-9.
- [17] Karkkainen MJ, Saaristo A, Jussila L, Karila KA, Lawrence EC, Pajusola K, Bueler H, Eichmann A, Kauppinen R, Kettunen MI, Yla-Herttuala S, Finegold DN, Ferrell RE, Alitalo K. A model for gene therapy of human hereditary lymphedema. *Proc Natl Acad Sci U S A* 2001; 98: 12677-82.
- [18] Evans AL, Bell R, Brice G, Comeglio P, Lipede C, Jeffery S, Mortimer P, Sarfarazi M, Child AH. Identification of eight novel VEGFR-3 mutations in families with primary congenital lymphoedema. *J Med Genet* 2003; 40: 697-703.
- [19] Gordon K, Spiden SL, Connell FC, Brice G, Cottrell S, Short J, Taylor R, Jeffery S, Mortimer PS, Mansour S, Ostergaard P. FLT4/VEGFR3 and Milroy disease: novel mutations, a review of published variants and database update. *Hum Mutat* 2013; 34: 23-31.
- [20] Butler MG, Dagenais SL, Rockson SG, Glover TW. A novel VEGFR3 mutation causes Milroy disease. *Am J Med Genet A* 2007; 143A: 1212-7.
- [21] Karkkainen MJ, Ferrell RE, Lawrence EC, Kimak MA, Levinson KL, McTigue MA, Alitalo K, Finegold DN. Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. *Nat Genet* 2000; 25: 153-9.
- [22] Spiegel R, Ghalamkarpour A, Daniel-Spiegel E, Vikkula M, Shalev SA. Wide clinical spectrum in a family with hereditary lymphedema type I due to a novel missense mutation in VEGFR3. *J Hum Genet* 2006; 51: 846-50.
- [23] Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. *Nat Methods* 2010; 7: 248-9.
- [24] Ramensky V, Bork P, Sunyaev S. Human non-synonymous SNPs: server and survey. *Nucleic Acids Res* 2002; 30: 3894-900.