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A novel mutation in the conserved sequence of vascular endothelial growth factor receptor 3 leads to primary lymphoedema

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Abstract

Objective: To investigate whether lymphoedema in a Chinese family showed the hereditary and clinical characteristics of Milroy disease, an autosomal dominant form of congenital lymphoedema, typically characterized by chronic lower limb tissue swelling due to abnormal lymphatic vasculature development, and to perform mutational analyses of vascular endothelial growth factor receptor (*VEGFR*)3.

Methods: Individuals from a three-generation family affected by congenital lymphoedema were clinically assessed for Milroy disease. Mutation analysis of *VEGFR3* was performed using DNA from family members and healthy controls.

Results: Out of 20 family members, eight were diagnosed with hereditary lymphoedema. Mutation analyses revealed a novel mutation site for c.3163 G>A, resulting in a p.1055D>N mutation in the second tyrosine kinase domain of *VEGFR3*, which was present in affected individuals only (absent in all unaffected family members and 130 healthy controls). Computed functional analyses showed the mutation may lead to structural alterations with a probability of 0.99999 of being disease causing.

Conclusion: A novel mutation associated with Milroy disease was identified in a Chinese family, expanding our knowledge of *VEGFR3* gene function and providing a potential molecular target for treating hereditary lymphoedema.

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Introduction

The lymphatic vasculature is essential to homeostasis of the circulatory and immune systems, and in maintaining body fluid composition. Developmental disorders of the lymphatic vasculature result in a failure to maintain a homeostatic capacity for fluid transport, a condition known as primary lymphoedema.[1] Primary lymphoedema involves chronic tissue swelling; most commonly present in a lower limb,[1] and other characteristics such as hydroceles, prominent veins and abnormal lymphoscintigraphy with functional aplasia.[2] The degree and distribution of swelling varies between patients, and lymphoedema may be peripubertally observed, occur in association with pregnancy or develop soon after.[3]

Lymphoedema can be clinically identified by hypoplasia of the lymphatic vasculature that results in swelling of the extremities and inflammation of affected tissues. Described in 1892, Milroy disease (hereditary lymphoedema type I) is a congenital, autosomal dominant type of primary lymphoedema with decreased penetrance of 80–90%, that affects the lower limbs.[4] In 1998, the gene associated with Milroy disease was mapped to 5q35.3,[4] and later, to the vascular endothelial growth factor receptor (*VEGFR*)3 gene.[5] A previous study showed that the mutation rate of *VEGFR3* was nearly 75% in typical patients with Milroy disease with an affected family cohort,[6] and Milroy disease is characterized by mutations in *VEGFR3* in many positive family cohorts.[7–9] The *VEGFR3* gene encodes a tyrosine kinase receptor, also called vascular endothelial growth factor receptor 3, and during early embryogenesis this kinase is expressed in all endothelial cells.[10] *VEGFR3* plays an important role in several processes, such as angiogenesis, endothelial cell migration, lymphangiogenesis, proliferation and survival, and lymphatic development.[11,12] The VEGFR3 receptor is activated by VEGF-C and VEGF-D proteins and is crucial for lymphatic development during embryogenesis.[13] For example, in a developmental investigation of the mouse embryo, VEGFR3 activation was shown to be associated with mechanotransduction and β1-integrin signalling, and was essential for normal development and fluid homeostasis.[14] Transgenic mice that are homozygous for *VEGFR3* mutations, die before birth as a result of severe developmental cardiovascular disorders.[10] In addition, Chy mice (VEGFR-3+/2) and mice in which the VEGFR3 receptor is blocked, develop swelling in each of the limbs due to a shortage of dermal lymphatic vessels.[15]

Mutations in *VEGFR3* that cause congenital lymphoedema are consistently located within the functional domain,[7] and whether this disease could be caused by mutations in other regions of *VEGFR3* remains unknown. Thus, the mutational analysis of *VEGFR3* is crucial to understanding the underlying molecular basis of hereditary lymphoedema. The aim of the present study was to investigate whether the presence of lymphoedema in a three-generation Chinese family displayed the hereditary and clinical characteristics of Milroy disease, and to perform mutational analyses of the *VEGFR3* gene in members this family (comprising affected and unaffected individuals), and in a healthy control population.

Patients and methods

Study population

This observational study was conducted in the department of Microsurgery, Trauma and Hand Surgery, the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China between June 2014 and December 2014. Following referral of an 11-year-old male (the proband), other individuals in this family were invited for clinical and genetic evaluation regarding lymphatic and venous disorders, and detailed examination and analysis indicated a hereditary pattern. Healthy individuals were randomly recruited from Guangzhou, Guangdong as control participants, and individuals with limb infection, limb swelling, varicose veins, or nail disease were excluded from the study. Venous blood samples from each family member and control participants were collected into heparin tubes and stored at –80 °C prior to DNA extraction. Genomic DNA was extracted from the blood samples for genetic analyses using a TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions, and stored at –80 °C prior to use.

All experiments were approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University (201401246521) and were performed in accordance with the Helsinki Declaration (1983). All family members and healthy control participants provided written informed consent to be included in the study.

Mutation analyses

Mutation analysis of exons 17–26 of *VEGFR3* was conducted using DNA samples from all family members and healthy controls, following the methods previously described,[16] and using previously published *VEGFR3* primer sequences.[17] Briefly, PCR was performed using a Super HiFi PCR Mix (TIANGEN) and a T100™ Thermal Cycler (Bio-Rad, Hercules, California, US), with the following *VEGFR3* primer sequences: Exon 17, forward CTGTCCTGGGCAAAGTTCTG CC, reverse ATGGAGGGATTCAGGCA CTCCG; Exon 18, forward TGTCTCC ACGCTCACCC, reverse CCGCTGACC CCACACCTT; Exon 19, forward TGC CTTAGCTAAGCGGCCAGTG, reverse GCTCCGCGTTTGCACCCGCG; Exon 20, forward CGCGGGTGCAAACGCGG AGC, reverse CGCAGAGGCGCCTCCA TTCC; Exon 21, forward GCCCTCGAG CCAGCTTCG, reverse AGGAAAAGGG AAGAGGCCAG; Exon 22, forward ACA TGCTTGTTAGCTGTTCCCTG, reverse CAAGCACTTCTTGGCGACTG GT; Exon 23, forward GAGTTGACCTCCC AAGGT, reverse TCTCCTGGACAGG CAGTC; Exon 24, forward TTCAA CCCAGGCAGAGGACCC, reverse CG TAACCTGCCGCCAGTGACC; Exon 25, forward CAGGACGGGGTGACTTGA, reverse GCCCAGGCCTGTCTACTG; and Exon 26, forward GGGCACTGAA GGTCCTGGGAC, reverse GCAGCTCG GGCCAGGGACC. The reaction involved a preliminary denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30s, and elongation at 68 °C for 45 s, and a final elongation step at 68 °C for 8 min. The PCR products were then sequenced using an automated ABI 3730XL DNA Analyser and associated reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequence analyses were performed in both the forward and reverse directions.

Clinical analyses

All family members were clinically evaluated (by XZ) for abnormalities in the lower extremities, including large calibre veins, keratosis, ski jump toenails; and a

qualitative assessment of aortic aneurysm (including subcutaneous thickening) was performed. Male family members underwent a genital examination (XZ) to confirm the presence of testicular hydrops.

Bioinformatic analyses

Potential molecular and functional effects of the detected mutation were analysed using MutationTaster software, version 2.0 (http://www.mutationtaster.org/) and PolyPhen2 software, version 2.0 (http://genetics.bwh.harvard.edu/pph2/) with Gene ID: 2324. The present sequences were aligned and compared with sequences from the following *Homo sapiens* receptor tyrosine kinases using ClustalW software (http://www.genome.jp/tools-bin/clustalw; UCD, Dublin, Ireland): platelet derived growth factor receptor beta (*PDGFRB*; accession number, AAH 32224), fibroblast growth factor receptor 1 (*FGFR1*; accession number, AAH 18128), ret proto-oncogene (*RET*; accession number, AA 04257), epidermal growth factor receptor (*EGFR*; accession number, BAD 92679) and EPH receptor A1 (*EPHA1*; accession number, AAI 30292). In addition, the VEGFR3 protein sequences were aligned with the following species: *Homo sapiens* (accession number, NP_891555.1), *Pan troglodytes* (accession number, XP_518160.4), *Coturnix* (accession number, CAA58267.1), *Canis familiaris* (accession number, XP_538585.2), *Mus musculus* (accession number, NP_032055.1), *Rattus norvegicus* (accession number, XP_579569.1) and *Danio rerio* (accession number, NP_571020.1).

Results

A total of 20 family members (mean age, 44.5 ± 22 years; 8 male/12 female) and 135 healthy control participants (mean age, 43.6 ± 13 years; 52 male/83 female) were included in the study.

Clinical results

Out of 20 members in three generations of a Chinese family, there were a total of eight individuals with congenital lymphoedema (Figure 1). The presence of lymphoedema in this family was verified at the evaluation of an 11-year-old boy (the proband), and detailed clinical examination and analyses of the family revealed a hereditary pattern. Other clinical symptoms, observed in all affected family members, included hydrocele, hyperkeratosis, ski jump toenails and papillomatosis. Additionally, preputial oedema was detected in the family. All male patients showed preputial oedema upon genital examination. Apart from congenital lymphoedema, no other abnormality was observed for other family members.

Results of genetic analyses

Exons 17–26 of *VEGFR3* were amplified and sequenced in 20 members of a family with congenital lymphoedema and 135 healthy controls. The results revealed a G-to-A substitution in exon 23, at position 3163 in the coding sequence (Figure 2a), resulting in an amino acid change from aspartic acid to asparagine in amino acid position 1055. This change was confirmed via reverse sequencing and was found to exist in all eight affected family members. This change was not observed in unaffected family members or in unrelated, unaffected control subjects.

Bioinformatic results

This mutation was not found in the Exome Aggregation Consortium database (ExAC; http://exac.broadinstitute.org/) or the 1000 genomes project '1000 genomes' browser (http://www.internationalgenome.org/1000-genomes-browsers). The potential mutation effect was analysed using MutationTaster and PolyPhen2 software, and results showed that the occurrence of

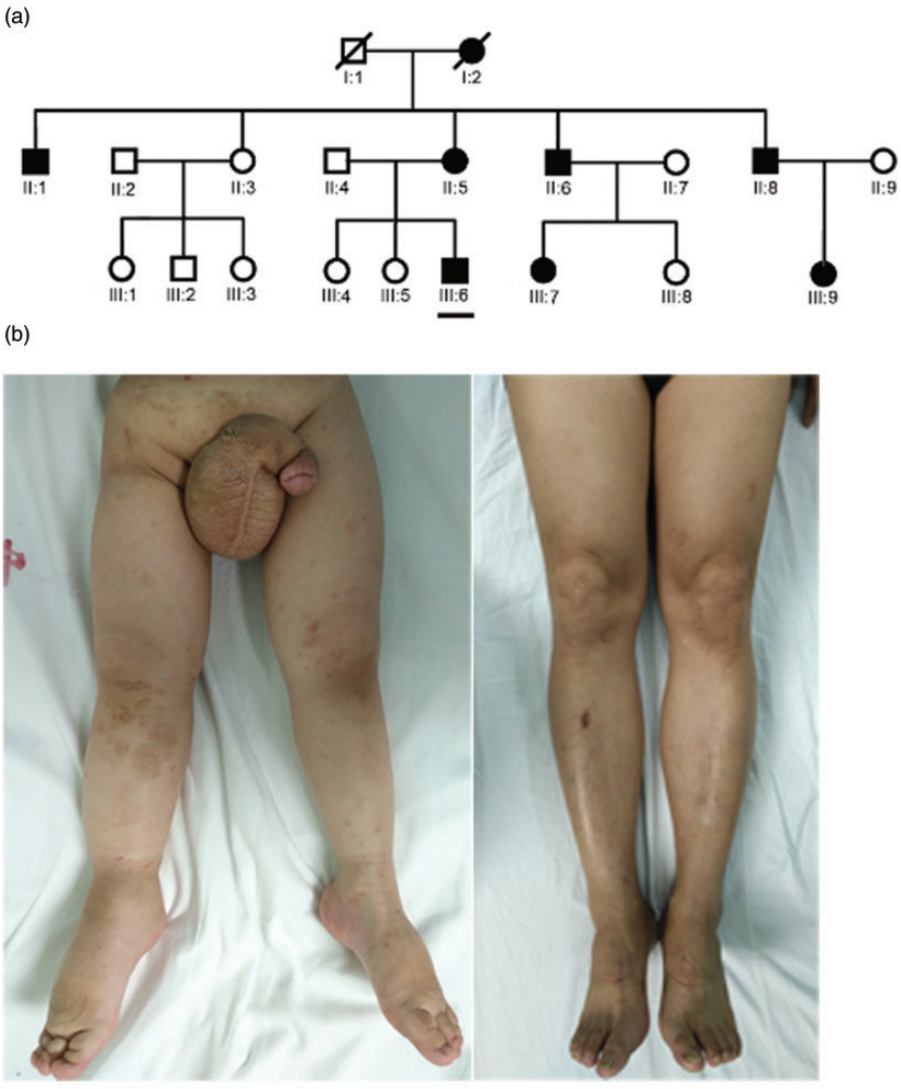

Figure 1. Pedigree and clinical characteristics in three generations of a Chinese family with hereditary congenital lymphoedema, showing: (a) pedigree results with the proband underlined, III:6 (key: squares, male; circles, female; solid black symbols, affected individuals; and open symbols, unaffected individuals); and (b) photograph of proband (III:6) illustrating lymphoedema in both legs and preputial oedema (left panel), which was observed in all of the male patients in this family, and photograph of III:5 with no oedema-related abnormality (right panel).

this novel mutation may lead to structural alterations. With a probability of 0.99999, the MutationTaster software predicted that the amino acid change might be disease causing. The PolyPhen2 software predicted that damage was a likely result of this mutation, with a score of 1.000 (sensitivity, 0.00; and specificity, 1.00). Cross-alignment

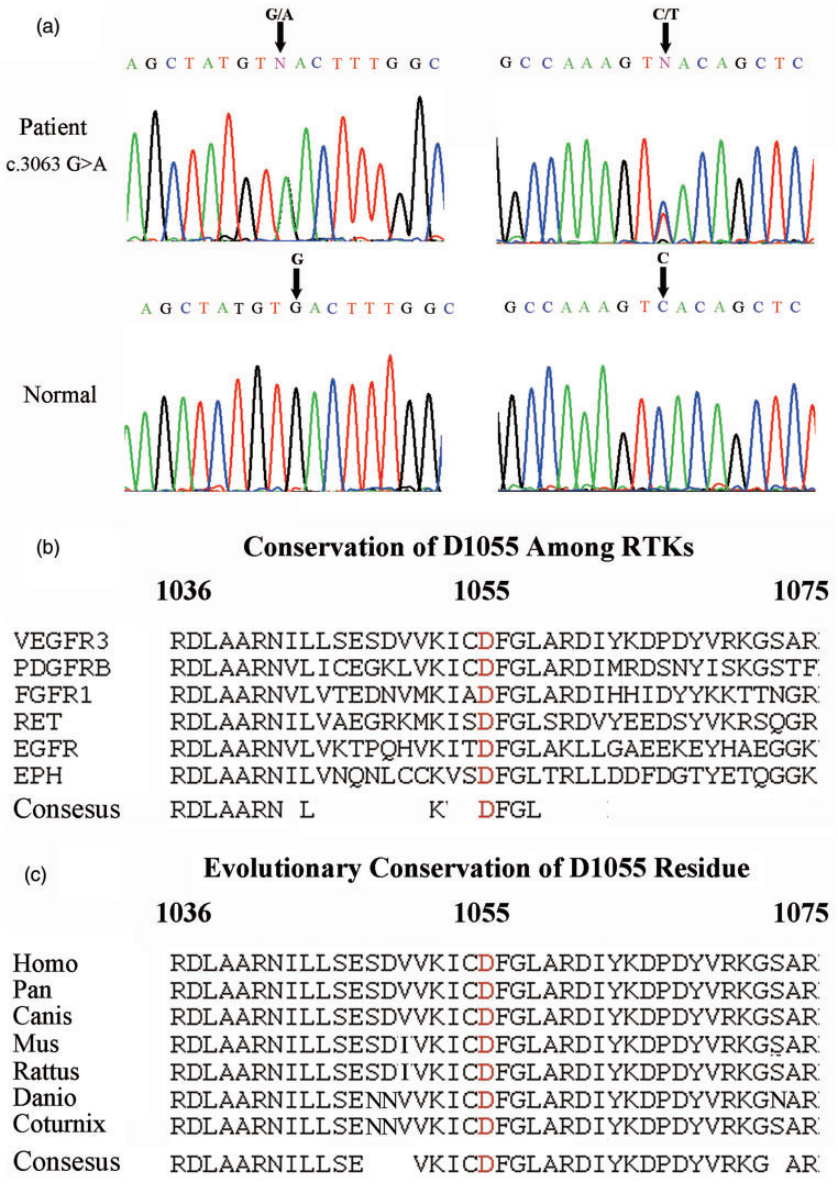

Figure 2. Novel missense mutations in the vascular endothelial growth factor receptor (*VEFGR*)*3* gene associated with Milroy disease: (a) Representative image showing a point mutation at position 3163 of the *VEGFR3* coding sequence, which results in a D1055N amino acid substitution. Genomic DNA sequencing indicates that this constitutes a missense mutation; (b) Results of cross-alignment studies using ClustalW software of other tyrosine receptor types in *Homo sapiens*, showing conservation of aspartic acid residues at position 1055 (highlighted in red); and (c) alignment of a portion of the VEGFR3 protein in six different species using ClustalW software, indicating the evolutionary conservation of aspartic acid residues at position 1055 (highlighted in red); Abbreviations: RTK, receptor tyrosine kinase; PDGFRB, platelet derived growth factor receptor beta; FGFR1, fibroblast growth factor receptor 1; RET, ret proto-oncogene; EGFR, epidermal growth factor receptor; EPH, EPH receptor A1; Homo, *Homo sapiens*; Pan, *Pan troglodytes*; Canis, *Canis familiaris*; Mus, *Mus musculus*; Rattus, *Rattus norvegicus*; and Danio, *Danio rerio*.

studies involving other types of tyrosine receptor demonstrated that this aspartic acid is highly conserved (Figure 2b). Alignment analyses of the VEGFR3 protein sequence indicated that this position is highly conserved among many different species (Figure 2c). Thus, all the results suggest that this amino acid exerts an important role in VEGFR3 protein function.

Discussion

Mutations in *VEGFR3* have been previously shown to cause Milroy disease and most *VEGFR3* mutations associated with congenital lymphoedema have been localized to the tyrosine kinase domain of the VEGFR3 receptor;[7] thus, the present investigation involved the amplification and sequencing of exons 17–26 of *VEGFR3*. The present report describes a novel *VEGFR3* mutation, c.3163 G>A, which results in the substitution of asparagine for aspartic acid in the tyrosine kinase domain. Analysis of individuals in a three-generation Chinese family revealed that all individuals affected by congenital lymphoedema carried the *VEGFR3* c.3163 G>A mutation. In contrast, the same mutation was not detected in any unaffected family members and was not found among 135 healthy controls.

Lymphoedema is a troubling disease that currently lacks an effective cure. Several studies have demonstrated the relationship between the *VEGFR3* mutation and primary lymphoedema.[7,17] The *VEGFR3* gene comprises 31 exons, transcribed as two alternatively spliced transcripts, and encodes proteins with seven immunoglobulin-like repeat domains and two tyrosine kinase domains.[18] *VEGFR* genes play a vital role in several processes, including endothelial cell migration, angiogenesis, lymphangiogenesis, proliferation and survival,[11,12] and VEGFR3 may be the most important receptor in lymphatic development.

A key regulator in endothelial cell function, VEGF has a significant role in vasculogenesis and physiological and pathological angiogenesis.[19] The VEGF family has several subtypes, such as VEGF-A, B, C, D, E, F and placental growth factor (PlGF), which specifically combine with VEGFRs, such as VEGFR1, 2, 3 and neuropilin (NRP)2. Of these isotypes, VEGF-C, VEGF-D and VEGFR-3 are the first and best studied regarding mechanisms of lymphatic-specific signalling.[17] The VEGF-C/D-VEGFR3/NRP2 axis is a lymphatic-specific biochemical axis that includes three essential parts, comprising extracellular VEGF-C/D as ligands, cell membrane VEGFR3/NRP2 as receptors, and extracellular or intracellular pathway-related molecules as executors.[20] The up and downstream pathway-related molecules of the VEGFC/D-VEGFR3/NRP2 axis form a complex biochemical network and act in synergy with lymphangiogenesis.[21] For example, the VEGFC/VEGFR3 axis upregulates the expression of contactin (*CNTN*)1 through activation of the proto-oncogene tyrosine-protein kinase Src (Src)- mitogen-activated protein kinase 14 (p38)/ mitogen-activated protein kinase (MAPK)-C/ emopamil binding protein (sterol isomerase) (EBP)-dependent signalling pathway, whereas CNTN1 can also reduce E-cadherin expression during gene transcription, by activating the Notch/ AKT serine/threonine kinase 1 (AKT) pathway and inhibiting the transcription factor snail family transcriptional repressor 2 (SNAI2).[10]

In the present Chinese family, the detected mutation triggered a c.3163 G>A in exon 23 of *VEGFR3*, leading to an amino acid p.1055D>N substitution, and the subsequent new amino acid may result in a structural change of the VEGFR3 protein. This mutation was not found in either the ExAC or 1000G databases. The MutationTaster program predicted that this novel mutation might cause disease with a

probability of 0.99999, and the PolyPhen2 program predicted that damage would likely result from this mutation, with a score of 1.000 (sensitivity: 0.00; specificity: 1.00).[22] Moreover, this residue was found to be located in a highly conserved domain among different species and different receptor tyrosine kinases (Figure 2). The high conservation of this residue suggests that it may exert an important structural role within a functional domain of the VEGFR3 protein.

An increase in interstitial protein rich fluid subsequently leads to insufficient lymphatic transport.[9,23] Milroy disease accounts for less than 10% of primary lymphoedema cases, with less than 200 cases reported in the literature.[7] The oedema associated with Milroy disease is typically painless and chronic, and as previously described, almost all patients are affected at birth, with precise family history.[7] The other associated symptoms are large calibre leg veins, papillomatosis, toenail abnormalities, and hydrocele in male patients.[24] Preputial oedema was detected in the present family, and to the best of the authors' knowledge, the present study is the first to report preputial oedema in a patient with lymphoedema. The main consequences of lymphatic failure are swelling and recurrent infection.[24]

Previously, one study identified an amino acid p.D1055V substitution in a four-generation Chinese family with hereditary lymphoedema type I,[25] and another study reported an amino acid p.D1055A mutation in an English patient with atypical Milroy disease, glaucoma and learning difficulties, without family history.[26] In the present family, the novel mutation relates to the same residue described by the previous two studies. Given the high conservation rate of this residue, this mutation site appears to be a mutation hot spot, and the mutation results will be useful for further genetic consultation and genetic diagnoses.

In summary, the present results, regarding a Chinese family with distinct clinical features characterized by primary lymphoedema with preputial oedema, will be useful for clinicians to further understand primary lymphoedema. The novel c.3163 G>A mutation (resulting in amino acid p. D1055A) was associated with the clinical phenotype, and the mutation was located in the second tyrosine kinase domain of the VEGFR3 receptor. Although a mutation occurred at the same residue described by two previous studies, the mutation found in the present family is novel, suggesting that this region may be a mutation hot spot. These findings have expanded our knowledge of the *VEGFR3* gene and will improve the current understanding of hereditary lymphoedema. The results may be useful for genetic consultation and genetic diagnoses; however, further studies are needed to explore the detailed mechanisms associated with the mutation.

Declaration of conflicting interests

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