

A Milroy Disease Family Caused by *FLT4* Gene Mutation of c.2774 T>A with Phenotypes Heterogeneity

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Abstract

Background: Milroy disease is a rare, autosomal dominant disorder. Mutations of *FLT4* (Fms Related Tyrosine Kinase 4) gene impaired tyrosine kinase signaling, and further cause symptoms of Milroy disease. In this research, we found a large Chinese MD family with phenotype heterogeneities. And we conducted Next Generation Sequencing analysis to explore possible genetic causative factors might be related to clinical heterogeneities among family members.

Methods: Sanger sequencing was conducted on the 17-26 exons of *FLT4* (NM_182925.4) gene. Primers were as follows: Forward: 5' CTTTCATCAGCGTCGAGTGG 3'; Reverse: 5' ATTATGGGCGGGTTCCTT 3'; Next-generation sequencing was conducted to explore pathogenic mutation might lead to phenotype heterogeneities. Then we conducted Sanger sequencing of the possible related genes. The *GIMAP7* gene amplification primers as follows: Forward primer: 5' ACCACCTGCAAGGAAATCAGCCGCT3'; Reverse primer: 5' GTTAGAGAAATACCTCCTCCCTT3'. The amplification system for two genes are as follows: 2×Biotech Power PCR Mix: 10µl; forward primer: 0.8µl (10µM); reverse primer: 0.8µl (10µM); DNA template: 1µl (50ng/µl); ddH₂O: 13.4µl. The effects of the mutations on the gene functions were evaluated with mutation taster and/or SIFT, PolyPhen.

Results: A heterozygous substitution mutation was detected in all patients (*FLT4* gene: c.2774 T>A, p.V925E). Meanwhile, a G deletion (c.826delG, p.Val276Phefs*29) of *GIMAP7* gene was detected in all patients but two patients with phenotype heterogeneities (I1, and II1). Both the two mutations were predicted to be pathogenic.

Conclusions: In this report, we described a large Milroy disease family caused by a missense mutation of the *FLT4* gene (c.2774 T>A, p.V925E). Meanwhile, a frame-shift mutation (c.826delG, p.Val276Phefs*29) of *GIMAP7* gene might be related to the clinical phenotype heterogeneities of the family.

1. Background:

Primary hereditary lymphoedema type IA (LMPH1A, OMIM#153100), also known as the Milroy disease (MD) is a rare, autosomal dominant disorder[1]. The phenotypes of Milroy disease (MD) is genetically heterogenous, the characteristic phenotypes including: painless and non-progressive lymphedema of uni- or bilateral edema of the legs and feet [2–4] and prominent venous vessels[2]. congenital hands edema [5], and persistent bilateral pleural high protein fluid effusion [2–4] and scrotum swelling[2]. The *FLT4* gene (Fms Related Tyrosine Kinase 4)—also known as the Vascular Endothelial Growth Factor Receptor 3 (*VEGFR3*)—encodes a receptor tyrosine kinase [6–9], is important for lymphatic endothelial cell survival, proliferation and migration[10, 11]. The *FLT4* gene mutations impaired tyrosine kinase signaling, and further cause symptoms of Milroy disease[12]. In this research, we found a Chinese MD family with a *FLT4* gene mutation and presented heterogeneities within the family members.

The proband with suspected primary lymphedema was self-referred to our research team and was evaluated by lymphological specialists and medical geneticist (Miao Jiang and Wei-tian Han). Then we obtained a complete family history. All participated family members underwent a series of clinical examinations for primary lymphedema. The recruiting criteria of patients are at least one of the following criterions: lower extremity lymphoedema, pitting edema, hyperkeratosis, subcutaneous thickening. We also assessed the morphology of the nail plates. Medical history and physical examination were conducted to exclude the secondary lymphedema: we asked the patient if they have filariasis, cancer, infection, radio-/chemo-therapy and surgery. Genetic counseling offered to family members when the pathogenic mutation was identified. Local research ethics committee approved the study protocol and consent forms. All family members enrolled in the study signed two informed consents: one for the genetic test; and second to make the clinical and genetic data available for research purpose.

2. Methods:

2.1 DNA Extraction:

Blood samples were obtained from all family members. We recruited 100 healthy individuals from the same geographical areas as the patients lived, to clarify whether new mutations were innocuous polymorphism or pathogenic mutations. Genomic DNA was extracted from the blood samples using a DNA Isolation Kit for Mammalian Blood (Tiangen Biotech, China).

2.2 PCR amplification and Sanger sequencing

2.2.1 The PCR amplification and Sanger sequencing of *FLT4* gene

We sequenced the 17–26 exons (tyrosine kinase coding domains) of *FLT4* gene in all family members (Sangon Biotech, Shanghai, China). The primers of PCR amplification systems and conditions set designed based on *FLT4* gene (NM_182925.4) are in supplementary materials (Table S10: PCR amplification system and conditions of tyrosine kinase coding domains of *FLT4* gene). Primer amplified exon 20 were as follows: Forward primer: 5' CTTTCATCAGCGTCGAGTGG 3'; Reverse Primer: 5' ATTATGGGCGGGTTCCTT 3'; The PCR amplification conditions for the *FLT4* gene exon 20 amplification were as follows: denaturing at 95°C for 5 min; 35 cycles of denaturing at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s; and final step for 7 min at 72°C. The amplified fragment is 176 bp. The amplification system is: 2 × Biotech Power PCR Mix: 10 µl; forward primer: 0.8 µl (10 µM); reverse primer: 0.8 µl (10 µM); DNA template: 1 µl (50 ng/µl); ddH₂O: 12.4 µl. The amplification system is 25 µl. The novel mutation was also ruled out as polymorphism by digestion with the restriction enzyme Hph I and the amplification primers are as follows: 5' AACCTCCTCGGGGCGTGACCAAGC 3', 5' GCGCAGGGGCTGAAGGCGTCCCG 3'; The amplification system is: 2 × Biotech Power PCR Mix: 10 µl; forward primer: 0.8 µl (10 µM); reverse primer: 0.8 µl (10 µM); DNA template: 1 µl (50 ng/µl); ddH₂O: 12.4 µl. The PCR amplification conditions for the *FLT4* gene is denaturing at 98°C for 30 s; 35 cycles of denaturing at 98°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s; and final step for 7 min at 72°C. The PCR amplification fragment is 262 bp.

2.2.2 PCR amplification and Sanger sequencing of the possible pathogenic genes

If any variant was included, we verified it within the family members. We conducted PCR amplification and Sanger sequencing in four patients (I1, II1, III3 and III4) and the healthy control (II7). PCR amplification and Sanger sequencing analysis based on the variant's information. The PCR amplification systems are listed in supplementary materials (Table S8: PCR amplification systems and conditions of possible pathogenic variants). The Sanger sequencing was conducted by Sangon Biotech, Shanghai, China.

2.2.3 Mutation analysis

A search performed for human FLT4 and GIMAP7 protein homologs using BLAST on the NCBI web site (<http://www.ncbi.nlm.nih.gov>). The identified proteins were aligned using ClustalW[13], and a phylogenetic tree was reconstructed with MEGA4 with neighbor-joining method[14]. The effect of the amino acid substitution on the protein function was evaluated with mutation taster (www.mutationtaster.com) and/or SIFT [15, 16], PolyPhen.

2.2.4 PPI network generation

First, the PPI networks of *FLT4* and *GIMAP7* gene were generated using string database (<https://string-db.org/cgi/input.pl>). Then we construct functional annotation maps to understand which protein was involved in the *FLT4* and *GIMAP7* gene functions. The interaction network were obtained from the STRING database version 10.5.

3. Results:

3.1 Description of the MD pedigree

The family history presents 7 affected patients (age ranged from 29–75 years old) of four generations (Fig. 1 Pedigree of the family). Table 1 overviewed the clinical features of all 7 patients (Table 1. Overview of clinical features of 7 patients). All the affected individuals presented with congenital bilateral lower limbs lymphedema at birth, and lymphedema extended from the toes to the upper foot calves, presented as different degrees of creases and brawny texture of the skin—depend on management of disease progression and compliancy with the medical treatment (Fig. 2 – 1, 2: bilateral lower limbs lymphedema of patient II1; and Fig. 2–3: bilateral lower limbs lymphedema of patient II6; and Fig. 2–4: bilateral lower limbs lymphedema of patient III3). Further, the propositus (III3) always feels a pain in her foot after taking a long walk, especially during the hot season. The swollen region is also warm to touch; the marks caused by the compression stockings is visible, and the edema is much lighter than that of her aunt (II1) (Fig. 2 – 1,2: bilateral lower limbs lymphedema of patient II1 and Fig. 2–4: bilateral lower limbs lymphedema of patient III3). The phenotypes of her male cousin (III4) are not quite distinguishable by naked eyes. On the contrary, patient I1 complained that the edema of his legs aggravated and extended to the roots of the thighs when he caught cold and fever from childhood, no other patients (II1, II2, II4, II6, III3, and III4) showed these phenotypes under the same condition within this family. They show lower levels of keratinization of the feet skin, and the swelling degree of lower limbs is also lighter than that of the two patients (I1 and II1). Meanwhile, Patient II1 complained that: as for adulthood, edema continued to the lower extremities beneath the knees (Fig. 2 – 1,2. bilateral lower limbs lymphedema of patient II1). During childhood, skin hyperkeratosis of the feet occurred, and the lymphedema caused the instep rise too high to wear shoes. The tissue of foot was filled with lymph fluid and caused local foot skin and tissue swelling and deforming, either the proliferation of adipose tissue and connective tissue (Fig. 2 – 1,2: bilateral lower limbs lymphedema of patient II1). The lymphedema phenotype is not visible now due to plastic surgery, but the heavier lower extremities edema and elephant-like appearance of her legs are obvious (Fig. 2 – 1,2: bilateral lower limbs lymphedema of patient II1).The toenails of patients within the family are hypoplastic with up-turned concavity, consistent with the “ski-jump” nails (Fig. 2–3: bilateral lower limbs lymphedema of patient II6). One patient in this family shows slight epicanthic folds and down-slanting palpebral fissures (Fig. 3: slight epicanthic folds and down-slanting palpebral fissures)[4]. No asymptomatic carrier, late onset or non-penetrance patient found within the family.

Table 1
Overview of clinical features of 7 patients

Patient	Onset at birth	congenital bilateral lower limbs lymphedema at birth	deep creases over the toes	congenital hands edema	brawny texture of the skin	small dysplastic toenails ('ski-jump')	swelling of the scrotum	Hypoproteinemia	Lymphedematous	Edema extends to the thigh	epicanthic folds down slanting palpebral fissure
I1	+	+	++	-	+	+	-	-	-	+	-
II1	+	+	++	-	+	+	-	-	-	-	-
II2	+	+	+	-	+	+	-	-	-	-	-
II4	+	+/-	+/-	-	+/-	+	-	-	-	-	+
II6	+	+	+	-	+	+	-	-	-	-	-
III3	+	+	+	-	+	+	-	-	-	-	-
III4	+	+/-	+/-	-	+/-	+	-	-	-	-	-

3.2 Mutation analyses

3.2.1 Identification of a novel mutation in the *FLT4* gene

The Sanger sequencing results 17–26 exons of *FLT4* gene (NM_182925.4) revealed a single nucleotide heterozygous substitution of T to A in all the patients at nucleotide position 2774 (c.2774 T > A, p.V925E) in exon 20, results in an amino acid change from Valine to Glutamic at the amino acid residue 925 (Fig. 4: Sanger sequencing result of the *FLT4* gene c.2774 T > A mutation.), and was detected in all affected patients (I1, II1, II2, II4, II6, III3 and III4) but not in unaffected family members (II3, II5, II7 II8, III1, III2, III5 and IV1). The novel mutation was also ruled out as polymorphism by Hph I digestion, the PCR amplification fragment is 262 bp. The restriction endonuclease cleavage site is 5'GGTGA(N)₈3'. If the mutation site presented, the fragment is cleaved and turn out to be two fragments: 198 bp and 64 bp, and analyzed by 8% polyacrylamide gel electrophoresis and silver staining. Using DL2000 as the marker (Takara). The polypropylene gel electrophoresis voltage is 400 Voltage and the electrophoresis time is 4 hours. (Fig.S1: Hph I restriction enzyme digestion results of *FLT4* gene), and no existence in 100 healthy controls showed a normal sequencing pattern. Using the SIFT program the normalized probability of the substitution of V to E was calculated to be 0.00, which was less than the threshold of 0.05. Thus, this mutation was predicted to be deleterious. The PolyPhen program predicted that this mutation was likely to be damaging with a score of 1.00. Meanwhile, the mutation taster website (<http://www.mutationtaster.org/>) also showed the substitution V925E is disease causing. Twelve proteins in the FLT4 subfamily were found by a BLAST search in NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) formed a cluster in the phylogenetic tree (Fig.S2: Polygen Tree of *FLT4* gene). FLT4 protein sequence alignment revealed the invariant valine in the tyrosine kinase domain and the wild type is conserved in a wide range of organisms, ranging from humans to *Danio rerio* (Fig.S3: Conserved motif of *FLT4* gene).

3.2.2 Identification of a novel deletion in the *GIMAP7* gene

For there are phenotypes heterogeneities within the family members (patients I1 and II1 compared to other patients), so we conducted the NGS analysis to explore whether there are possible pathogenic variants might be related to phenotypes. The flow chart of NGS screening result presented in supplementary material (Fig S4: flow chart of data analysis of NGS results; The raw NGS results are in supplementary materials: Table S1-S5). After data cleaning and collating there are several variants left. The functions of the possible pathogenic genes are in the supplementary materials (Table S6: variants shared by patients I1 and II1 but not by patient II7; Table S7: variants shared by patient III3 and III4 but not by patient II7). According to the gene functions we selected 19 genes including 30 variants as the possible pathogenic variants. Then we sequenced the possible pathogenic variants among all family members (Table S8. PCR amplification systems and conditions of the possible pathogenic variants). After we sequenced all the possible pathogenic variants, we found a G deletion at the c. 826 nucleotide site (c.826delG, p.Val276Phefs*29) of the *GIMAP7* gene (GTPase, IMAP FAMILY, MEMBER 7, NM_153236.3) which caused a frame-shift mutation and loss of the original stop code (Fig. 5, Sanger sequencing results of *GIMAP7* gene c.826delG, p.Val276Phefs*29). This deletion was detected in all affected patients (II2, II4, II6, III3 and III4) except two patients (I1, II1), and not detected in unaffected family members (II3, II5, II7 II8, III1, III2, III5 and IV1). Also, no existence in 100 healthy controls showed a normal sequencing pattern. Mutation taster website (<http://www.mutationtaster.org/>) showed the nucleotide c.826G deletion (p.Val276Phefs*29) is disease causing. Seven proteins of the *GIMAP7* subfamily were found by a BLAST search that formed a cluster in the phylogenetic tree (Fig.S5: Polygenic tree of *GIMAP7* gene). *GIMAP7* protein sequence alignment revealed the presence of nucleotide G is conserved in a wide range of organisms, especially within the Primates, ranging from humans to macaca nemestrna (Fig.S6: conserved motif of *GIMAP7* gene).

4. Discussion:

Primary congenital lymphedema (PCL), also called Milroy disease (OMIM:#153100, MD) is caused by developmental lymphatic vascular anomalies, with an estimated prevalence of 1 in 160,000 individuals[17]. We reviewed the reported phenotypes of the Milroy disease, (Table S9. Review of clinical phenotypes of Milroy disease) that MD patients usually exhibit lymphedema at birth with swelling of the lower-limb, and most are bilateral[2]. They often have brawny texture and hyperkeratosis of foot skin; (Table S9: Review of clinical phenotypes of Milroy disease). The other phenotypes associated with MD including: Hydrocoele of male (37%), "ski jump" toenails (14%) and bilateral pleural effusion[2] (Table S9: Review of clinical phenotypes of Milroy disease). Although below knees lymphedema is the most common phenotypes of MD, edema of some patients extends to thighs [2, 18]. Meanwhile, hydrocoele is more common in male patients [2, 3, 19–22].

The *FLT4* (FMS-LIKE TYROSINE KINASE 4; FLT4; NM_182925.4) gene mutations cause kinase-inactivation and Milroy disease [6, 7, 22–27]. to date, all mutations located in either of two intracellular kinase domains [6, 7]. In this research, we sequenced the tyrosine kinase coding domains of *FLT4* gene in a large hereditary congenital lymphedema family which presents Milroy disease phenotypes, and found a missense mutation of c.T2774A which leads to the Valine to Glutamic acid substitution exchange (Fig. 4 Sanger sequencing result of the *FLT4* gene c.2774 T-A mutation. p.V925E). This missense mutation causes the major phenotypes of Milroy disease in our pedigree members.

Interestingly, two patients suffered for specific clinical phenotypes compared with other patients within the family: patients I1 complaint that edema of his bi-legs aggravated and extend to the roots of the thighs when he caught cold and fever from childhood; Meanwhile, patient II1 complained that: as for childhood, edema continued to the lower extremities beneath the knees, and lymph fluid caused thinning and deforming of skin tissue, combined with proliferation of adipose and connective tissue, lead to swelling and bulky of feet. The lymphedema phenotype is not visible now due to plastic surgery, but the heavier lower extremities edema and "elephant-like" skin hyperkeratosis appearance of her legs is obvious (Fig. 2 – 1,2: bilateral lower limbs lymphedema of patient II1) [4, 28, 29]. On the contrary, the phenotypes of the other patients (except I1 and II1) are not like these two patients: lighter below knees lymphedema could be found in most of the patients within our family (Fig. 2–3: bilateral lower limbs lymphedema of patient II6; Fig. 2–4: bilateral lower limbs lymphedema of patient III3), some of the patients' lymphedema is hard to see (II2, II4 and III4), neither the brawny texture of the skin; No hydrocoele has found within the male members.

As for now, reporters believed that the major pathologic changes of Milroy disease are aplastic, hypoplastic and dysfunctional cutaneous lymphatic vessels [6, 12], which fail to transport the fluid into the venous circulation, result in a lymphatic fluid stasis and swelling of the extremities [30–33] That is, lymphatic vessels malformation triggers the increase of interstitial protein rich fluid, results in insufficient lymphatic drainage and transport [34]. As a result, large

amount of protein-rich fluid accumulates in tissue interstitial spaces, makes skin, subcutaneous tissue, fibrous tissues hyperplasia, and oppress of lymphatics more difficult to reflux for lymphatic; the edematous fluid and adipose tissue accumulate subcutaneously, and followed by inflammatory response developing and forming a vicious cycle that aggravating the formation of edema [19, 35]. Meanwhile, slowed down of lymphatic flow incites lipogenesis and fat deposition and leads to increased fibrocyte activation and connective tissue overgrowth [36–38]. Firmer subcutaneous tissue as fibrosis, hypertrophy of adipose tissue presents, these pathologic changes manifest initially as swelling of the affected limb or region, described as soft and pitting, but later progress to a more indurated state. The skin thickened, hardened, getting rough and bulky, and forming “elephant skin” swelling over time.

In our pedigree, we found two elder patients suffered for fever-related edema (I1) and heavier lower extremities edema and “elephant-like” skin hyperkeratosis appearance (II1), but phenotypes of other patients are not like them. The Heterogeneities of symptoms drove us to conduct NGS analysis to explore possible variants might be related to the phenotypes. Then we found a G deletion (c.826delG, p.Val276Phefs*29) of *GIMAP7* gene in all patients except two (I1 and II1), neither in all the healthy controls. To date, there have been very few reports on the gene function of *GIMAP7* gene. *GIMAP7* gene belongs to the GTPase family, which is a family of immuno-related proteins (GIMAPs, GTPase of immunity-associated proteins), is widely expressed in the immune system and is differentially regulated during early human Th-cell differentiation[39]. The GIMAPs have been closely linked with cell survival of lymphoid cells[40]. Regulation of cell viability of death is likely to be regulated through GIMAP family members in immune cells. And the expression of *GIMAP7* gene in normal tissues is higher than that in tumor tissues and may play a regulatory role in the immune response regulation in coordination with other molecules[41]. We speculated that the G deletion (c.826delG, p.Val276Phefs*29) of *GIMAP7* gene caused the malfunction and its coordination with other molecules, and finally jeopardized immune response of the body, then the patients in this MD family both showed different symptoms compared to the patients I1 and II1: eg. The immune related— such as cold and fever related edema, and serious “elephant skin appearance” of foot skin even the hyperplasia of foot tissue. On the contrary, in Milroy disease patients— just like the patients I1 and II1 and other reported MD patients— protein-rich fluid accumulates in tissue interstitial spaces and followed by inflammatory response developing—forming a vicious cycle that aggravate the formation of edema and hyperplasia, leads to “hyperkeratosis appearance” of foot skin[19, 35] and below knees edema. According to the PPI network, the FLT4 and GIMAP7 protein is not directly related, and several proteins were involved in the protein-protein interaction network (Fig. 6 Protein-Protein interaction network of GIMAP7 and FLT4 gene). And some of the involved gene function is immune-related. Eg. the LILRB2 gene. So we speculated that immune associated gene malfunction and protein interactions might be the main reason for the phenotype heterogeneities. Based on our findings, our genetics and research team suggested that: Further research on *GIMAP7* gene function should be conducted to testify our hypothesis. Two mutations occurred within one family and orchestrated together to present phenotype differences is a new perspective to explore heterogeneities, and need further research and attention. *GIMAP7* gene mutation can be used as a potential molecular target for future exploration.

5. Conclusion:

In this report, we described a large family with the manifestations of Milroy disease which caused by a missense mutation of *FLT4* gene (c.2774 T > A, p.V925E). All the patients within the family presented with lighter phenotypes except two (I1 and II1): Patient I1 suffered from cold and fever-related edema and extended to the thigh, and patient II1 complained the hyperkeratosis and hyperplasia of foot skin from childhood. To explore the possible sources of clinical heterogeneities among family members, we conducted the Next Generation Analysis and found a frame shift mutation (c.826delG, p.Val276Phefs*29) in an immune-related gene : the *GIMAP7* gene. Patients carried the frame shift mutation of *GIMAP7* gene both have lighter clinical symptoms; So we postulated that the malfunctioned *GIMAP7* gene caused by the frame-shift mutation (c.826delG, p.Val276Phefs*29) might influence immune response, and changed the phenotypes of lymphedema caused by the missense mutation of the *FLT4* gene (c.2774 T > A, p.V925E). And further exploration of *GIMAP7* gene function should be conducted to testify such assumption.

Abbreviations

MD
Milroy disease
FLT4
Fms related tyrosine kinase 4
VEGFR3
Vascular endothelial growth factor receptor 3
GIMAP7
GTPase, IMAP family, Member 7
PCR
polymerase chain reaction
PCL
primary congenital lymphedema
NGS
Next generation sequence

Declarations

- Ethics approval and consent to participate

The research was performed with the approval of the Ethics committee of Reproductive Health of the Liaoning province and China Medical University, participants gave written consent to participate after being informed of the nature of the research.

Consent for publication

Signed informed consent was obtained from all members of the studied family for publication of personal and clinical information (images included) in this research.

- Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

- Competing interest

The authors declared that there is no competing interest in this research.

- Funding

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Authors' contributions

MJ and YS designed the project; YS write papers; YS and MJ collected clinical data; YS, MNL and YPL organized experimental results; YS and YPL interpreted the data, MJ, WTH and YS evaluated the phenotypes for this hereditary lymphedema family. XN and HL conducted the PCR experiments and sequencing analysis. XRC and YPL interpreted the NGS analyses data.

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References

1. Milroy WF. *An undescribed variety of hereditary oedema*. N Y Med J, 1892. **Nov** (5).
2. Brice G, et al. Milroy disease and the VEGFR-3 mutation phenotype. J Med Genet. 2005;42(2):98–102.
3. Gordon K, et al. FLT4/VEGFR3 and Milroy disease: novel mutations, a review of published variants and database update. Hum Mutat. 2013;34(1):23–31.
4. Connell F, et al. A new classification system for primary lymphatic dysplasias based on phenotype. Clin Genet. 2010;77(5):438–52.
5. Esterly JR. CONGENITAL HEREDITARY LYMPHOEDEMA. J Med Genet. 1965;2(2):93–8.
6. Karkkainen MJ, et al. Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. Nat Genet. 2000;25(2):153–9.
7. Irrthum A, et al. Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. Am J Hum Genet. 2000;67(2):295–301.
8. Kaipainen A, et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A. 1995;92(8):3566–70.
9. Partanen TA, et al. VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. FASEB J. 2000;14(13):2087–96.
10. Lohela M, et al. Lymphangiogenic growth factors, receptors and therapies. Thromb Haemost. 2003;90(2):167–84.
11. Coso S, Bovay E, Petrova TV. Pressing the right buttons: signaling in lymphangiogenesis. Blood. 2014;123(17):2614–24.
12. Mellor RH, et al. Lymphatic dysfunction, not aplasia, underlies Milroy disease. Microcirculation. 2010;17(4):281–96.
13. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22(22):4673–80.
14. Tamura K, et al. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24(8):1596–9.
15. Ng PC, Henikoff S. Predicting deleterious amino acid substitutions. Genome Res. 2001;11(5):863–74.
16. Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Res. 2003;31(13):3812–4.
17. Smeltzer DM, Stickler GB, Schirger A. Primary lymphedema in children and adolescents: a follow-up study and review. Pediatrics. 1985;76(2):206–18.
18. Hurwitz PA, Pinals DJ. *PLEURAL EFFUSION IN CHRONIC HEREDITARY LYMPHEDEMA (NONNE, MILROY, MEIGE'S DISEASE). REPORT OF TWO CASES*. Radiology, 1964. 82: p. 246–8.
19. Gezginc K, Yazici F, Gok D. Prenatal diagnosis of Milroy's primary congenital lymphedema. Taiwan J Obstet Gynecol. 2012;51(2):280–2.
20. Dai T, et al. A novel mutation in the conserved sequence of vascular endothelial growth factor receptor 3 leads to primary lymphoedema. J Int Med Res. 2018;46(8):3162–71.
21. Butler MG, et al. A novel VEGFR3 mutation causes Milroy disease. Am J Med Genet A. 2007;143A(11):1212–7.
22. Spiegel R, et al. Wide clinical spectrum in a family with hereditary lymphedema type I due to a novel missense mutation in VEGFR3. J Hum Genet. 2006;51(10):846–50.

23. Ferrell RE, et al. Hereditary lymphedema: evidence for linkage and genetic heterogeneity. *Hum Mol Genet.* 1998;7(13):2073–8.
24. Evans AL, et al. Identification of eight novel VEGFR-3 mutations in families with primary congenital lymphoedema. *J Med Genet.* 2003;40(9):697–703.
25. Daniel-Spiegel E, et al. Hydrops fetalis: an unusual prenatal presentation of hereditary congenital lymphedema. *Prenat Diagn.* 2005;25(11):1015–8.
26. Mizuno S, et al. Clinical variability in a Japanese hereditary lymphedema type I family with an FLT4 mutation. *Congenit Anom (Kyoto).* 2005;45(2):59–61.
27. Ghalamkarpour A, et al. Hereditary lymphedema type I associated with VEGFR3 mutation: the first de novo case and atypical presentations. *Clin Genet.* 2006;70(4):330–5.
28. Oduber CE, van der Horst CM, Hennekam RC. Klippel-Trenaunay syndrome: diagnostic criteria and hypothesis on etiology. *Ann Plast Surg.* 2008;60(2):217–23.
29. Biesecker L. The challenges of Proteus syndrome: diagnosis and management. *Eur J Hum Genet.* 2006;14(11):1151–7.
30. Karkkainen MJ, et al. A model for gene therapy of human hereditary lymphedema. *Proc Natl Acad Sci U S A.* 2001;98(22):12677–82.
31. Jussila L, Alitalo K. Vascular growth factors and lymphangiogenesis. *Physiol Rev.* 2002;82(3):673–700.
32. Ji RC. Lymphatic endothelial cells, lymphangiogenesis, and extracellular matrix. *Lymphat Res Biol.* 2006;4(2):83–100.
33. Zhang S, et al. Immunohistochemical Evaluation of Histological Change in a Chinese Milroy Disease Family With Venous and Skin Abnormities. *Front Genet.* 2019;10:206.
34. Kitsiou-Tzeli S, et al. Milroy's primary congenital lymphedema in a male infant and review of the literature. *Vivo.* 2010;24(3):309–14.
35. Tammela T, Alitalo K. Lymphangiogenesis: Molecular mechanisms and future promise. *Cell.* 2010;140(4):460–76.
36. Smahel J. Adipose tissue in plastic surgery. *Ann Plast Surg.* 1986;16(5):444–53.
37. Ryan TJ. Lymphatics and adipose tissue. *Clin Dermatol.* 1995;13(5):493–8.
38. Gaffney RM, Casley-Smith JR. Excess plasma proteins as a cause of chronic inflammation and lymphoedema: biochemical estimations. *J Pathol.* 1981;133(3):229–42.
39. Krücken J, et al. Comparative analysis of the human gimap gene cluster encoding a novel GTPase family. *Gene.* 2004;341:291–304.
40. Nitta T, et al. IAN family critically regulates survival and development of T lymphocytes. *PLoS Biol.* 2006;4(4):e103.
41. Song Y, Pan Y, Liu J. The relevance between the immune response-related gene module and clinical traits in head and neck squamous cell carcinoma. *Cancer management research.* 2019;11:7455.

Figures

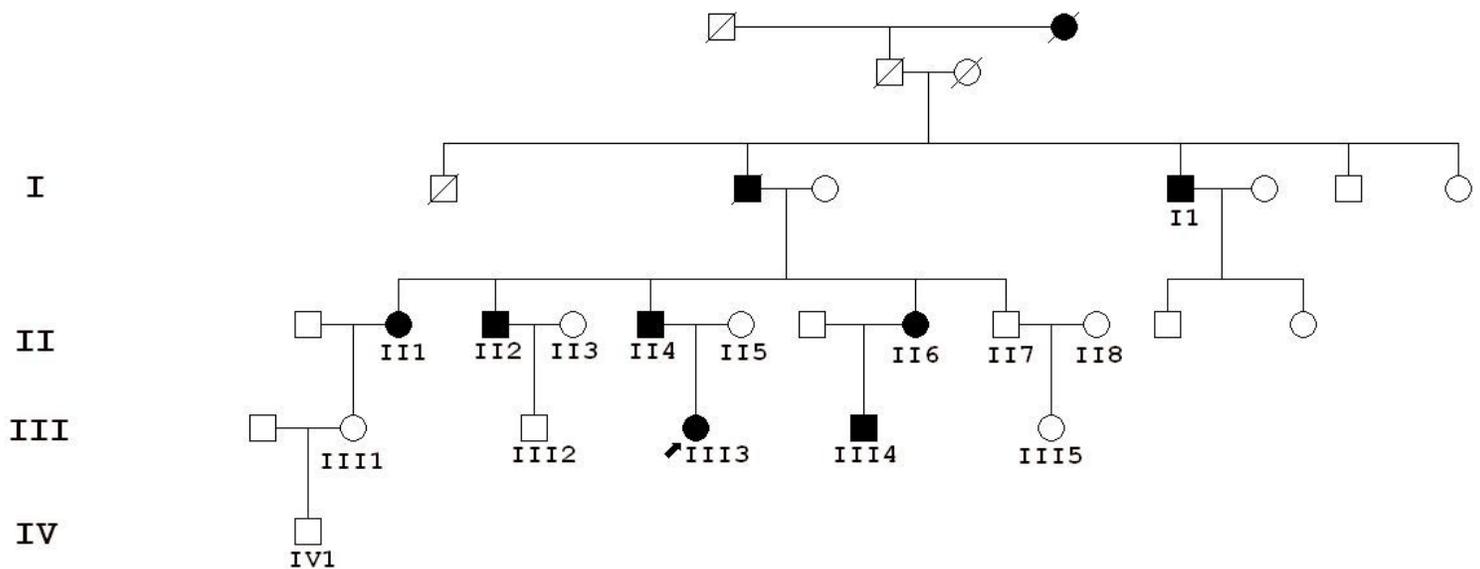


Figure 1

Pedigree of the family



Figure 2
bilateral lower limbs lymphedema of patient II1; and Fig 2-3: bilateral lower limbs lymphedema of patient II6; and Fig 2-4: bilateral lower limbs lymphedema of patient III3 bilateral lower limbs lymphedema of patient II1 and Fig 2-4: bilateral lower limbs lymphedema of patient III3 bilateral lower limbs lymphedema of patient II1 bilateral lower limbs lymphedema of patient II1 bilateral lower limbs lymphedema of patient II1 bilateral lower limbs lymphedema of patient II6



Figure 3

slight epicanthic folds and down-slanting palpebral fissures

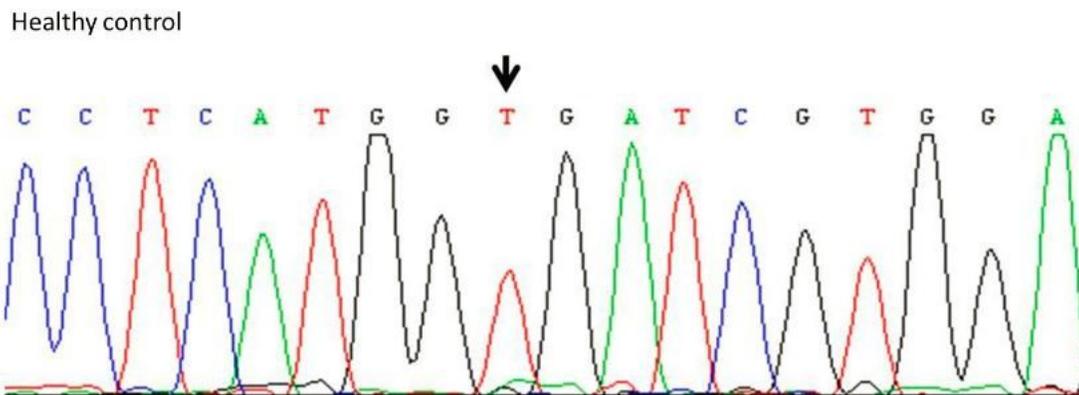
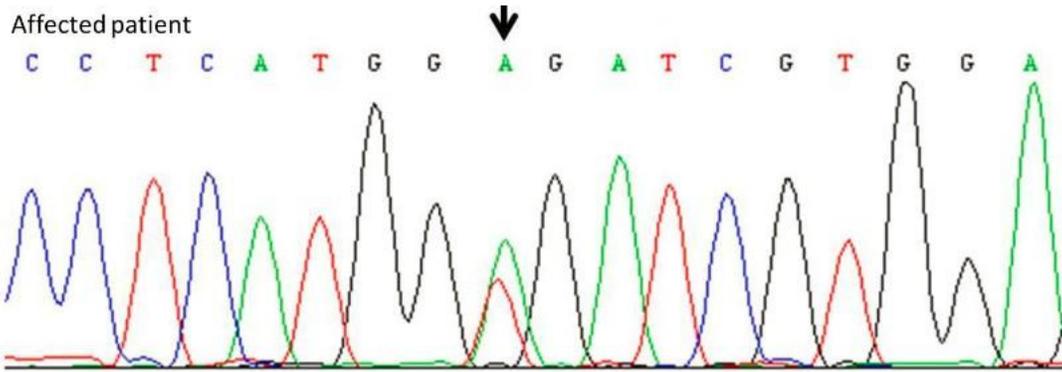


Figure 4

Sanger sequencing result of the FLT4 gene c.2774 T>A mutation.

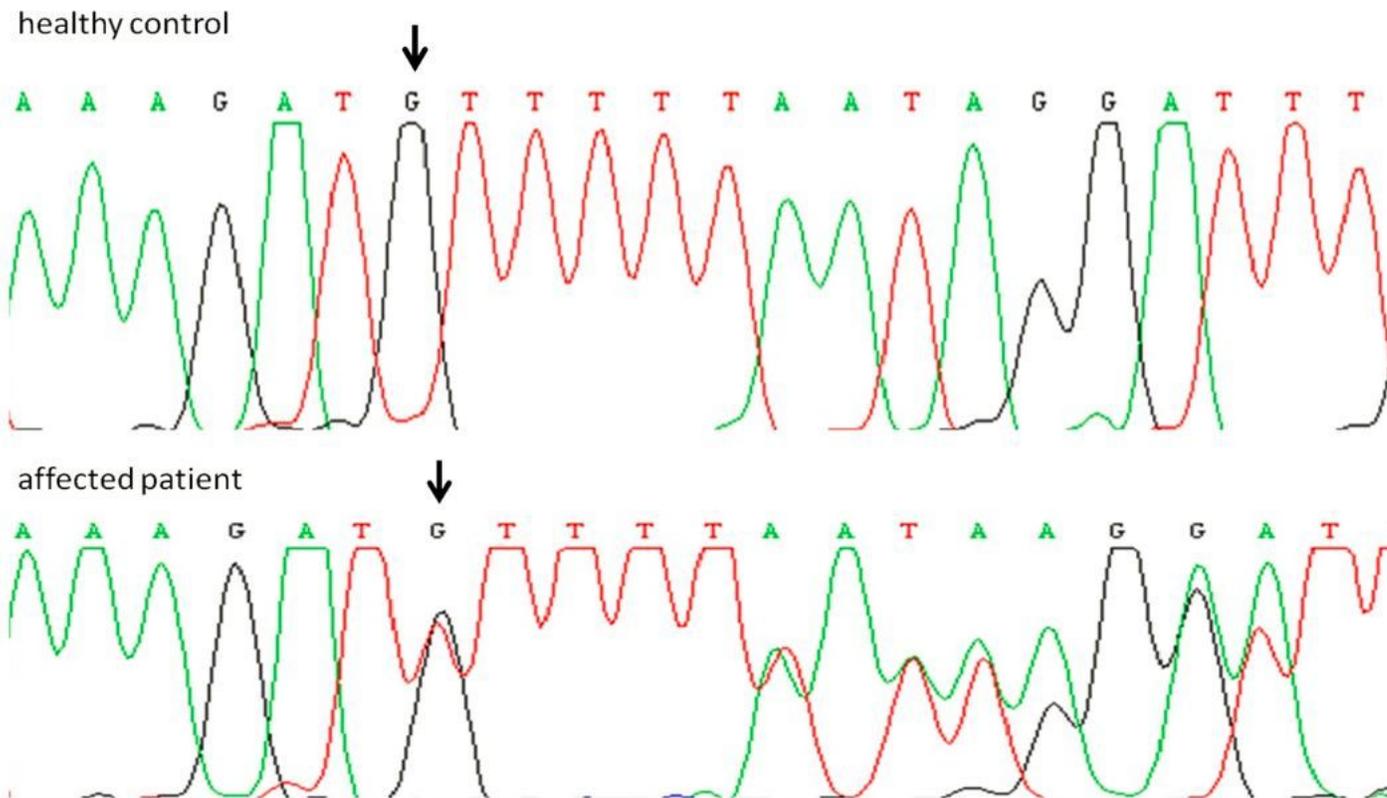


Figure 5

Sanger sequencing results of GIMAP7 gene c.826delG, p.Val276Phefs*29

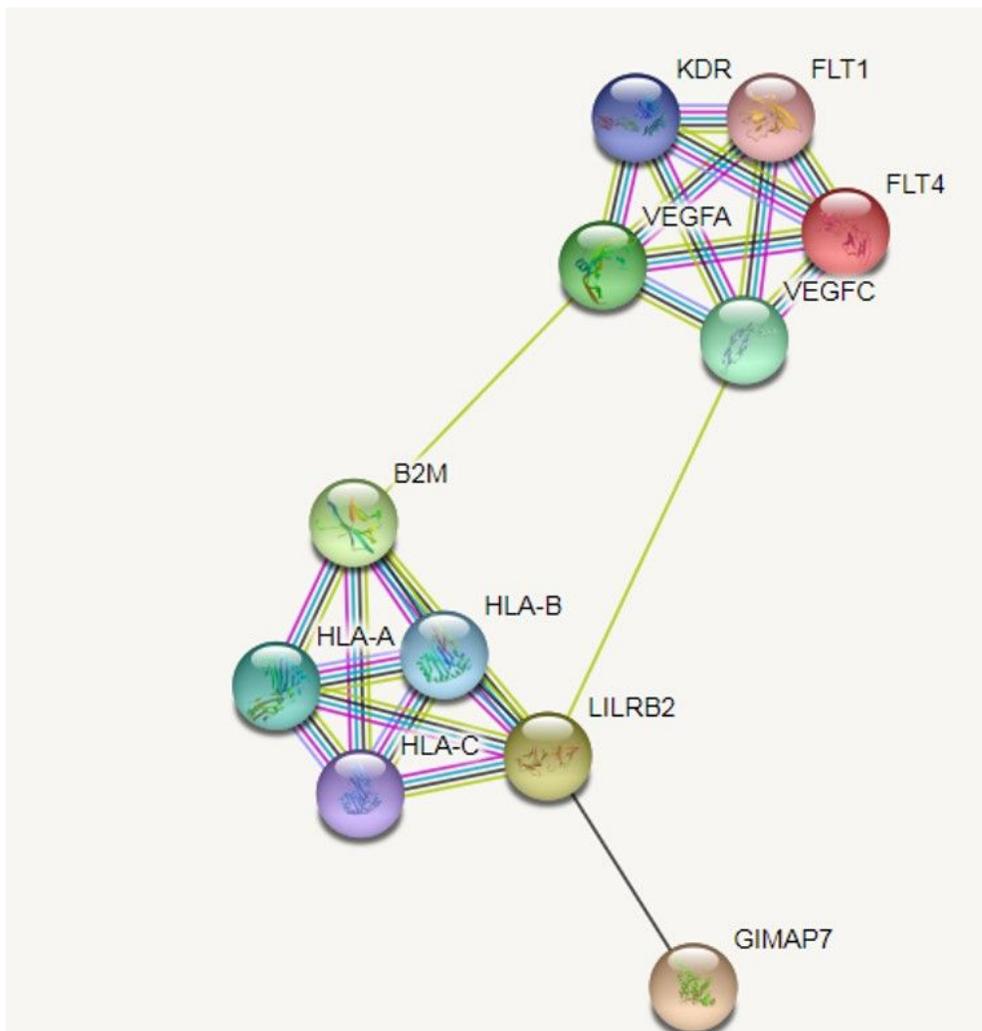


Figure 6

Protein-Protein interaction network of GIMAP7 and FLT4 gene

Supplementary Files

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- [TableS10.PCRamplificationofFLT4gene.docx](#)
- [TableS5.NGSresultsofhealthycontrolII7.xlsx](#)
- [TableS8PCRamplificationsystemsandconditions.docx](#)
- [TableS4.NGSresultsofPatientIII4.xlsx](#)
- [TableS7.variantssharedbyIII3III4butnotII7.docx](#)
- [TableS3.NGSresultsofPatientIII3.xlsx](#)
- [TableS6.variantssharedbyI1andII1butnotII7.docx](#)
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- [Fig.S5PolygenictreeofGIMAP7gene.JPG](#)
- [Fig.S4.flowchartofdataanalysisofNGSresults.jpg](#)
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