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AN UPDATED CLASSIFICATION OF PRIMARY LYMPHEDEMA BASED ON AGE OF ONSET, LYMPHATIC ANOMALIES, AND GENETICS

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ABSTRACT

Primary lymphedema (PLE) is a chronic disease caused by lymphatic dysplasia and progresses to irreversible tissue edema and hypertrophy. Understanding of PLE has been hitherto limited. The aim of this study is to devise an updated classification system for PLE of 1013 patients with PLE of lower limb were enrolled. Sex, age of onset, location, family history and morbidity were documented. The lymphatic imaging findings of magnetic resonance lymphography (MRL), indocyanine green lymphography (ICGL) and lymphoscintigraphy (LSG), skin tissue immunohistochemical staining, whole exome sequencing and the correlation of genotype-phenotype were evaluated. Patients were divided into a congenital onset category and a late onset category. The late onset category was further divided according to developmental age. The ratio of congenital-onset to late-onset PLE was 1:4 and that the highest incidence was in adolescence. The sex ratio was 1.04:1 and 1.5:1 in congenital-onset and late-onset groups, respectively. Three major lymphatic anomalies were identified, in which segmental lymphatic dysfunction, characterized by delayed or partial demonstration of lymph vessels, is the most common and associated with FLT4*,* GJC2*,* CELSR1*, and* PTPN14 *mutations. The next most common type is lymphatic hyperplasia, which is associated with* FOXC2 *and* GATA2 *variants, followed by initial lymphatic aplasia or dysfunction, which is more common in patients with congenital PLE and associated with* FLT4 *mutation. A functional and structural combined classification of lymphatic anomalies is proposed, which includes segmental lymphatic dysfunction, lymphatic hyperplasia and initial lymphatic aplasia or dysfunction.*

Keywords: primary lymphedema classification, lymphatic dysfunction, initial lymphatic aplasia/dysfunction, lymphatic imaging, pathogenic genes of primary lymphedema, lymphatic anomalies

The lymphatic circulation is a unidirectional circulatory system that consists of initial lymphatic, pre-collectors and collectors, afferent and efferent vessels to and from the lymph nodes, and lymph trunks, which finally connect with the central veins (1). Primary lymphedema (PLE) is a chronic disease characterized by tissue edema, fibrosis, deposition of fat, and inflammation caused by lymphatic dysplasia and may occur anywhere along this continuous pathway (2). The traditional classification of PLE is based on age of onset and includes congenital meaning present at birth, praecox developing up to the age of 35 years

and tarda developing after the age of 35 years, in which praecox covers infants, children, adolescents, and adults (3). This classification does not have a biological basis and lacks clinical practicability. Another classification system for PLE proposed decades ago was lymphatic hyperplasia, lymphatic hypoplasia, and lymphatic aplasia, based on the anomalies seen on direct lymphangiography (4), whereby contrast is injected directly into the lumen of a vessel. However, this imaging method only allows observation of morphological changes in the large lymphatics, namely, the lymph collectors, and not those in the lymphatic capillaries and pre-collectors, and cannot monitor the functional status of lymphatic vessels.

PLE has a wide variety of presentations. It occurs most often in the lower limb but may also occur in the arm, face, external genitalia, or multiple locations or be combined with other features in syndromes (5). Thus far, nearly 50 genes have been associated with PLE (6), which indicates that the pathological mechanism of the disease is complex. Recent study showed that PLE may be site-specific because none of the genes that were pathogenic in PLE of the lower limb was identified in patients with congenital upper limb lymphedema (7). Recent years have seen the emergence of indirect lymphography techniques, whereby contrast is injected via the intradermal route. These techniques include magnetic resonance lymphography (MRL), which allows high-resolution imaging of both the superficial and deep lymphatic system (8,9), and indocyanine green lymphography (ICGL) which allows observation of the superficial lymph pathways in real time (10,11). Combined with traditional lymphoscintigraphy (LSG) (12), these examinations can complement each other technically and functionally and detect abnormalities of the peripheral lymphatic vessels at all levels.

The aim of this study was to devise an updated classification system for PLE based on a comprehensive analysis of age of onset, imaging and histological abnormalities in the lymphatic vessels, genetic screening, and the genotype-phenotype correlation in patients with lower limb lymphedema, which is the most common type of PLE.

METHODS

Patients

The subjects in this prospective study were 1013 patients with PLE in a lower extremity who visited our clinic between 2007 and 2021. Patients with arm, multisite, syndromic or syndromic/visceral lymphedema were excluded. The diagnosis was based on history, physical examination, lymphatic imaging, skin biopsy, and DNA screening of peripheral blood. The trial was approved by the Ethics Committee of the Shanghai Ninth People's Hospital (ethical approval code 201428).

Lymphatic Imaging

Magnetic resonance lymphography

MRL was performed as described previously (8). In brief, MRL was performed with a 3·0-T magnetic resonance unit (Philips Medical System, Best, The Netherlands). First, three-dimensional heavily T2-weighted magnetic resonance imaging was performed. After intradermal injection of the paramagnetic contrast agent gadobenate dimeglumine (MultiHance, Bracco, Milan, Italy) into the first and second digital web spaces (0.7-0.8 mL per point), three-dimensional fast spoiled gradient-recalled echo T1-weighted images were acquired with a fat saturation technique at consecutive time points for dynamic observation.

Indocyanine green lymphography

ICGL was performed in a manner described elsewhere (13). Briefly, after intradermal injection of the contrast agent (ICG 2.5 mg/mL) into the web spaces between the toes (two to three points per limb, 0.05 mL per point), a fluorescence-locating instrument (Photodynamic Eye, Hamamatsu Photonics, Hamamatsu, Japan) was used for dynamic observation of lymph flow immediately and

120 min after injection. Further injections were added in the lower leg or thigh in some subjects when lymphatics were not visualized in the dorsum of the affected foot.

Lymphoscintigraphy

LSG was performed following a previously described protocol (14). Briefly, 99Tc-labelled dextran (Syncor International Corporation, Shanghai, China) was injected intradermally into the first and second digital web spaces (1-2 mCi or 37-74 MBq) of both feet. Patients were then asked to walk. Images were obtained (Hawkeye SPECT, GE Healthcare, Milwaukee, Wisconsin, USA) 15 min and 2 h after injection.

Immunohistochemical staining

Immunohistochemical staining was performed as described elsewhere (13). In brief, whole-thickness skin sections (2 × 1 cm^2) were obtained from lymphedematous feet. Lymphatic and blood vascular staining was performed using primary anti-podoplanin (1:50; AngioBio, San Diego, CA, USA) and rabbit anti-human CD-31 (1:200; Abcam, Cambridge, UK) antibodies. Secondary Alexa Fluor555 goat anti-mouse (1:300; Invitrogen, San Diego, CA, USA) and Alexa Fluor 488 goat anti-rabbit (1:300; Invitrogen) antibodies were used to visualize the signal. Photography and whole-slide image construction were performed using a confocal microscope (LSM 710; Carl Zeiss, Jena, Germany).

Whole-exome sequencing

Whole-exome sequencing was performed using the procedure described by a previous report (13) with the SureSelectXT Human All Exon V6 kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced with a HiSeq system (Illumina Inc., San Diego, CA, USA). Sequences were aligned to the human reference genome (Hg19) using the Burrows-Wheeler Aligner (version 0.7.17); variants were called with SAMtools and the Genome Analysis Toolkit (version 4.0) and annotated with ANNOVAR software. Perl scripts were used to filter single-nucleotide variants with respect to variants present in the dbSNP150 database. The clinical impact of the variants was assessed following the guidelines of the American College of Medical Genetics and Genomics for variant interpretation (15), with the help of the online tool Varsome Premium (https://varsome.com/, last accessed on 14 November 2023). The following genes known to be associated with lymphedema were specifically analyzed: *CCBE1, FOXC2, FLT4, KIF11, GATA2, SOX18, FAT4, PTPN14, GJC2, VEGFC, PIEZO1, PTPN4, CELSR1, HGF, MET, EPHB4, RASA1, ITGA9, PIK3CA, HRAS, SOS1, RAF1, RIT1, ADAMTS3,* and *ANGPT2.*

RESULTS

Age of Onset

According to age of onset, 1013 patients were divided into a congenital onset category (younger than 1 year) and a late onset category (one year or older). The late onset category was divided further into six subgroups: >1-10 years (childhood); 11-20 years (adolescence); 21-30 years (early adulthood); 31-40 years (middle adulthood); 41-50 years (late adulthood); and 51-60 years (old age).

In total, 204 (20%) of the 1013 cases were identified to be congenital. The sex ratio was 1.04:1 (male, n=104; female, n=100). One hundred and thirty patients had unilateral lower limb involvement and 74 had bilateral lower limb involvement. Twenty-six patients (12.7%) had a relevant family history (*Fig. 1*). The remaining 809 cases (80%) were late-onset (male, n=487; female, n=322; sex ratio 1.5:1). Fifty-three (6.5%) had a family history of PLE. The highest incidence was in the group aged 11-20 years, and the numbers aged 20-30 years and 30-40 years were very similar. The incidence decreased by half after the age of 40 years and by half again after the age of 50 years. In each age group, the number of cases with unilateral involvement was at least twice (1.3-3.4 times) as high as the number with bilateral involvement (*Fig. 1).*

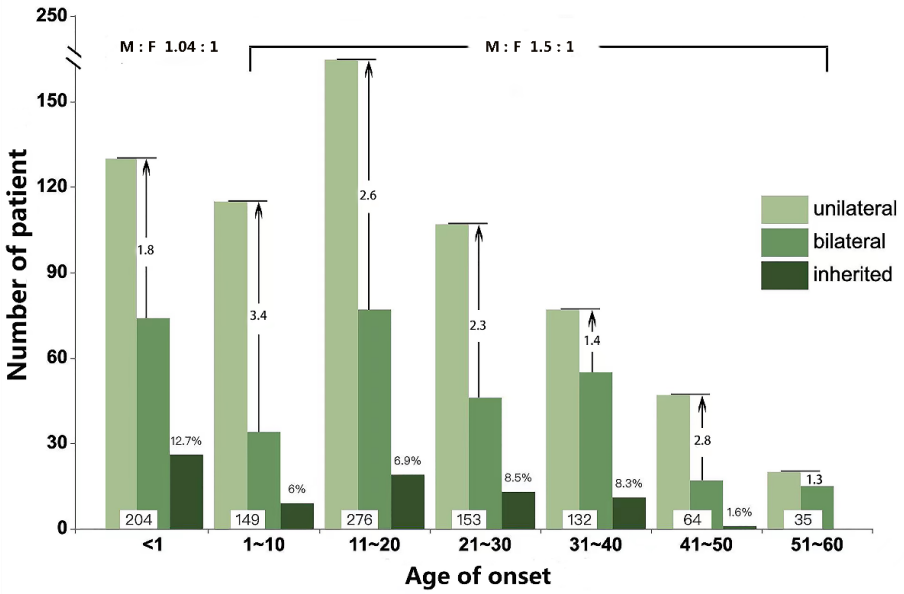

Fig. 1 *A graph that shows incidence of primary lymphedema (PLE) of the lower limb classified by age of onset. M = male; F = female*

Whole-Exome Sequencing

Blood samples were available for 211 patients. Whole-exome sequencing of these samples identified 38 pathogenic variants of five genes in 37 patients, representing 18% of all tested cases. Twenty-two patients were found to have 22 *FLT4* variants, all of which have previously been reported to be pathogenic (13,16-19). *FOXC2* variants were identified in eight patients. Other identified pathogenic variants were *GATA2* mutations in one patient, *CELSR1* variants in three, *GJC2* variants in two, and *PTPN14* mutation in one. All identified *FOXC2, GATA2, CELSR1, GJC2,* and *PTPN14* variants were loss-of-function *mutations and pathogenic (Table 1*). Two *FOXC2*, one *GJC2* and one *GATA2* variants were previously reported (20-23).

Lymphatic Imaging

Well-reported lymphatic imaging data were available for 929 patients (92%), who were examined using one or more imaging modalities. Lymphatic function and morphologic abnormalities were recorded simultaneously and identified the three following major types of lymphatic abnormalities.

Segmental Lymphatic Dysfunction

After intradermal injection of the contrast agent, lymph flow increased very slowly and the lymph trunks were visualized only partially in the distal portion of the limb in 102 (52%) of the 198 patients with congenital PLE and in 548 (75%) of the 731 with late-onset PLE. Fine reticular lymphatic vessels around the injection site were common, suggesting that it was difficult for lymph to ascend. The contrast-enhanced lymph collectors were demonstrated below the ankle, knee, thigh, and, in rare cases, above the inguinal region (*Fig. 2a,d; Fig. 3a*).

Lymphatic Hyperplasia

Lower limb lymphatic hyperplasia was identified in 46 (23%) of the 198 patients with congenital PLE and in 146 (20%) of the 731 with late-onset PLE. Characteristic mor-

TABLE 1
Clinical and Genetic Findings in 37 Patients with 5 Genes Mutations

ID	Sex/ Age	Onset	Family history	Nucleotide change	Amino acid change	Classification	Skin histology	Imaging findings
FLT4								
s1	M/2	Birth	Yes	c.3315G>C	p.(W1105C)	LP	NA	Lymphatic below knee
s2	M/1	Birth	Yes	c.3295T>C	p.(S1099P)	LP	NA	Lymphatic below knee
s3	M/1	Birth	Yes	c.3296C>T	p.(S1099F)	LP	NA	Lymphatic below ankle
s4	F/6	Birth	Yes	c.3121C>T	p.(R1041W)	P	NA	No lymphatic visualized
s5	M/2	Birth	Yes	c.3122G>A	p.(R1041Q)	P	NA	lymphatic below ankle
s6	F/30	Birth	Yes	c.3111C>G	p.(D1037E)	LP	NA	NA
s7	F/1	Birth	Yes	c.3163G>C	p.(D1055H)	P	NA	Lymphatic below ankle
s8	M/1	Birth	Yes	c.2748C>G	p.(C916W)	LP	NA	No lymphatic visualized
s9	M/5	Birth	Yes	c.2531G>C	p.(R844P)	LP	No dermal lymphatic was visualized	No lymphatic visualized
s10	M/27	Birth	Yes	c.3410C>T	p.(P1137L)	P	NA	No lymphatic visualized
s11	F/3	Birth	Yes	c.2632G>A	p.(V878M)	P	NA	Lymphatic below ankle
s12	F/28	Birth	Yes	c.3122G>A	p.(R1041Q)	P	NA	Lymphatic below knee
s13	F/18	Birth	Yes	c.3175G>C	p.(A1059P)	LP	Lymphatic visualized in the skin	Lymphatic below knee
s14	F/7	Birth	Yes	c.2554G>T	p.(G852C)	P	No dermal lymphatic was visualized	No lymphatic visualized
s15	F/30	13	Yes	c.3122G>A	p.(R1041Q)	P	No dermal lymphatic was visualized	No lymphatic visualized
s16	M/12	Birth	No	c.2587T>C	p.(S863P)	LP	NA	Lymphatic below knee
s17	M/1	Birth	No	c.3316G>C	p.(E1106Q)	LP	NA	No lymphatic visualized
s18	M/1	Birth	No	c.3341C>T	p.(P1114L)	P	NA	NA
s19	M/1	Birth	No	c.3073A>T	p.(M1025L)	LP	NA	NA
s20	F/2	Birth	No	c.3323_3325 del	p.(F1108_1109del)	P	NA	Lymphatic below ankle
s21	F/1	Birth	No	c.2740G>C	p.(G914R)	LP	NA	Lymphatic below ankle
s22	M/12	Birth	No	c.3230C>T	p.(P1077L)	LP	NA	No lymphatic visualized
FOXC2								
s23	M/10	9	Yes	†c.931_932insGCCGCCG	p.(A311Gfs*154)	LP	Slightly dilated dermal lymphatics	Dilated lymphatics
s24	M/20	4	Yes	†c.348G>A	p.(W116*)	LP	NA	Dilated lymphatics
s25	M/15	4	Yes	†c.574_583dup	p.(P195Qfs*271)	LP	NA	Dilated and tortuous lymph vessels
s26	M/11	9	Yes	†c.556del	p.(P186Rfs*15)	LP	NA	Tortuous lymphatic with lymph reflux
s27	M	19	Yes	c.347G>A	p.(W116*)	P	NA	Varicose lymph collectors

s28	F/30	7	No	†c.687_732del	p.(T230Pfs*32)	LP	Dermal lymphatic capillary was visualized	Dilated collecting lymphatics
s29	M/19	14	No	†c.802_803insT	p.(P268Lfs*195)	LP	Obviously dilated dermal lymphatics	Tortuous lymphatic with lymph reflux
s30	F/23	20	No	c.1238_1253del	p.(Q413Rfs*15)	P	NA	Varicose lymph collectors
GATA2								
s31	M/13	Birth	Yes	c.818dupG	p.(P274Tfs*8)	P	NA	Dilated lymphatics
CELSR1								
s32	F/23	Birth	No	†c.2737C>T	p.(Q913*)	LP	NA	Dorsal tortuous lymphatic with skin contrast diffusion
				†c.5544dup	p.(T1849Dfs*23)	LP		
s33	F/32	20	Yes	†c.4247del	p.(G1416Afs*4)	LP	NA	Tortuous lymphatics in the dorsum of foot
s34	F/22	4	No	†c.79C>T	p.(R27*)	LP	NA	Tortuous lymphatic vessels rising to the mid-calf
GJC2								
s35	F	Birth	Yes	c.143C>T	p.(S48L)	LP	Dilated dermal lymphatics with increased number	Discontinuous lymphatic with skin contrast stagnant
s36	F/14	6	No	c.143C>T	p.(S48L)	LP	NA	Unclear lymphatic with skin contrast diffusion
PTPN14								
s37	F/27	Birth	No	†c.220C>T	p.(R74*)	LP		Dorsal indistinct lymphatic with skin contrast diffusion

ID = patient identification code; S = subject; M = Male; F = Female; †= not reported; LP = Likely pathogenic; P = Pathogenic; NA = Not available

phological changes included numerous bead-like, tortuous lymphatic vessels and a few thick lymphatic trunks with no increase in number (*Fig. 2b,d*).

Initial Lymphatic Aplasia or Dysfunction

No lymphatic vessels were visualized in 50 (25%) of the 198 patients with congenital PLE or in 37 (5%) of the 731 patients with late-onset PLE after intradermal injection of the contrast agent into the dorsum of the foot *(Fig. 2c,d)*.

Genotype-Phenotype Correlations Between Pathogenic Variants, Lymphatic Imaging Results, and Skin Histology

FLT4, CELSR1, GJC2, and *PTPN14* variants were identified in patients with segmental lymphatic dysfunction. The common features seen on lymphatic images were signif-

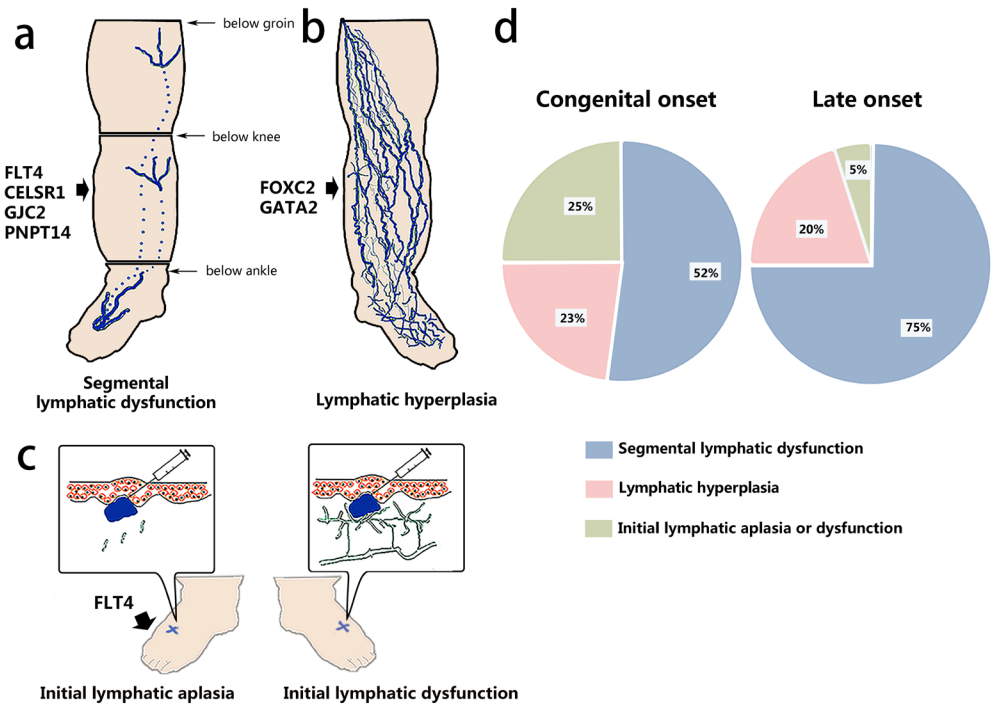

Fig. 2 *A schematic representation of classification of lymphatic anomalies in PLE: a. Segmental lymphatic dysfunction with associated causal genes variants; b. Lymphatic hyperplasia with associated causal gene mutations; c. Initial lymphatic aplasia or dysfunction with causal genes mutations; d. Three type of lymphatic anomalies in congenital and late-onset PLE.*

icantly delayed contrast enhancement and irregular lymphatic vessels on the dorsum/sole of the foot or below the knee with contrast diffusion in the skin. Histological examination of the skin revealed that the initial lymphatics in the dermis of the affected limb were dilated slightly in carriers of the FLT4 variant and markedly in carriers of the GJC2 variant (*Fig. 3b-3d*) (*Table 1*).

FOXC2 and GATA2 mutations were identified in patients with lymphatic hyperplasia. Magnetic resonance lymphangiography clearly showed large tortuous superficial lymphatic trunks in patients with the *FOXC2* variant (*Fig. 3e*, right panel) and tortuosity of both superficial and deep lymphatics in those with the GATA2 mutation (*Fig. 3e*, left panel).

FLT4 mutation with initial lymphatic aplasia or dysfunction was identified in nine of 22 patients in whom no contrast-enhanced lymphatics or dermal backflow in the dorsum of the foot were visualized on imaging. Skin biopsies were available for three patients, and none showed any initial lymphatic vessels in the dermis.

DISCUSSION

We advocate that PLE be classified as congenital or late-onset according to age of onset (24) and that late-onset PLE be defined further according to developmental age (19). Twenty percent of the cases investigated in this study were found to have congenital PLE and 80% developed the disease after one year of age. Adolescence was the most common age of onset, with at least twice as many cases detected in this age group than in the other late-onset age groups. The incidence decreased by half after the age of 40 years and by half

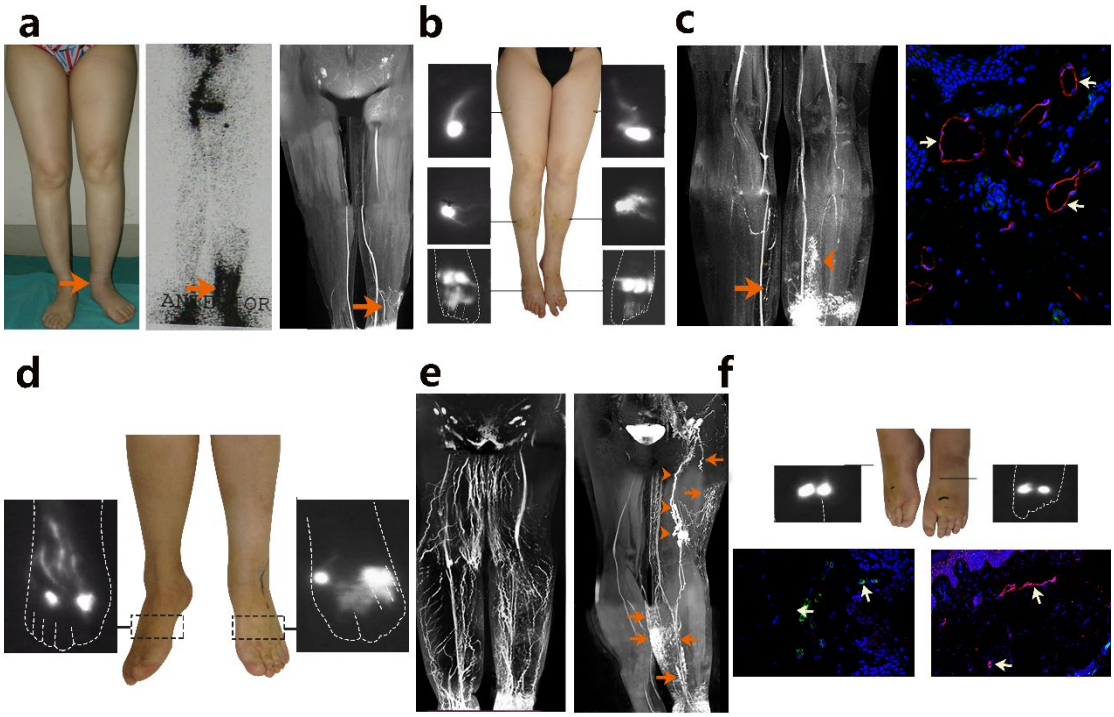

Fig. 3 *Imaging and histological findings in patients of this study. a. Unilateral primary lymphedema (left), Lymphscentigram (LSG) only showing extensive tracer retention in the distal part of the leg (arrow, middle), MR lymphangiogarm (MRL) clearly showing lymphatics up to the ankle region (arrow, right). b. Bilateral PLE of lower limbs with CELSR1 mutation (S33 in Table 1), Indocyanine green lymphogram (ICGL) showing unclear small lymphatics around the injection sites and local contrast retention in the dorsum of feet and lower legs and clear lymph collectors in both thighs. c. Bilateral congenital PLE of lower limbs with GJC2 mutation (S35 in Table 1), MRL showing discontinuous lymphatic (arrow) in the right and contrast retention in the left legs (arrowhead), histological staining showing obviously dilated dermal lymphatics (white arrows). d. PLE of left leg with PTPN14 mutation (S37 in Table 1), ICGL showing contrast diffusion in the dorsum of affected foot and clear lymphatics in the right foot. e. MRL (right) showing tortuous and dilated lymphatics in bilateral lower limbs of a patient with FOXC2 mutation (S28 in Table 1), MRL (left) showing tortuous and dilated superficial (arrows) and deep (arrow heads) lymphatics in a patient with PLE of left lower limb and GATA2 mutation (S31 in Table 1). f. ICGL (top) showing no enhanced lymphatic in the dorsum of feet of a patient (S15 in Table 1) with FLT4 mutation, no dermal lymphatic was identified and only blood capillaries were detected (arrows) in the dorsum skin (bottom right), dermal lymph capillaries (arrows) in normal skin (bottom left).*

again after the age of 50 years. Considering that the proportion of hereditary cases in each late-onset period before the ages of 40 was consistent at around 6%-8%, the marked increase in morbidity during puberty is most likely to be associated with an increase in growth hormone rather than a higher genetic penetrance. Adolescence may be a critical or vulnerable period for maturation of the lymphatic system. It is not clear why PLE has a variable latent period, even in patients with a family history, and the trigger for onset of the disease needs to be explored.

We propose a combined functional and structural classification of lymphatic anomalies that includes the following three categories: segmental lymphatic dysfunction, lymphatic hyperplasia, and initial lymphatic aplasia or dysfunction. The segmental lymphatic dysfunction appears to be the most common of

the three types of lymphatic anomalies. It is common for lymphatic imaging to show no or delayed transit of contrast when the defective lymph vessel is located in the dorsum of the foot, and this is considered to indicate dysfunction or "functional aplasia" in patients with Milroy disease and *FLT4* mutation (13,19,25). Our study is the first to demonstrate that *CELSR1, GJC2,* and *PTPN14* mutations are associated with segmental lymphatic dysfunction in individuals with PLE. *CELSR1* is a member of the cadherin superfamily (26) while *GJC2* belongs to the gap junction pro-tein family (27). Both of these molecules are involved in valve formation and important for lymphatic function in terms of controlling flow of lymph and mediating spontaneous contractions of lymphatic vessels (28-30). *PTPN14* encodes a nonreceptor tyrosine phosphatase. *PTPN14* deficiency was found to cause hyperplasia in the lymphatic capillaries of the skin and lymphedema in an animal study (31). The four pathogenic genes discovered thus far may represent only a small number; however, they may provide some clues to guide exploration of the complex pathogenesis of this type of lymphatic anomaly in the future.

To the best of our knowledge, lymphatic hyperplasia is limited to valvular insufficiency caused by developmental defects (6,19). *FOXC2* is a well-known disease-causing gene (32). Our present study is the first to provide evidence of an association between *GATA2* mutation and lymphatic hyperplasia in patients with nonsyndromic lower limb lymphedema. It is worth noting that although both *FOXC2* and *GATA2* are important for development of the lymphatic valves (33,34), the clinical phenotypes of patients with pathogenic variants in these two genes are different. First, patients with a *FOXC2* variant develop the disease during childhood or adolescence while those with a *GATA2* mutation have the disease at birth. Second, only superficial vessels are affected in *FOXC2* mutation whereas both superficial and deep lymph collectors are involved in patients with the *GATA2* variant, who have more severe malformations of the deep lymphatics. This may reflect the fact that *GATA2* may have a more pronounced effect on development of lymphatic vessels (35).

We have included initiating lymphatic aplasia/dysfunction as a separate category instead of the previous item "lymphatic aplasia" for two reasons. First, the initial lymphatic vessels have a unique structure and function. They are composed of a single layer of endothelial cells, which may function as a primary valve system for absorbing and transporting tissue fluids (36). Second, a growing body of evidence suggests that defects in cutaneous lymphatic capillaries play an important role in initiating PLE. For example, dermal lymphatic capillary aplasia in the leg has been confirmed in Milroy disease (13,37), dysfunction of the initial lymphatics in the dermis has been found to cause congenital arm lymphedema (7), and "functional aplasia" of the initial lymphatic vessels causes nonsyndromic and syndromic lymphedema of the limbs (25,39). Therefore, an initial lymphatic defect is an important component of the lymphatic anomalies in PLE and can initiate the disease.

In summary, we propose a functional and structural combined classification of lymphatic anomalies, which includes segmental lymphatic dysfunction, lymphatic hyperplasia and initial lymphatic aplasia or dysfunction. An updated understanding of PLE will help to improve the accuracy of diagnosis and lead to development of targeted therapies.

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CONFLICT OF INTEREST AND DISCLOSURE

The authors declare no competing financial interests exist.

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