Lymphology 49 (2016) 192-204

RARE VARIANTS IN LAMA5 GENE ASSOCIATED WITH FLT4 AND FOXC2 MUTATIONS IN PRIMARY LYMPHEDEMA MAY CONTRIBUTE TO SEVERITY

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ABSTRACT

*Mutations in the Fms-related tyrosine kinase 4 (FLT4) and forkhead box protein C2 (FOXC2) genes cause Milroy disease (MD) and lymphedema-distichiasis syndrome (LDS), respectively, but the mechanism underlying disease pathology remains unclear. Applying whole-exome sequencing to two families with MD, one LDS family, and one sporadic LDS case, we identified four rare variants in the laminin subunit alpha-5 gene (*LAMA5*) in subjects carrying novel and known missense* FLT4 *mutations and a 7-bp duplication and 1-bp insertion in* FOXC2*. Phenotyping was expanded in some individuals using magnetic resonance lymphangiography, indiocyanine green fluorescence lymphography, and immunofluorescent lymphatic staining of skin tissue. Skin lymphatic staining showed the existence of dermal lymphatic vasculature in the MD case. Significant lymphatic dysfunction was observed in both MD and LDS patients. In the MD patient, tortuous lymphatics in the dorsum of the foot were slowly enhanced on indocyanine green fluorescent lymphography (ICG) imaging. Dilated lymph collectors with disruption and lymph leakage were observed in the familial LDS case on magnetic resonance lymphangiography (MRL). Numerous tortuous lymph collectors were visualized along the entire length of affected lower limbs on MRL imaging, and retrograde lymph flow was observed in the lymph collectors during ICG lymphography in the isolated LDS case. The finding of rare* LAMA5 *variants together with* FLT4 *and* FOXC2 *mutations suggests that these mutations may be co-responsible for these disorders and most likely interfere with the function of lymphatics. Further, larger studies are needed to confirm these results.*

Keywords: Milroy disease, lymphedema-distichiasis, *LAMA5*, whole exome sequencing, lymphatic dysfunction, magnetic resonance lymphangiography, ICG lymphography

Primary lymphedema is a rare genetic condition with both autosomal dominant and autosomal recessive modes of inheritance caused by mutations in specific genes that are involved in lymphatic development and function. So far, germline mutations have been identified in at least 20 human primary lymphedema genes (1). *FLT4* (*VEGFR3*, encoding the vascular endothelial growth receptor) was the first gene to be identified as causative of Milroy disease (MD; MIM 153100) (2-4) and *FOXC2* was the first gene found to be causative for lymphedema-distichiasis syndrome (LDS; MIM 153400) (5-7). MD is characterized by congenital bilateral lymphedema of the lower limbs, and heterogeneous *FLT4* mutations are responsible for the majority of MD cases. Studies have shown that mutations within

the tyrosine kinase domains of tyrosine kinase receptors were sufficient to reduce tyrosine kinase activity and thereby affect lymphatic development (4). Since initial dermal lymphatics have been reported in the affected skin and in some MD patients, lymph collectors are observed in the affected lower limbs, it is proposed that lymphatic dysfunction, not aplasia, underlies MD (8). Clinical phenotyping and genomic studies have demonstrated that primary lymphedema is highly heterogeneous with marked inter- and intrafamilial variation in the degree of symptoms. Comparisons of the clinical features of patients and their families with the same mutations have revealed incomplete penetrance and variable expression, making genotype-phenotype correlations difficult (2). Therefore, it has not been confirmed whether *FLT4* mutations alter lymphatic function alone or in combination with other mutations.

LDS is an inherited or sporadic primary lymphedema characterized by bilateral or unilateral lower limb lymphedema presenting after the onset of puberty. Affected individuals also usually have an abnormal second row of eyelashes. *FOXC2* is a forkhead box transcription factor gene and is the only gene known to be involved in LDS (6,9,10), and it plays a central role in lymphatic vessel development and lymphatic valve formation (11). Reflux of lymph and lymphatic valve failure was suspected in the lower limbs of individuals with *FOXC2* mutations based on low uptake of isotopic tracer in the ilioinguinal nodes in the lymphoscintigram (12). However, the pathological mechanisms underlying lymphatic valve defects caused by *FOXC2* mutations remain unclear. In families with *FOXC2* mutations, there appears to be no clear genotype-phenotype correlation, and the expression of the disease varies widely even within families (7). It is speculated that intra- and interfamilial variation is the result of stochastic effects or interaction with other genes in the *FOXC2* pathway (7).

We identified genetic mutations that were causative of primary lymphedema associated with mutations in *LAMA5*, which were identified together with *FLT4* mutations in two familial cases of MD and with *FOXC2* in a familial and a sporadic case of LDS. *LAMA5* is a component of the extracellular matrix (ECM) of lymphatic valve leaflets (13) and the basement membrane of the lymphatic wall (14) but *LAMA5* mutations have not been previously reported in cases of human primary lymphedema. In this study, we present the detailed genotype and phenotype of *FLT4*/*LAMA5* and *FOXC2*/*LAMA5* co-mutations in MD and LDS.

METHODS

Patients and Samples

A total of nine subjects were recruited for the study. These included four from two clinically diagnosed MD families, four from an LDS family, and one sporadic LDS case without any family history of the disease. The pedigree of the two MD families and the LDS family are shown in *Figs. 1A, 2A, and 3A*. Lymphedema of the lower extremities appeared at birth in subjects I:1 and VI:1 in MD family1. Subject I:1 had unilateral lymphedema and subject VI.1 exhibited bilateral lower leg lymphedema with a prominent great saphenous vein (*Fig. 1B*) No clinical symptoms of lymphedema were found in subjects II:1 and III:1. In MD family 2, subjects I:2, II:3, and II:5 all exhibited lymphedema in the left lower extremity and varicose veins. Subjects of the third generation (III:1) did not show signs of edema. Subjects of the fourth generation (VI:1) exhibited bilateral lymphedema of the lower limbs with enlarged saphenous vein (*Fig. 2B*).

The familial LDS case exhibited typical symptoms of LDS, including double row of eyelashes (*Figs. 3B,C*), and lower extremity lymphedema with onset of edema was noted during the teen years. Subjects I:1, II:2, and II:3 exhibited bilateral advanced lymphedema (*Fig. 3D, left and Fig. 3E*), and subject VI:1 had lymphedema only in the left leg (*Fig. 3D,*

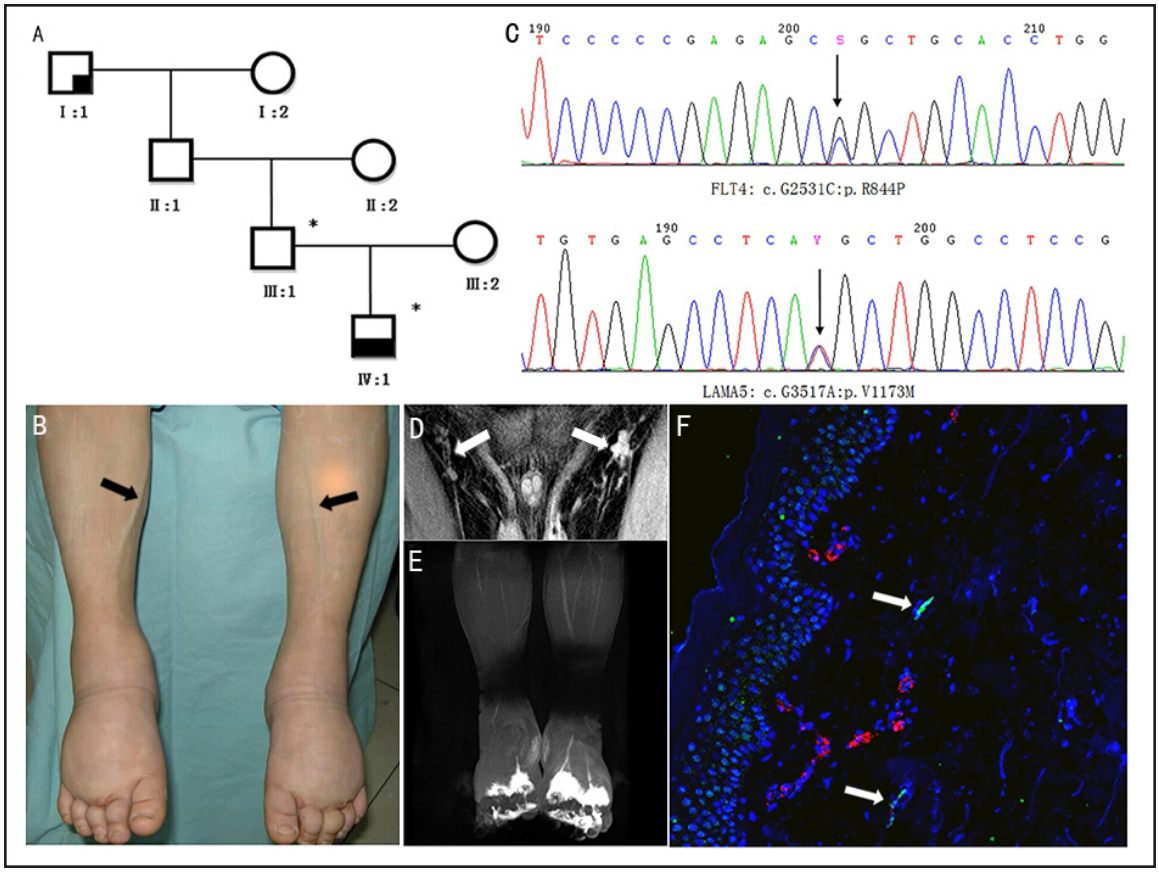

Fig. 1. Phenotype and genotype of FLT4/LAMA5 *mutations in MD family 1. A: Pedigree of the family, half-filled shape indicates affected individuals with bilateral lower extremity lymphedema, a quarter filled shape indicates unilateral (left) lower extremity lymphedema; * indicates mutation carrier; B: Bilateral lymphedema of lower extremity with prominent great saphenous veins (arrows) of IV:1; C: DNA sequencing analysis: the top panel shows a missense mutation of c. 2531G>C in one allele of* FLT4, *and the bottom panel shows one missense mutation c.3517 G>A in one allele of* LAMA5 *identified in III:1 and IV:1; D: MRL imaging shows inguinal lymph nodes (arrows) in IV:1; E: No lymph collector was visualized in the affected lower limbs on MRL imaging; F: Dermal initial lymphatic vessels (arrows) stained with podoplanin IV:1.*

right). The isolated LDS case exhibited double eyelashes (*Fig. 4A*), bilateral lower leg lymphedema (*Fig. 4B*), and venous valve insufficiency of the saphenofemoral, superficial femoral, and popliteal veins determined by Doppler ultrasonography.

Blood samples were collected from the nine subjects with a familial history of MD and LDS for at least two generations and a sporadic case of LDS. DNA was prepared for second-generation sequencing. Full-thickness skin biopsies from lymphedematous limbs were collected from the dorsa of the feet of one MD patient from family1 (II:1), one familial LDS patient (III:1), and the sporadic LDS case. Control biopsies (n=8) were obtained from healthy volunteers.

All subjects provided informed consent. This study was granted approval from Shanghai Ninth People's Hospital Medical Ethics Committee.

Exome Capture, Library Construction and Sequencing

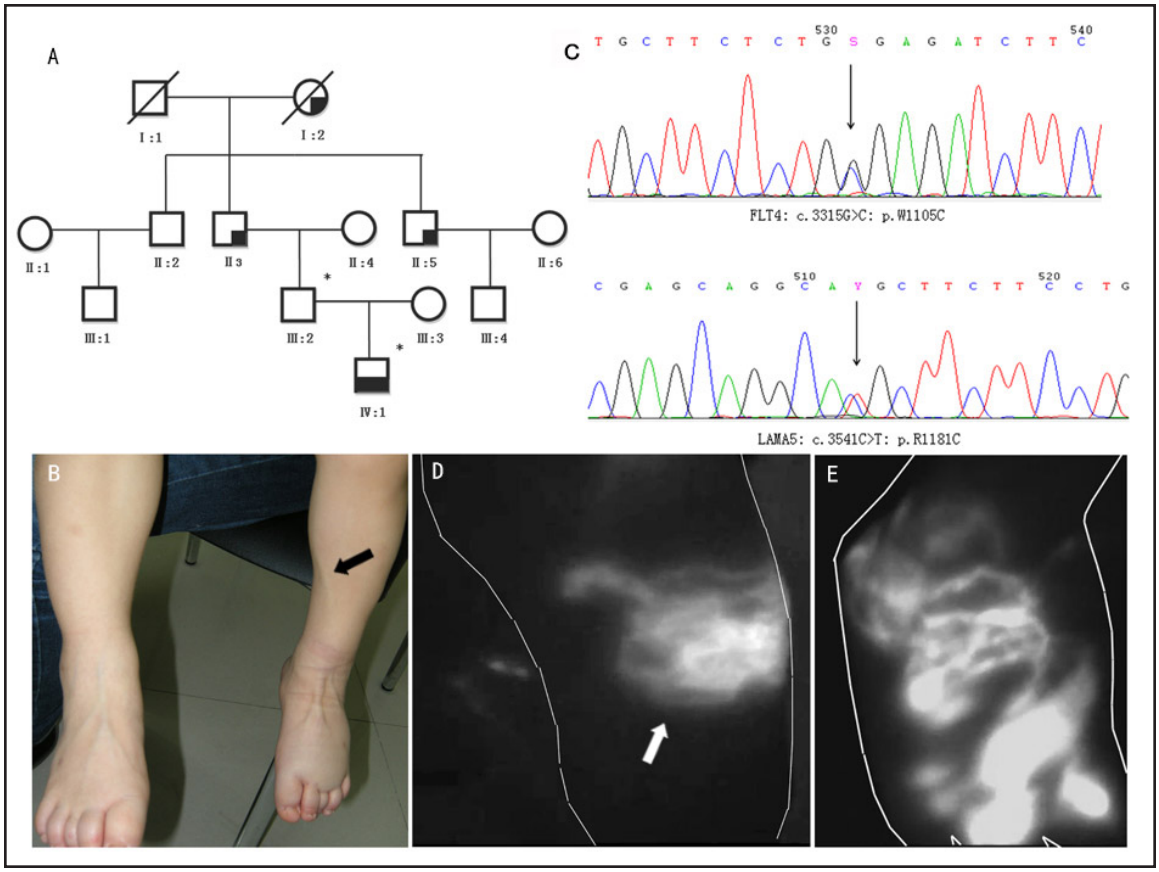

Fig. 2. Phenotype and genotype of FLT4/LAMA5 *mutations in MD family 2. A: Pedigree of the family, half-filled shape indicates affected individuals with bilateral lower extremity lymphedema, a quarter filled shape indicates unilateral (left) lower extremity lymphedema; B: Bilateral lymphedema of lower extremity of VI:1. The arrow points to prominent great saphenous veins; C: DNA sequencing results: the top panel shows a missense mutation of c.3315G>C in one allele of* FLT4*, and the bottom panel shows one missense mutation of c.3541C>T in one allele of* LAMA5 *identified in III:1 and IV:1; D: ICG lymphogram shows few enhanced collecting lymph vessels at the bottom of left foot (arrow), and E shows tortuous lymph collectors in the dorsum of the right foot of IV:1.*

Exome capture was performed by Illumina using Agilent Sure Select in-solution target enrichment technology (Agilent Technologies). Libraries were constructed following the Illumina Paired-End Sequencing Library Preparation Protocol version 1.0.1 from the SureSelect Human All Exon kit, with an added gel purification step for insert size selection. The kit contains a pool of RNA-based 120-mer capture oligomers (or baits) targeting 37,640,396 bases of 165,637 consensus coding sequence exons and their flanking regions. Exome enriched shotgun libraries were sequenced on the Illumina Hiseq2000 platform, and pairend reads with an average targeted insert size of ~180 bp were generated. Image analysis and base calling were performed with Illumina CAVSAV version 1.8, using default parameters. Raw sequencing data were demultiplexed into individual FastQ read files with Illumina's bcl2fastq v2.16.0.10 based on unique index pairs Exact Match. Low quality (Q<15) reads/bases were trimmed using

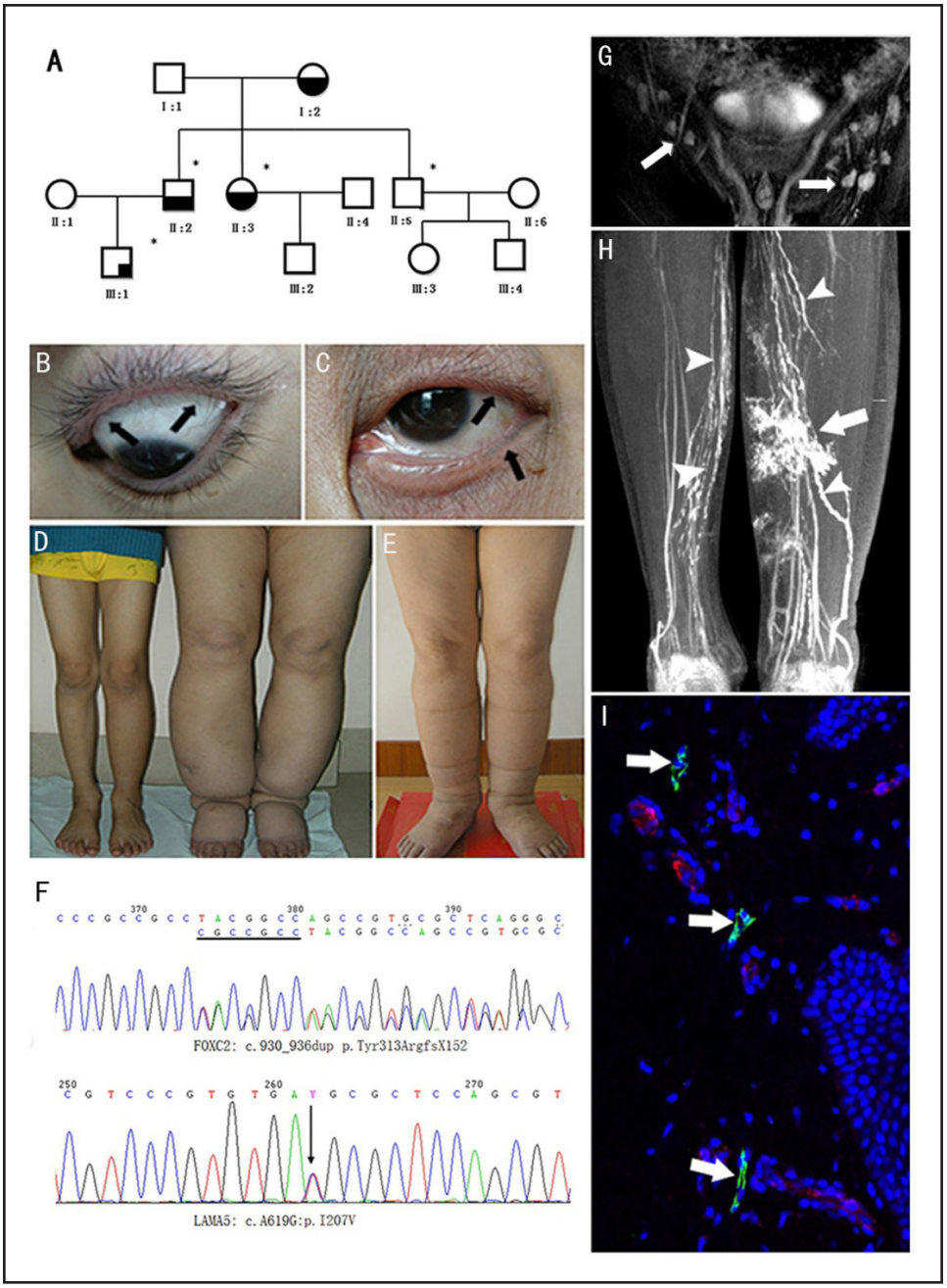

Fig. 3. Phenotype and genotype of FOXC2/LAMA5 *mutations in LDS family. A: Pedigree of the family, half-filled shape indicates affected individuals with bilateral lower extremity lymphedema, a quarter filled shape indicates unilateral lower extremity lymphedema; B&C: Aberrant eyelashes-distichiasis (arrows) in III: 1 and II:3; D& E: Lymphedema of the legs in III: 1 and II:3 and II:2; F: DNA sequencing analysis: the top panel shows a 7-bp duplication, c.930_936dup of* FOXC2 *and the bottom panel shows one missense mutation c.619A>G of* LAMA5 *identified in II:2, II:3, and III:1; G: Inguinal lymph nodes (arrows) on MRL imaging; H: dilated collecting lymphatics (arrowheads) and lymphatic disruption and lymph leakage (arrow) in affected left leg of III:1; I: Slightly dilated dermal initial lymphatics in the skin of III:1 (arrows).*

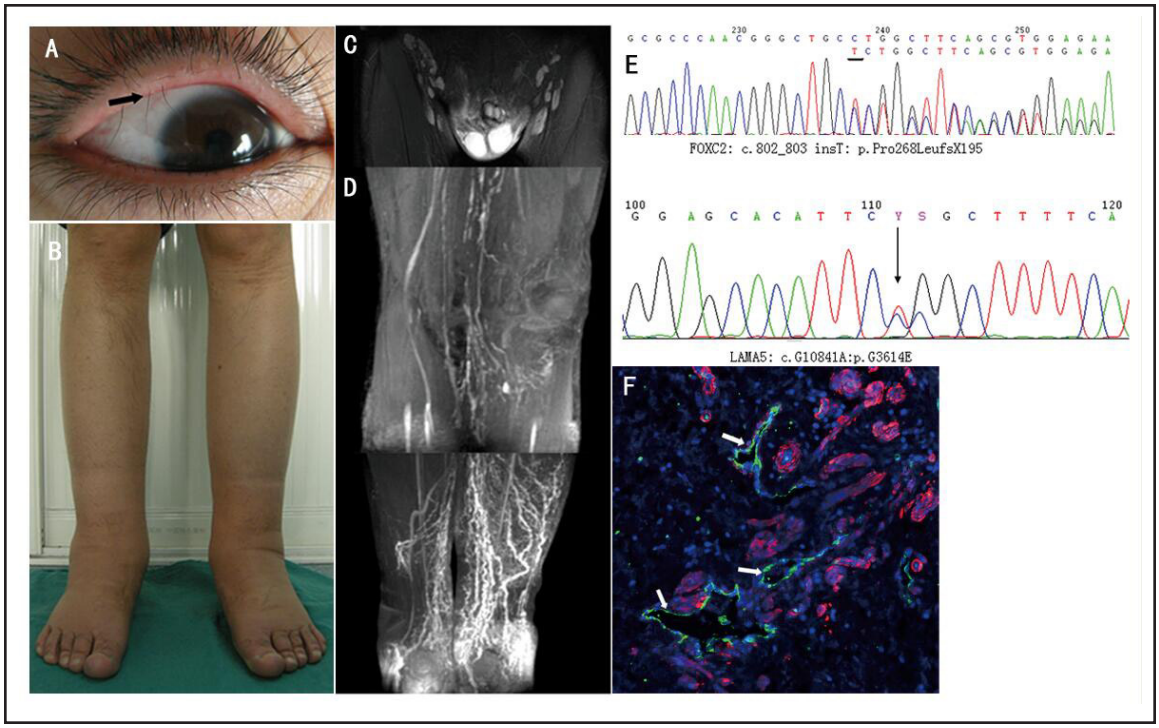

Fig. 4. Phenotype and genotype of FOXC2/LAMA5 *mutations in the isolated LDS case. A: Double eyelashes (arrow); B: Bilateral lower leg lymphedema; C: Inguinal lymph node with normal structure and slightly increase in size; D: Numerous, bead-like and tortuous lymphatics in the bilateral lower limbs with more severe pathology in the left lymphedematous limb; E: DNA sequencing analysis: the top panel shows one-bp insertion mutation (c.802_803insT) of* FOXC2 *and the bottom panel shows a nonsynonymous changes of* LAMA5 *c.10841G>A; F: Obviously dilated dermal lymphatics were found in the skin (arrows).*

Fastx, high quality reads were aligned to the NCBI human reference genome (hg19) using the Burrows Wheeler Aligner (BWA) software that can build assemblies by mapping short reads to a reference genome using default parameters. To identify potential mutations, we performed local realignments of the BWA aligned reads using the Genome Analysis Toolkit (GATK) (15). The pileup file of all variations detected in each sample was first compared to all variations annotated in dbSNP147 along with data from the 1000 Genomes Project. After this analysis, all newly identified variations were fully annotated. All filtering and annotation was performed using ANNOVAR (16). To predict the effect the nonsynonymous mutations might have on the encoded proteins, we used dbNSFP31, which collates the outputs from the prediction programs SIFT, Polyphen2, LRT, MutationTaster and PhyloP (17-19).

The bioinformatics analysis identified single nucleotide variations (SNVs) and short Insertion/deletions (INDELs) in this set of samples. The genomic variations were annotated against a collection of comprehensive functional annotation databases, including gene/protein structure, germline variations (dbSNP, 1000 Human Genome Project, GWAS), functional consequence of amino acid change (VISIFT), known somatic mutations (COSMIC), and functional elements (transcription binding sites, microRNA targets, conserved elements), to help understand and prioritize the SNVs and INDELs for further studies. Any SNV recorded in

dbSNP147 and with a minor allele frequency of ≥1% in Chinese from 1000 genome database was considered as benign polymorphisms and therefore removed for subsequent analysis. The SNV in a gene which has not been reported in dbSNP and 1000 Human Genome Project databases with frequency <0.001 and with high suspicion for a connection with primary lymphedema was further tested in 200 normal controls.

Sanger Sequencing

High attention was paid to the known lymphedema related genes as well as the genes which had not been reported as mutated in lymphedema but contained multiple SNVs that potentially change the protein sequences within a minimum of 2 families with primary lymphedema. The suspected mutated genes from the secondary generation sequencing were further tested and verified by the first generation sequence. The mutant fragments of suspect gene were amplified by PCR, and purified PCR products were submitted for Sanger sequencing (ABI, 3730XL, Perkin Elmer, Foster City, CA, USA).

Magnetic Resonance Lymphangiography (MRL) and Indocyanine Green Fluorescent (ICG) Lymphography

MRL was performed in subject VI:1 from MD family 1, subject III:1 with familial LDS, and the sporadic LDS patient using a 3.0T MR unit (Philips Medical Systems, Best, the Netherlands). Contrast medium gadobenate dimeglumine (Gd - BOPTA, MultiHance Bracco, Milan, Italy) was injected intradermally into the interdigital webs of the dorsal foot. To image the lymphatic channels of the lower limbs, 3D fast spoiled gradient-recalled echo T1-weighted images were taken using a fat saturation technique (20,21).

ICG lymphography was performed in subject VI:1 with familial MD and in the sporadic LDS case. ICG contrast agent (2.5 mg/ml) was injected intradermally into the toe web spaces (three points for each limb, 0.05 ml/point). After injection, lymph flow imaging was performed immediately and 30 min after injection using a photodynamic camera (Hamamatsu Photonics, Hamamatsu, Japan) (22).

Immunofluorescence Staining of Skin Tissue

Skin biopsies were collected from subject VI:1 with familial MD, subject III:1 with familial LDS, and the sporadic LDS case. Skin biopsies were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 5-µm serial sections. Sections were incubated in mouse anti-human podoplanin antibody (1:50 AngioBio, San Diego, CA, USA) and rabbit anti-human α-SMA (1:300 Abcam, Cambridge, UK) overnight at 4°C. Then, samples were labeled with the secondary antibodies Alexa Fluor 555 goat anti-mouse (1:300 Invitrogen, San Diego, CA, USA) and Alexa Fluor 488 goat anti-rabbit (1:300 Invitrogen) for 1 h at 37°C. Photography and whole-slide image construction were performed using a confocal microscope (Zeiss Confocal LSM 710 microscope, Carl Zeiss, Jena, Germany).The number of podoplanin positive vessels was calculated and the total number of vessels identified in the total section area was the lymphatic vessel density.

RESULTS

Gene Mutations

In this study, one known missense mutation c. 2531G >C (p.R844P) in exon 17 of *FLT4* (23) was identified in III:1 and IV:1 (father and the proband) of familial MD 1 (*Fig. 1C*). One novel missense mutation (c.3315G>C, p.W1105C) in exon 24 of *FLT4* was found in III:1 and IV:1of familial MD 2 (*Fig. 2C*). Both of the distinct identified mutations of *FLT4* are located in the tyrosine kinase domains. In addition, *LAMA5*

TABLE 1
Frequency and Validation of Identified LAMA5 Mutations

Sample	Region	HGVS cDNA	HGVS Protein	NCBI dbSNP	Freq (1000)	200 Control
Family MD 1	exon28	c.3517G>A	p.V1173M			0
Family MD 2	exon28	c.3541C>T	p.R1181C	rs73598381	0.0005	0
Family LDS	exon4	c.619A>G	p.I207V			0
Sporadic LDS	exon79	c.10841G>A	p.G3614E			0

mutations were also identified in those *FLT4* mutated subjects. One missense mutation (c.3517 G >A, p.V1173M) in exon 28 of *LAMA5* was found in III:1 and IV:1 of familial MD 1 (*Fig. 1C*) and another missense mutation (c.3541C>T, p.R1181C) in exon 28 of *LAMA5* was identified in III:1 and IV:1 of familial MD 2 (*Fig. 2D*). Although both mutations are located outside of the domain regions of laminin subunit alpha-5 protein, c.3517 G >A and c.3541C>T mutations were predicted damaging with SIFT scores lower than 0.05. Furthermore, both mutations were predicted to induce splice site changes with MutationTaster. These changes, in turn, might result in the expression of different *LAMA5* mRNA variants that affect disease susceptibility and severity.

In the familial LDS cases, one known mutation (7-bp duplication, c.930_936dup) occurring in a GC-rich genomic region (c.893-930) known to be prone to mutations (11) was identified (*Fig. 3F*). A one-bp insertion mutation (c.802_803insT), which would create 195 novel amino acids before truncating the protein, lies in the carboxy terminal region after the forkhead domain was identified in the isolated LDS case (22) (*Fig. 4E*). Both mutations are responsible for truncation of the mature protein in the C-terminal region and consequently lead to the elimination of alpha-helical domains required for the interaction of FOXC2 with the transcription complex (24). Thus, mutations identified in *FOXC2* in this study are very likely to abolish its role in regulating transcriptional activation of multiple key downstream genes. Among those subjects with familial LDS, one missense mutation c.619A>G (p.I207V) in exon4 was identified in N-terminal domain (IPR008211) of *LAMA5* (*Fig. 3F*). In the isolated LDS cases, a nonsynonymous changes of *LAMA5* c.10841G>A in exon79 (p.G3614E) (*Fig. 4E*), which is located in Concanavalin A-like lectin/glucanase domain (IPR013320), was identified. None of the identified SNVs in the *LAMA5* have been found in 200 normal controls (*Table 1*). As laminins are trimeric molecules, the identified mutations within those domains may interfere with self-assembly, binding to other matrix macromolecules and their receptor-integrins, and cell interactions (PMID: 10842354, PMID: 15363809). The location of detected *LAMA5* mutations in this study is summarized in *Fig. 5*.

Imaging Findings

MD patients

MRL imaging of subject II:1 in MD family 1 revealed no lymphatic vessels in the affected lower limbs. The inguinal lymph nodes had a normal size and shape in MRL images *(Figs. 1D,E)*.

ICG imaging of subject IV:1 in MD family 2 revealed a few enhanced lymphatics at the bottom of the left foot (*Fig. 2D*) and tortuous lymphatics that were slowly enhanced and distributed as a network in the dorsum of the right foot to the ankle region

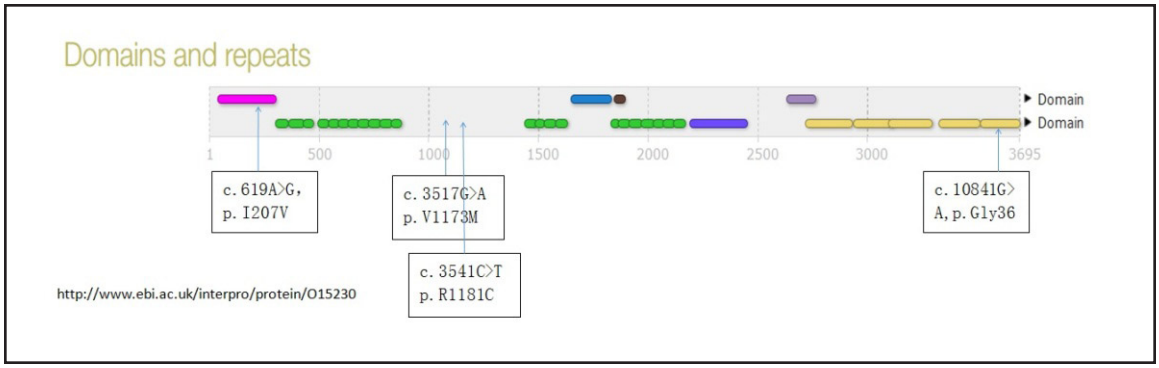

Fig. 5. Locations of detected LAMA5 *mutations in this study.*

(*Fig. 2E*). No lymph node was observed during the ICG imaging.

LDS patients

MRL imaging of subject III:1 with familial LDS revealed straight collecting lymph vessels on the affected left lower leg that were significantly dilated. The inguinal lymph nodes were slightly larger and the efferent and afferent lymph vessels of the lymph nodes were more prominent (*Fig. 3G*). The lymph collector was disrupted and lymph leakage was observed in the anterior tibial area of the affected leg (*Fig. 3H*). This finding may be indicative of high intraluminal pressure.

MRL imaging of the isolated LDS case revealed varicose lymphatic vessels that were numerous, bead-like, and tortuous in the bilateral lower limbs with more severe pathology in the left limb (*Fig. 4D*). The inguinal lymph nodes were relatively large but had a normal structure (*Fig. 4C*). During ICG imaging of the same LDS patient, the contrast quickly diffused into the dermal skin of the dorsum of the foot. The collector vessels were gradually enhanced and visualized along the whole length of lower limbs. Intriguingly, spontaneous constriction of the tortuous collecting vessels with a frequency of 3-5/min was observed, and retrograde lymph flow was clearly observed in the lymph collectors of the lower leg during real-time observation with the patient in a supine position.

Skin Lymphatic Staining

In control skin tissue, most podoplanin-positive initial dermal lymphatics were collapsed with a mean lymphatic vessel density of 3.48/mm^2 (data not shown).

In the dorsum skin of subject III:1 from MD family 1, podoplanin-positive initial dermal lymphatics were identified which were collapsed (*Fig. 1F*) with a density of 1.05/mm^2 (10 in 9.52mm^2). Slightly dilated dermal lymphatics were seen in the skin of familial LDS case (*Fig. 3I*), and obviously dilated dermal lymphatics were found in the isolated LDS skin (*Fig. 4F*). The density of dermal lymphatic vessels was 3.97/mm^2 (51 in 12.8mm^2) and 5/mm^2 (148 in 29.62mm^2), respectively. These results revealed that initial dermal lymphatics were present in the affected skin of familial MD patients but at a lower density compared with the skin of control and LDS subjects.

DISCUSSION

In this study, rare mutations in the *LAMA5* gene were identified in subjects with MD and LDS who had clinical phenotypes of lymphedema and carried *FLT4* and *FOXC2*

mutations. The finding of missense mutations in one allele of *LAMA5* together with *FLT4* or *FOXC2* mutations in familial and sporadic lymphedema subjects suggests that these mutations may be co-responsible for these disorders. LAMA5 belongs to a family of extracellular matrix glycoproteins, which contain three non-identical chains: laminin alpha, beta, and gamma (14). *LAMA5* encodes one of the vertebrate laminin alpha chains, which is an important matrix component of ECM core leaflets of the lymph collector valve, required for lymphatic function. Collecting lymphatics contain numerous intraluminal valves, which open and close in response to pressure changes and ensure a unidirectional flow of lymph without reflux, and this action is critical for the function of the lymphatic vascular system (4). The ECM core has a unique composition, containing high levels of LAMA5, fibronectin-EIIIA/EDA (FN-EIIIA), and collagen IV, onto which endothelial cells attach (13). The ECM controls endothelial cell signaling and may provide structural integrity during lymphatic valve morphogenesis (25). Morphological and functional defects of lymphatic valves have been observed in mice lacking the EIIIA domain of FN (13) and a similar defect has also been observed in mice deficient for integrin-9, the receptor of FN-EIIIA, confirming an important role of the ECM in lymphatic valve morphogenesis (15). LAMA5 has been used as a lymphatic valve marker but its function in valve morphogenesis has not been determined. Our study shows for the first time that *LAMA5* mutations are associated with human primary lymphedema, suggesting that LAMA5 is involved in lymphatic dysplasia.

LAMA5 are the major noncollagenous constituents of the basement membranes (BMs) (26). *Lama5* knockout mice showed defects of BMs in varies tissues (27,28). BMs are present in all calibers of lymphatic vessels (29). Laminin deficient vessels have wider lumens and altered BM composition, which may compromise the stability of the vessels in vivo (30). LAMA5 is an important component of the ECM core of lymphatic valve leaflets and the BMs of the lymphatic wall, which may explain why *LAMA5* mutations could cause lymphatic defects. These observations suggest that *LAMA5* is a candidate causative gene for human lymphedema and should be investigated further.

FOXC2 is the only gene known to be involved in LDS and is important for lymphatic development and lymphatic valve formation. The *FOXC2* mutations identified in this study included a 7bp duplication and a missense mutation, and all occurred within the forkhead domain; they are therefore likely to disrupt DNA binding (25). The morphology of lymphatic collectors was altered in carriers of *FOXC2* mutations in our study. The lymphatics were normal in number and shape in the familial LDS subject and showed typical hyperplasia in the isolated LDS case. Strikingly, significant lymphatic dysfunction was demonstrated in both familial and isolated LDS subjects. In the familial LDS subject, the collecting lymph vessel was dilated and disrupted in the lower leg. This phenomenon is more commonly observed in obstructive secondary lymphedema (31), suggesting that the vessel was under high intraluminal pressure. This abnormality is likely caused by incompetent valve function and retrograde lymph flow. During a real-time image test, we observed retrograde lymph flow in the tortuous and significantly dilated collecting lymphatic with active constriction in the lower leg of the isolated LDS subject; this finding indicated a failure of the valve function in the lymph collectors of this individual.

FOXC2 mutations may exert their actions through haploinsufficiency (32), but other genetic or environmental factors are likely to be involved. *Foxc2* -/- embryos have a complete absence of lymphatic valves (12), while 25% of *Foxc2* +/- mice develop lymphatic vascular defects (32). In humans, not all *FOXC2* mutation carriers exhibit lymphedema (13). *Foxc2* expression and

Lama5 deposition occur close together during embryonic development of the lymphatic valve. Formation of lymphatic valves is initiated around E16.0, and Prox1and Foxc2 expression is upregulated in lymphatic valve-forming cells (LVCs) (33). LVC clusters produce *Lama5* during valve initiation and support the further development of the valve leaflets (33). *Foxc2* signaling is likely to be upstream of the *Lama5* signaling pathway and *Foxc2* mutations presumably influence the formation of LVCs. *Lama5* mutations may also affect the deposition of ECM in the leaflets of lymphatic valves, resulting in deformity and dysfunction of the lymphatic valves. Alternatively, they may interfere with lymphatic endothelial signaling in the ECM of the leaflets. The combined influence of *Foxc2* and *Connexin37* causes severe defects in the lymphatic vessels and a lack of lymphatic valves in animal models (34). Therefore, lymphatic valve dysplasia may be a consequence of multiple gene mutations. Whether *FOXC2* and *LAMA5* mutations interact or cooperate in human lymphatic malformation and dysfunction needs further investigation.

Our study supports the current understanding that MD is caused by lymphatic dysfunction. This conclusion is based on the following: (1) initial lymphatic capillaries were present in the affected skin but no collecting lymphatics were observed on MRL imaging in one MD subject; and (2) 4-5 tortuous lymphatic collectors were observed in the bilateral lymphedematous feet of another MD patient on the ICG lymphogram. It is noteworthy that lymphatic enhancement was delayed and that enhanced lymph flow rose very slowly and stopped below the ankle. In addition, no contrast-enhanced inguinal lymph node was seen in our MD patient, indicating poor lymph transport. Another observation worth noting is the distinct difference between lymphatic dysfunction in MD and LDS limbs. In LDS limbs, dilated and tortuous lymphatic collectors were visualized along the whole limb and inguinal lymph nodes were observed after contrast injection suggesting that initial capillary lymphatics absorb lymph from the interstitial space and transport it through small vessels. The retrograde lymph flow observed was caused by impaired valve function in the collecting vessels. In contrast, the collecting lymphatics in MD were either not present or the enhancement was delayed and only the lymphatic in the distal region of the lower limb was identified. This suggests an impaired absorbing function of the initial lymphatics and impaired transport in the vessel. *FLT4* (*VEGFR-3*) signaling is responsible for capillary sprouting (35); therefore, *FLT4* mutations may influence the absorption and transport of lymph by initial and precollecting lymphatic vessels at the onset of lymph circulation. The biological significance of the *FLT4* and *LAMA5* interaction in lymphatic development is unclear. It is also not clear how they are associated with MD. Multiple germline mutations have been identified in at least 20 genes of the RAS/MAPK and PI3K/AKT pathways in patients with lymphatic anomalies (1). FLT4 belongs to signaling pathways that are characterized by cytokine-cytokine receptor interactions (hsa04060), while LAMA5 interacts with ECM receptors (hsa04512). FLT4 and LAMA5 pathways regulate downstream RAS/MAPK and PI3K/AKT pathways through tyrosine kinase receptors and transmembrane molecules, mainly integrins. LAMA5 plays a crucial role in both epithelial and mesenchymal cell behavior by activating PI3K signaling (28). Mutations in *LAMA5* and *FLT4* might abolish these interactions in the normal lymphatic structure. It is therefore tempting to suggest that *LAMA5* mutations cause dysfunction of precollecting lymphatic semilunar valves and lymph capillary walls independently or in combination with *FLT4* in MD.

Primary lymphedema is likely to have multiple genetic causes despite its apparent transmission as a “single-gene” autosomal dominant disease, as in MD. The present

study demonstrated that *LAMA5* mutations may be associated with *FLT4* and *FOXC2* mutations in non-syndromic and syndromic lymphedema as well as in inherited and non-inherited subjects. Therefore, lymphatic abnormalities may be caused by several genes, similar to other disorders. The multiple phenotypes that arise in single individuals are the cumulative result of multiple genetic and environmental influences. Future studies on primary lymphedema should identify additional disease-causing genes and the factors that influence or regulate the clinical phenotype of the disease.

ACKNOWLEDGMENTS

We would like to thank Genesky Biotechnologies Inc., (Shanghai, China) for the DNA sequencing and the bioinformation analysis. This study was supported by Chinese National Science Foundation (grant number 81272146), National Key Specialty of Clinical Plastic Surgery Project, Shanghai Key Specialty of Clinical Reconstructive Surgery Project.

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