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A LYVE-1/CRSBP-1 MUTATION IN INHERITED PRIMARY LYMPHEDEMA

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ABSTRACT

*Primary lymphedema is clinically and genetically heterogeneous with germline mutations identified in approximately 20 primary lymphedema genes. The lymphatic vessel endothelial hyaluronan receptor-1 (*LYVE-1*) gene, also known as cell-surface retention sequence binding protein-1 (*CRSBP-1*), encodes the major hyaluronan receptor in lymphatic endothelia and is one of the most specific lymphatic vessel markers. However, the role of this lymphatic endothelial-specific protein in the development of the lymphatic system and lymphatic diseases remains unclear. Here, we report a missense mutation c.18C>G (p.S6R) in exon 1 within the N-terminal extension domain (outside the hyaluronan binding region) of* LYVE-1 *in three generations of an inherited lymphedema family with or without clinical symptoms. Lymphatic imaging revealed a partial, weak and delayed enhancement of tortuous lymph collectors in the distal part of the lymph-edematous lower limb. Our findings revealed that* LYVE-1/CRSBP-1 *mutation in primary lymphedema cases is connected with both structural and functional lymphatic defects.*

Keywords: primary lymphedema, *LYVE-1/CRSBP-1*, gene mutation, whole-exome sequencing, lymphatic, ICG lymphography

Primary lymphedema is a clinical peripheral edema condition resulting from stagnation of lymph fluid in tissues caused by lymphatic malformation and/or dysfunction (1). Primary lymphedema is clinically and genetically heterogeneous (2) with at least 20 independent human germline mutations identified (3). The lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), also known as cell-surface retention sequence (CRS) binding protein-1 (CRSBP-1) (4), is a type I transmembrane glycoprotein and the major hyaluronan (HA) receptor in lymphatic endothelia (5,6). It is expressed on both the luminal and basolateral surfaces of the lymphatic endothelium and has been proposed to mediate the uptake of intermediate and high molecular weight HA (6). LYVE-1 is known as one of the most specific lymphatic vessel markers in the skin and is specifically expressed by lymphatic endothelial cells (LECs) (7). Previous studies in *Lyve-1*/*Crsbp-1* knock-out mice found no specific phenotype with regard to lymphangiogenesis (8-9) and the possible role of this specific lymphatic endothelial protein in the development of the lymphatic system remains unclear.

This study presents, for the first time, a missense mutation in the *LYVE-1* gene in three generations of an inherited lymphedema family, together with the detailed clinical phenotypes of the patients. Additionally, we discuss the potential underlying mechanisms of the occurrence of this *LYVE-1* mutation in hereditary primary lymphedema.

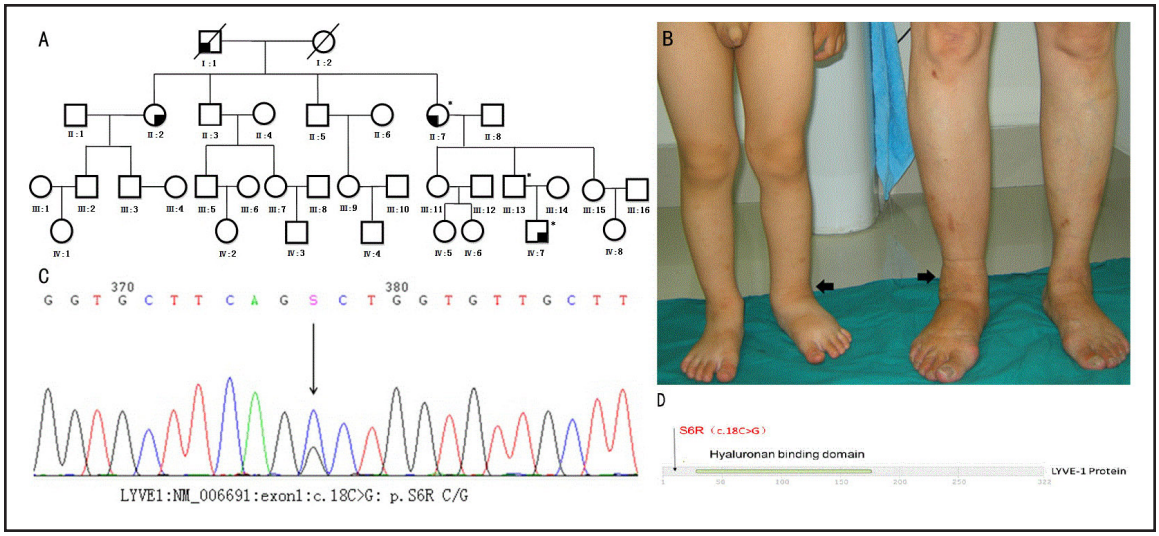

*Fig. 1. Phenotype and genotype of LYVE-1/CRSBP-1 mutations in a family with primary lymphedema. A: Pedigree of the family, a quarter filled shape indicates unilateral (right or left) lower extremity lymphedema; * indicates mutation carrier. B: Unilateral lymphedema of lower extremity (arrows) of IV:7 (left) and II:7 (right). C: DNA sequencing analysis: the panel shows a missense mutation of c.18C>G (p.S6R) in exon 1 of LYVE-1/CRSBP-1 changing amino acid # 6 from serine to arginine in three generations of the family. D: Locations of detected LYVE-1/CRSBP-1 mutation in this family.*

PATIENTS AND METHODS

Patients

The pedigree of the family is shown in *Fig. 1A*. Blood samples were collected from the proband (IV:7), his father (III:13) and grandmother (II:7). Patient I:1 passed away and had lymphedema of his right lower limb with time of onset unknown. Among his children, two daughters exhibited unilateral lymphedema of the leg. Lymphedema appeared in the right lower leg of one of his daughters (II:7) during the pregnancy of her second son (III:13) while she was in her 20s. The below knee edema (*Fig. 1B*) became obvious when she was tired, and she suffered recurrent erysipelas attacks. Venous valve insufficiency of the femoral and popliteal veins was detected in her bilateral lower limbs by Doppler ultrasonography. None of her children including III:13 (father of proband IV:7) exhibited clinical symptoms of lymphedema. Unilateral lymphedema was noted in her grandson (proband IV:7) at the age of 11 months. He exhibited below knee pitting edema of his left lower leg with slight skin fibrosis of the toes (*Fig. 1B*) when he was examined at age 3.

Whole-Exome Sequencing and Data Analysis

Blood samples were drawn and whole exome sequencing was performed on DNA samples from three generations of the family (II:7, III:13 and IV:7). DNA capture was prepared using Agilent SureSelecTarget Enrichment Kit by following the manufacturer guide. The libraries were sequenced on the Illumina Hiseq2000 platform, and pair-end reads with an average targeted insert size of ~180 bp were generated.

High quality reads were aligned to the NCBI human reference genome (hg19) using the Burrows Wheeler Aligner (BWA) software. Potential mutations were called using the Genome Analysis Toolkit (GATK) and 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). 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. PolyPhen2, SIFT, MutationTaster, CADD, and Dann were used to predict mutation functional importance. Phobius program, a server for prediction of transmembrane topology and signal peptides was explored to predict the impact of mutation on subcellular localization of protein.

The mutant fragments of suspect genes were amplified by PCR, and purified PCR products were submitted for Sanger sequencing (ABI, 3730XL, Perkin Elmer, Foster City, CA, USA).

Indocyanine Green Fluorescent (ICG) Lymphography

ICG lymphography was performed in subject II:7 with contrast agent (2.5 mg/ml) injected intradermally into 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).

RESULTS

We identified one missense mutation c.18C>G (p.S6R) in exon 1 of *LYVE-1* in all samples (II:7, III:13 and IV:7) from three generations of this primary lymphedema family (*Fig. 1C*), among whom, the proband (IV:7) and his grandmother (II:7) exhibited clinical signs of lymphedema, while his father (III:13) had no clinical symptoms. The LYVE-1/CRSBP-1 protein includes three domains: the HA binding domain, a membrane proximal domain, and a cytoplasmic tail. The single HA binding domain is at the N-terminus of the protein, corresponding to amino acids 29-176 of exon 1. In this study, the detected missense mutation c.18C>G (p.S6R) occurs outside of the HA binding region (*Fig. 1D*) and maps to the signal peptide of the protein (between positions 1-19), it corresponds to amino acid 6, changing the encoded amino acid from serine to arginine. This base pair change was not found in dbSNP, the 1000 genome project (http://www.1000genomes.org), or the Yanhuang project (http://yh.genomics.org.cn). Although this mutation localizes in the signal peptide-encoding region, it should not affect the subcellular localization of the protein according to the Phobius program.

Five *in silico* programs were used to predict mutation functional importance. The variation is predicted to be neutral by Mutation Taster, possibly damaging by Polyphen V2 (score=0.815), tolerated by SIFT (score=0.159), no deleterious effects by CADD (score=2.28), and pathogenic by Dann (score=0.991). Although these prediction algorithms imply that this mutation might be pathogenic, the inconsistent observations across these prediction algorithms further illustrate the need for functional examination of the mutation. No other mutation was found in known lymphedema related genes (3).

Indocyanine green (ICG) lymphography in patient II:7 demonstrated two slowly and faintly enhanced tortuous lymphatics with undefined walls in the dorsum of the lymphedematous right foot. Contraction of the lymph vessel was visualized when the patient wiggled her toes. These indistinct lymph vessels did not terminate until the ankle region (*Fig. 2A*) 30 min and 1 hr later. The right inguinal lymph node was not visualized during the test. Four tortuous lymphatics in the dorsum of the non-edematous left foot were visualized immediately after contrast injection, and

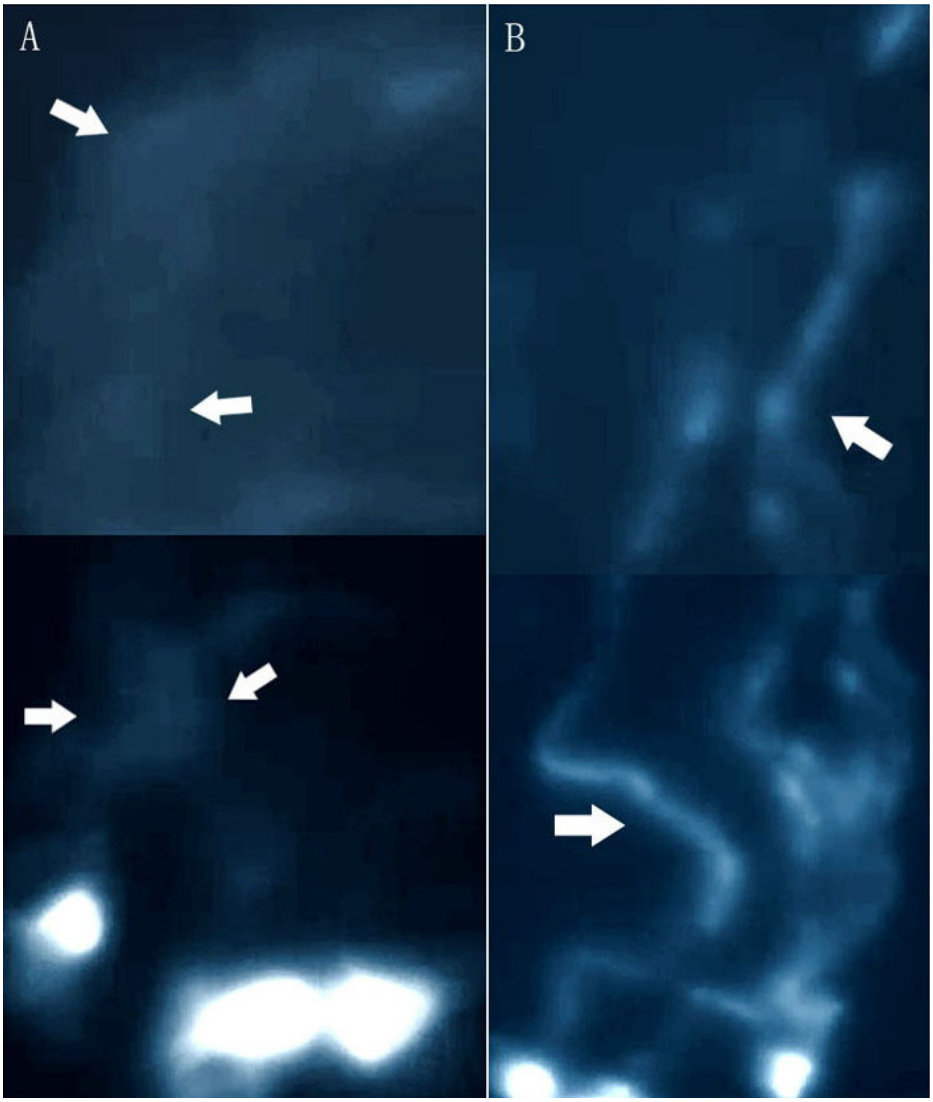

Fig. 2. Imaging results of ICG lymphography of subject II:7. A: ICG lymphogram shows faintly enhanced tortuous lymphatics (arrows) with undefined shape in the lymphedematous right foot (bottom) and ankle (top) regions. B: ICG lymphogram shows strongly enhanced lymph collectors (arrows) with clear shape in the dorsum (bottom) and ankle (top) regions of the non-edematous left foot.

voluntary contraction of the collecting lymphatic was also noticed during inspection (*Fig. 2B*). The enhanced lymph collectors in the left lower leg and thigh as well as the inguinal lymph nodes were identified 30 min afterwards (data not shown).

DISCUSSION

The functional role of LYVE-1/CRSBP-1 protein is not firmly established and remains controversial. A growing literature suggests that LYVE-1/CRSBP-1 is important for both physiological and pathological lymphangiogenesis with binding of low molecular weight HA to LYVE-1 activating intracellular signaling which promotes lymphangiogenesis

in vitro (10). Furthermore, an *in vivo* study showed that LYVE-1 is a direct substrate of the membrane type 1-matrix metalloproteinase (MT1-MMP), which is an endogenous negative regulator of lymphangiogenesis and sheds cell surface-bound LYVE-1 on LECs, restricting lymphangiogenic potential. *Mt1-Mmp*-deficient mice exhibit spontaneous corneal lymphangiogenesis without concomitant changes in angiogenesis (11). The c.18C>G missense mutation in exon 1 of *LYVE-1* in our study was mapped to the structure of the LYVE-1 N-terminal extension domain which is very close to the MT1-MMP cleavage sites of LYVE-1. Therefore, this mutation might interfere with the physiological balance of lymphangiogenesis. Another study revealed that LYVE-1 is proteolytically cleaved from LECs in response to VEGF-A and therefore might contribute to promoting pathological lymphangiogenesis (12).

It is well established that fibroblast growth factor 2 (FGF2) induces lymphangiogenesis (13-14). Furthermore, a previous study revealed that FGF2 can directly interact with LYVE-1 and regulate its glycosylation. Treatment with soluble LYVE-1 or knockdown of *LYVE-1* in LECs impaired FGF2 signaling and functions (15). Thus, LYVE-1 may be involved in modulating VEGF-C-independent lymphangiogenesis by directly interacting with critical lymphangiogenesis factors, such as FGF2 or PDGF-BB (15)

Lyve-1/*Crsbp-1*- null mice are overtly normal and fertile but exhibit identifiable morphologic phenotypes such as distended lymphatic vessel lumens in some tissues including the liver and intestine, which are marked by a constitutively increased transit of fluid from the interstitial space into lymphatic vessel lumens. CRSBP-1 ligands (PDGF-BB, VEGF-A165, PDGF peptide) can increase the interstitial-lymphatic transit of fluid in wild-type mice but not in *Crsbp-1* knockout mutants (8). Both the downregulation of CRSBP-1 protein by transfecting cells with CRSBP-1 siRNA or the *Crsbp-1* null mutation in mice cause constitutive contractions of LECs, increased permeability of LEC monolayers, the formation of distended lymphatic vessel lumens (openings), and an increase in interstitial-lymphatic transit in whole animals (8,16). Furthermore, LYVE-1/CRSBP-1 has been shown to be involved in the formation of VE-cadherin-mediated "button"-like structures at lymphatic intercellular junctions by experiments that demonstrated CRSBP-1 null mutations and binding of CRSBP-1 by its cognate ligands (PDGF-BB, VEGF-A165 and HA) result in the loss and "unbuttoning" of VE-cadherin-mediated buttons leading to the opening of lymphatic intercellular junctions (16). Thus, LYVE-1/CRSBP-1 has multiple functions in regulating lymphangiogenesis and maintaining the normal lymph vessel structure and its transport functions.

Our study is the first report of a *LYVE-1*/*CRSBP-1* mutation in hereditary lymphedema. The causal relationship between the missense mutation in *LYVE-1*/*CRSBP-1* and the onset of lymphedema as well as the clinical phenotypes are unclear. The mutation is localized in the signal peptide-encoding region. Although the mutation was predicted to have no effect on the subcellular localization of the protein according to the Phobius program, there is still the possibility that it could be a gain of function mutation. Discrepancies in mutation prediction by the programs (sequence conservation vs. altered protein structure) has been found. In silico program predictors provide a fast and inexpensive way to define functional annotation and to predict the effects of mutations and could theoretically be employed to assist in the selection of the functional mutations. However, different predictors have been designed based on different algorithms and trained using different sets of functional and neutral mutations. Because of the differences in the underlying methodology, these predictors often return dissimilar or even contradictory results. Thus, defining whether a mutation is biologically and/or clinically relevant is a trivial task, in particular for

missense mutations. The possibility of the involvement of other genes in the disease can not be excluded as primary lymphedema is likely to have multiple genetic causes (17). Further laborious functional assays need to be performed.

While collecting lymph vessels in the affected limb were present in our lymphedema patients with *LYVE-1* mutations, these vessels were dilated and tortuous with an undefined shape. These distinct morphological changes in the lymphatics were accompanied by obvious functional disability, such as delayed and partially enhanced lymphatics in the distal part of the lymphedematous limb during real-time lymphatic imaging. This faint enhancement phenomenon might be due to a diffusion of the contrast through the walls of the lymphatics as a result from defects in the lymphatic vascular system. The delayed transport of the contrast-enhanced lymph flow in the lymphedematous limb compared with the non-edematous limb revealed dysfunctions of the lymphatic system either due to impaired lymph absorption by the initial lymphatics and/or failure in the transporting capacity of the lymph collectors. The structural basis of the dysfunction of the affected lymphatic is unclear and might be due to an opening of the lymphatic endothelial junction, lymphatic valve insufficiency, or lymph vessel contractile dysfunction.

In conclusion, we identified a missense mutation c.18C>G (p.S6R) in exon 1 of *LYVE-1*/*CRSBP-1* in three generations of familial primary lymphedema. Clinical investigations revealed that while the lymphatic collectors exist, they exhibited distinct morphological and functional disorders in the affected limb, as evidenced by the delayed and faint enhancement of tortuous lymph collectors with undefined wall shape in the affected lower leg. Our findings revealed that a *LYVE-1*/*CRSBP-1* mutation in primary lymphedema cases may be connected with both structural and functional defects of the lymphatics. Although the results of functional importance prediction implies that this mutation might be pathogenic, further study (including *in vitro* validation) is needed for functional examination of the mutation.

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