

***NRAS* mutation in a central conducting lymphatic anomaly and *PPFIBP1::ROS1* fusion in a Gorham-stout disease patient**

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Abstract

Lymphatic malformations (LMs) can lead to severe clinical complications, including disfigurement and even death. While genomic alterations have been identified in LMs, the genomic landscape of complex LMs remains poorly defined due to their rarity. In this study, we report two novel findings: an *NRAS* p.Q61R mutation in central conducting lymphatic anomaly (CCLA) and a *PPFIBP1::ROS1* fusion in Gorham–Stout disease (GSD), both described for the first time in their respective LM subtypes. The discovery of the *PPFIBP1::ROS1* fusion provided a unique opportunity to localize the somatic event to a specific cell type. Using serial tissue sections from the same specimen, we observed that the fusion signal was spatially associated with lymphatic endothelial cells, based on serial section analysis with D2–40 staining. Given that both *NRAS* mutations and *PPFIBP1::ROS1* fusions have also been identified in other LM subtypes, our findings support the hypothesis that LMs may share common molecular mechanisms. We propose that phenotypic diversity among LM subtypes may arise from differences in developmental timing, anatomic location, and microenvironmental context at the time the somatic mutation occurs.

Keywords Gorham-stout disease, *NRAS* mutation, *PPFIBP1::ROS1* fusion, lymphangiomatosis

Introduction

Lymphatic malformations (LMs) are classified under the 2025 ISSVA Classification into two categories: isolated lymphatic malformations and complex lymphatic malformations. Complex LMs are further subdivided into generalized lymphatic anomaly (GLA), kaposiform lymphangiomatosis (KLA), Gorham–Stout disease (GSD), and central conducting lymphatic anomaly (CCLA) [1].

GLA is characterized by diffuse, multi-organ lymphatic proliferation, often involving the bones, lungs, and soft tissues. KLA represents a more aggressive variant of GLA, distinguished by the presence of spindle cell clusters that trap platelets, resulting in thrombocytopenia and coagulopathy. GSD is characterized by lymphatic proliferation within bone, leading to progressive bone resorption and disintegration [2]. CCLA involves structural and functional abnormalities of the

central lymphatic system, particularly the thoracic duct, leading to obstruction or leakage. Although GLA, KLA, and GSD can all affect bones, their patterns of involvement differ. In GLA and KLA, the cortical bone is typically spared. In GSD, however, lymphatic proliferation invades the cortical bone, leading to bone destruction and disappearance [3].

Recent advances in genomic profiling have identified somatic mutations in various LMs. *PIK3CA* mutations are predominantly associated with isolated LMs. For example, in a cohort from Boston Children's Hospital, 16 of 17 isolated LM cases harbored somatic *PIK3CA* mutations, with five hotspot variants accounting for approximately 80% of cases. Similar *PIK3CA* mutations have also been found in 5 of 9 GLA patients [4]. *NRAS* mutation c.182A > G p.Q61R was identified in 10 of 11 KLA cases [5]. In CCLA, mutations in *EPHB4*, a tyrosine kinase

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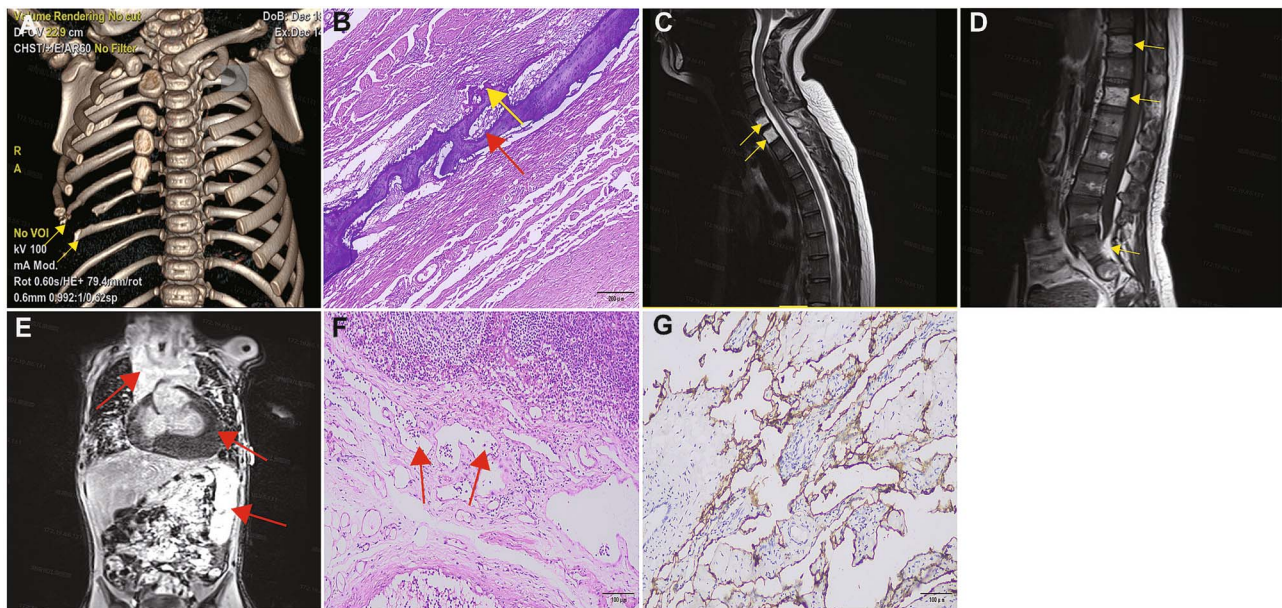


Figure 1 Radiological and histological findings in patients with complex lymphatic malformations. (A) Three-dimensional computed tomography (3D-CT) of the chest in patient 1 (GSD), demonstrating irregular bone destruction and resorption of the right 7th to 9th ribs (arrows), with no evident periosteal reaction. (B) Patient 1 (GSD, rib biopsy): H&E stain reveals trabecular bone destruction and deformity with characteristic 'moth-eaten' resorption (red arrows); the normal osteoblastic/osteoclastic lining is obscured, with an infiltrating network of thin-walled vessels and delicate fibrous tissue invading the bone matrix (yellow arrows). (C, D) sagittal T2-weighted magnetic resonance imaging (MRI) of the spine in patient 2 (KLA), showing abnormal marrow signal within the C7 and T1 (C) as well as the T10, T12, and L5 (D) vertebral bodies (arrows). (E) Coronal T2-weighted MRI of the chest and abdomen in patient 3 (CCLA) revealing extensive mediastinal lesions, pleural and pericardial effusions, and splenomegaly with abnormal signals (arrows). (F) Patient 3 (CCLA, lymph node biopsy): H&E stain reveals prominent dilated and irregular lymphatic channels (arrows) within the perinodal tissue, situated adjacent to normal lymphoid follicles. (G) D2–40 immunohistochemistry (IHC) in patient 3.

receptor essential for venous and lymphatic vessel separation and remodeling during embryonic development, have been identified [6]. For GSD, no large-cohort genomic studies are reported to date. However, several case studies have identified diverse mutations, including *KRAS* c.35G > T p.G12V, *EML4::ALK* fusion, and *ATG101::SLC4A8* fusion, each reported in a single case [4, 7–10].

In the present study, we evaluated genomic alterations in 3 pediatric complex LM patients. Two of the 3 harbored somatic alterations: one with an *NRAS* c.182A > G p.Q61R in a CCLA and the other with a *PPFIBP1::ROS1* fusion in a GSD. These findings represent the first report of *NRAS* p.Q61R in CCLA and the first identification of a *PPFIBP1::ROS1* fusion in GSD [11]. Notably, *PPFIBP1* expression is highest in endothelial cells, suggesting that fusion with *ROS1* may result in aberrant *ROS1* expression and kinase activation in lymphatic endothelial cells. Correlative analyses using *ROS1* fluorescence in situ hybridization (FISH), *ROS1* immunohistochemistry (IHC), and D2–40 (a lymphatic endothelial marker) suggest that the *ROS1* fusion is localized to lymphatic endothelial cells, providing supportive evidence that these somatic alterations arise in lymphatic endothelial cells.

Results

Clinical, imaging, and histologic findings in three patients with complex LMs

Patient 1 (GSD)

An 11-month-old boy presented with progressive shortness of breath for one week. Chest CT revealed irregular bone destruction of the right 7th to 9th ribs, without evident periosteal reaction (Fig. 1A). In the corresponding area, an irregular soft tissue density was noted,

showing marked heterogeneous enhancement on contrast imaging. Bilateral pleural effusions were also observed.

A biopsy of the affected rib was performed. Hematoxylin and Eosin (H&E) staining demonstrated a fragment of lamellar bone undergoing active resorption, surrounded by dense fibrovascular tissue that replaced the normal marrow (Fig. 1B). Immunohistochemistry (IHC) showed strong D2–40 (Podoplanin) positivity in the irregular lymphatic channels. Additionally, numerous CD68-positive macrophages and osteoclast-like cells were concentrated at sites of active bone erosion and scattered throughout the stroma, underscoring the cellular basis of osteolysis. Based on the clinical, imaging, and histological findings, the patient was diagnosed with Gorham-Stout disease (GSD).

The patient underwent thoracic drainage, and oral sirolimus therapy was initiated. As of the most recent follow-up [2025-8-22], the child has been on sirolimus (0.25 mg/day) for 1 year and 7 months and continues the treatment. Disodium pamidronate infusions (1 mg/kg/day) are also administered over 3 consecutive days every 3 months. A recent chest X-ray showed persistent bone destruction of the 7th to 9th ribs, but the pleural effusion has resolved.

Patient 2 (KLA/CCLA)

An 11-year-old girl presented with intermittent cough and shortness of breath for > 2 months, with worsening over the recent week. The cough was non-spasmodic, mild, and intermittent, without a clear trigger. Over the same 2-month period, she had > 10 nocturnal episodes of abruptly waking from sleep, sitting upright, and gasping for air for ~10–20 seconds, then returning to sleep.

Laboratory testing revealed evidence of coagulation dysfunction, including an elevated D-dimer level of 6.60 $\mu\text{g/mL}$ (normal range < 0.5 $\mu\text{g/mL}$), prolonged prothrombin time (PT) of 15.8 seconds

(normal 10–14 seconds), decreased fibrinogen (FIB) at 158 mg/dL (normal 200–400 mg/dL), and increased fibrin degradation products (FDP) at 23.30 $\mu\text{g/mL}$ (normal $< 5 \mu\text{g/mL}$). Chest CT demonstrated a patchy, mixed-density lesion in the thymic region with enhancement (infection favored), mediastinal air and fluid, fluid within the left main and segmental bronchi, narrowing at the left main bronchial origin, a large left pleural effusion with compressive atelectasis of the left lung, pericardial effusion, and soft-tissue swelling of the left chest wall. Spine MRI showed scoliosis; abnormal marrow signal in C7, T1, T10, T12, and L5 vertebral bodies (fatty infiltration favored) (Fig. 1C and D); multiple small cysts in the sacral canal; a right pelvic cystic lesion (possible ovarian origin); and diffuse punctate, mildly T2-hyperintense foci in the spleen.

Pericardial effusion was evaluated via cytology, revealing a large number of red blood cells, a significant presence of segmented nuclear neutrophils, and a small number of lymphocytes and monocytes. No nuclear atypical cells were identified. Given the clinical presentation, coagulopathy, and imaging evidence of multi-organ involvement, a provisional diagnosis of KLA was considered. However, CCLA remained in the differential diagnosis, though no tissue was available for detailed histologic confirmation.

The patient underwent median sternotomy with exploratory thoracotomy, pericardial stripping, and pericardial window. Intraoperatively, a large fibrinous exudate filled the pericardial space; no structural injury was identified. Pericardial and thoracic drains were placed, yet high-volume effusions persisted. Chylothorax was subsequently suspected. Lymphangiography followed by thoracic duct embolization demonstrated obstruction of the thoracic duct near its cardiac termination with substantial chyle leakage into the pericardial cavity. With supportive management, including bowel rest and a low-fat diet, the patient improved, and pleural drainage came under control.

Patient 3 (CCLA)

A 4-year-and-5-month-old male presented with a cough for over 10 days, worsening with wheezing and shortness of breath for 8 days. Chest CT revealed pericardial effusion, bilateral pleural effusion, right axillary soft tissue swelling, right cervical lymph node enlargement, and splenomegaly with abnormal density shadows. Chest and abdominal MRI showed abnormal mediastinal lesions with enhancement, indistinct boundaries with the thymus and thyroid, splenomegaly with abnormal signals, pericardial effusion, bilateral pleural effusion, and minimal ascites (Fig. 1E). Cervical lymph node biopsy indicated dilated, irregular channels in the perinodal tissue (Fig. 1F). IHC showed the presence of lymphatic vessels (D2–40 positive) and macrophages within the stromal tissue (Fig. 1G). Based on the clinical, imaging, and histological findings, the patient was diagnosed with CCLA.

The patient underwent closed left pleural drainage, pericardial puncture drainage, and lymphatic fistula adhesion surgery. Intraoperative findings revealed a thickened, tortuous thoracic duct with distal outlet obstruction. Lipiodol was seen entering the mediastinum retrogradely from the thoracic inlet. After sealing the thoracic duct with glue, drainage gradually decreased. A telephone follow-up approximately 18 months after sealing of the thoracic duct indicated a near-complete recovery.

Genomic findings

A NGS panel targeting 638 cancer-related genes, along with whole-genome CNV analysis, was performed on the 3 patients with complex

LMS. Distinct genomic alterations were identified in two of the three cases.

Patient 1 (GSD)

DNA NGS evaluation on the rib biopsy found a *PPFIBP1::ROS1* fusion as the sole aberration. The chromosome breakpoints were at intron 15 of *PPFIBP1* and intron 35 of *ROS1*. The predicted fusion transcript has an intact reading frame that encodes the coiled coil domain from *PPFIBP1* and an intact kinase domain from *ROS1* (Fig. 2A). The *PPFIBP1::ROS1* fusion transcript was confirmed by a RT-PCR with primers specific for *PPFIBP1* (*PPFIBP1*-F1: 5'-CCAGCAACTGTAAGCATGGA; *PPFIBP1*-F2 (nested): 5'-TCCTAAGCCAGAGACTTCATTG) and *ROS1* (*ROS1*-R1: 5'-CTGGTCTGTGGAACCTTCT; *ROS1*-R2 (nested): 5'-GCAAGAGACGACAGTCACT), which amplified a product of the expected size (Fig. 2B). Subsequent Sanger sequencing of the PCR product confirmed the *PPFIBP1::ROS1* fusion (Fig. 2C).

Patient 2 (KLA/CCLA)

DNA NGS detected no genomic alterations in the DNA isolated from the patient's pericardial effusion; unfortunately, no affected tissue was available for additional testing. Therefore, this case was deemed non-informative for the genomic analysis and was included here for clinical completeness only.

Patient 3 (CCLA)

DNA NGS evaluation of the cervical lymph node showed an *NRAS* c.182A>G (p.Q61R) in 7 of 1223 reads (VAF 0.6%) as the sole aberration. To confirm this finding, a duplex Amplification Refractory Mutation System (ARMS) ddPCR was performed, which contains a mutation-specific primer set (*NRAS* Q61R ARMS F: AZALTGGATACAGLTGGACP, *NRAS* Q61R ARMS R: TTCCCCATAAAGATTGAGAACACAAAGATC) and a VIC-tagged probe (probe Yakima: VIC-ALATGAGGALAGCGAAGGC-BHQ1) to detect the *NRAS* Q61R allele, and a FAM-tagged reference gene assay (control F: AGGACACCGAGGAAGAGGACTT, control R: GGAATCACCTTCTGTCTTCATT, control probe: FAM-CTGCLTPAZGAGGGGAA-BHQ1) for normalization of total alleles (symbols L, P, and Z denote locked nucleic acid (LNA) modifications: L=LNA-modified C, P=LNA-modified G, and Z=LNA-modified T). This highly sensitive assay confirmed the *NRAS* c.182A>G p.Q61R mutation with a variant allele frequency of 0.44% (calculated via Poisson statistics yielding 4 mutant copies relative to 900.8 wild-type copies) in the same sample used for DNA NGS (Supplementary Fig. 1). This value remained above the assay's estimated limit of detection (LoD) of 0.08%. Crucially, parallel wild-type and no-template controls yielded zero mutant droplets, supporting that this low-frequency signal represents genuine somatic mosaicism rather than technical noise. The *NRAS* p.Q61R variant was identified in a cervical lymph node rather than thoracic-duct or cisterna chyli most directly representative of the primary CCLA lesion. Although the lymph node showed histologic evidence of abnormal perinodal lymphatic channels consistent with CCLA involvement, this source does not allow definitive attribution of the variant to the central conducting lymphatic pathology itself.

Localization of *ROS1* rearrangement in lymphatic endothelial cells

The identification of a *PPFIBP1::ROS1* fusion in a patient with GSD provides a unique opportunity to track this rearrangement in specific

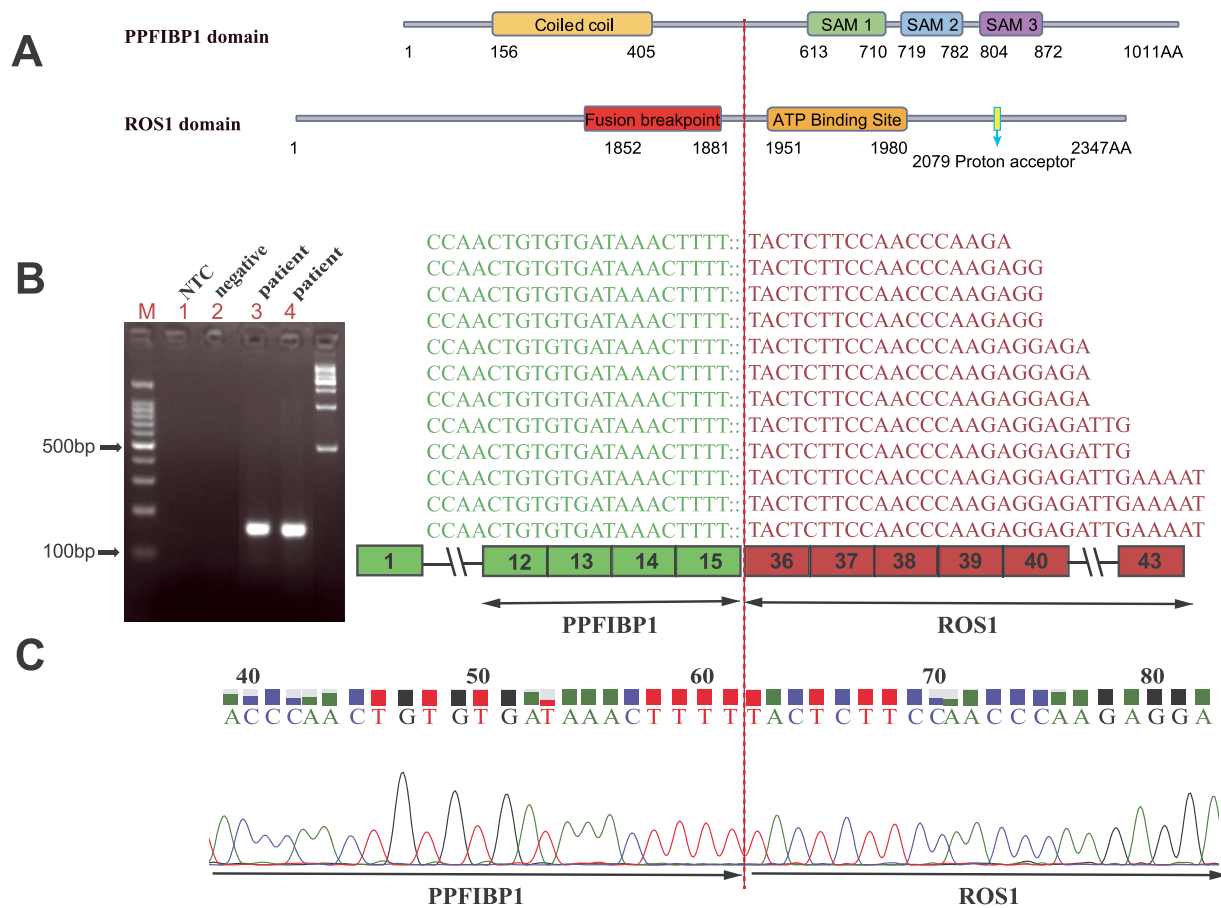


Figure 2 Characterization of the *PPFIBP1::ROS1* fusion. (A) Schematic representation of the *PPFIBP1::ROS1* fusion, showing the fusion of the 5' region of PPFIBP1 to the 3' region of ROS1, retaining the N-terminal coiled coil domain of PPFIBP1 and the kinase domain of ROS1. The vertical dotted line indicates the breakpoint. (B) RT-PCR using *PPFIBP1* and *ROS1* gene-specific primers amplified a product of the expected size from the *PPFIBP1::ROS1* fusion. (C) Sanger sequencing of the RT-PCR product confirmed the fusion junction.

cell types using FISH technology. Three consecutive tissue sections from the same block were used for *ROS1* split-apart FISH and immunohistochemistry (IHC) for D2-40 (a marker for lymphatic endothelial cells) and CD34 (a marker for vascular endothelial cells). As shown in Fig. 3A-F, *ROS1* rearrangement was detected in lymphatic endothelial cells but not in vascular endothelial cells. Interestingly, only a subset of lymphatic endothelial cells showed the *ROS1* rearrangement, suggesting a mosaic pattern of somatic alteration in the lesion. This finding is consistent with observations in animal models of lymphatic malformation [12].

In addition, IHC confirmed aberrant *ROS1* expression in lymphatic endothelial cells in the GSD specimen (Fig. 3E), which is not normally expressed in these cells. We hypothesize that the *PPFIBP1* promoter drives the expression of the *PPFIBP1::ROS1* fusion. To investigate this, we examined publicly available gene expression datasets and found that *PPFIBP1* is highly expressed in endothelial cells, with the highest levels observed in lymphatic endothelial cells (Fig. 4A). We then evaluated the expression of several genes commonly associated with lymphatic malformations, including *PTEN*, *PPP1R1B*, *PIK3CA*, *NRAS*, *KRAS*, *GNAQ*, and *GNA11*, across lymphatic endothelial cells from different organs and tissues (Fig. 4B). While these genes were generally expressed, their expression levels varied by tissue of origin, highlighting tissue-specific expression patterns that may underlie the clinical and pathological heterogeneity of these related disorders.

Notably, while *PPP1R1B* was highly expressed in lymphatic endothelial cells from all tissue types, *ROS1* was not detected in any of them. This expression pattern is consistent with the working hypothesis that the *ROS1* expression observed in the GSD lesion is fusion-driven rather than reflecting native expression in lymphatic endothelial cells. A limitation of this interpretation, however, is that it is based on a reference dataset derived from publicly available normal tissues rather than from the patient's own cells. Physiologically, *ROS1* expression is generally absent in normal endothelial cells and is normally restricted to specific epithelial cell subsets, such as alveolar type II cells and renal tubular cells. Therefore, the detection of *ROS1* expression in our lesional tissue appears biologically aberrant relative to its normal physiologic expression pattern. We acknowledge that the interpretation of cell-type localization in this study was based on spatial concordance observed across serial tissue sections. Direct single-cell colocalization approaches, such as dual-label FISH, would be required to more definitively establish cell-type specificity. Additional tissue material was unavailable for further analysis.

Discussion

We report an *NRAS* activation mutation in a CCLA and a *PPFIBP1::ROS1* fusion in a GSD. While *NRAS* mutations have been previously described in the majority of KLA cases, this is the first report of such

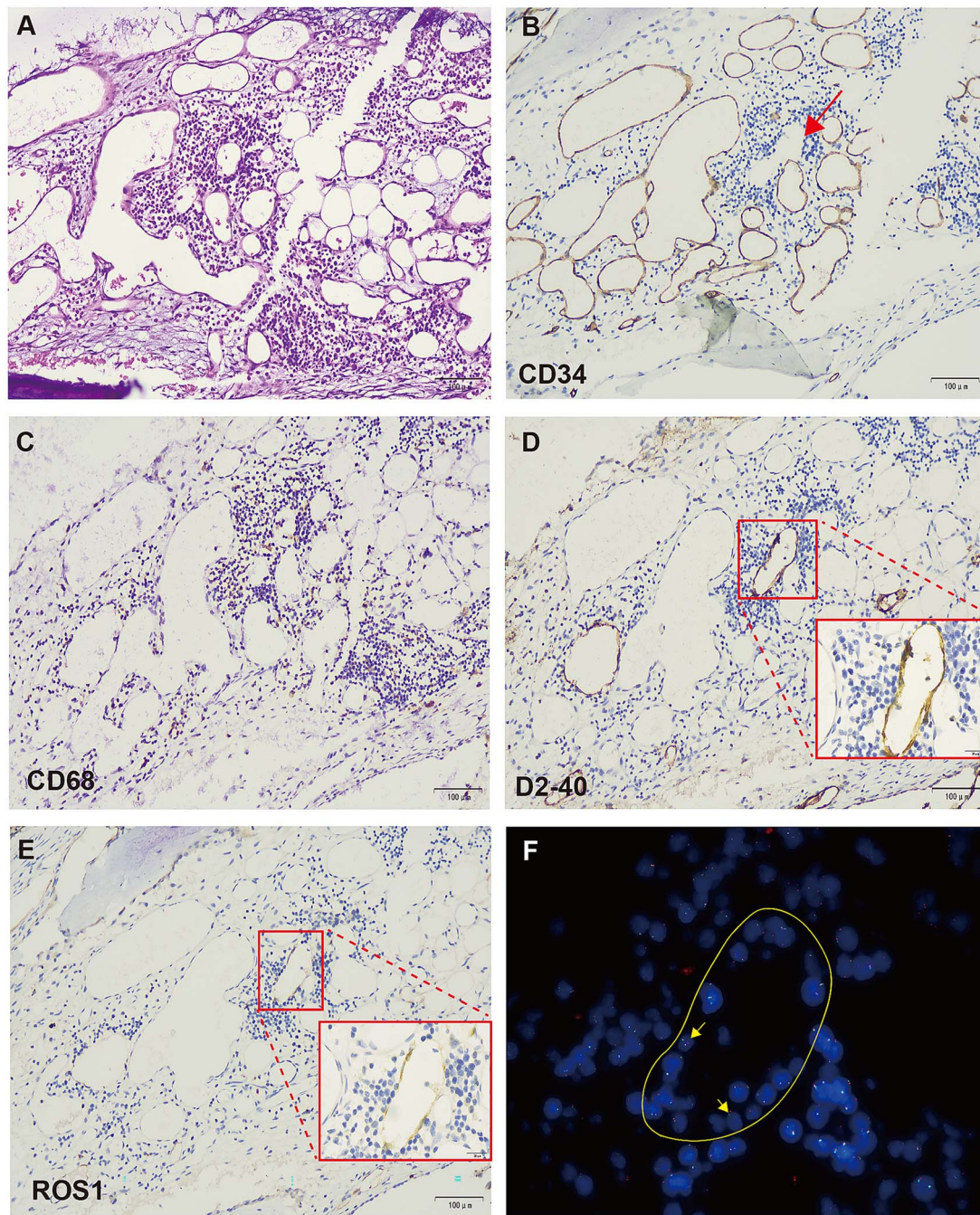


Figure 3 In situ localization of *ROS1* rearrangement in lymphatic endothelial cells in a rib biopsy from patient 1 (GSD). Serial sections were analyzed by H&E staining (A), CD34 IHC for vascular endothelial cells (B), and CD68 IHC for macrophages/osteoclasts (C). The arrow in (B) indicates the region of interest, which is negative for CD34 but positive for D2–40 (D), confirming its lymphatic endothelial cell origin. These lymphatic endothelial cells are positive for *ROS1* (E) and contain *ROS1* rearrangements by FISH (F; split-apart signals indicated by arrowheads).

a mutation in *CCLA*. Similarly, although the *PPFIBP1::ROS1* fusion has been identified in GLA [11], this is the first time it has been observed in GSD. These findings raise several intriguing questions on LMs:

Do all LMs share fundamental molecular abnormalities?

To date, genes mutated in LMs include *PIK3CA*, *NRAS*, *KRAS*, *BRAF*, *ARAF*, *EPHB4*, *PTEN*, *EML4::ALK*, and *PPFIBP1::ROS1* fusion [1, 8, 13–

16]. These genes involve two major signaling pathways: the PI3K–AKT–mTOR pathway and the RAS–MAPK pathway. Interestingly, the same gene mutations have been reported across different LM subtypes [15]. For example, *PIK3CA* mutations, predominantly seen in isolated LMs, are also frequently found in GLA [4, 17]. *EML4::ALK* fusions have been identified in both GSD and GLA [8], and the *PPFIBP1::ROS1* fusion, with identical breakpoints to those found in our GSD case, was previously reported in a GLA patient [11]. These observations suggest that the molecular pathogenesis of LMs is likely shared across different subtypes, despite their clinical and anatomical diversity.

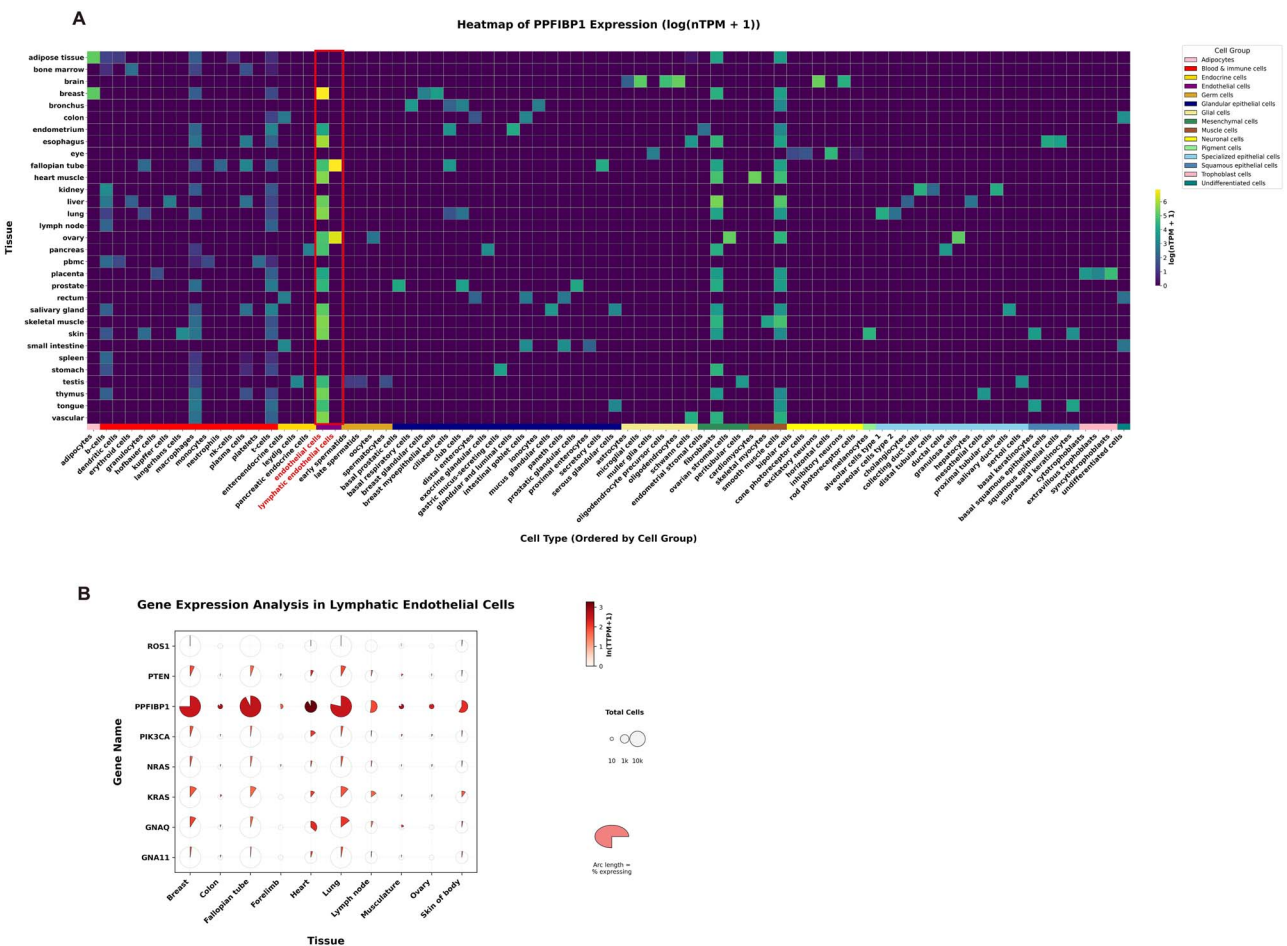


Figure 4 Analysis of publicly available single-cell RNA sequencing data for lymphatic malformation–related gene expression. (A) Heatmap demonstrating the highest expression of *PPFIBP1* (logTPM +1) in endothelial cells across multiple tissues and cell types (box). (B) Circular plot illustrating expression levels of genes commonly altered in GSD (*PTEN*, *PPP1R1B*, *PIK3CA*, *NRAS*, *KRAS*, *GNAQ*, *GNA11*, and *ROS1*) in 10 tissue types. Arc length represents the percentage of expressing cells, while the color gradient (0–3 TPM) indicates expression intensity.

Why do the same mutations cause different types of LMs?

Several mechanisms may contribute to this phenomenon. One key factor is the developmental timing of the mutation [18]. Early embryonic mutations may affect a large pool of progenitor cells, potentially resulting in complex LMs with multi-organ involvement. In contrast, later-occurring mutations are likely to affect a more localized cell population, leading to isolated LMs. Another important factor is the anatomic location and tissue microenvironment. Local cues, such as extracellular matrix composition or growth factor availability, could influence how mutant endothelial cells behave and differentiate, thereby shaping the final phenotype. A striking feature of these disorders is the wide range of variant allele frequencies (VAFs) reported, from less than 1% to as high as 35% [19]. This variability likely reflects differences in mosaicism and mutation burden, which may influence both the severity and type of LM that develops.

Why do mutations occur in endothelial cells?

Our case involving the *PPFIBP1::ROS1* fusion supports localization of the somatic rearrangement to lymphatic endothelial cells. Somatic mutations primarily arise during DNA replication in the S phase

of the cell cycle, with an estimated 1–10 new mutations per cell division. During development, the lymphatic system undergoes substantial expansion, with total lymphatic vessel length and surface area increasing dramatically from infancy to adulthood. This growth requires ongoing division and proliferation of lymphatic endothelial cells, providing ample opportunities for the accumulation of somatic mutations over time.

An interesting phenomenon is observed in cases of GSD following bone fractures. Unlike non-trauma-associated GSD, these cases are typically localized to the site of injury or adjacent bones. Patients with post-injury GSD also tend to be older (average age of 29.6 years) compared to those with non-injury-related GSD (average age of 14.3 years). This suggests that somatic mutations may occur in endothelial cells during bone healing, a process that involves lymphangiogenesis and tissue remodeling, potentially triggering localized pathological lymphatic proliferation [6].

The role of PPFIBP1 in the activation of PPFIBP1::ROS1

PPFIBP1 (PTPRF Interacting Protein, Binding Protein 1) is a scaffold protein that anchors protein complexes at focal adhesions and cell–cell junctions. It interacts with integrins and cytoskeletal components,

stabilizing adhesion sites across various cell types, including endothelial and neuronal cells. Knockdown of *PPFIBP1* disrupts lymphatic structures in *Xenopus* tadpoles, highlighting its role in lymphangiogenesis. Additionally, *PPFIBP1* regulates cell migration by assembling signaling complexes involving FAK (focal adhesion kinase), Src, and JNK pathways. It also binds to PTPRF (LAR), modulating tyrosine phosphorylation and downstream signaling, including growth factor signaling and cytoskeletal dynamics.

The functional domains of *PPFIBP1* include an N-terminal Coiled-Coil Domain for multimerization and complex assembly, and three C-terminal SAM (Sterile Alpha Motif) Domains for protein interactions. In the *PPFIBP1::ROS1* fusion, the N-terminal portion of *PPFIBP1* including the Coiled-Coil Domain is retained, whereas the SAM Domains lie distal to the breakpoint (intron 15) and are not incorporated into the fusion protein. A common mechanism in the activation of kinases from rearranged tyrosine kinase fusion proteins involves multimerization domains contributed by fusion partners. These domains enable ligand-independent dimerization of the chimeric fusion protein, bringing the kinase domains into close proximity, which leads to cross-autophosphorylation and activation. Thus, the Coiled-Coil Domain of *PPFIBP1::ROS1* likely contributes to kinase activation through ligand-independent dimerization. Furthermore, while *ROS1* is not typically expressed in lymphatic endothelial cells, *ROS1* protein expression was observed by IHC in lymphatic endothelial cells of the GSD lesion (Fig. 3E). Although our clinical and genomic data support an association between the *PPFIBP1::ROS1* fusion and the GSD phenotype, without functional validation, its precise pathogenic role remains hypothesis-generating. Future studies utilizing *in vitro* or *in vivo* functional assays are necessary to determine its pathogenic contribution.

Targeted therapy for LMs

Genomic alterations in LMs lead to activation of the PI3K–AKT–mTOR and RAS–MAPK signaling pathways. Thanks to advances in cancer research, several FDA-approved drugs targeting these pathways are now available. Clinically, multiple case reports have demonstrated therapeutic benefit from inhibitors directed at either the PI3K–AKT–mTOR or RAS–MAPK pathways [6, 10, 19–23]. An important question arises when considering rearranged tyrosine kinases including *PPFIBP1::ROS1*: Could selective inhibition of the *ROS1* kinase provide effective blockade of aberrant signaling while minimizing off-target effects? Many growth factor-related pathways converge on PI3K–AKT–mTOR and RAS–MAPK signaling, so broad inhibitors may also impair normal developmental signaling, raising concerns about potential developmental side effects, especially in pediatric patients, who represent the majority of LM cases. In this context, clinically available kinase inhibitors that specifically target *ROS1* or *ALK* may offer a more precise therapeutic approach, with the potential to suppress pathological signaling while better preserving normal physiological processes.

Materials and methods

Patient cohort and sample collection

This study included pediatric patients diagnosed with complex LMs, including GSD, KLA, and CCLA. Biopsied tissue samples were obtained from affected sites for downstream analysis. The study was conducted

in accordance with ethical guidelines, and informed consent for publication was obtained from the legal guardians.

Cytochemistry

Tissue specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin, and cut into 3–5 μm sections for analysis. A subset of sections was stained with Hematoxylin and Eosin (H&E) for morphological assessment of tissue architecture and cellular infiltration. For immunohistochemistry, sections were deparaffinized, rehydrated, and underwent antigen retrieval. Slides were then incubated with primary antibodies against CD31, CD34, the lymphatic endothelial marker D2–40, and *ROS1*. A secondary antibody conjugated to a detection polymer was used, followed by a chromogen. Slides were counterstained with Hematoxylin and analyzed under a brightfield microscope.

Next-generation sequencing (NGS) and bioinformatic analysis

Genomic DNA was extracted from FFPE tissue specimens from the three patients using the AllPrep DNA/RNA FFPE Kit (Cat. 80 234, Qiagen, Germany). This protocol utilizes optimized lysis conditions and a high-temperature incubation to partially reverse formaldehyde modification of nucleic acids. Following extraction, genomic DNA was physically fragmented using an ultrasonic disruptor (Bioruptor Pico, Diagenode). DNA libraries were constructed using the VAHTS High-Efficiency DNA Library Prep Kit (Cat. ND612–02, Vazyme, China) and subsequently subjected to targeted sequencing via hybridization capture with NadPrep ES Hybrid Capture Reagents (Cat. 1 005 401, Nanodigm, China) and custom-designed target-specific probes. The enriched libraries were then sequenced on an Illumina platform. The bioinformatic analysis began with raw data quality control using **fastp (v0.23.2)** to remove adapters and low-quality reads. The cleaned reads were then aligned to the **hg19 reference genome** using **BWA-mem (v0.7.17)**. The resulting BAM files were sorted, and duplicate reads arising from PCR amplification were marked and removed using **sambamba (v0.8.2)**. Base quality scores were subsequently recalibrated using **GATK's (v4.2.0.0)** BaseRecalibrator and ApplyBQSR tools. Single nucleotide variants (SNVs) and short insertions/deletions (indels) were called using **VarDict (v1.8.2)** and annotated with **ANNOVAR**. Structural Variants (SVs), including the *PPFIBP1::ROS1* fusion, were detected using **seeksv**, an algorithm specifically designed to identify structural rearrangements by evaluating split-read alignments and discordant paired-end reads.

Validation of genetic alterations

To validate the expression of the alterations identified by NGS, total RNA was first reverse transcribed into cDNA using the **SuperScript™ III Reverse Transcriptase kit (ABI)** according to the manufacturer's protocol. Gene fusions were subsequently confirmed by RT-PCR amplification followed by Sanger sequencing of the products to determine the exact fusion junction. Point mutations were validated and quantified using a droplet digital PCR (ddPCR) assay on a QX200 system (Bio-Rad) with target-specific primers and probes. The analytical sensitivity and estimated limit of detection (LoD) of this ddPCR assay were approximately 0.08%.

Fluorescence In situ hybridization (FISH)

To confirm gene rearrangements at the chromosomal level, FISH was performed on 4 μ m thick FFPE tissue sections. Following baking, slides were deparaffinized in xylene and rehydrated through a graded ethanol series. Tissue sections then underwent pre-treatment involving boiling in water and digestion with a pepsin solution. After dehydration, a specific break-apart probe for the *ROS1* gene was applied, and the slides were co-denatured and hybridized overnight. Following stringent post-hybridization washes, nuclei were counterstained with DAPI. Slides were analyzed using an Olympus BX43 fluorescence microscope, where non-overlapping tumor cell nuclei in the target region were scored by two independent analysts.

Single-cell gene expression analysis

To investigate the cell-type-specific biological context of the identified fusion, the expression profiles of *PPFIBP1* and other GSD-related genes were analyzed using publicly available single-cell RNA-sequencing (scRNA-seq) datasets. Cell-type-resolved expression data for *PPFIBP1* across normal human tissues were obtained from the Human Protein Atlas Single Cell Type resource (<https://www.proteinatlas.org/>). Expression profiles of LM-associated genes in lymphatic endothelial cells across organs were retrieved from the CZ CELLxGENE Discover Gene Expression portal (<https://cellxgene.cziscience.com/gene-expression>). These reference datasets were derived from publicly available normal human tissues rather than from the patients' own cells.

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Author contributions

Guangxian Yang (Conceptualization, Investigation, Writing—original draft, Writing—review & editing), Haoran Ren (Methodology, Writing—original draft, Writing—review & editing), Jinghua Wang (Investigation, Writing—review & editing), Weijian Chen (Investigation, Writing—review & editing), Xiaoming Li (Investigation, Writing—review & editing), Si Chen (Methodology, Writing—review & editing), Huafei Chen (Methodology, Writing—review & editing), Lina Zhao (Methodology, Writing—review & editing), Wenwen Fan (Conceptualization, Supervision, Writing—review & editing), Sheng Xiao (Conceptualization, Supervision, Writing—review & editing).

Supplementary material

Supplementary material is available at *Human Molecular Genetics* online.

Declaration of interests

The authors declare no conflict of interest, financial or otherwise.

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Data availability

The data and materials of this study will be available from the corresponding author upon reasonable request.

Standard of reporting

CARE guidelines were followed.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Affiliated Children's Hospital of Xiangya School of Medicine (Hunan Children's Hospital), Central South University (Approval No. HCHLL-2026-244). The study was conducted in accordance with the Declaration of Helsinki. Written informed consent to participate was obtained from the legal guardians of all pediatric patients.

Consent for publication

Written informed consent for publication of the clinical, imaging, histologic, and genomic data presented in this manuscript was obtained from the legal guardians of all pediatric patients.

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