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*Original Article*

# Upregulation of the Four and a Half LIM Domains 1 linked with familial venous dysplasia in a familial genetic examination

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**Abstract:** Background: This study aimed to analyze the mutation site in a family diagnosed with venous dysplasia to identify possible pathogenic genes. Methods: A 15-year-old female presented with lower extremity venous tortuosity aggravated by ulceration. Only the young sister exhibited similar symptoms within the immediate family of the proband. Whole genome sequencing (WGS) was used to evaluate the mutation sites and chromosome copy number variations (CNV) within the family. The possible pathogenic genes located in the region with CNVs were identified, and the expression of the possible pathogenic genes was verified via quantitative polymerase chain reaction (Q-PCR) and western blotting (WB) analysis. In-vitro models were used to verify the role of possible pathogenic genes linked with the development of venous dysplasia. Results: The high-resolution karyotype analysis of the chromosomes found no abnormalities. The results of the WGS indicated that the proband and her sister shared the CNV events, including a microdeletion on chromosomes X: 13580000-1358555000 and microduplications of chromosome X: 136055000-136290000, chromosome X: 136475000-13671000. The results of the Q-PCR and WB showed that FHL1 was highly expressed in the proband and her sister, indicating that mutations of the FHL1 may have an important role in the development of vein malformations. The results of the in vitro experiments showed that FHL1 overexpression could inhibit venous development. Conclusion: The CNV in the Xq26 region (136054501-136288300) was found to be linked with the development of venous malformations in this family. However, further studies are required to evaluate the genetic mechanisms involved in the development of venous malformations.

**Keywords:** Whole genome sequencing, copy number variation, FHL1, venous development

## Introduction

Venous dysplasia is a rare condition characterized by the abnormal development of veins that can lead to swelling, pain, and disfigurement [1, 2]. These malformations may cause functional impairment and limit a person's ability to perform everyday activities. Additionally, the psychological impact of visible deformities and disfigurement can cause significant mental distress for patients and may affect their quality of life [3, 4]. There is no definitive cure for venous dysplasia. The current treatment options for venous dysplasia aim to control the symptoms of the disease and may include medications to relieve pain and swelling, compression therapy, and surgery to remove or repair the affected veins [5]. Although numerous studies have been conducted on venous dysplasia, its etiology is still poorly understood [6]. Therefore, there is a need to understand the pathogenesis of this disease to develop appropriate therapeutic interventions. Previous studies [7] showed that the Four and a Half LIM Domain 1 (FHL1) gene may inhibit the Hypoxia-Inducible Factor 1 (HIF1) protein transcriptional activity and HIF1-mediated Vascular endothelial growth factor (VEGF) expression by blocking the heterodimerization of HIF1A to HIF1B. This process is necessary for the recruitment of VEGF pro-

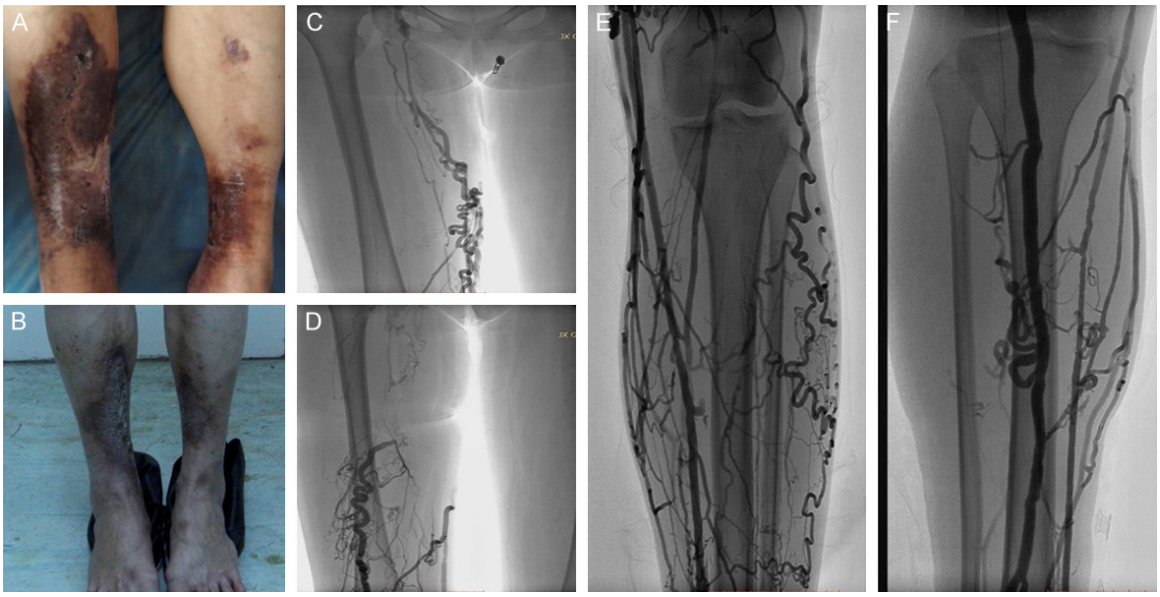


**Figure 1.** Images of the proband and her sister. A. Photograph of the proband illustrating bilateral lower extremity ulcers. B. Photograph of the proband's sister illustrating bilateral lower extremity ulcers. C. CTV of the proband showing bilateral lower extremity deep veins absence. D. CTV of the proband's sister with bilateral lower extremity deep veins absence. E. CTV of the proband with bilateral lower extremity deep veins absence. E. CTV of the proband with venous dysplasia in both lower limbs. F. CTV of the proband's sister with venous dysplasia in both lower limbs.

moters. Zhou and colleagues [8] transfected Hepg2 cells with VEGF-Luc reporter gene, SMAD Family Member 4 (SMAD4) and FHL1, or SMAD4 and FHL1 siRNA, and found that the overexpression of FHL1 and SMAD4 synergistically inhibited VEGF promoter activity, mRNA expression, and secretion. On the other hand, the down-regulation of endogenous SMAD4 and FHL1 had the opposite effect. Furthermore, the reduction of endogenous SMAD4 abrogated the FHL1-mediated inhibition of VEGF promoter activity. These findings suggest that FHL1 and SMAD4 may co-regulate the VEGF signaling pathway and affect angiogenesis and development.

Genetic studies have shown that venous malformations could be linked to rare congenital mutations [9]. In this case study, we evaluated a rare case of two sisters simultaneously diagnosed with venous dysplasia. Both sisters presented with recurrent lower extremity venous ulcers with superficial venous tortuous dilatations. A magnetic resonance image (MRI) examination of the lower limb confirmed the absence of the femoral and iliac veins. These findings suggested that this condition was hereditary.

Next-generation sequencing technologies such as whole genome sequencing (WGS) and quantitative polymerase chain reaction (Q-PCR) have revolutionized genetic research by allowing researchers to analyze large amounts of genetic data quickly and efficiently [10]. Therefore, in this study, we aimed to conduct Q-PCR and WGS of the 2 sisters and their immediate family to identify the mutation sites and copy number variations (CNV) linked with the development of their venous dysplasia. In addition, in vitro models were used to verify the role of specific genetic variants or mutations on vascular development and function.

## Materials and methods

### *Presentation of the disease*

The proband was 22 years old at the time of this study. The woman first presented with lower extremity venous tortuosity at age 15, aggravated by ulceration (**Figure 1A**). The diagnosis of venous dysplasia is based on a thorough history, physical exam, and imaging examination including; computed tomography venogram (CTV) greyscale, color doppler and spectral ultrasound (US) or magnetic resonance imaging (MRI). CTV showed extensive pelvic varices, bilateral thickening of the ascending lumbar veins, and the absence of the deep veins in both legs (**Figure 1C**). In addition, the

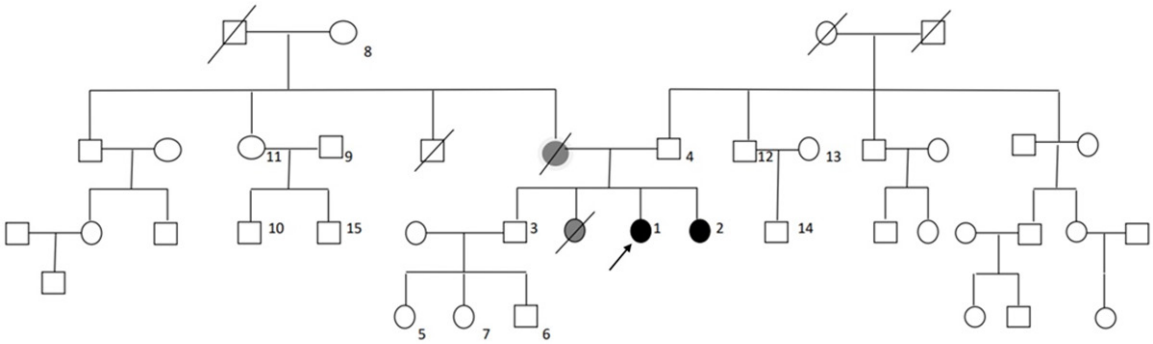


**Figure 2.** Pedigree of the family with venous dysplasia. The arrow indicates the proband. The circles denote the female relatives, and the squares denote the male relatives. The black and white symbols represent the clinically affected and unaffected individuals, respectively. The grey symbol indicates the subject with minor signs of the disease.

**Table 1.** Clinical characteristics of the proband and her family members

| Subject | Actual relation to the patient | Sex | Age at diagnosis/ investigation | Clinically affected and unaffected individual |
|---|---|---|---|---|
| 1 | Younger sister | F | 21 | + |
| 2 | Proband | F | 25 | + |
| 3 | Elder brother | M | 31 | - |
| 4 | Father | M | 42 | - |
| 5 | The brother's offspring | F | 8 | - |
| 6 | The brother's offspring | M | 1 | - |
| 7 | The brother's offspring | F | 3 | - |
| 8 | Grandmother | F | 80 | - |
| 9 | The aunt's husband | M | 45 | - |
| 10 | The aunt's offspring | M | 12 | - |
| 11 | Aunt | F | 44 | - |
| 12 | Uncle | F | 52 | - |
| 13 | The uncle's wife | M | 50 | - |
| 14 | The uncle's offspring | M | 25 | - |
| 15 | The aunt's offspring | M | 22 | - |

pelvic, upper and lower extremities, chest wall, and superficial pelvic wall veins were compressed to compensate for the tortuous dilatation (**Figure 1E**). The younger sister of the proband had similar symptoms (**Figure 1B**, **1D**, **1F**). The proband's maternal grandmother, father, uncle, spouse, elder brother, and the brother's offspring did not present with the same symptoms. According to information provided by the proband and her family, the mother of the proband had a history of both lower extremity venous tortuosity and ulcer formation. However, she was never officially diagnosed with congenital venous dysplasia and was treated in a regular-level hospital. Unfortunately, the relevant medical records could not be obtained to confirm the diagnosis. The pedigree of the Proband family is illustrated in **Figure 2**. The clinical characteristics of the proband and family members are summarized in **Table 1**.

*High-resolution karyotype analysis of peripheral blood chromosomes*

About 3 to 5 ml of peripheral blood samples were collected from the proband, her younger sister, father, and elder brother. The supernatant was first isolated from the blood samples by centrifugation at 2000 per min. Five mL of the supernatant was cultured in a medium without lectin, 300 μl of thymine, and 50 μl of

ethidium bromide for 4 hours. Thereafter 300 μl of colchicine was added, and the cells were fixed, harvested, and used to prepare the high-resolution banded chromosome samples. A GSL120 full-automatic scanner was used to acquire high-resolution images of the banded chromosome samples. The karyotype analysis software (VideoTesT-Karyo 3.1) was used to analyze 7 karyotypes in 20 cells in accordance with the International System of human cytogenetics nomenclature (2016).

*Peripheral blood whole genome sequencing and analysis*

The venous blood samples of the proband (1), the proband's sister (2), and their family members (3-15) were collected (**Figure 2**). The genomic DNA of the peripheral blood was extracted using the DNA extraction kit (Qiagen Company, Germany) under aseptic conditions. The extracted DNA was then broken down by ultrasound and blunt ends by end repair enzyme. Subsequently, the DNA was added to the 'A' tail and linker to construct the sequencing library. The samples were quantitatively controlled. Several libraries were sequenced using the index mark and BGISEQ-500 sequencing platform. The obtained sequence fragments were aligned with the human reference genome (GRCH37/HG19) for data analysis. The single nucleotide polymorphism (SNP) insertions or deletions (Indel) were filtered to identify the high-quality variants most likely to be true positive mutations. A single nucleotide variant (SNV) Indel analysis of the filtered variants was performed to identify the common mutations in the proband and her sister. A CNV kit (https://cnvkit.readthedocs.io/en/stable/) was used to investigate and locate the unique CNV events in the proband and his sister for each chromosome. The significance of the identified CNV events was assessed by comparing them with those available in the human genome of chromosomal imbalance and phenotype in humans using Ensembl resources (DECIPHER, https://www.deciphergenomics.org), University of California SANTA CRUZ database (UCSC database, https://genome.ucsc.edu), the Online Mendelian Inheritance in Man Database (OMIM, https://www.omim.org), the Database of Genomic Variants (DGV, https://ngdc.cncb.ac.cn/databasecommons/), and the database of chromosomal imbalance and phenotype in humans. The possible biological functions of all the genes in the chromosome duplication and deletion regions were analyzed, and the pathogenic genes that might lead to abnormal vascular development were identified.

*Q-PCR and WB analysis*

The venous blood samples of the proband (1), the proband's sister (2), and their family members (3-15) were collected (**Figure 2**). The genomic DNA of the peripheral blood was extracted with a DNA extraction kit (Qiagen Company, Germany) under aseptic conditions. The Has21 and Xq26 primers were designed using HBB as the reference gene. Subsequently, Q-PCR was performed to verify the possible pathogenic gene in the Xq26 chromosome. Similarly, the protein extraction kit (Qiagen Company, Germany) was used to extract the total peripheral blood proteins under aseptic conditions. The possible pathogenic genes in the XQ26 repeat region were verified by WB analysis.

*Analysis of genes linked with endothelium angiogenesis in vitro*

Based on the CNV analysis, the effect of genes linked with endothelial angiogenesis was studied on HUVEC-T1 cells in vitro. After the HUVEC-T1 cells were Lentiviral transfected with the gene, the migration and invasion of HUVEC-T1 cells were detected, and the endothelium angiogenesis ability of HUVEC-T1 cells was observed.

*Statistical analysis*

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software version 22.0. The continuous variables were compared using the t-test or the non-parametric Mann-Whitney U test, as appropriate. A *p*-value below 0.05 was deemed statistically significant.

## Results

*Chromosome high-resolution karyotype*

The chromosome high-resolution karyotype analysis revealed no abnormal karyotypes in the proband (**Figure 3A**) and her sister (**Figure 3B**).

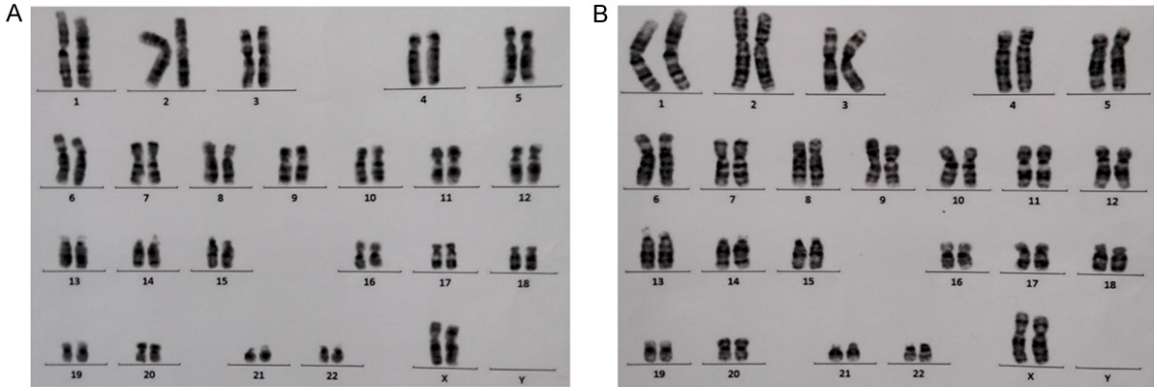


**Figure 3.** The chromosome high-resolution karyotype analysis did not indicate any abnormal karyotypes. (A) Proband karyotype analysis, (B) proband's sister karyotype analysis.

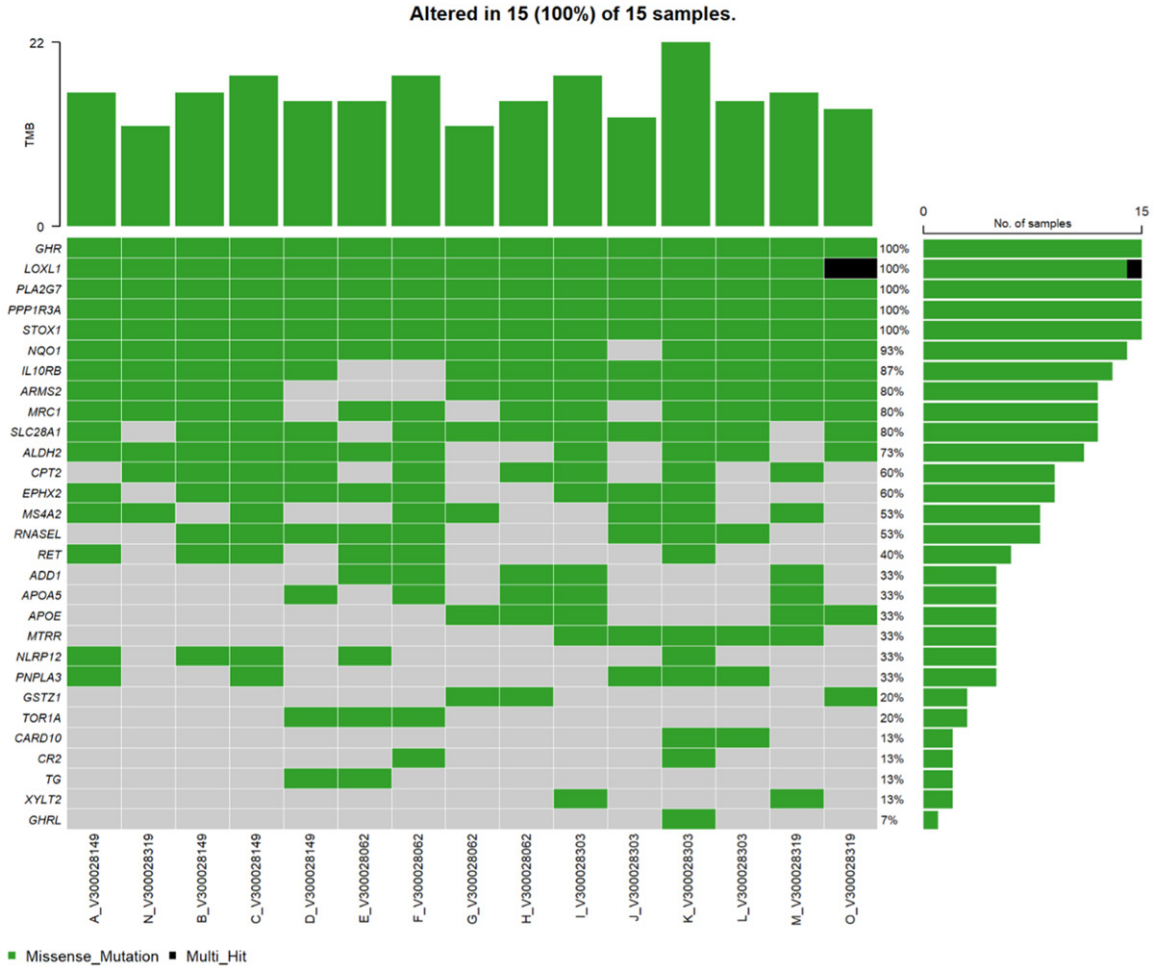


**Figure 4.** Single Nucleotide Variant/Insertion-Deletion (SNV/INDEL) results showed that no common variant site was shared by the proband (A), the proband's sister (N), and the other family members.

*SNV/INDEL results*

The mutation annotation format (MAF) files for the sequencing analysis of all samples were merged to visualize the variant sites present in all samples and the variant sites shared by the proband and her sister (**Figure 4**). The results of the SNV/INDEL analysis did not identify any

common variant site between the proband, the proband's sister, and the other family members.

*CNV results*

**Figure 5** illustrates the results of the CNVkit in the genome IGV browser for the proband, the proband's sister (N), and the other family members. The CNV analysis revealed CNV events in the x-stained region 26 band in the 2 sisters but not the other family members.

In addition, in the local view, 3 CNV events were identified in the proband and her sister in the X chromosome 2q6 region. These events included a microdeletion at chr X: 13580000-135855000 and chr X: 136055000-136290000 and a microrepeat at chr X: 136475000-13671000 (**Figure 6A**, **6B**). After comparing the results with the UCSC database, this region was linked with FHL1, MAP7D3, BRS3, HTATSF1, VGLL1, CD40LG, and ARHGEF6 genes. None of the family members with the normal phenotype (3-15) had the above variations (**Figure 6C**). The functional analysis of the gene in the CNV region identified the FHL1 gene as having a key role in vascular development and was therefore analyzed further.

*Q-PCR and WB analysis of the FHL1 gene*

The Q-PCR and WB analysis showed that the FHL1 gene was significantly upregulated in the proband and her sister (**Figure 7**).

*FHL1 gene overexpression on angiogenesis in the in vitro model*

The Huvec-T-FHL1 KI cell line was successfully constructed by transfecting the FHL1 gene into HUVEC-T1 cells. The tube-formation experiments showed that the HUVEC-T-FHL1KI cell line had a significant reduction in the formation of branch lengths, meshes, nodes, and junction tube points compared with the control group (**Figure 8A**, **8B**). The HUVEC-T1 cells transfected with the FHL1 gene significantly reduced the cell migration ability compared to the control group. These findings were further confirmed by the transwell experiments (**Figure 8C-E**), which also revealed a significant reduction in the migration ability of the HUVEC-T1 cells transfected with the FHL1 gene compared with the control group. The WB experiments showed that the vascular endothelial growth factor (VEGF-65) expression in the Huvec-T-FHL1 KI cell line was significantly down-regulated (**Figure 9**).

## Discussion

The pathogenesis of vascular dysplasia is still poorly understood. In this case study, we reported a rare case of familial inherited vascular dysplasia in 2 sisters. In order to identify the genes that could be linked with this disease, we used WGS to locate the mutation sites and the CNV events in the proband, the proband's sister, and other family members [11]. Our findings showed that the proband and her sister had common CNV events in chromosome 2q6 (chr X: 135800000-135855000 microdeletions, chr X: 136055000-136290000 and chr X: 136475000-136710000 microreplication).

The XQ26 region on the X chromosome is a likely pathogenic (LP) disease-causing variant. Numerous studies reported that CNV within this region is linked with human growth, and mutations might lead to several diseases, such as macrosomia or microsomia [12]. Trivellin and colleagues described a case of a 4-year-old boy with developmental delay who was found to carry chromosome Xq26 microduplication [13]. However, the role of this mutation in the development of various diseases remains unclear [14]. Population studies show that more than 99% of benign CNVs are inherited, and most inherited CNVs are much smaller than 500 kb [15]. Moreover, most pathogenic copy number alterations consist of de novo mutations greater than 1 MB [16]. Although genetic CNVs may be pathogenic, they may also have incomplete penetrance and variable expression capacity [17]. The genetic mechanisms that influence the expression of traits also have different clinical implications [18]. Whether CNV causes an abnormal phenotype depends on several factors, including; gene size and content, previous evidence of pathogenic CNVs in the region, types of CNVs (deletion or duplication), genetic patterns, and their frequency in the healthy population [19].

The CNV size is a key factor leading to the development of the disease [20]. Larger CNV fragments are more likely to be classified as pathogenic [21]. CNVs with fragment sizes of about 900 KB were found in our study. However,

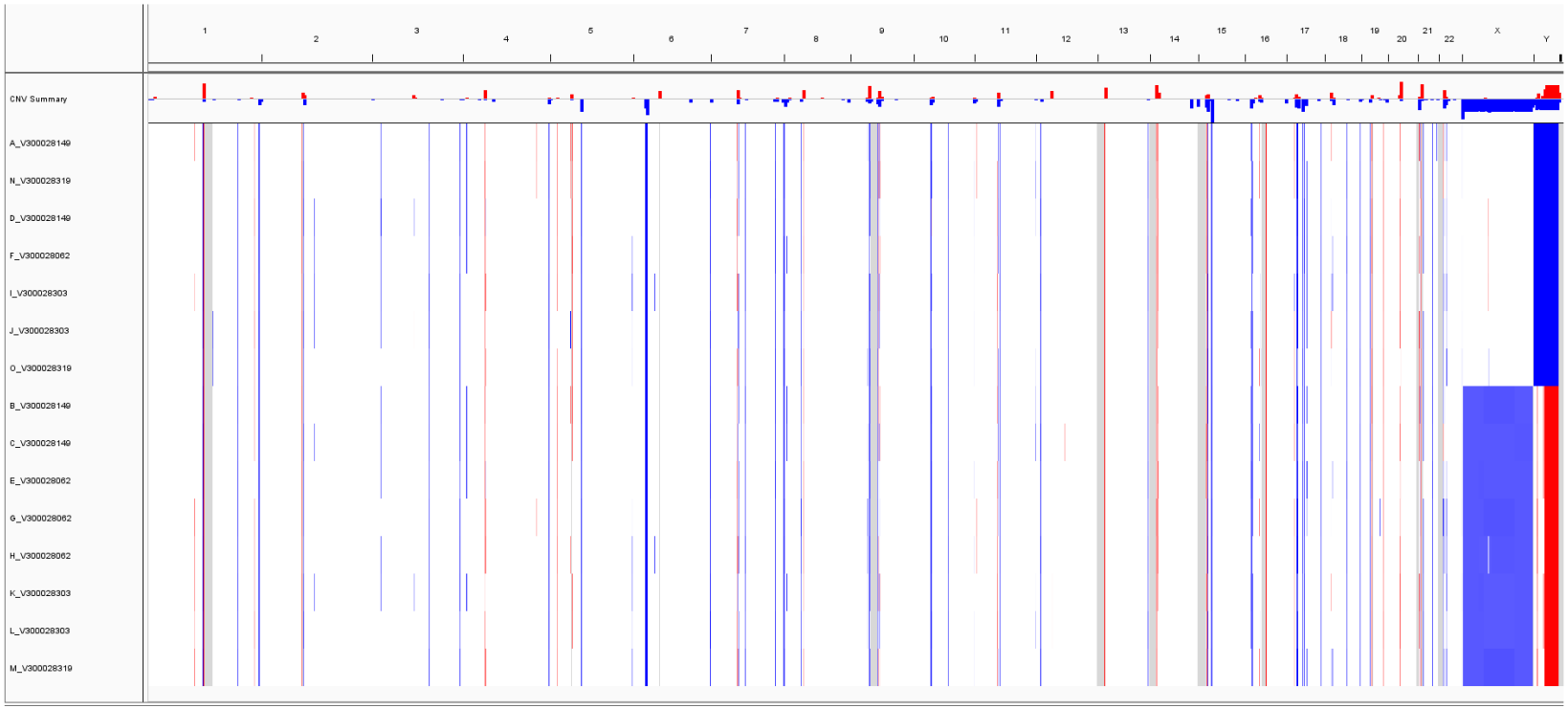


**Figure 5.** The Copy Number Variant (CNV) analysis revealed a CNV event shared by the proband and her sister but not by the rest of the family members.

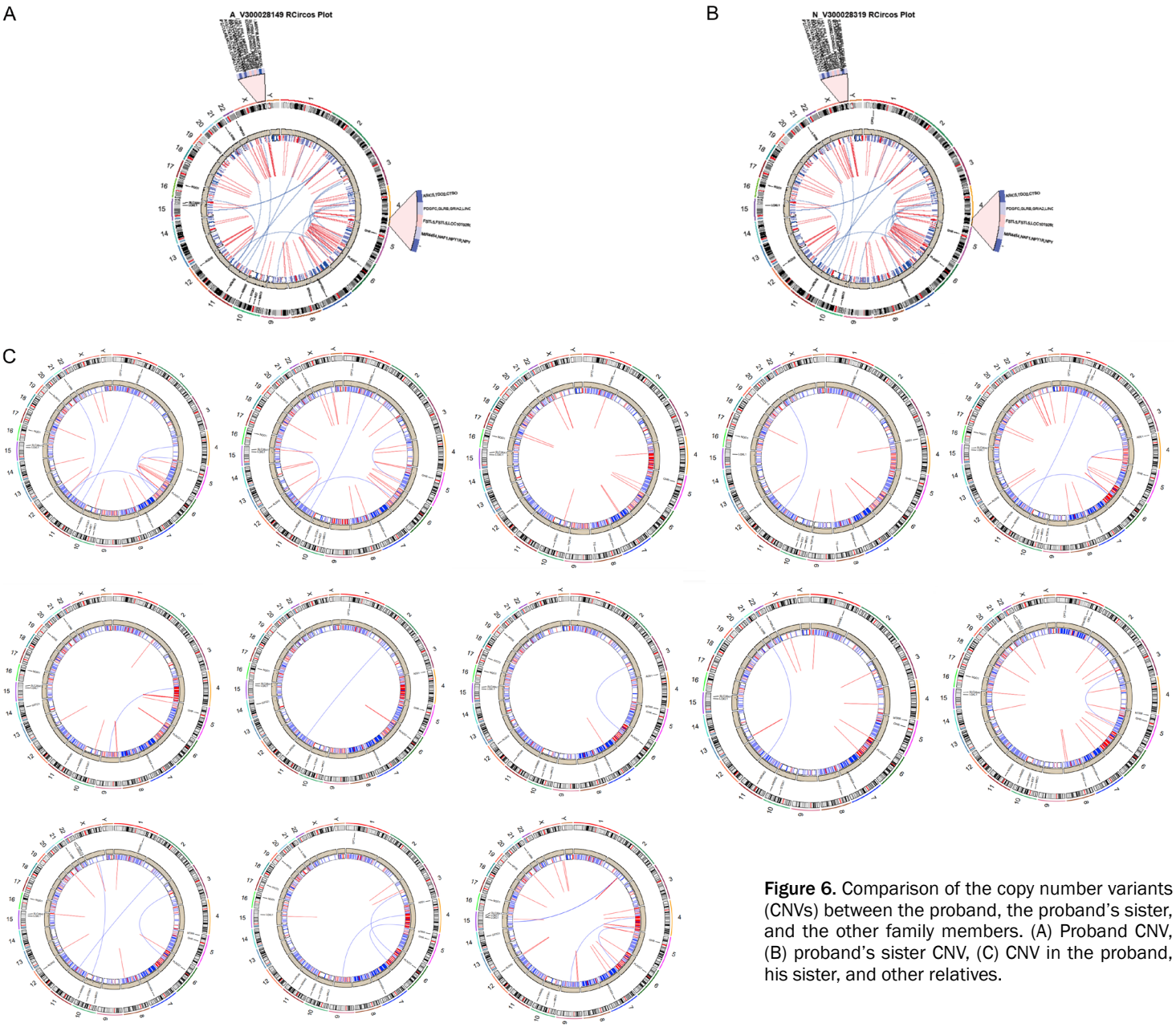


**Figure 6.** Comparison of the copy number variants (CNVs) between the proband, the proband's sister, and the other family members. (A) Proband CNV, (B) proband's sister CNV, (C) CNV in the proband, his sister, and other relatives.

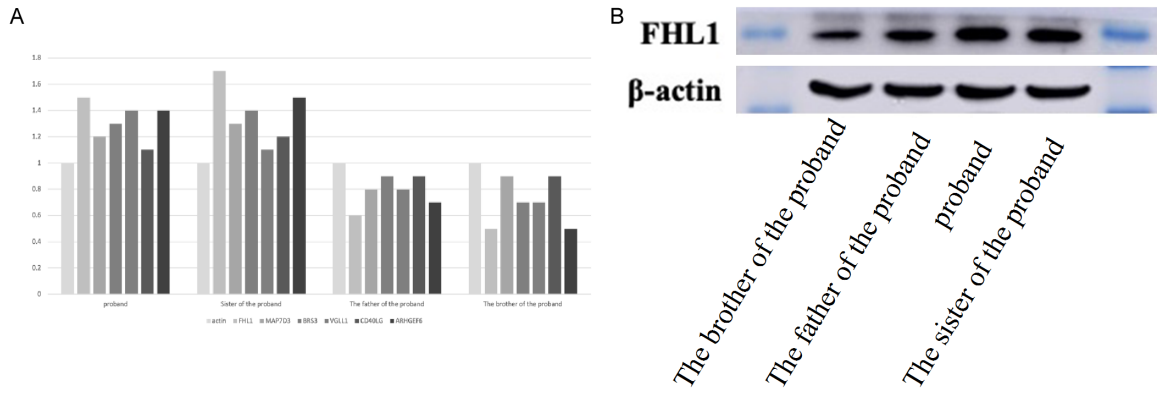


**Figure 7.** Results of the quantitative polymerase chain reaction (A) and Western blotting (B) analysis showing that the FHL1 gene was significantly upregulated in the proband and her sister.

after comparing these CNVs with the DECIPHER databases (DECIPHER, https://www.deciphergenomics.org), no CNVs in the Xq26 region were associated with vascular malformations in the lower extremities and vascular dysplasia [22].

The FHL1 gene mediates the interaction of transcriptional regulators, kinases, and structural proteins through the LIM domain and can influence cell differentiation and development [22]. The FHL1 protein is highly expressed in the skeletal and heart muscles and is associated with some skeletal muscle and heart diseases [23]. Recent studies have shown that the FHL1 protein regulates the activity of various transcription factors, including SMAD4 and HIF1 [24]. The FHL1 protein interacts with HIF1α and inhibits the HIF1α-induced VEGF promoter activity and the VEGF expression by blocking the HIF1α-HIF1β heterodimerization [7]. Previous studies have shown that FHL1 and SMAD4 have a synergistic transcriptional regulation effect on VEGF signaling [8, 25]. In our study, these CNV events were located in the FHL1 gene segment in both the proband and her sister, suggesting that the FHL1 gene may be involved in regulating vascular development. Based on these findings, we conducted Q-PCR and WB analysis to evaluate the function of the FHL1 gene in regulating vascular development. Our findings confirmed that the FHL1 gene was highly expressed in the two sisters. These findings were confirmed by the in-vitro analysis, which also showed that the overexpression of FHL1 could regulate the proliferation, migration, and capillary tube formation in HUVEC cells. Therefore, we speculated that the overexpression of FHL1 may lead to abnormal vascular development. Recent studies have shown that the FHL1 protein has been shown to regulate the activity of transcription factors, including SMAD4 and HIF1 [8, 26]. FHL1 interacts with HIF1α and inhibits HIF1α-induced VEGF promoter activity and VEGF expression by blocking HIF1α-HIF1β heterodimerization. In addition, FHL1 and SMAD4 were found to have a synergistic transcriptional regulation effect on VEGF signaling and may provide a new therapeutic target for VEGF signaling-related diseases [8]. Similarly, the expression profiles of FHL1 and P21 were found to be altered in PASMCs of HPH neonatal rats. Furthermore, FHL1 and P21 were found to have an important role in pulmonary vascular remodeling [27]. Consistent with our findings, these studies confirm that FHL1 may play an important role in the development of blood vessels. However, further research is required to understand the genetic mechanisms involved in the development of vascular dysplasia.

This study has some limitations that have to be acknowledged. Since congenital venous dysplasia is rare, and the disease only affected 2 sisters within the family evaluated in this study, we could not perform a large sample study to confirm these findings. In addition, we could not confirm the phenotypic effect of the FHL1 gene on vascular development with other studies. Further studies are required to understand the link between the FHL1 gene and the development of venous abnormalities.

## Conclusion

This report presented a rare case of 2 sisters diagnosed with congenital vascular dysplasia.

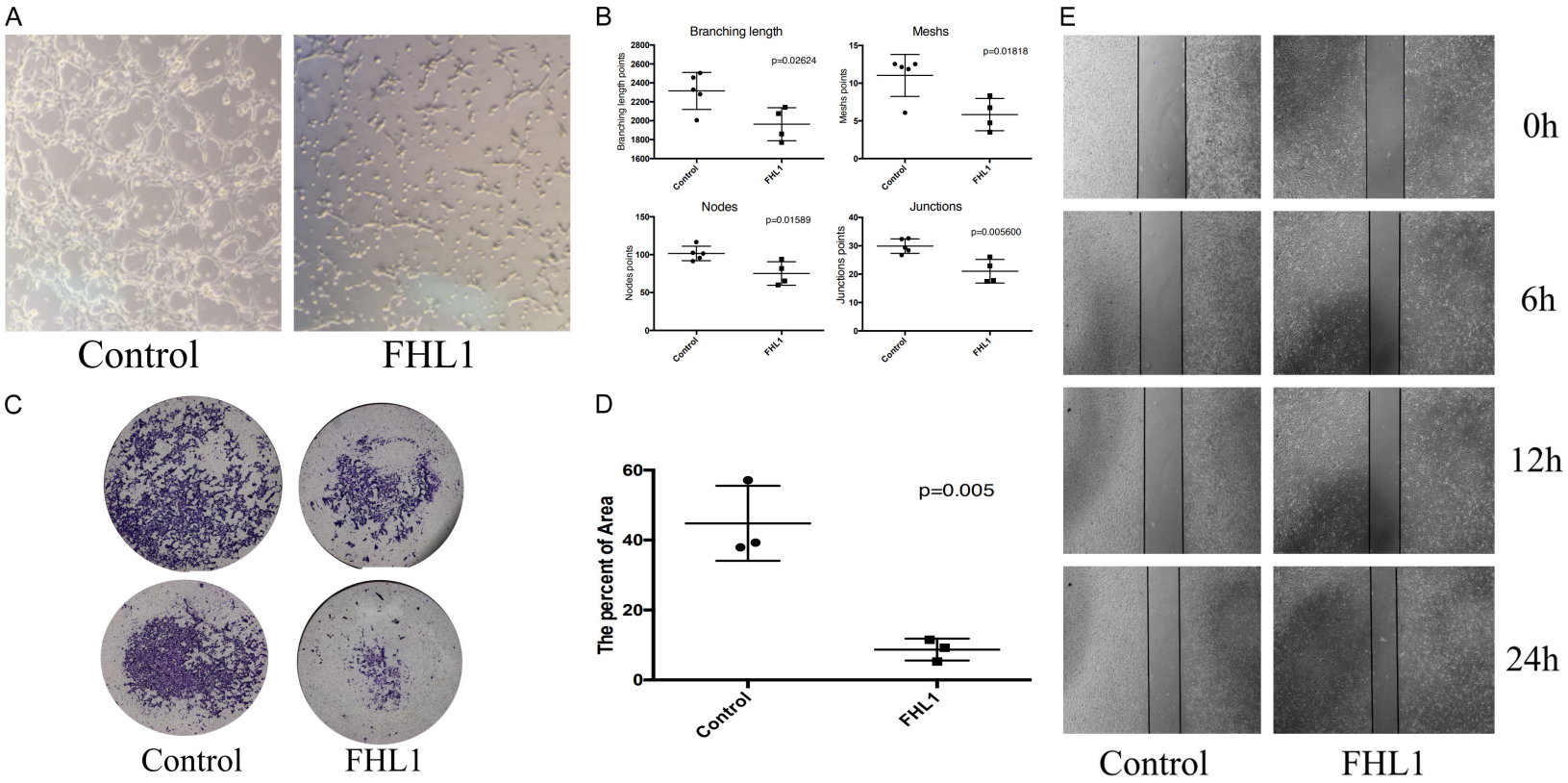


**Figure 8.** Results of the in vitro model for the control, Huvec-T-FHL1 KI (A, B) tube experiment, the control, the Huvec-T-FHL1 KI (C, D) transwell experiment, and the Huvec-T-FHL1 KI (E) migration experiment. All experiments showed that the overexpression of the FHL1 gene inhibited angiogenesis.

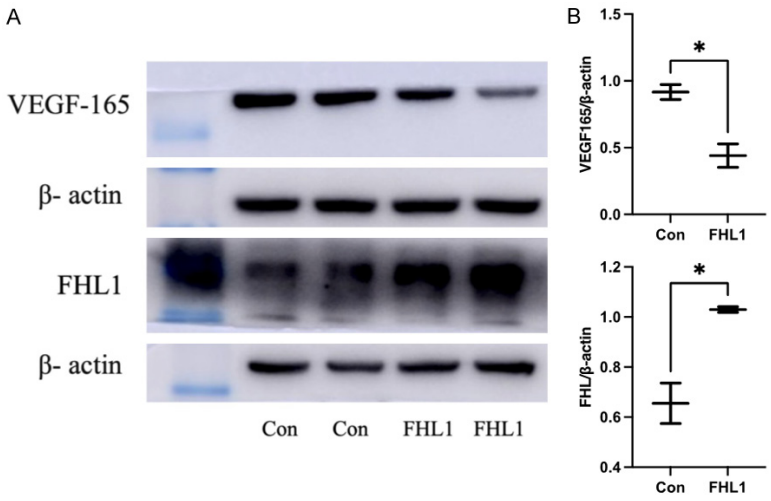


**Figure 9.** Control (Con) and Huvec-T-FHL1 KI western blotting results. A. The expression of the FHl1 gene in the Huvec-T-FHL1 KI was significantly higher than that of the control group. The expression of vascular endothelial growth factor-165 (VEGF-165) in the Huvec-T-FHL1 KI was significantly higher than in the control group. B. Results of the western blotting analyses for the VEGF-165 and FHL1 proteins. $^{*}P < 0.05$, n = 2 per group, statistical significance was determined by the unpaired t-test.

Through WGS analysis, we found that CNV in the Xq26 region (136054501-136288300) contributed to the development of venous malformations in this family. The functional analysis of the genes in the CNV region identified the FHL1 gene as having a key role in vascular development. The role of the FHL1 gene in developing vascular malformations was further confirmed by the in vitro experiments.

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## Disclosure of conflict of interest

None.

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