Janus-faced EPHB4-associated disorders: novel pathogenic variants and unreported intrafamilial overlapping phenotypes

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PURPOSE: Several clinical phenotypes including fetal hydrops, central conducting lymphatic anomaly or capillary malformations with arteriovenous malformations 2 (CM-AVM2) have been associated with EPHB4 (Ephrin type B receptor 4) variants, demanding new approaches for deciphering pathogenesis of novel variants of uncertain significance (VUS) identified in EPHB4, and for the identification of differentiated disease mechanisms at the molecular level.

METHODS: Ten index cases with various phenotypes, either fetal hydrops, CM-AVM2, or peripheral lower limb lymphedema, whose distinct clinical phenotypes are described in detail in this study, presented with a variant in EPHB4. In vitro functional studies were performed to confirm pathogenicity.

RESULTS: Pathogenicity was demonstrated for six of the seven novel EPHB4 VUS investigated. A heterogeneity of molecular disease mechanisms was identified, from loss of protein production or aberrant subcellular localization to total reduction of the phosphorylation capability of the receptor. There was some phenotype–genotype correlation; however, previously unreported intrafamilial overlapping phenotypes such as lymphatic-related fetal hydrops (LRFH) and CM-AVM2 in the same family were observed.

CONCLUSION: This study highlights the usefulness of protein expression and subcellular localization studies to predict EPHB4 variant pathogenesis. Our accurate clinical phenotyping expands our interpretation of the Janus-faced spectrum of EPHB4-related disorders, introducing the discovery of cases with overlapping phenotypes.

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INTRODUCTION

The EPHB4 gene encodes a receptor tyrosine kinase protein, EPHB4, that binds to its ligand EphrinB2, to initiate complex contact-dependent bidirectional signaling cascades, controlling cellular fate during embryonic angiogenesis and essential cellular processes such as adhesion, migration, and proliferation, in both blood and lymphatic endothelial cells.

We previously reported that monoallelic missense variants in the intracellular tyrosine kinase domain of EPHB4 cause a form of in utero primary lymphatic anomaly coined lymphatic-related fetal hydrops with/without atrial septal defect (LRFH) (OMIM 617300). The study included two families (GLD UK and GLD NOR) with a history of fetal hydrops and/or atrial septal defects (ASD). Supporting evidence from mouse models lacking Ephb4 expression during specific stages of development, or genetically modified to alter only the Ephb4 dependent forward signaling, show edema, blood filled lymphatic vessels with defective collector valves, and lymphovenous valves, which suggested the EPHB4-associated hydrops in the two families was caused by a lymphatic-related fault. Li et al. identified an in-frame insertion in EPHB4 in a family with a history of fetal hydrops and lymphovenous dysfunction characterized by edema of the lower extremities, venous stasis, and variable chylous effusions. This work confirmed the important role of EPHB4 in the function and development of the lymphatic system and the association of EPHB4 variants with fetal hydrops of lymphovenous origin.

Other research groups have reported that loss-of-function, monoallelic variants in the same gene can cause various vascular pathologies. Under the umbrella of capillary malformation–arteriovenous malformation 2 (CM-AVM2) (OMIM 618196) are included several vascular pathologies such as isolated multifocal...
capillary malformations, telangiectasia, or high flow complex arteriovenous malformations. Patients with telangiectases consistent with hereditary hemorrhagic telangiectasia (HHT) but negative for the classical HHT-associated genes have been positively screened for EPHB4 variants suggesting that the spectrum of the CM-AVM2 phenotype should be expanded.

EPHB4 variants have been detected in patients with cutaneous telangiectases, initially suspected to have HHT, but negative for variants in the classical HHT-associated genes. Upon review of these cases regarding specific location, number, and appearance of the telangiectases, they were noted to be distinct from those seen in patients with HHT. The cutaneous telangiectases in these patients with an EPHB4 variant had an earlier onset (childhood) and were often innumerable in a given location. Some of the larger lesions (labeled as capillary malformations [CMs]) were “haloed” and occurred in locations (trunk and extremities) not typical of HHT. These CMs probably represent larger dermal AVMs.

The increasing number of EPHB4 variants of uncertain significance (VUS) identified through exome sequencing demands rapid and effective tools to confirm pathogenicity to enable an accurate genetic diagnosis. No clear phenotype–genotype correlation associated with EPHB4 variants has yet been identified and our understanding of the disease mechanism of the known causal variants that could assist in clinical decision making is limited.

In this study we aimed to functionally investigate seven novel EPHB4 VUS (from eight unreported index cases) associated with a disease phenotype to predict their pathogenicity and attempted to unravel the molecular disease mechanisms that could explain the variable phenotypes. For comparison, three variants from previously reported cases and three control variants selected from gnomAD were also included in this investigation. We demonstrated the pathogenic effect of six of the seven variants, showing a variety of EPHB4-related disease mechanisms at a molecular level.

We present an increasing number of variable or overlapping phenotypes associated with EPHB4 variants. These include a dominant form of primary lymphedema without fetal hydrops, ASD, or cutaneous malformation, and an intrafamilial overlapping phenotype with capillary malformation (telangiectasia) and ASD, with other members of the family presenting with fetal hydrops but no telangiectasia, i.e., a dual (Janus-faced) phenotype. This highlights that a detailed clinical evaluation of the affected families and continuous follow-up are critical. Functional validation of the causative variants was undertaken, and various disease mechanisms put forward. This knowledge will help in understanding any phenotype–genotype correlations, and aid clinical practice.

MATERIALS AND METHODS

Patient selection, EPHB4 variant detection, and lymphoscintigraphy

Ten index cases and affected family members with variants in EPHB4 were included in the study. Two were previously described, and eight were novel, unreported referrals. Of those, five cases were direct referrals from clinicians, two were identified through the Prenatal Assessment of Genomes and Exomes (PAGE) study, and one was identified through GeneMatcher.

See Supplementary Information, Supplementary Table 1, and Supplementary Figs. 1–3 for detailed clinical information of the recruited cases. EPHB4 variants were detected by next-generation sequencing (NGS) in the respective molecular genetics services and confirmed by Sanger sequencing.

Patients from the GLDUK, GLDNOR, and PL1 families underwent lower local procedure by injecting radioactive isotope (technetium-99m-nanocol) into the web spaces between the toes for localisation of radioactivity. Images were taken at 15 minutes and 2 hours postinjection with a gamma camera. Quantification figures 2 hours postinjection were calculated, where possible, as percentage (%) of tracer retention within right and left foot, and tracer uptake in the ilioinguinal nodes.

In silico analysis

The relative genomic and protein positions of EPHB4 reported here correspond to the transcript EPHB4-001 (RefSeq: NM_004444, Ensembl: ENST00000358173.3) and P54760 Uniprot protein accession ID. The reported genomic coordinates refer to the GRC38/hg38 human genome reference. Putative changes in the gene structure and/or amino acid sequence caused by the reported variants were retrieved from the University of California–Santa Cruz (UCSC) Refgene database. Allele frequencies (AF) were checked in gnomAD databases. Pathogenicity was predicted by the Combined Annotation Dependent Depletion (CADD) tool, MutationTaster, and PolyPhen-2. The protein model of EPHB4 was generated in R using the Bioconductor trackViewer package.

Multiple sequence alignments of EPHB4 protein from different species were performed using the T-Coffee tool.

Site-directed mutagenesis of EPHB4 constructs

Fifty nanograms of the EPHB4 mammalian expression vector Myc-DDK-tagged pCMV6-Entry-EPHB4 (RC208559, Origene) was used as template with the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). All primers (Supplementary Table 2) were designed with QuikChange Primer Design (Agilent) and all constructs verified by Sanger sequencing.

Cell culture, transfection, and activation of EPHB4 receptor

Human dermal lymphatic endothelial cells (LECs) (C-12217, PromoCell) were cultured in supplemented endothelial growth medium MV2 (C-22022, PromoCell) containing 50 ng/ml VEGF-C (9199-VC-025, Bio-Technne). The cells are routinely analyzed by flow cytometric analysis and immunofluorescent staining: >95% of the cells are CD31 positive and podoplanin positive. All experiments with LECs were repeated n ≥ 3 with cells isolated from independent single donors.

The day before transfection 2×10^5 cells/well were seeded in fibronectin-coated (F1141, Sigma-Aldrich) 6-well plates. They were then transfected with 2 µg of the EPHB4 constructs and Viomer Yellow transfection reagent (VY-01LB-01, Lipocalyx) or 1 µg of the EPHB4 constructs and Lipofectamine 3000 transfection reagent (Life Technologies). Ligand activation of EPHB4 receptor was performed as previously described.

Immunoprecipitation and western blot

Twenty-four hours post-transfection LECs were harvested in lysis buffer and immunoprecipitation performed as previously described. Immuno-precipitates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-FD PVDF membranes (Millipore). Membranes were blocked with TBS blocking solution (Odyssey Blocking Buffer diluted 1:1 in TBS, LI-COR Biosciences) and incubated with β-actin antihuman EPHB4 (AF3038, Bio-Technne) and mouse antiphosphotyrosine (clone 4G10, 05–321, Millipore) antibodies diluted in TBS blocking solution containing 0.2% Tween 20 (Sigma-Aldrich).

After incubation with IRDye 680RD donkey antigoat IgG (925–68074, LI-COR Biosciences) and IRDye 800CW donkey antimouse IgG (925–32212, LI-COR Biosciences) antibodies diluted in TBS blocking solution containing 0.2% Tween 20 (Sigma-Aldrich) and 0.01% SDS (Sigma-Aldrich), membranes were scanned with the Odyssey infrared imaging system (LI-COR Biosciences).

For the detection of EPHB4 expression levels, after transfection, cell lysates were subjected to western blotting as above but instead, EPHB4 expression was detected with C-term specific mouse anti-DDK (clone 4C5, TAA0011, OriGene), and mouse anti-GAPDH (clone 6C5, MAB374, Millipore) was used as an internal control. Uncropped western blots are shown in Supplementary Figure 7.

Immunofluorescence and microscopy

Human dermal LECs were seeded on fibronectin-coated glass coverslips prior to lipofectamine-mediated transfection with EPHB4 variants. After 24 hours transfection, cells were fixed with 3% paraformaldehyde (PFA), then permeabilized and quenched with 0.2% saponin/50 mM NH4Cl. Mouse anti-DDK and goat anti-VE-Cadherin (sc-6458, Santa Cruz Biotechnology) were used as primary antibodies. Alexafluor488 anti-mouse (A21206, Invitrogen) and Alexafluor565 anti-goat (A32816, Invitrogen) were used as fluorophore-conjugated secondary antibodies. All antibody dilutions were prepared in PBS containing 0.2% (w/v) fish skin gelatin, 0.02% (w/v) saponin, and 0.02% (w/v) Na3P, DAPI (Sigma-Aldrich) was used for nuclei detection and VECTASHIELD® (Vector Laboratories) was used as mounting medium.
RESULTS

Novel EPHB4 variants related to fetal hydrops of unknown etiology

Since the first report of two pathogenic EPHB4 missense variants associated with LRFH, four additional cases of fetal hydrops (FH1:II.2, FH2:II.1, FH4:II.1, and FH5:II.2) and one case presenting with bilateral pleural effusions (FH3:II.7) with variants in EPHB4 have come to our attention (Fig. 1a–c). Including other family members, who carried an EPHB4 variant, a total of seven individuals are reported all presenting with lymphovenous problems and/or congenital heart defects (CHD); for full clinical details and pedigrees see the Supplementary Information, Supplementary Table 1, and Supplementary Figs. 1, 2.

The two pathogenic variants reported by Martin-Almedina et al. are located in the intracellular tyrosine kinase domain. Of the five new referrals, four carried missense EPHB4 VUS also located in the same region (Supplementary Figure 4) and conservation analysis revealed that all the variants alter highly conserved residues in the tyrosine kinase domain of EPHB4 (Supplementary Figure 5A). The p. R744H variant was reported in two unrelated cases (FH1:II.2 and FH2:II.1) (Table 1) and is located in a highly conserved region in close proximity to the catalytic loop HRD (His-Arg-Asp), also strongly conserved in most protein kinases (Supplementary Figure 5B) and predicted to control the active conformation of the kinase domain.21,22 FH4:II.1 carried an indel (c.760_761insC) predicted to cause a frameshift resulting in the premature truncation of the EPHB4 protein (p.S254Tfs*10), which would lack several functional domains including the tyrosine kinase domain (Supplementary Figure 4). None of the variants were reported in gnomAD and in silico analysis predicts them to be nonfunctional (Table 1).

Novel EPHB4 variants related to a vascular anomaly phenotype

The findings from Amyere et al. and Wooderchak-Donahue et al. led to the identification in our genetics clinic of two index cases presenting with familial telangiectasia with novel variants in EPHB4. There were five affected individuals from two families (VA1 and VA2) who presented with HHT-like features (Fig. 1d, e). For the full clinical details see Supplementary Information, Supplementary Table 1, and Supplementary Figure 3. Affected family members of VA1 carry a

Fig. 1 Clinical findings in individuals with EPHB4 variants. (a) Antenatal ultrasound scan (transverse plane) demonstrating bilateral pleural effusions at gestational week 30 + 4 in FH2:II.1. (b) Baby in the neonatal period with fetal hydrops (GLDUK:II.6). (c) Persistent peripheral lymphedema in the feet of FH5:II.2 at age 4 years. (d) Capillary malformation in the midline of the neck in VA1:II.2. (e) Multiple telangiectasia along the vermillion border of the upper lip and the mucous membrane of the lower lip in VA2:II.1 (inside boxed areas). (f) Early onset and extensive lower limb varicose veins in GLDUK:II.2. (g) GLDUK:II.4 with multiple telangiectasia with a propensity for the vermillion border of the lips (inside boxed area). (h) Dermatoscopic image of telangiectasia on the left cheek confirming the presence of dilated linear and branching capillary vessels in GLDUK:II.4.

medium. Images were taken at 20x magnification with the EVOS™ M5000 imaging system (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed in GIMP (www.gimp.org).
Novel EPHB4 variant related to isolated persistent peripheral lymphedema

After the screening of a patient (PL1:II.3) with primary lymphedema on the primary lymphedema gene panel through South West Thames Regional Genetic Services, a VUS in EPHB4 was identified. All three affected family members in PL1 carried this EPHB4 variant and the main clinical feature was early onset (around age of 2 years) swelling of the lower limbs. There was no history of fetal hydrops, CHD, CM, AVM, or telangiectases. Lymphoscintigraphy imaging was consistent with primary lymphedema (Fig. 2a, b). The full clinical details and the pedigree are presented in the Supplementary Information, Supplementary Table 1, and Supplementary Figure 3.

The EPHB4 VUS identified, p.N410K, is located in the extracellular fibronectin domain (Supplementary Figure 4) in a highly conserved residue (Supplementary Figures 4, 5A). None of the variants are in gnomAD and in silico analysis predicts them to be nonfunctional (Table 1).

Update on clinical findings in previously published EPHB4-associated LRHF cases

Since the first report in 2016, the two families, GLD\textsubscript{UK} and GLD\textsubscript{DOR}, have been periodically evaluated at the respective specialist centers. Despite no apparent clinical signs of persistent peripheral lymphedema, three individuals underwent lymphoscintigraphy imaging, which confirmed abnormal lower limb lymphatic function (Fig. 2c, d, f). Lymphoscintigraphy in the GLD\textsubscript{DOR} and GLD\textsubscript{UK} cases shared features of abnormal lymphatic drainage via tortuous tracts with superficial rerouting similar to previous findings in the GLD\textsubscript{UK} family (Fig. 2e, g). Features suggestive of venous hypertension contributing to impaired lymphatic function were also observed. This is in keeping with the observation of often extensive varicose veins from a young age in all five adults investigated in the two families (Fig. 1f). Updated clinical details are given in the Supplementary Information, Supplementary Table 1, and Supplementary Figure 1.

At the time of the original publication, none of the individuals in the GLD\textsubscript{UK} and GLD\textsubscript{DOR} families were assessed for telangiectases or capillary malformations. At a recent visit, GLD\textsubscript{UK:III.2} reported repeated nose bleeds and on further inspection several telangiectases were identified around his mouth. It was also noticed that his mother (GLD\textsubscript{UK:II.4}) had multiple, diffuse telangiectases around the mouth and on her hands (Fig. 1g, h). Both of these individuals have ASDs (III.2 had multiple ASDs) and neither were hydropic or had pleural effusions unlike other affected family members. Brain and spine magnetic resonance images (MRIs) were entirely normal, no AVMs were identified, and there were no capillary malformations. No pathogenic variants in the HHT genes (ENG, ACVR1L, GDF2, and SMAD4) or RASA1 were identified in the exome data of GLD\textsubscript{UK:II.4} so it is highly likely that the EPHB4 variant is causing the overlapping features in the two.

Analysis of protein expression levels, kinase activity, and subcellular localization of EPHB4 mutated proteins expressed in LECs

Functional analysis of the two pathogenic missense variants originally reported to cause LRHF demonstrated normal EPHB4 protein expression levels but reduced protein activity. The analysis of 4 of the 20 missense variants reported to cause CM-AVM2 by Amyere et al. showed largely reduced protein expression levels with EPHB4 protein aggregated in intracellular inclusions.

These findings led us to carry out a functional classification of the seven new variants for FH1–FH5, VA1–VA2, and PL1. As a reference, the two previously published missense variants, p.R739Q and p.I782S, were included, and missense variant, p.D802G, was included as a representation of the CM-AVM2 phenotype. All three are located in the tyrosine kinase domain of EPHB4 in highly conserved residues and are predicted to be pathogenic by in silico analysis (Table 1, Supplementary Figures 4, 5). They are all novel, except p.D802G, which has been reported previously in a dominantly inherited CM-AVM2.

Table 1. Functional annotation of the thirteen EPHB4 variants investigated in this study.

| ID | Genomic coordinates (GRCH38) | Nucleotide change | Predicted protein change | Domain affected | CADD Phred score | Polyphen2 HVAR rankscore | MutationTaster prediction | gnomAD v2.1 AF homozygotes | gnomAD v2.1 AF heterozygotes | gnomAD v3 AF homozygotes | gnomAD v3 AF heterozygotes |
|----|-----------------------------|------------------|--------------------------|----------------|------------------|--------------------------|--------------------------|-----------------|-----------------------------|-----------------|-----------------------------|
| GLD\textsubscript{UK} | chr7:1008493-1008493 c.2126G>A | R739Q | TKD | 35 | 0.916 | Pathogenic | - | - | - | - | - |
| GLD\textsubscript{DOR} | chr7:10086559-10086559 c.2340T>G | I782S | TKD | 26.5 | 0.971 | Pathogenic | - | - | - | - | - |
| FH1 | chr7:10087468-10087468 c.2231G>A | R744H | TKD | 31 | 0.916 | Pathogenic | - | - | - | - | - |
| FH2 | chr7:10087468-10087468 c.2231G>A | R744H | TKD | 31 | 0.916 | Pathogenic | - | - | - | - | - |
| FH3 | chr7:10087372-10087372 c.2327G>A | S791N | TKD | 29.2 | 0.749 | Pathogenic | - | - | - | - | - |
| FH4 | chr7:10082218-10082218 c.760_761insC | S254Tfs*10 | TKD | - | - | Pathogenic | - | - | - | - | - |
| FH5 | chr7:10085525-10085525 c.2654A>G | K885R | TKD | 24.3 | 0.769 | Pathogenic | - | - | - | - | - |
| VA1 | chr7:10087568-10087568 c.2131C>T | Q711X | TKD | 38 | - | Pathogenic | - | - | - | - | - |
| VA2 | chr7:10087372-10087372 c.2327G>A | S791N | TKD | 29.2 | 0.749 | Pathogenic | - | - | - | - | - |
| CM-AVM2 | chr7:10084949-10084949 c.2670G>T | E890D | TKD | 32 | 0.971 | Pathogenic | 0.000004 | 0 | 0 | 0 | 0 |
| PL1 | chr7:100819624-100819624 c.1230C>G | N410K | TKD | 23.2 | 0.971 | Pathogenic | - | - | - | - | - |
| SNP | chr7:10087375-10087375 c.2131C>T | Q711X | TKD | 38 | - | Pathogenic | - | - | - | - | - |
| SNP | chr7:100805535-100805535 c.2640G>A | A862T | TKD | 23.5 | 0.639 | Pathogenic | 0.000115 | 3 | 0.003831 | 5 | - |
| SNP | chr7:10085509-10085510 c.2670G>T | E890D | TKD | 14.98 | 0.121 | Benign | 0.005635 | 6 | 0.005392 | 2 | - |

Genomic coordinates, nucleotide and protein changes, predicted pathogenicity, and population allele frequencies are summarized. All previously reported EPHB4 variants for the GLD\textsubscript{UK}, GLD\textsubscript{DOR}, and CM-AVM2 cases and EPHB4 variants of uncertain significance (VUS) for the FH1–FH5, VA1–VA2, and PL1 cases are either not reported in gnomAD databases or their allele frequency is infinitesimal, supporting the argument that they are extremely rare in the general population, while they are all reported to be pathogenic by all three prediction tools used (CADD, PolyPhen-2, MutationTaster). The three EPHB4 variants used as controls (SNP) in this study are reported as rare variants (AF < 0.01) in gnomAD databases. Two of them are found in homozygotes and/or are predicted as pathogenic. GLD\textsubscript{UK} and GLD\textsubscript{DOR} published in Martin-Almedina et al. and CM-AVM2 in Yu et al. AF allele frequency, CM-AVM capillary malformation–arteriovenous malformation, FH fetal hydrops, FND fibronectin domain, LBD ligand binding domain, PL primary lymphedema, SNP single-nucleotide polymorphism (likely benign variant), TKD tyrosine kinase domain, VA vascular anomalies.
once in gnomAD, but is extremely rare (minor allele frequency [MAF] = 0.000004).

In addition, three variants located in the tyrosine kinase domain and not reported to be associated with disease were identified in gnomAD and included as controls. Two of the variants have been reported in homozygotes, they all had a low MAF and included as controls. Two of the variants have been predicted to be pathogenic (Table 1, Genetics in Medicine (2021) 23:1315 – 1324).

We generated mammalian expression vectors for the wild-type (WT) form of EPHB4 and for each of the 13 variants listed in Table 1 by site-directed mutagenesis. The constructs were transfected into LECs and the effects of the variants on EPHB4 protein expression levels were analyzed by western blot. Most of the variants tested resulted in expression levels comparable with the WT protein (Fig. 3a). However, the three variants, p.T110P, p.N410K, and p.D802G, showed reduced expression of protein, and what seems to be a shorter protein (compared with the predicted 108-kDa full-size protein). Variants p.Q711X and p.S254Tfs*10 could not be detected with the C-terminal specific antibody, indicating a loss of full-size protein expression. The in silico prediction from MutationTaster suggests the two variants lead to nonsense-mediated decay (NMD) due to the introduction of premature stop codons.

To investigate the possible impact of the variants on EPHB4 function, the tyrosine kinase activity of the receptor was analyzed by immunoprecipitation and western blot after EphrinB2 stimulation. We also included the three control variants in this analysis. Stimulation with preclustered EphrinB2 (EphrinB2-Fc) for 30 minutes increased the tyrosine phosphorylation level of the receptor after its binding in the WT form while tyrosine phosphorylation was reduced or not detected for most variants (Fig. 3b). The three control variants displayed tyrosine phosphorylation activity after EphrinB2 treatment; so did one VUS (p.K885R).

Immunofluorescence studies were performed to investigate the subcellular localization of the generated variants. Exogenous EPHB4 expression was detected with an anti-DDK antibody, while VE-cadherin expression was used as a marker of cell–cell membrane contact. Five of the variants investigated were detected in the cell membrane, confirmed by the localization in proximity to VE-cadherin, showing a similar expression localization pattern to the WT protein. On the other hand, the three variants, p.T110P, p.N410K, and p.D802G, showed absent or much reduced membrane localization and a distinct intracellular expression pattern. In some cells, increased reticular staining and/or intracellular aggregates that could be accumulations of misfolded proteins were detected when transfecting these three variants (Fig. 3c). A summary of our interpretation on the effect of the EPHB4 variants regarding the expression levels, kinase activity, and subcellular localization obtained from the functional assays is shown in Supplementary Table 3.

DISCUSSION

Since the initial reports of germline variants in EPHB4 associated with a disease phenotype, new cases with EPHB4 VUS have come to our attention from several clinicians looking for additional evidence to confirm diagnosis. To verify the pathogenicity of all the new variants, we investigated protein expression levels, tyrosine kinase activity, and subcellular localization of the EPHB4 receptor. We showed that six of the seven novel variants under
investigation resulted in a reduction or total absence of EPHB4 kinase activity, due to the presence of a dysfunctional receptor or total loss of protein expression. Thus, for most variants we were able to report a damaging effect on the protein function to the clinicians, suggesting that the VUS was pathogenic and causative of the disease. However, the p.K885R variant in family FH5 performed similarly to the WT protein in our functional assays. Based on our results, this variant appears benign and we cannot
conclude it was the cause of the LRFH in this family; therefore, more extensive functional work must be done to confirm its pathogenicity.

We phenotypically describe eight new families with variable lymphatic and vascular phenotypes associated with novel EPHB4 variants (summarized in Table 2) and provide a more detailed clinical report for the two families first published in 2016. Until now, our understanding has pointed toward the existence of two phenotypically differentiated autosomal dominantly inherited disease entities caused by germline variants in EPHB4: LRFH and CM-AVM2. Based on the evaluation of the new families, individuals in families VA1 and VA2 could be classified under the umbrella of CM-AVM2 as their presentation is similar to that given by Wooderchak-Donahue et al., who reported a mucocutaneous telangiectasia in a series of atypical HHT cases with EPHB4 variants.9

Five of the new cases were more difficult to interpret. They had fetal hydrodrops (FH1–FH3 and FH5) or pleural effusions (FH4), which were suspected to be lymphatic-related in FH1 and FH5 as they had chylous (“milky”) fluid drained postnatally. However, the lack of clinical information for the other cases does not allow us to conclude whether lymphatic dysfunction is the source of the fetal hydrodrops phenotype.

Of particular significance is the discovery of intrafamilial overlapping phenotypes with individuals presenting with features of both LRFH and CM-AVM2 (Table 2). An increasing number of allelic conditions have now been shown to have features of two different phenotypes in the same individual or family—the Janus (after the ancient Roman god with two faces) or dual phenotype.23,24 Furthermore, overlapping lymphatic and vascular features have previously been reported in patients with RASA1-associated CM-AVM type 1.25

Three individuals from the PL1 family presented with peripheral primary lymphedema but no history of fetal hydrodrops or ASD. The onset of lymphedema was around the age of 2 years and the lymphoscintigraphy was distinct from those of the LRFH families. PL1 also differs from the CM-AVM2 cases as there were no CM or AVMs. This is the first family to be described with just primary lymphedema, which showed no overlap with LRFH and CM-AVM2, the disease phenotype spectrum needs expanding to include primary lymphedema.

Our results show different possible molecular disease mechanisms associated with EPHB4 pathogenesis and highlight the importance of further research into the functional and clinical consequences of the disruption of the EPHB4 forward and reverse signaling cascades. For some variants, we have shown that the EPHB4 mutant protein is expressed and presented on the membrane of LECs, but that tyrosine kinase activity is reduced, presumably altering the forward signaling (Fig. 3d, left). Reverse signaling might be unaffected although this was not investigated here. The reduced forward signaling could be due to an antimorphic disease mechanism with a dominant negative effect of the EPHB4 mutant on the WT protein activity. This dominant negative disease mechanism has been suggested for some VEGFR3 variants associated with primary lymphedema.26 The variants in this category predominantly associated with LRFH with fetal hydrodrops, lymphovenous abnormalities, and ASD, some with very mild symptoms (e.g., FH3).

For other variants, we detected a loss of EPHB4 protein, probably due to either nonsense-mediated messenger RNA (mRNA) decay or protein degradation. Any EPHB4 mutant protein detected was of a shorter size and was trapped intracellularly, which could be a sign of the protein undergoing degradation. These EPHB4 mutants are most likely amorphic, acting as null alleles (Fig. 3d, right). Lower EPHB4 receptor levels will lead to an imbalance in the ratio of EphrinB2 ligand to EPHB4 receptor, which promotes cell dysfunction in normal microvascular endothelial cells.27 Variants in this category predominantly associated with CM-AVM2, but there were exceptions (e.g., the PL1 family). Bidirectional Eph–Ephrin signaling is complex and we can only speculate on how downstream forward or reverse signaling is affected by the EPHB4 variants as this was not investigated in this study. The existence of receptor–ligand oligomerization and bidirectional endocytosis of receptor–ligand complexes add another level of intricacy, which supports the need for more research into the field to fully understand the contribution of both forward and reverse signaling to the arteriovenous and lymphovenous compartments.

Further studies are also needed to explain why the phenotype within families can be so variable. Several authors have suggested that a postzygotic de novo variant in the other allele (“second hit hypothesis”) would be required for the development of the focal lesions associated with the CM-AVM2 disease phenotype.6–8 However, variable expression of the disease phenotype could also be due to genetic modifiers or polygenic inheritance, therefore, it is necessary to understand whether one or two EPHB4 null alleles will lead to disease.

In conclusion, several reports have shown that monoallelic EPHB4 variants can cause a spectrum of arteriovenous and lymphovenous disorders, but our findings add more complexity to this. We introduce a possible new phenotype, dominant primary lymphedema, which showed no overlap with LRFH and CM-AVM2, the EPHB4-associated disease classifications might need a major revision. Regardless, a change of clinical practice is required as cases thought to be CM-AVM2 should undergo echocardiography, while cases thought to be LRFH should also undergo...
careful skin examination and consideration of brain and spine MRI to check for arteriovenous malformations. Furthermore, *EPHB4* variant pathogenicity was demonstrated in this study for six of the seven novel VUS investigated and highlights the usefulness of protein expression and subcellular localization studies to predict the pathogenesis of VUS. With NGS as a routine method for the diagnosis of inherited disorders, the detection rate for rare and novel variants is growing and inferring the pathogenicity of the identified variants can be challenging. Therefore, as suggested by the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines, a comparable approach is essential for other genes/diseases to maximize diagnostic rates. Our study also highlights different *EPHB4*-related disease mechanisms; however, further research is necessary to expand our knowledge on how they operate at a cellular and molecular level to fully understand the intricate *EPHB4* clinical enigma.

**DATA AVAILABILITY**

The most recent version of the scripts used for the described analysis can be found online: [Preprocessing/Alignment: https://github.com/sgul-genetics-centre-bioninformatics/Next-Generation-Sequencing-Pipelines; Unpaired Somatic Calling: https://github.com/digrigor/Unpaired_somatic_variant_calling. Archived version of this software can be found here (https://figshare.com/s/efc5dcb85b7420be4938)].

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**Table 2. Clinical summary.**

| ID | Case | Sex | Age (yr) | Fetal hydrops (wks) | Congenital heart defect | Persistent peripheral lymphedema | Varicose veins | Telangiectasia | Predicted protein change |
|----|------|-----|----------|---------------------|------------------------|-----------------------------|---------------|----------------|------------------------|
|    |      |     |          |                     |                        |                             |               |               |                        |
| GLDUK | I.2 | M | 60 | Yes | Yes | Yes |             |               |               |                        |
|     | II.2 | F | 35 | Yes | Yes | (Yes) | Yes |               |               |                        |
|     | II.4 | F | 29 | Yes | (Yes) | Yes | Yes |               |               |                        |
|     | II.6 | M | ND 21d | Yes | Normal |               |               |               |                        |
|     | II.7 | F | IUD 26wks | Yes | Normal |               |               |               |                        |
|     | III.1 | M | 13 | Yes | Yes |               |               |               |                        |
|     | III.2 | M | 9 | Yes | Yes |               |               |               |                        |
| GLDNOR | II.2* | F | 42 | ? | Normal | (Yes) | Yes |               |               |                        |
|     | III.5 | M | ND 1.5d | Yes | Yes |               |               |               |                        |
|     | III.9 | M | 11 | Yes | Yes |               |               |               |                        |
| FH1 | II.2 | M | ND 36d | Yes | Yes |               |               |               |                        |
| FH2 | II.1 | F | ND 9wk | Yes | Yes |               |               |               |                        |
| FH3 | II.7 | F | only PE | Normal |               |               |               |               |                        |
| FH4 | II.1 | M | IUD? | Yes | Yes |               |               |               |                        |
|     | I.2 | F | 40 | Normal | Yes |               |               |               |                        |
|     | II.1 | F | 8 | ? | Normal |               |               |               |                        |
|     | II.2 | M | 4 | Yes | Yes |               |               |               |                        |
| VA1 | I.2 | F | 44 |               |               |               |               |               |                        |
|     | II.1 | F | 17 |               |               |               |               |               |                        |
|     | II.2 | F | 16 |               |               |               |               |               |                        |
| VA2 | I.1 | M | 54 |               |               |               |               |               |                        |
|     | II.1 | M | 14 |               |               |               |               |               |                        |
| PL1 | II.2 | F |               | Yes |               |               |               |               |                        |
|     | III.3 | F | 14 |               | Yes |               |               |               |                        |
|     | III.1 | F | 6 |               | Yes |               |               |               |                        |

Overview of the phenotype of the eight new index cases and affected family members with an *EPHB4* VUS (FH1–FH5, VA1–VA2, and PL1) included in this study. Clinical details for the *EPHB4* index cases and affected family members previously reported by Martin-Almedina et al. (GLDUK and GLDNOR) have been updated and included for comparison. Telangiectases are indicated in teal. Features originally described as associated with lymphatic-related fetal hydrops (LRFH) are indicated with a pale yellow. The lymphedema observed in PL1 is different on lymphoscintigraphy to that of individuals from the GLDUK and GLDNOR families, thus a darker shading has been used to indicate it. Refer to the Supplementary Information and Supplementary Table 1 for detailed clinical description of each case. "?” in the fetal hydrops column indicates that there were hydropic features at birth. "Normal" in the congenital heart defect column indicates that a normal echocardiogram was obtained. "(Yes)" in the persistent peripheral lymphedema column indicates that the individual is clinically normal, but abnormal lymphatic drainage was demonstrated on lymphoscintigraphy. Blank fields indicate the relevant procedure was not carried out or the information was not available.

*Female, FH fetal hydrops, GLD generalized lymphatic dysplasia, IUD intrauterine death, M male, ND neonatal death, PE pleural effusions, PL primary lymphedema, VA vascular anomaly.

*aMonzygotic twins.

*bThe type of *EPHB4* variant suggests this could be a capillary malformation–arteriovenous malformation 2 (CM-AVM2) case, but telangiectasia was not confirmed.
