

Exploring EPHB4 in Gait Disorders: VUS Detection and Phenotypic Characterization in a Toe-Walking Cohort

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Abstract

Background:

Idiopathic toe walking (ITW) is a gait anomaly with suspected genetic origins, though underlying mechanisms remain poorly understood. Although the established role of ephrin type-B receptor 4 (EPHB4) involves mainly vascular development, in this study we focused whether the detected variants might correlate with clinical findings in idiopathic toe walkers cohort where EPHB4 variants were identified.

Methods:

We retrospectively analyzed clinical and genetic data from 20 toe-walking patients harboring EPHB4 variants detected using 49-gene Next-Generation Sequencing (NGS) panel.

Results:

Genetic analysis identified 18 distinct EPHB4 variants, all classified as variants of uncertain significance (VUS) per American College of Medical Genetics and Genomics (ACMG) guidelines

Common clinical features included:

- Pes cavus (40%)
- Clinodactyly/brachydactyly (40%)
- Speech/language delays (40%)

Notably, only 10% showed venous anomalies typically associated with EPHB4 mutations.

Conclusions:

While this study reports an unexpected frequency of EPHB4 variants in ITW patients, these findings should be interpreted cautiously given:

- The VUS classification of all variants
- Lack of clear genotype-phenotype correlation
- Potential ascertainment bias
- Further investigation through functional studies and familial segregation analysis is needed to determine whether these observations represent a true biological signal or incidental findings. This work highlights the complexity of studying genetic factors in common pediatric conditions like ITW.

Clinical trial registration: not applicable. Retrospective cohort analysis of existing clinical data. Not prospectively registered as no interventions were performed.

Background

The Eph receptor protein tyrosine kinase family, the largest subgroup of receptor tyrosine kinases (RTKs), plays crucial roles in cell-cell communication, tissue patterning, and developmental processes by binding to membrane-anchored ephrin ligands¹. These receptors are divided into two subclasses -EphA (binding ephrin-A ligands) and EphB (binding ephrin-B ligands)- based on their ligand affinities and structural features². Among the Eph receptors, EPHB4 (Ephrin type-B receptor 4) stands out due to its specialized role in vascular development and its emerging significance in oncology³.

EPHB4, a member of the EphB subclass, preferentially binds ephrin-B2 and mediates bidirectional signaling to regulate cell adhesion, migration, and tissue boundary formation³. Structurally, EPHB4 consists of an extracellular ephrin-binding domain, a transmembrane region, and an intracellular tyrosine kinase domain, enabling it to activate downstream pathways such as PI3K/AKT and MAPK⁴. Notably, EPHB4 is essential for venous specification during embryogenesis, where its interaction with ephrin-B2 establishes arterial-venous differentiation⁵.

Beyond development, EPHB4 is increasingly recognized for its role in disease, particularly in cancer progression and pathological angiogenesis. In vascular disorders, for instance, EPHB4 mutations have been implicated in several diseases with heterozygous carriers exhibiting distinct pathological manifestations such as capillary malformation-arteriovenous malformation syndrome (CM-AVM)⁶, vein of Galen aneurysmal malformation⁷, and venous valve aplasia⁸. Moreover, in cancer, EphB4's interaction with its ligand, ephrin-B2, can either promote or inhibit tumor growth, leading to a complex and sometimes paradoxical understanding of its role in cancer. Given its multifaceted involvement in disease mechanisms, EPHB4 has garnered interest as a potential therapeutic target⁹.

Beyond vascular and oncologic disorders, emerging evidence suggests EPHB4 may play underappreciated roles in neural development. Recent in vitro studies demonstrate that EPHB4 regulates self-renewal, proliferation, and neuronal differentiation of human embryonic neural stem cells through the Abl-CyclinD1 pathway¹⁰.

Habitual toe walking represents another phenotype with suspected genetic origins¹¹. As a symptom, it has been associated with several hereditary disorders such as Charcot Marie Tooth, cerebral palsy and autism spectrum¹². Habitual toe walking, defined as persistent plantar-flexion gait beyond two to three years of age without any underlying cause²⁵. In a previous study of children with persistent non idiopathic toe walking (n-ITW), we analyzed a selected cohort for genetic causes using a targeted 49-gene NGS panel. All genes included in this panel have been reported to lead to hereditary neuro/myopathies associated with gait abnormalities²³.

Within our targeted sequencing analysis, heterozygous variants in EPHB4 – a gene not previously associated with gait disorders – were identified in a subset of patients. Here we describe the clinical characteristics of these variant carriers as an exploratory observation to inform future hypothesis-driven studies.

Materials and Methods

This four-year investigation examined genetic contributors to persistent idiopathic toe walking (ITW) in pediatric patients referred for gait analysis. Eligible participants met the following criteria:

1. Parental confirmation of toe-walking for >50% of the day.
2. Completion of a dedicated 49-gene next-generation sequencing (NGS) panel
3. Absence of diagnosed neurological conditions (e.g., cerebral palsy, tethered cord syndrome, ASD) or significant musculoskeletal abnormalities including limb length discrepancies, axis deformities, scoliosis or severe foot deformities.

All participants underwent a standardized assessment, including a detailed questionnaire and physical examination. The following parameters were systematically recorded in our clinic (Table1):

Table 1: Clinical and Anamnestic Parameters Assessed in Patients

Patient History

- Perinatal history
- Age of onset of TW
- Onset of walking (OoW)
- Family history of toe walking or related neuropathies
- Speech and language difficulties (e.g., dysarthria)¹
- Social development¹
- Visual impairments¹
- Pain localization¹
- Muscle-related symptoms (myalgia, cramps, fatigue)¹
- Comorbidities

Toe walking Diagnosis

- Visual gait analysis
- Ankle joint mobility²
- Ability to balance on heels
- Heel walking test
- Foot drop test (toe lifting)

Physical Symptoms and Abnormalities

- Spinal abnormalities (lumbar spine hyperlordosis)³
- Structural abnormalities (pes cavus, clinodactyly, brachydactyly, pectus excavatum)³
- Reflex anomalies⁴
- Presence of tremor⁵

Notes: ¹Parent/Caregiver-reported data; ²Ankle mobility assessed via goniometry (neutral/0° method; restricted = dorsiflexion <20° or plantarflexion <50°); ³Morphological inspection; ⁴Patellar reflex evaluation; ⁵Tremor assessed with arms extended forward (palms down), provoked by repeated hand opening-closing; rhythmic oscillations post-movement were considered positive.

Statistical Analysis:

Given the exploratory nature of this retrospective cohort study, statistical analysis was limited to descriptive statistics. All calculations were performed using LibreOffice Calc to:

1. Characterize the study population:

Demographic parameters (age distribution, sex ratio)

Variant classification frequencies

2. Quantify clinical feature prevalence:

Musculoskeletal abnormalities (pes cavus, digit anomalies)

Developmental markers (speech/language delays)

Vascular findings (venous anomalies)

Family history patterns

For categorical variables (e.g., presence of pes cavus), we reported:

- Absolute counts (n)
- Percentages of the total cohort ($n/N \times 100$)

Continuous variables (age) were expressed as:

- Mean $\pm$ standard deviation
- Range (minimum-maximum)

Note: No comparative statistical tests were performed due to:

- The absence of a control group
- All variants being classified as VUS
- The study's hypothesis-generating design

Genetic Analysis Protocol

This study focused on patients who underwent genetic testing using a specialized 49-gene next-generation sequencing (NGS) panel designed to identify hereditary neuropathies and myopathies potentially associated with toe walking. Individuals tested using alternative methods, such as exome sequencing, were not included. Testing was conducted as part of the diagnostic process, utilizing saliva samples collected during standard clinical care.

The NGS panel, developed based on the Human Phenotype Ontology (HPO) database, examined 49 neuromuscular-related genes. Sequencing was performed on the Ion Torrent platform, covering coding exons and adjacent intronic regions (± 10 base pairs). Variants were classified according to the American College of Medical Genetics and Genomics (ACMG) criteria, with only pathogenic (P), likely pathogenic (LP), or variants of uncertain significance (VUS) included in the analysis. Benign variants were excluded

but could be accessed upon request. Interpretation was supported by in silico tools (e.g., MutationTaster) and databases like HGMD and ClinVar.

The method demonstrated high sensitivity (>96%) for identifying clinically relevant variants.

Results

Study Population:

From an initial cohort of 1,300 pediatric patients screened for toe walking, we identified 20 EPHB4 heterozygous carriers. The final study population comprised – 10 males and 10 females- with a mean age of 7 years old (range: 2–17 years old).

Genetic Testing results:

Our cohort of 20 patients harbored exclusively variants of uncertain significance (VUS) in EPHB4, comprising 18 distinct genetic variants, all interpreted according to the clinical knowledge and classification standards available at the time of each laboratory report. Variant assessment incorporated evidence from biomedical literature, population databases (ClinVar, gnomAD, HGMD), and in silico prediction tools (PolyPhen-2, SIFT, MutationTaster). Table 2 details all identified variants with the respective frequencies as reported on the specified dates.

Table 2
Summary of EPHB4 Variants and Population Frequencies in Study Cohort

Patient ID	Age	Gender	Variant c. HGVS	Frequencies	Report date
1	2	m	c.1085G > A	0.0029%	17.03.2021
2	2	m	c.1144G > A	0.0602%	10.10.2022
3	13	m	c.118G > A	0.0012%	10.08.2022
4	9	m	c.1205A > G	Not available	25.08.2023
5	17	f	c.1295A > T	Not available	13.07.2022
6	3	f	c.1429G > A	0.0056%	10.02.2023
7	6	f			10.09.2021
8	7	f	c.1561G > A	Not available	27.11.2020
9	5	m	c.1692-6C > A	0.0652%	09.08.2022
10	3	m	c.1905G > T	0.0044%	01.09.2023
11	7	m	c.236C > T	0.012%	27.11.2020
12	5	f	c.2417A > T	Not available	21.06.2023
13	11	m	c.2596C > T	0.0004%	16.07.2024
14	10	f	c.280A > G	0.0016%	06.06.2024
15	5	m	c.2826C > G	Not available	04.11.2021
16	8	f	c.691C > T	0,02%	14.06.2021
17	3	f			19.01.2021
18	7	f	c.703C > T	0.154%	18.11.2020
19	7	f	c.817C > A	Not available	21.12.2022
20	4	f	c.1405G > A	0.01%	25.02.2019

Clinical Findings

Patients exhibited a range of clinical features, six patients reported muscle pain affecting the back, knees, calves, legs, feet, or heels. Four individuals experienced muscle fatigue, while one reported muscle cramps. Physical examination revealed pes cavus in eight patients and eight with clinodactyly and brachydactyly. Three individuals presented with pectus excavatum, and only one patient exhibited essential tremor upon provoking it. One case had lumbar lordosis. A family history of similar symptoms was present in four cases (two paternal, one maternal, and one sibling). Parental reports indicated speech difficulties in eight cases.

Discussion

The identification of EPHB4 variants of uncertain significance (VUS) in this toe-walking cohort offers an interesting avenue for further investigation. While EPHB4 is primarily associated with vascular development, its detection here invites consideration of potential non-canonical roles. The shared clinical features among variant carriers suggest a possible correlation worth exploring. However, several factors merit careful consideration when interpreting these results:

Clinical Context

The observed features (pes cavus, tremor, speech delays) are not unique to EPHB4-related conditions and could stem from other genetic or developmental factors. Vascular abnormalities however are typically seen with pathogenic EPHB4 mutations. A speculative hypothesis proposes that venous malformations in calf muscles might alter ambulation biomechanics. Isolated case reports describe toe-walking secondary to venous malformations in calf muscles, including: (1) a 13-year-old with unilateral then bilateral toe-walking showing intramuscular venous malformation on MRI , and (2) three additional cases of equinus deformity associated with vascular anomalies .

These reports demonstrate that structural venous anomalies can alter gait biomechanics through muscle contracture. Notably, one patient carrying c.1405G > A in our cohort exhibited a complex presentation of toe-walking secondary to right-sided Achilles tendon shortening and gastrocnemius-soleus complex contracture, compounded by a diagnosed ipsilateral venous malformation (VM) infiltrating the calf musculature (Fig. 1). Key clinical features included:

- persistent unilateral equinus gait (right > left), with inability to achieve heel strike or neutral ankle dorsiflexion (0° at knee extension).
- asymmetric calf involvement: Palpable firmness and functional limitation on the right.
- concurrent bilateral vascular malformations (right dominant), managed previously with image-guided sclerotherapy.

We identified another illustrative case of toe-walking with suspected vascular involvement in our cohort, this patient carrying c.1085G > A variant, presented with unilateral calf asymmetry and prominent plantar venous patterning shown in Figs. 2³.

Text 1: Clinical photograph showing anteroposterior cutaneous discoloration.

Text 2: Clinical image showing left-right asymmetry in calf musculature. Such changes may suggest underlying venous malformation potentially affecting gait biomechanics.

Text 3: Prominent subcutaneous venous patterning on the plantar surface, possibly reflecting venous ectasia or malformation.

Genetic Interpretation

As all variants are classified as VUS, their clinical significance remains uncertain. Current guidelines emphasize that VUS require additional evidence—such as segregation in families, functional validation, or recurrence in affected populations—before being deemed pathogenic. While the recurrence of EPHB4 VUS in our cohort is notable, this alone cannot establish causality per ACMG guidelines .

Biological Plausibility

While EPHB4's primary role involves vascular development, its potential influence on neuromuscular function appears indirect -mediated through vascular effects on nerve/muscle perfusion or blood pressure regulation. The gene's established functions in vascular smooth muscle contractility could theoretically affect peripheral neuromuscular performance, though no direct role in motor neuron or muscle development has been demonstrated. This would require targeted investigation, including:

- Expression studies in human or model organism neural tissues
- Functional assays assessing variant impacts on protein behavior
- Segregation analysis in familial cases of toe walking

Limitations:

This study has several limitations. First, all identified EPHB4 variants were classified as variants of uncertain significance (VUS), precluding definitive conclusions about pathogenicity without functional validation or segregation studies. Second, the small cohort size limits statistical power to detect potential genotype-phenotype correlations. Third, the retrospective design relied on existing clinical records, which may have introduced inconsistent phenotyping across providers, underreporting of subtle vascular features, and missing familial segregation data.

Technical limitations of the 49-gene panel included its inability to assess non-coding regions, copy number variants, or low-level mosaicism, and its restriction to known neuromuscular genes (potentially missing novel associations). Additionally, phenotypic overlap with neuropathies (e.g., pes cavus, speech delays) complicates differentiation between primary EPHB4-related effects, comorbid undiagnosed conditions, and idiopathic presentations. Finally, the absence of a matched control group without toe walking prevents determination of whether EPHB4 VUS prevalence differs from background population rates.

Conclusion

This study reports the incidental identification of EPHB4 variants of uncertain significance in a selected cohort of toe-walking patients. While the recurrence of variants across multiple cases is notable, the VUS classifications and absence of characteristic vascular phenotypes -except for two cases- prevent definitive claims of causality. The clinical features observed, though consistent among carriers, overlap

with the excluded neuropathies and connective tissue disorders, necessitating cautious interpretation. The emerging evidence of EPHB4's roles in neural development offers a plausible but unproven avenue for future investigation. Rigorous functional validation and segregation studies still remain essential before any pathophysiology link between EPHB4 and gait disorders can be established. These findings should be considered exploratory rather than demonstrative of clinical association. Rigorous validation in independent cohorts and functional investigations will be required to determine whether this association reflects biological significance or ascertainment bias.

Abbreviations

ITW = Idiopathic toe walking / NGS = Next-Generation Sequencing / EPHB4 = Ephrin type-B receptor 4 / VUS = Variants of uncertain significance/ P = Pathogenic / LP = Likely pathogenic /ACMG = American College of Medical Genetics and Genomics / OoW = Onset of walking / HPO = Human Phenotype Ontology / VM = Venous malformation

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the ethical board of Deutschen Verbandes für Physiotherapie an der Physio-Akademie in Wremen, Germany, project number 2025-02. For participants under 16 years of age, written informed consent was obtained from their parents or legal guardians, and all data were anonymized.

Consent for publication

Not applicable (no individual patient images or identifiable data are included).

Availability of data and materials

The datasets generated during this study (variant classifications and clinical phenotypes) are included in the manuscript. Raw sequencing data may be made available from the corresponding author upon reasonable request.

The authors declare that they have no competing interests.

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Authors' contributions

D.P. conceived the study, collected clinical data, performed genetic analysis, and drafted the manuscript.

K.R. critically revised the manuscript for intellectual content and methodology.

B.F. assisted with clinical interpretation and literature review.

J.O.S. contributed to data organization and preliminary analysis.

A.Z. provided technical support for work design.

All authors reviewed and approved the final manuscript.

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Figures



Figure 1

Right calf venous malformation with associated equinus gait



Figure 2

Asymmetric calf contour in a child with toe walking.



Figure 3

Plantar venous markings in a child with toe walking.