

ORIGINAL ARTICLE

Identification of candidate variants in *NEB*, *OFD1*, and *SLCO2A1* in a product of conception from a family with recurrent miscarriages

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ABSTRACT

Background: Recurrent fetal loss can arise from a wide range of genetic abnormalities, many of which remain undetected in the absence of comprehensive molecular testing. Trio-based clinical exome sequencing enables the systematic identification of candidate mutations and variants of uncertain significance (VUS) that underlie fetal loss and supports accurate diagnosis and informed genetic counselling for affected families.

Methods: Genomic DNA was extracted from fetal tissue and peripheral blood leukocytes of the couple, siblings, and grandparents. Trio-clinical exome sequencing was performed, followed by Sanger sequencing to confirm candidate variants. The variants were evaluated for pathogenicity and the pattern of inheritance.

Results: Trio-clinical exome sequencing identified two homozygous nonsynonymous variants in the *NEB* gene, one in exon 6 (NM_001164507.2: c.367C>T; p.Arg123Cys) and another in exon 133 (NM_001164507.2: c.20279A>G; p.Asp6760Gly) in the proband. In addition, a hemizygous nonsynonymous variant in exon 10 of the X-linked *OFD1* gene (NM_003611.3: c.1035G>T; p.Lys345Asn) and a homozygous nonsynonymous variant in exon 8 of the *SLCO2A1* gene (NM_005630.3: c.1105G>T; p.Gly369Cys) were observed in the proband. The *NEB* and *SLCO2A1* variants were heterozygous in the couple, and only the mother was heterozygous for the *OFD1* variant.

Conclusion: This study identified two candidate *NEB* gene variants (classified as VUS) as the most likely candidate contributors to recurrent fetal loss. The findings provide the basis for informed genetic counselling and reproductive risk assessment.

Keywords: Clinical exome sequencing, fetal tissue, *NEB*, recurrent pregnancy loss, variants of uncertain significance, consanguinity.

Introduction

Recurrent pregnancy loss (RPL), defined as two or more consecutive pregnancy losses, is one of the most common complications encountered during pregnancy, with fetal anomalies representing a significant contributing factor. Approximately 70% of human conceptions fail to achieve viability, and nearly 50% of pregnancies end in miscarriage before the clinical recognition of a missed period or the detection of embryonic cardiac activity (1). Preimplantation genetic diagnosis (PGD) for aneuploidy screening may be considered in couples with unexplained RPL (2,3). Genetic analysis of fetal tissue from couples

experiencing more than two miscarriages can elucidate the underlying etiology and support genetic counselling

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Received: 06 August 2026 | **Accepted:** 20 August 2026

to inform prognosis for subsequent pregnancies (2). Genetic factors, particularly chromosomal abnormalities, are among the most common identifiable causes of RPL, although a significant proportion of cases remain unexplained or are attributable to non-genetic causes (1). Nongenetic factors include advanced maternal age, alcohol consumption, cigarette smoking, drug use, heavy caffeine intake, chronic maternal diseases, uterine malformations, previous induced abortions, structural reproductive tract abnormalities, and prior miscarriages (4).

Pregnant individuals with a history of genetic disorders can be tested for single-gene disorders, chromosomal abnormalities, multifactorial or complex disorders, and teratogenic conditions to enable early diagnosis and intervention. Prenatal genetic defects can be detected through techniques such as karyotyping, chromosomal microarray analysis (CMA), and whole-exome sequencing (WES) of fetal DNA. Next-generation sequencing (NGS) has emerged as an invaluable tool, enabling not only the identification of novel disease-associated genes but also the clinical diagnosis of previously unexplained pathologies, thereby improving prenatal diagnostic accuracy (5).

WES is increasingly applied in the evaluation of RPL, although its diagnostic yield remains relatively low in unselected cases (~2%-5%), particularly in the absence of structural abnormalities. However, the yield improves in carefully selected cases with suspected monogenic etiologies. In contrast, prenatal exome sequencing has demonstrated higher diagnostic utility in fetuses with structural anomalies, with an additional yield of approximately 30%-36% following negative CMA (6). In cases of recurrent fetal anomalies, the diagnostic yield may reach up to ~40%, highlighting the importance of phenotype-driven testing (7).

Genetic variations in Nebulin (*NEB*), Oral-Facial-Digital Syndrome Type 1 (*OFDI*), and Solute Carrier Organic Anion Transporter Family Member 2A1 (*SLCO2A1*) have been associated with fetal anomalies and recurrent spontaneous miscarriages. Variants in *NEB* lead to muscle weakness and nemaline myopathy (8). The *NEB* gene (OMIM#161650), located on chromosome 2q23.3, causes nemaline myopathy 2 (OMIM#256030), an autosomal recessive skeletal muscle disorder. Variants in *OFDI* (OMIM#300170), located on chromosome Xp22.2, are responsible for Joubert syndrome 10 (OMIM#300804) and oral-facial-digital syndrome type 1 (*OFDI*; OMIM#311200). Joubert syndrome is characterized by abnormal hindbrain development, hypotonia, cerebellar ataxia, dysregulated breathing, and developmental delay. In contrast, oral-facial-digital syndrome presents with craniofacial, oral, and digital malformations and follows an X-linked dominant inheritance pattern with male lethality (9). Variants in *SLCO2A1* (OMIM#601460), located on chromosome 3q22.1, cause primary hypertrophic osteoarthropathy 2—*PHOAR2* (OMIM#614441) in the autosomal recessive form, while heterozygous variants result in the autosomal dominant form of *PHOAR2* (OMIM#167100). *PHOAR2* is a rare disorder characterized by digital

clubbing, pachydermia, and periostosis, with more severe manifestations observed in males (10,11).

This study reports the identification of homozygous and hemizygous variants in three genes (*NEB*, *SLCO2A1*, and *OFDI*, respectively) in a fetus from a couple with a history of multiple miscarriages, culminating in intrauterine fetal demise (IUFD).

Materials and Methods

Ethical considerations and informed consent

The Institutional Ethics Committee of Madras Medical College (MMC) approved the study (Approval No: 34042021; Dated: 07.04.2021), and written informed consent was obtained from all participants.

Clinical history of the family

A 28-year-old female with a history of multiple miscarriages resulting in IUFD was referred to the Department of Genetics, Dr ALM Post-Graduate Institute of Basic Medical Sciences, University of Madras, for diagnosis and genetic counseling. The couple was related by a consanguineous marriage (first cousins), and the mother had a poor obstetric history. During the first pregnancy, the fetus was male (confirmed by fetal/perinatal autopsy) at 13-14 weeks gestational age (by LMP), presenting with cardiac anomalies and bradycardia on ultrasound scan. Fetal autopsy revealed facial dysmorphism including hypertelorism (inner intercanthal distance 0.5 cm), flat nose with anteverted nares, long philtrum, elongated neck, and cardiomegaly with dilated ascending aorta and aortic arch with four systemic branches. The lung parenchyma was completely autolyzed and could not be assessed. Histopathological examination of the placenta revealed diffuse villitis, subchorionic hemorrhage, and microinfarctions; the pathologist noted that placental infection had probably contributed to IUFD. Skeletal muscle histopathology was not possible due to autolytic changes at 13-14 weeks gestational age. Chromosomal aneuploidy screening of the fetal tissue was performed using the KaryoLite BoBs assay, and no aneuploidy was detected in any chromosome, including X/Y. In the second pregnancy, an antenatal scan identified a cardiac anomaly, resulting in IUFD at 14 weeks of gestation. Cytogenetic analysis revealed normal karyotypes for the couple. Fetal tissue from the third pregnancy, along with peripheral blood samples from the couple, their siblings (B1 and B2: paternal siblings of the father; B3: maternal sibling of the mother), and the couple's parents (maternal grandmother, maternal grandfather, paternal grandmother, paternal grandfather), was collected for further genetic analysis.

Pregnancy timeline

Pregnancy 1: 13-14 weeks gestation (by LMP); male fetus (confirmed by autopsy); cardiac anomalies and bradycardia on ultrasound; fetal autopsy performed (facial dysmorphism, cardiomegaly with dilated ascending aorta; lung parenchyma autolyzed; skeletal

muscle histopathology not possible due to autolysis); placental histopathology showed villitis, subchorionic haemorrhage, and microinfarctions; chromosomal aneuploidy screening (KaryoLite BoBs) on fetal tissue was normal, including X/Y; outcome: IUFD.

Pregnancy 2: 14 weeks gestation; cardiac anomaly identified on antenatal ultrasound; parental cytogenetic (karyotype) analysis was normal for both partners; no fetal autopsy or fetal genetic testing performed; outcome: IUFD.

Pregnancy 3 (proband): Evaluated at 12-13 weeks of gestation (by ultrasound crown-rump length; CRL 62 mm, corresponding to 12 weeks 4 days); non-immune hydrops fetalis (generalized edema, pleural effusion, ascites) with absent cardiac activity on ultrasound; early gestational age and autolytic changes precluded detailed autopsy and skeletal muscle histopathology; no dedicated aneuploidy assay (e.g., KaryoLite BoBs) was performed on this pregnancy; this fetus was the proband, which underwent trio-clinical exome sequencing (with the parents), maternal cell contamination (MCC) testing, and Sanger confirmation of candidate variants

DNA extraction

Genomic DNA was extracted from fetal tissue and whole blood samples using a modified phenol–chloroform–isoamyl alcohol (P:C:I) method (12). The concentration and purity of the extracted DNA were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, DE, USA). DNA integrity was assessed by electrophoresis on a 0.8% agarose gel using a Mupid Gel Electrophoresis System (Takara, Japan) and visualized with a UV Gel Doc system (Syngene, G: Box Chemi-XX6).

Trio-clinical exome sequencing

Targeted sequencing was performed using a custom-designed clinical exome capture kit covering approximately 6,670 disease-associated genes. Genomic DNA was extracted from peripheral blood samples and subjected to selective enrichment of protein-coding regions, followed by library preparation. Sequencing was performed on the Illumina platform, generating high-quality data with an average sequencing depth of 241X, an average on-target depth of 129.3X, and >92% of target bases covered at $\geq 30X$. MCC testing was performed separately on the DNA extracted from the fetal tissue sample at MedGenome Labs Ltd., Bangalore and was confirmed to be negative. As per the Pre-Natal Diagnostic Techniques (Regulation and Prevention of Misuse) Rules, 1994, the laboratory does not determine fetal sex from sequencing data; fetal sex was inferred from the hemizygous state of the X-linked *OFDI* variant in the proband. Raw reads were aligned to the human reference genome (GRCh38.p13) using the Sentieon aligner, following GATK best practices. Post-alignment processing included duplicate removal, base quality score recalibration, and indel realignment (13,14).

Variant calling and copy number detection

Variants were called using the Sentieon Haplotype Caller, generating an initial set of single nucleotide variants (SNVs) and small insertions/deletions (indels). Copy number variants (CNVs) were detected from the targeted sequencing data using ExomeDepth (v1.1.10), which identifies rare CNVs by comparing read depths to a matched aggregate reference dataset. The coding region coverage of *NEB* was 90.60%, and *SLCO2A1* was 100.00% (clinical exome report). The CNV detection sensitivity of the assay is 70%-75% for deletions/duplications greater than 10 bp; no large CNVs were identified in *NEB* or *SLCO2A1*. However, approximately 9.4% of the *NEB* coding region was not covered by the assay, and deep intronic variants, repeat expansions, or structural variants below the detection threshold may not have been identified.

Variant filtering and annotation

A stepwise variant filtering workflow was employed. All 6,670 genes in the clinical exome panel were screened for the given clinical indications. The non-immune hydrops panel genes were fully covered and analyzed; coverage of critical genes including *NEB* (90.60%) and *SLCO2A1* (100.00%) is provided in the supplementary appendix of the clinical exome report. Low-quality and low-depth variants were first removed. Common variants were filtered based on allele frequencies in public population databases, including 1000 Genomes Phase 3, gnomAD (v2.1), EVS, dbSNP (v151), 1000 Japanese Genome, and an internal Indian database. Functional filtering retained only protein-altering variants, including non-synonymous and splice-site changes, while synonymous variants were excluded. Variants were annotated using Ensembl VEP against Ensembl release 99 and cross-referenced with ClinVar, OMIM, HGMD, GWAS, and SwissVar databases.

Functional impact and clinical interpretation

The functional impact of nonsynonymous variants was assessed using multiple in silico prediction tools, including *SIFT*, *PolyPhen-2*, *MutationTaster2*, *LRT*, *CADD*, and *REVEL*. Clinically relevant variants were prioritized based on the patient phenotype and interpreted according to ACMG/AMP guidelines. Only actionable variants in coding regions and splice sites were reported, while silent variants without predicted functional impact were excluded.

Confirmation of trio-clinical exome sequencing-identified variants by Sanger sequencing

The candidate gene variants identified by trio-clinical exome sequencing were validated using Sanger sequencing. Selected exons of *NEB* (exons 6 and 133), *OFDI* (exon 10), and *SLCO2A1* (exon 8) were amplified using gene-specific primers. PCR primers were designed using the reference sequences from the NCBI database, and Primer3 was used to generate variant-specific primers. Sanger sequencing was performed using the ABI BigDye Terminator Cycle Sequencing Kit v3.1

(Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Sequencing was carried out on an ABI PRISM 3500 Genetic Analyzer (Tokyo, Japan), and the results were analyzed using BioEdit and FinchTV software.

Functional analysis of the impact of identified variants

The functional impact of the identified nonsynonymous variants was evaluated using multiple in silico prediction tools. Sorting Intolerant From Tolerant (*SIFT*) predicts whether amino acid substitutions are likely to affect protein function based on sequence homology and the physical properties of amino acids (Kumar et al., 2009), while Polymorphism Phenotyping v2 (*PolyPhen-2*) assesses the potential impact of these substitutions on protein structure and stability using sequence, phylogenetic, and structural information (<http://genetics.bwh.harvard.edu/pph2/>).

Combined Annotation Dependent Depletion (CADD) scores were obtained to estimate the deleteriousness of SNVs by integrating multiple genomic annotations (<https://cadd.bihealth.org/snv/>), and Rare Exome Variant Ensemble Learner (REVEL) was used as an ensemble method combining several computational tools to predict the pathogenicity of missense variants (<https://sites.google.com/site/revelgenomics/contact>).

Population frequency data were retrieved from publicly available databases, including the 1000 Genomes Project (1KGP) and gnomAD, and gene-level constraint metrics such as missense Z-scores from gnomAD were used to assess intolerance to variation. Additional details on prediction tool parameters and score interpretation have been described previously (Richards et al., 2015; Genet Med. 17:405–424).

Results

Trio-clinical exome sequencing identifies novel variants in the *NEB*, *OFD1*, and *SLCO2A1* genes

Trio-clinical exome sequencing of the proband and couple revealed two nonsynonymous variants in *NEB* (NM_001164507.2) and one variant each in *OFD1* (NM_003611.3) and *SLCO2A1* (NM_005630.3). In *NEB*, the proband carried two homozygous nonsynonymous variants: One in codon 123, c.367C>T (p.Arg123Cys) and the second in codon 6760, c.20279A>G (p.Asp6760Gly), corresponding to genomic positions chr2:g.151725488G>A and chr2:g.151547517T>C. Both variants were present in a heterozygous state in the couple. Interestingly, a hemizygous nonsynonymous variant, c.1035G>T (p.Lys345Asn), mapping to chrX:g.13751348G>T was in codon 345 of the X-linked *OFD1* gene in the proband; this variant was absent in the father but present in the mother in a heterozygous state. Additionally, the proband carried a homozygous nonsynonymous variant, c.1105G>T (p.Gly369Cys), corresponding to chr3:133948536C>A in *SLCO2A1* (NM_005630.3), with the couple being heterozygous

carriers. All identified variants were confirmed by Sanger sequencing (Figure 1).

Analysis of *NEB*, *OFD1*, and *SLCO2A1* variants in extended family members

Sequencing of extended family members, including siblings and grandparents, revealed that the maternal grandmother, paternal grandfather, and siblings B2 (paternal sibling of the father) and B3 (maternal sibling of the mother) were heterozygous carriers of *NEB* and *SLCO2A1* variants. The unrelated maternal grandfather, paternal grandmother, and paternal sibling (B1) did not carry any variants. The maternal grandmother was also found to be heterozygous for the X-linked *OFD1* variant (Figure 2).

The amino acid residues affected in *NEB* (p.Arg123 and p.Asp6760), *OFD1* (p.Lys345), and *SLCO2A1* (p.Gly369) were highly conserved across species, indicating that these variants may disrupt critical functional regions of the proteins (Figure 3).

Variant interpretation

The two homozygous variants identified in the *NEB* gene (p.Arg123Cys and p.Asp6760Gly) showed high *PolyPhen-2* scores (0.975 and 0.917, respectively), indicating probable damaging effects, along with elevated CADD scores (26.7 and 32), suggestive of functional impact. Although *SIFT* scores (0.269 and 0.424) were within the tolerated range, the combined interpretation of multiple tools, including REVEL scores (0.269 and 0.397), supports a possible deleterious effect. Additionally, the *NEB* gene demonstrated moderate intolerance to missense variation (Z-score = 2.25), further indicating that amino acid substitutions in this gene are less likely to be tolerated and may have functional consequences.

The hemizygous variant in the *OFD1* gene (p.Lys345Asn) showed a high *PolyPhen-2* score (0.966) and moderate REVEL score (0.501), suggesting a potentially damaging effect, while the *SIFT* score (0.682) indicated tolerance. The missense Z-score for *OFD1* was 0, suggesting that the gene is relatively tolerant to missense variation, thereby reducing the strength of computational evidence supporting pathogenicity.

Similarly, the homozygous variant in the *SLCO2A1* gene (p.Gly369Cys) demonstrated a high *PolyPhen-2* score (1.000), elevated CADD score (35), and high REVEL score (0.726), indicating a potentially damaging effect, although the *SIFT* score (0.726) suggested tolerance. The missense Z-score for *SLCO2A1* was 1.11, indicating only mild constraint, which limits the strength of evidence for functional intolerance.

Overall, while all variants exhibited computational evidence suggestive of potential deleterious effects, the combination of multiple prediction tools along with gene-level constraint metrics indicates that the *NEB* variants have comparatively stronger support for functional impact. In contrast, the *OFD1* and *SLCO2A1* variants show less consistent evidence, particularly due to

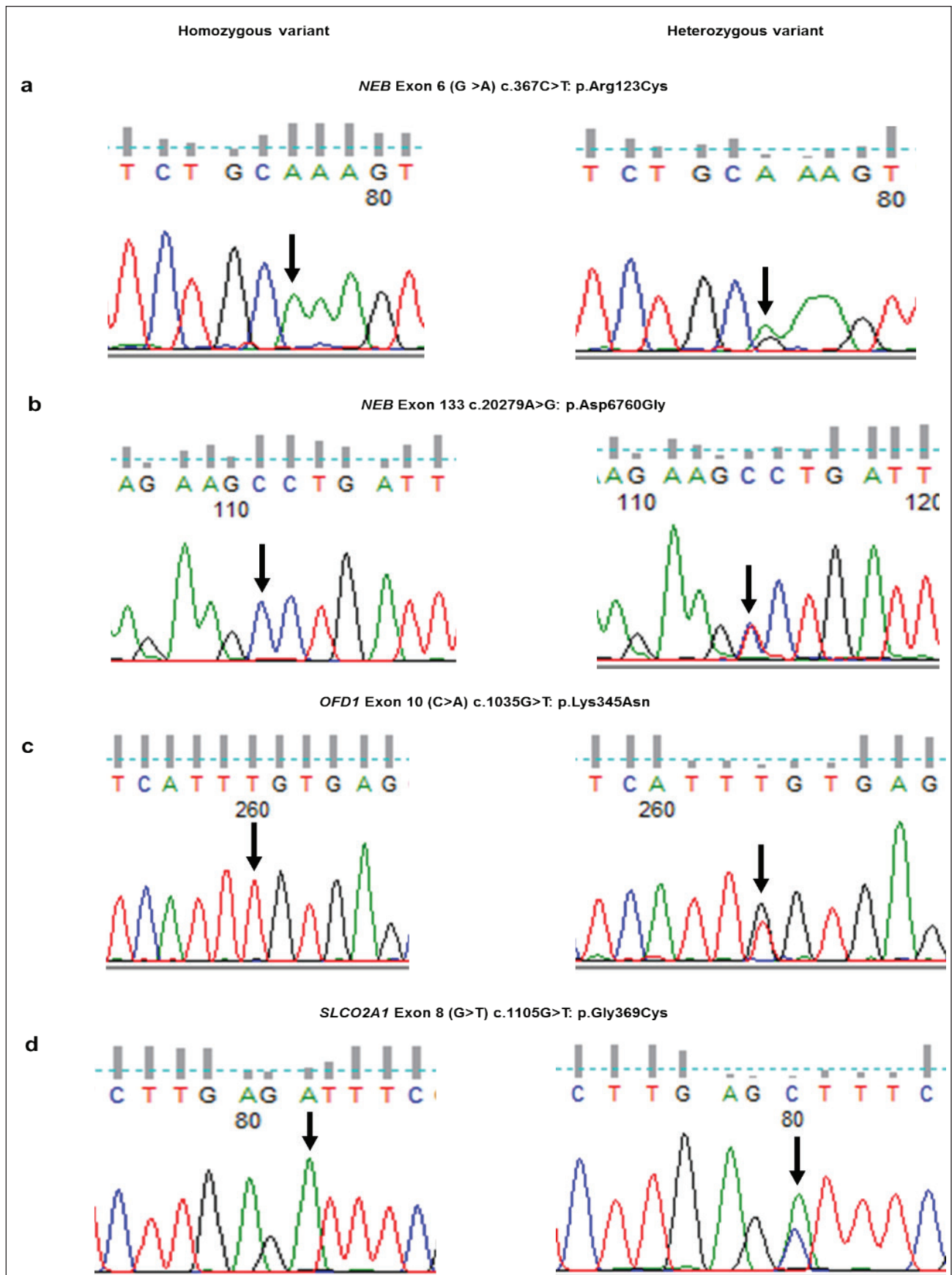


Figure 1. Sanger sequencing chromatograms confirming variants identified by trio-clinical exome sequencing in the proband and couple. a. A homozygous nonsynonymous variant in exon 6 of the *NEB* gene, c.367C>T, was identified in the proband, and the couple were heterozygous. b. Exon 133 of the *NEB* gene c.20279A>G variant was homozygous in the proband, and the couple were heterozygous. c. The nonsynonymous variant identified in exon 10 of the *OFD1* gene c.1035G>T was in a hemizygous state in the proband. The mother was heterozygous for this variant. No variant was detected in the proband's father. d. A homozygous nonsynonymous variant in exon 8 of the *SLCO2A1* gene c.1105G>T, was detected in the proband, and the couple were carriers.

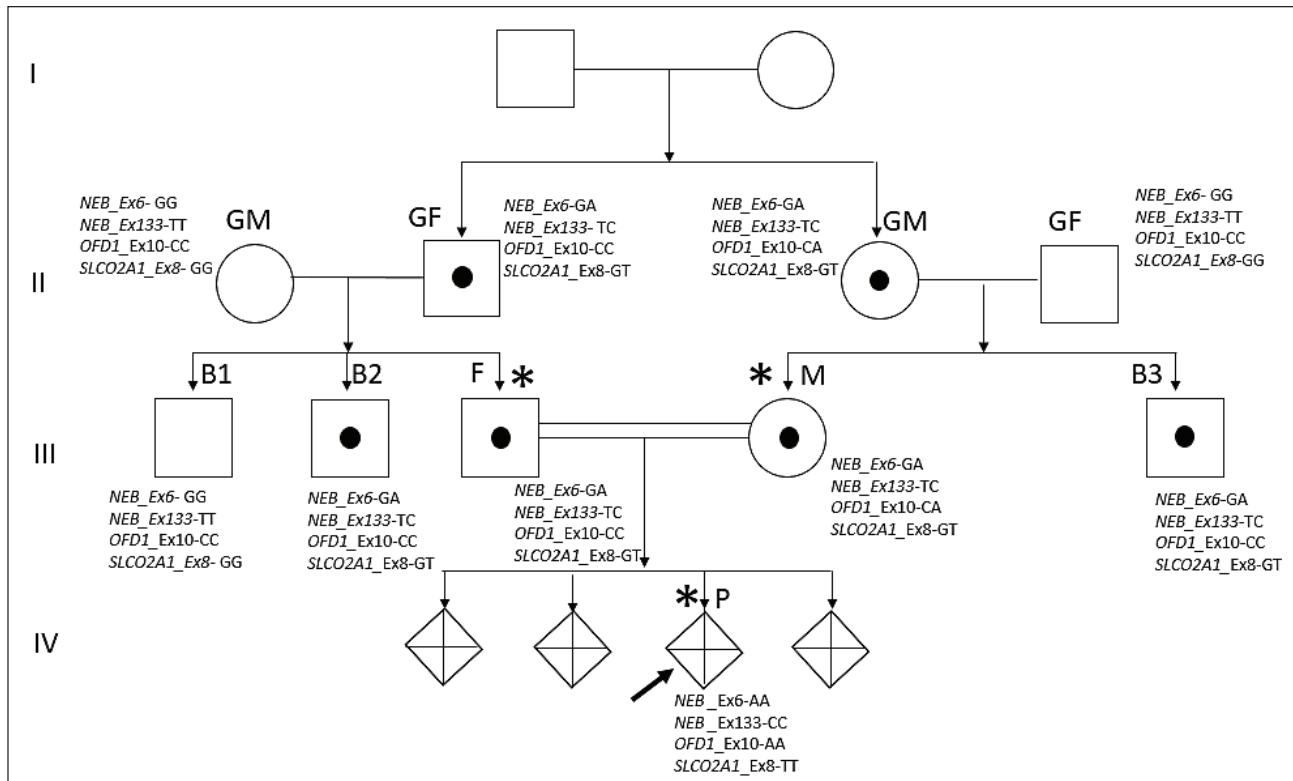


Figure 2. Pedigree chart of the proband's family showing segregation of identified variants. The black dotted circles (females) and squares (males) indicate family members carrying the gene variants, whereas the blank circles or squares indicate unaffected members. Asterisks indicate the DNA samples subjected to exome sequencing. The arrow indicates the proband. Abbreviations: P: Proband; F: Father; M: Mother; B1, B2: Paternal siblings (father's siblings); B3: Maternal sibling (mother's sibling); GF: Grandfather; GM: Grandmother.

gene-level tolerance and limited phenotypic correlation (Tables 1 and 2).

Phenotype–genotype correlation

The fetus was evaluated at 13 weeks of gestation and presented with non-immune hydrops fetalis, including generalized edema, pleural effusion, ascites, and absent cardiac activity. Early gestational age and autolytic changes precluded detailed autopsy and histopathological assessment, including confirmation of skeletal muscle abnormalities. Despite these limitations, the combination of recurrent IUFD, early-onset hydrops, consanguinity, and candidate *NEB* variants (VUS) are possibly consistent with a severe prenatal nemaline myopathy phenotype. It is acknowledged that hydrops fetalis and IUFD alone are not pathognomonic for *NEB*-related disease, and the PP4 criterion is applied with caution in the absence of histopathological confirmation of nemaline rods. Overall, even with limited histological data, the observed clinical pattern is compatible with *NEB*-related disease, providing a supportive phenotype–genotype correlation that is consistent with, but not diagnostic of, *NEB*-related prenatal myopathy. Histopathological confirmation of nemaline rods was not possible due to complete autolysis of fetal tissue at 13–14 weeks gestational age; this represents a key limitation of the phenotypic interpretation.

Discussion and Conclusion

We investigated the genetic cause of fetal loss in a proband from a 28-year-old woman with a history of multiple miscarriages. As the molecular basis of this unique miscarriage case was previously unknown, we performed trio-based clinical exome sequencing, which identified two novel homozygous nonsynonymous variants, p.Arg123Cys and p.Asp6760Gly, in the *NEB* gene. Both parents were confirmed to be carriers of these variants. Nebulin plays an essential role in actin filament-based movement, cardiac muscle cell development, muscle contraction, and muscle tissue formation. The histopathological assessment was not done.

Previous studies have linked *NEB* variants with Arthrogryposis Multiplex Congenita-6 (AMC6), a severe congenital myopathy characterized by fetal akinesia and multiple joint contractures (15). Yonath et al. (2012) reported abnormal prenatal ultrasound findings in fetuses affected by AMC6, while Abdalla et al. (2017) demonstrated that *NEB* variants contribute to fetal akinesia deformation sequence and lethal multiple pterygium syndrome (15,16). Ahmed et al. (2018) and Rocha et al. (2021) further confirmed the association of homozygous or compound heterozygous *NEB* variants with severe prenatal phenotypes (17,18). Formal haplotype phasing of the two *NEB* variants was not performed in this study; given the consanguineous background, these variants may reside on the same ancestral haplotype (identity

a. NEB c.367C>T: p.Arg123Cys			b. NEB c.20279A>G: p.Asp6760Gly			c. OFD1 c.1035G>T: p.Lys345Asn			d. SLC02A1 c.1105G>T: p.Gly369Cys		
Consensus	Conservation	DTPELRRIKKV	Consensus	Conservation	LIYKSDFFKMQ	Consensus	Conservation	ETYDRKLLKNEL	Consensus	Conservation	ANFLIGA VNL P
Homo sapiens	113	DTPELRRIKKV 123	Homo sapiens	6755	LIYKSDFFKMQ 6765	Homo sapiens	340	ETYDRKLLKNEL 350	Lemur catta	355	ANFLIGA VNL P 365
Pan paniscus	117	DTPELRRIKKV 128	Pan troglodytes	7241	LIYKSDFFKMQ 7251	Pan troglodytes	292	ETYDRKLLKNEL 302	Cavia porcellus	537	ANFLIGA VNL P 547
Pan troglodytes	117	DTPELRRIKKV 128	Gorilla gorilla gorilla	6755	LIYKSDFFKMQ 6765	Pan paniscus	340	ETYDRKLLKNEL 350	Rhinopithecus bieti	288	ANFLIGA VNL P 298
Gorilla gorilla gorilla	117	DTPELRRIKKV 128	Pan paniscus	6755	LIYKSDFFKMQ 6765	Cercopithecus atys	341	ETYDRKLLKNEL 351	Heterocephalus glaber	364	ANFLIGA VNL P 374
Pongo abelii	117	DTPELRRIKKV 128	Nomascus leucogenys	6264	LIYKSDFFKMQ 6274	Nomascus leucogenys	341	ETYDRKLLKNEL 351	Callithrix jacchus	364	ANFLIGA VNL P 374
Trachypithecus francoisi	117	DTPELRRIKKV 128	Pongo abelii	6750	LIYKSDFFKMQ 6760	Hyllobates moloch	340	ETYDRKLLKNEL 350	Acomys russatus	363	ANFLIGA VNL P 373
Rhinopithecus roxellana	117	DTPELRRIKKV 128	Rhinolophus ferrumequinum	5781	LIYKSDFFKMQ 5791	Gorilla gorilla gorilla	292	ETYDRKLLKNEL 302	Tupaia chinensis	362	ANFLIGA VNL P 401
Nomascus leucogenys	113	DTPELRRIKKV 123	Aotus nancymae	5778	LIYKSDFFKMQ 5788	Pongo abelii	292	ETYDRKLLKNEL 302	Cebus imitator	364	ANFLIGA VNL P 374
Ptilocolobus tephrosceles	117	DTPELRRIKKV 128	Trachypithecus francoisi	5783	LIYKSDFFKMQ 5793	Macaca thibetana thibetana	340	ETYDRKLLKNEL 350	Octodon degus	353	ANFLIGA VNL P 392
Colobus angolensis palliatus	117	DTPELRRIKKV 128	Rhinopithecus roxellana	5783	LIYKSDFFKMQ 5793	Rhinopithecus roxellana	292	ETYDRKLLKNEL 302	Propithecus coquereli	364	ANFLIGA VNL P 374
			Callithrix jacchus	5778	LIYKSDFFKMQ 5788	Macaca fascicularis	233	ETYDRKLLKNEL 243	Sciurus carolinensis	364	ANFLIGA VNL P 374
			Puma yagouaroundi	5787	LIYKSDFFKMQ 5797	Macaca mulatta	200	ETYDRKLLKNEL 210	Sapajus apella	364	ANFLIGA VNL P 374
			Panthera pardus	6273	LIYKSDFFKMQ 6283	Theropithecus gelada	371	ETYDRKLLKNEL 381	Aotus nancymae	364	ANFLIGA VNL P 374
			Macaca mulatta	5783	LIYKSDFFKMQ 5793	Macaca nemestrina	200	ETYDRKLLKNEL 210	Pongo abelii	364	ANFLIGA VNL P 374
			Macaca thibetana thibetana	5783	LIYKSDFFKMQ 5793	Mandrilus leucophaeus	341	ETYDRKLLKNEL 351	Colobus angolensis palliatus	362	ANFLIGA VNL P 372
			Chlorocebus sabaeus	5783	LIYKSDFFKMQ 5793	Trachypithecus francoisi	340	ETYDRKLLKNEL 350	Microcebus murinus	364	ANFLIGA VNL P 374
			Macaca fascicularis	5783	LIYKSDFFKMQ 5793	Rhinopithecus bieti	340	ETYDRKLLKNEL 350	Chlorocebus sabaeus	364	ANFLIGA VNL P 374
						Sapajus apella	340	ETYDRKLLKNEL 350	Macaca mulatta	364	ANFLIGA VNL P 374
						Saimiri boliviensis boliviensis	340	ETYDRKLLKNEL 350	Papio anubis	364	ANFLIGA VNL P 374
						Aotus nancymae	233	ETYDRKLLKNEL 243	Ptilocolobus tephrosceles	364	ANFLIGA VNL P 374
						Chlorocebus sabaeus	339	ETYDRKLLKNEL 349	Mandrilus leucophaeus	364	ANFLIGA VNL P 374
						Cebus imitator	340	ETYDRKLLKNEL 350	Macaca nemestrina	364	ANFLIGA VNL P 374
						Ptilocolobus tephrosceles	233	ETYDRKLLKNEL243	Oryctolagus cuniculus	363	ANFLIGA VNL P 373
						Callithrix jacchus	346	ETYDRKLLKNEL 356	Rhinopithecus roxellana	364	ANFLIGA VNL P 374
									Pan paniscus	364	ANFLIGA VNL P 374
									Hipposideros armiger	364	ANFLIGA VNL P 374
									Pan troglodytes	364	ANFLIGA VNL P 374
									Trachypithecus francoisi	334	ANFLIGA VNL P 344
									Gorilla gorilla gorilla	364	ANFLIGA VNL P 374
									Cercopithecus atys	364	ANFLIGA VNL P 374
									Nomascus leucogenys	364	ANFLIGA VNL P 374

Figure 3. Evolutionary conservation of mutated amino acid residues in NEB, OFD1, and SLC02A1. Clustal alignment showing 100% conservation of the amino acid Arginine (R) in codon 123 and Asparagine (D) in codon 6760 of the NEB gene (a & b), in the OFD1 gene amino acid residue Lysine (K) in codon 345 (c), in the SLC02A1 gene amino acid Glycine (G) in codon 369 (d) across the vertebrate species. Conserved amino acid residues are marked in red.

Table 1. Genetic variants identified by trio-clinical exome sequencing.

Gene	Ref Seq transcript / Protein ID	cDNA change	Protein change	Chromosome position/ dbSNP	Zygosity	Inheritance	Variant type
<i>NEB</i> (Exon 6)	NM_001164507.2/ NP_001157980.2	c.367C>T	p.Arg123Cys	Chr2:151725488/ rs200555425	Homozygous	Autosomal recessive	Nonsynonymous
<i>NEB</i> (Exon 133)	NM_001164507.2/ NP_001157980.2	c.20279A>G	p.Asp6760Gly	Chr2:151547517	Homozygous	Autosomal recessive	Nonsynonymous
<i>OFD1</i> (Exon 10)	NM_003611.3/ NP_003602.1	c.1035G>T	p.Lys345Asn	ChrX:13751348	Hemizygous	X-linked	Nonsynonymous
<i>SLCO2A1</i> (Exon 8)	NM_005630.3/ NP_005621.2	c.1105G>T	p.Gly369Cys	Chr3:133948536	Homozygous	Autosomal recessive	Nonsynonymous

Trio clinical exome sequencing was performed in the proband and both parents. This table summarizes the identified variants, including gene name, RefSeq transcript and protein identifiers (NM/NP), cDNA and protein changes, chromosomal position, zygosity, inheritance pattern, and variant type. All chromosomal positions are reported according to the human reference genome GRCh38.p13. NM, RefSeq mRNA accession number; NP, RefSeq protein accession number; cDNA, complementary DNA

by descent) or separate haplotypes, which cannot be determined from the current exome data.

In addition, we detected a hemizygous nonsynonymous variant (p.Lys345Asn) in exon 10 of the *OFD1* gene in the proband. The mother was heterozygous for this variant, while the father carried no variant, suggesting an X-linked inheritance pattern. The *OFD1* gene is known to play a crucial role in cilium assembly, membrane docking, and organelle localization via membrane tethering, and variants in this gene are often associated with X-linked male lethality. *OFD1*-related male lethality typically occurs during the first or second trimester, as previously reported in women with recurrent male miscarriages (9). However, given the absence of *OFD1*-specific phenotypic features in the proband, the *OFD1* variant identified in this study is interpreted as a possible contributory or modifying factor of uncertain relevance, noted for completeness and for genetic counselling purposes, rather than as a contributor to male fetal lethality. In our study, the hemizygous variant was maternally inherited; fetal sex was not determined by the sequencing laboratory as per regulatory requirements (Pre-Natal Diagnostic Techniques Rules, 1994), but is inferred to be male based on the hemizygous state of this X-linked variant.

Clinical features such as macrocephaly, nasal bridge abnormalities (depressed or broad), and lip malformations (cleft lip, pseudo-cleft lip, full lips, and prominent philtrum) have been observed in families carrying *OFD1* variants (9). Moreover, orofacioidigital syndrome type I (*OFD1*) is inherited as an X-linked dominant condition with male lethality, usually manifesting in the first or second trimester. The variant identified in our case was most likely maternally inherited. The *OFD1* gene lies within regions that escape X-chromosome inactivation (XCI), resulting in higher gene expression levels in females, approximately twice that of males (19). Therefore, heterozygosity for this gene in females is typically non-lethal.

In addition, we have also identified a novel homozygous nonsynonymous variant (p.Gly369Cys) in the *SLCO2A1* gene in the proband, which was heterozygous in the couple. Homozygous or compound heterozygous variants in *SLCO2A1* have been frequently associated with autosomal recessive primary hypertrophic osteoarthropathy-2 (*PHOAR2*) and autosomal dominant primary hypertrophic osteoarthropathy (*PHOAD*), characterized by digital clubbing, periostosis, and pachydermia (11,20). However, none of these phenotypic features were observed in the proband or the couple in the present case. This suggests that the identified *SLCO2A1* variant may have heterogeneous phenotypic effects and could be involved in later developmental stages rather than early embryogenesis.

Several studies have validated the functional importance of *NEB*. Bang et al. (2006) demonstrated that *NEB* knockout mice (*NEB*^{-/-}) exhibited 25% shorter skeletal muscle thin filaments, and isometric contractility assays revealed significantly reduced force generation in nebulin-deficient skeletal muscles, leading to

Table 2. *In-silico prediction, population frequency, and ACMG classification of variants identified by trio clinical exome sequencing.*

Gene	PolyPhen-2	SIFT	CADD	REVEL	SAS	gnomAD MAF	ACeMG Evidence	ACMG Classifi- cation	Inheritance/current phenotype
NEB (Exon 6)	0.975	0.269	26.7	0.269	0.00006601	0.00005476	PM2 + PP3 + PP1+PP4	VUS	Not reported in ClinVar; dbSNP rs200555425. Autosomal recessive, homozygous in proband, heterozygous in parents. VUS – candidate contributor to phenotype.
NEB (Exon 133)	0.917	0.424	32	0.397	0.0000112	0.000001376	PM2 + PP3 + PP1 + PP4	VUS	Not reported in ClinVar or dbSNP (novel). Autosomal recessive, homozygous in proband, heterozygous in parents. VUS – candidate contributor to phenotype.
OFD1 (Exon 10)	0.966	0.682	21.8	0.501	Novel	Novel	PM2 + PP3	VUS	Not reported in ClinVar or dbSNP (novel). X-linked, hemizygous in proband, heterozygous in mother. VUS—uncertain relevance to current phenotype; noted for completeness and genetic counselling.
SLCO2A1 (Exon 8)	1.000	0.726	35	0.726	Novel	Novel	PM2 + PP3	VUS	Not reported in 1000 Genomes, gnomAD, or internal MedVarDb. Autosomal recessive, homozygous in proband, heterozygous in parents. VUS—incidental finding; no PHOAR2 features observed.

This table presents in silico prediction scores (PolyPhen-2, SIFT, CADD, and REVEL), population frequencies from publicly available databases including the 1000 Genomes Project (1KGP) and gnomAD, and variant classification according to guidelines of the American College of Medical Genetics and Genomics. ACMG evidence codes applied include PM2 (low or absent population frequency), PP3 (computational evidence), PP1 (co-segregation with disease), and PP4 (phenotype specificity). All variants were classified as variants of uncertain significance (VUS).

PolyPhen-2, Polymorphism Phenotyping v2; SIFT, Sorting Intolerant From Tolerant; CADD, Combined Annotation Dependent Depletion; REVEL, Rare Exome Variant Ensemble Learner; SAS, South Asian population frequency (gnomAD/1000 Genomes); 1KGP-1000 Genomes Project; gnomAD-Genome Aggregation Database; ACMG, American College of Medical Genetics and Genomics; PM, moderate evidence of pathogenicity; PP, supporting evidence of pathogenicity; MAF, minor allele frequency; VUS, variant of uncertain significance.

compromised cardiac contractile function and death of the knockout mice (21).

Based on the genetic findings in the context of inheritance pattern, gene function, and phenotypic correlation, we consider the two homozygous variants identified in the *NEB* gene (c.367C>T; p.Arg123Cys and c.20279A>G; p.Asp6760Gly) to be the most likely candidate contributors to the observed phenotype, rather than an established primary cause, given that both remain variants of uncertain significance (VUS) and the functional testing or confirmatory skeletal muscle histopathology was not available. These variants are consistent with an autosomal recessive mode of inheritance, segregate appropriately within the family (homozygous in the proband and heterozygous in both parents), and are supported by established literature linking *NEB* variants to severe congenital myopathies, including fetal akinesia and IUFD.

The hemizygous variant in the *OFD1* gene (c.1035G>T; p.Lys345Asn), inherited from the mother, is noted given its association with X-linked disorders and male lethality. However, due to the absence of definitive phenotypic features specific to *OFD1*-related conditions in the proband, its role in the present case remains uncertain and is therefore interpreted as a possible contributory or modifying factor rather than a primary cause.

In addition, a homozygous variant (c.1105G>T; p.Gly369Cys) in the *SLCO2A1* gene was identified in the proband, with both parents being heterozygous carriers. Although variants in *SLCO2A1* are known to be associated with primary hypertrophic osteoarthropathy, the classical phenotype typically manifests during childhood or adolescence rather than in the prenatal period. In the present case, no clinical features consistent with this condition were observed in the proband or the parents, suggesting a lack of phenotypic correlation. Therefore, the direct role of this variant in the observed IUFD remains uncertain. It is possible that this variant exhibits variable expressivity or incomplete penetrance, or that it may not be causally related to the fetal outcome. Accordingly, this variant is reported as a variant of uncertain clinical significance in the context of the current phenotype and is presented as an incidental finding, as no phenotypic features consistent with *PHOAR2* were identified in the proband or the couple.

Overall, *in silico* analysis of the identified variants suggests a potentially deleterious effect for the two homozygous variants identified in the *NEB* gene and demonstrates the strongest evidence for pathogenic relevance, supported by their segregation pattern, extremely low population frequency, and their well-established association with severe congenital myopathies and fetal akinesia leading to IUFD. In contrast, the variants identified in *OFD1* and *SLCO2A1*, although predicted to be damaging, lack consistent phenotypic correlation and are typically associated with conditions manifesting later in development or with distinct clinical features not observed in the present case.

Therefore, based on the combined *in silico* predictions, inheritance pattern, and genotype–phenotype correlation, the *NEB* gene variants are the most likely contributors

to the observed fetal phenotype, while the *OFD1* and *SLCO2A1* variants are interpreted as variants of uncertain or possible modifying significance. The findings enabled informed genetic counseling and supported reproductive risk assessment and decision-making for the affected couple.

Acknowledgments

We are grateful to all the participants in this study. We thank Dr. Bharath Govindaswamy for his contribution in improving the manuscript. We gratefully acknowledge Department of Science and Technology—Fund for Improvement of S&T Infrastructure (DST-FIST) and University Grants Commission—Special Assistance Programme (UGC-SAP) for the infrastructural facilities and the Department of Health Research—Multidisciplinary Research Unit (DHR-MRU) for the sequencing facility.

List of Abbreviations

1KGP	1000 Genomes Project
GnomAD	Genome Aggregation Database
ACMG	American College of Medical Genetics and Genomics
AMC6	Arthrogryposis Multiplex Congenita-6
CADD	Combined Annotation Dependent Depletion
cDNA	complementary DNA
CMA	chromosomal microarray analysis
CNV	copy number variation
DNA	deoxyribonucleic acid
HGMD	Human gene mutation database
IUFD	Intrauterine fetal demise
LMP	Last menstrual period
MAF	Minor allele frequency
MCC	Maternal cell contamination
NEB	Nebulin
NGS	Next-generation sequencing
NM	Reference sequence mRNA accession number
NP	Reference sequence protein accession number
OFD1	Oral-Facial-Digital Syndrome Type 1
PCR	Polymerase chain reaction
PGD	Preimplantation Genetic Diagnosis
PHOAD	Primary Hypertrophic Osteoarthropathy, Autosomal Dominant
PHOAR2	Primary Hypertrophic Osteoarthropathy-2
PM	moderate evidence of pathogenicity
PolyPhen-2	polymorphism phenotyping v2
PP	supporting evidence of pathogenicity
REVEL	Rare Exome Variant Ensemble Learner
SAS	South Asian population frequency (gnomAD/1000 Genomes)
SIFT	Sorting Intolerant from Tolerant
SLCO2A1	Solute Carrier Organic Anion Transporter Family Member 2A1
SNV	single nucleotide variants
VEP	variant effect predictor
VUS	variant of uncertain significance
WES	whole-exome sequencing

Conflict of interest

The authors of this article have no affiliations with or involvement in any organization or entity with any financial

interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Funding

This study was supported by research grants from the Department of Health Research (File No. V.25011/570(i)/2010-HR; Dated: 28.03.2014) and the Department of Biotechnology (File No. BT/PR45460/MED/12/952/2022; Dated: 30.03.2022 Government of India).

Consent to participate

The Institutional Ethics Committee of Madras Medical College (MMC) approved the study (Approval No: 34042021; Dated: 07.04.2021). Written informed consent was obtained from the couple for genetic testing of fetal tissue, and separately from all extended family members (siblings and grandparents) who provided blood samples for segregation analysis.

Ethical approval

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Consent for publication

Written informed consent for publication of the fetal clinical, autopsy, pedigree, and genetic findings, including identifying pedigree information, was obtained from the proband's parent.

Author contributions

Gopinath Padmavathi and Arasambattu Kannan Munirajan were involved in the study conception and design. Gopinath Padmavathi, Ravi Sudesh, and Arasambattu Kannan Munirajan assisted in the analysis and interpretation of the data. Gopinath Padmavathi, Arasambattu Kannan Munirajan, and Avaniyapuram Kannan Murugan drafted and edited the manuscript.

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