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GENETIC VARIANTS IN GENES CORRELATED TO THE PI3K/AKT PATHWAY: THE ROLE OF *ARAP3*, *CDH5*, *KIF11* AND *RELN* IN PRIMARY LYMPHEDEMA

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ABSTRACT

Genetic anomalies affecting lymphatic development and function can lead to lymphatic dysfunction, which could manifest as lymphedema. Understanding the signaling pathways governing lymphatics function is crucial for developing targeted diagnostic and therapeutic interventions. This study aims to characterize genetic variants in genes involved in the PI3K/AKT signaling pathway, which plays a critical role in lymphangiogenesis. 408 patients diagnosed with primary lymphedema were sequenced using a next-generation sequencing (NGS) gene panel composed of 28 diagnostic genes and 71 candidate genes. The analysis revealed six variants in genes RELN, ARAP3, CDH5, *and* KIF11. *Five of these variants have never been reported in the literature. All these genes have been correlated to lymphatic activity and are involved in the PI3K/AKT pathway. As the PI3K/AKT signaling pathway plays an essential role in lymphangiogenesis and lymphatic function, genetic variants in genes correlated to this pathway could lead to lymphedema. Our findings underscore the potential of the PI3K/AKT pathway in lymphedema pathogenesis, supporting the role of* RELN, ARAP3, CDH5, *and* KIF11 *as diagnostic and therapeutic targets.*

Keywords: ARAP3, CDH5, RELN, PI3K/AKT pathway, primary lymphedema

The lymphatic system, crucial for tissue homeostasis, forms a network of vessels connecting lymphoid organs such as lymph nodes, tonsils, thymus, and spleen. Its primary role is to drain excess interstitial fluid from interstitial tissues. Defects in its development or function lead to lymphatic anomalies, notably lymphedema, a chronic condition characterized by swelling and reduced function due to impaired lymphatic fluid clearance and chronic inflammation (1-3). Lymphedema is classified as primary, resulting from hereditary or genetic abnormalities, or secondary, acquired post-injury, infection, or surgery (4,5). The prevalence of primary lymphedema is approximately 1/100,000, while secondary lymphedema is more common, with a prevalence of about 1/1000 (3,5). The true incidence and prevalence of primary lymphedema are largely unknown due to under-reporting and under-recognition. In adults, primary lymphedema is significantly less common than secondary, comprising less than one percent of all cases of lymphedema. In the pediatric population, however, primary lymphedema is significantly more common, encompassing over 90% of cases (5,6).

The majority of primary lymphedema cases are sporadic, often with unknown mutations. Over thirty genes and loci have been identified and implicated in lymphangiodysplasia or lymphangiogenesis. Identified genes implicated in primary lymphedema include *FLT4* (Fms-related tyrosine kinase 4; OMIM 136352), *VEGFC* (Vascular endothelial growth factor C; OMIM 601528), and *FOXC2* (Forkhead box C2; OMIM 602402) (3,6). *FLT4* encodes a tyrosine kinase receptor for vascular endothelial growth factors C and D (VEGFR-3), which controls lymphatic system formation and maintenance of the lymphatic endothelium (7). Vascular endothelial growth factor C (VEGF-C), which binds to VEGFRs, is the key molecule that directs proliferation and migration of LECs during embryogenesis (8). VEGFC-VEGFR-3 signaling primarily affect the RAS/MAPK and PI3K/AKT pathways, with the latter being pivotal for lymphangiogenesis (3,9). The PI3K/AKT pathway governs various physiological processes, including inflammation, cell proliferation, apoptosis, and lymphangiogenesis (3,10). Cell signaling starts with activation of PI3KA by receptor tyrosine kinases, such as VEGFRs, by growth factors, such as VEGFs, and continues with AKT activation (3,10). Many genes that are correlated to lymphedema or lymphatics development participate in the PI3K/AKT pathway, such as *ARAP3* (Arf-GAP with Rho-GAP domain; OMIM 606647), *RELN* (Reelin; OMIM 600514), *CDH5* (Cadherin 5; OMIM 601120) and *KIF11* (Kinesis Family member 11; OMIM 148760) (3).

This study aims to comprehensively describe the pathogenic or likely pathogenic genetic variants identified in genes belonging to the PI3K/AKT pathway in a cohort of lymphedema patients. By conducting genetic analysis, this study aims to unravel the molecular mechanisms underlying lymphedema and to identify potential targets for therapeutic intervention.

MATERIALS AND METHODS

Subjects and Samples

We analyzed a cohort of Italian patients suffering from lymphedema. All patients underwent pre-test genetic counseling, during which clinical data including personal and family history were collected. The patients were informed about the significance of genetic testing, and we obtained written informed consent from all of them, in accordance with the Declaration of Helsinki. Ethical approval and clearance were received from the Ethical Committee of Azienda Sanitaria dell'Alto Adige, Italy (Approval No. 132-2020). Genomic DNA was extracted from saliva samples or peripheral blood samples using a commercial kit (SaMag Blood DNA Extraction Kit (Sacace Biotechnologies, Como, Italy)) following the manufacturer's instructions.

Panel Design and Sequencing

We designed a comprehensive NGS panel of 99 genes, comprising 28 diagnostic genes and 71 candidate genes, as described in

Bonetti et al (3). The genes included in the panel were retrieved from various databases including the Human Gene Mutation Database (HGMD Professional), Online Mendelian Inheritance in Man (OMIM), UniProt, GeneReviews, and PubMed. The custom DNA probes were designed using Twist Bioscience technology (https://www. twistbioscience.com/ (accessed on 1 September 2022)) and the targets includedcoding exons with 15 bp flanking regions of each exon. DNA samples were processed before the analysis as in Bonetti et al (3). DNA sequencing was carried out using a MiSeq personal sequencer (Illumina, San Diego, CA, USA). Primer sequences, PCR reaction conditions, and sequencing conditions are available on request.

Bioinformatics

Following sequencing, Fastq (forward-reverse) files were produced. These reads were aligned to the reference genome using the Burrow-Wheeler Aligner (BWA) software, version 0.7.17-r1188. Duplicate sequences were removed for clearer downstream analysis using SAMBAMBA (version 0.6.7) and MarkDuplicates from the GATK toolkit (version 4.0.0.0). The BAM alignment files were further refined by employing local realignment and base quality score recalibration with GATK's RealignerTargetCreator and IndelRealigner. Minor allele frequencies (MAF) were extracted from the Genome Aggregation Database (GnomAD), an essential tool for studying genetic variant frequencies in populations. VarSome was utilized for in silico predictions of nucleotide changes' deleterious effects, offering insights into the genetic variants' potential functional impacts. Variants were categorized following the American College of Medical Genetics (ACMG) guidelines into pathogenic, likely pathogenic, variants of unknown significance

TABLE 1
Characteristics of the Probands Analyzed for this Study and in which Genetic Variants were Identified

Characteristics		Case Subjects (n=6)
Age	Mean Median	42 ± 12 39
Gender	Females/Males	5/1 (83% / 17%)
Period of onset	Childhood (1-10 years) Youth (11-17 years)	4 (67%) 2 (33%)
Age of onset	Mean Median UNKNOWN	8 ± 4 10 1
Familiarity	Sporadic Familiar	3 (50%) 3 (50%)
Location	Lower limbs	6 (100%)
Gender	Females/Males	5/1 (83% / 17%)
Period of onset	Childhood (1-10 years) Youth (11-17 years)	4 (67%) 2 (33%)
Age of onset	Mean Median UNKNOWN	8 ± 4 10 1
Familiarity	Sporadic Familiar	3 (50%) 3 (50%)
Location	Lower limbs	6 (100%)

TABLE 2
Genetic Variants Identified in Genes Involved in PI3K/AKT Signaling

Gene	RefSeq	Nucleotide Change	dbSNP	Amino Acid Change	Zygosity	ACMG	Frequency in GnomAD	Reference
RELN	NM_173054	c.3334G>T	rs1472749720	p.(Ala1112Ser)	Het	LP	0.00000636	NA
RELN	NM_173054	c.8608C>T	rs753769576	p.(Gln2870Ter)	Het	P	NA	NA
CDH5	NM_001795	c.836_839del	rs1961063700	p.(Ser279CysfsTer13)	Het	P	0.00000821	NA
ARAP3	NM_022481	c.4634_4635insT	NA	p.(Ter1545Cysext*7)	Het	LP	NA	NA
KIF11	NM_004523	c.1159C>T	rs387906641	p.(Arg387Ter)	Het	P	NA	Ostergaard et al., 2012
KIF11	NM_004523	c.177_181del	NA	p.(Asp59GlufsTer9)	Het	LP	NA	NA

(P = Pathogenic; LP = Likely Pathogenic; NA = Missing data; Het = Heterozygous).

(VUS), likely benign, or benign classes (Available online: https://www.acmg.net/acmg/medical-genetics-practice-resources/practice-guidelines.aspx), as reported in Cristofoli et al (11). The study ultimately focused on reporting variants identified as either pathogenic or likely pathogenic.

RESULTS

A cohort of 408 patients was analyzed with a NGS panel comprising 99 diagnostic or candidate genes for lymphedema. *Table 1* reports the characteristics of the probands analyzed for this study and in which genetic variants were identified. All the patients presented lower limbs lymphedema and developed lymphedema earlier in life. *Table 2* reports the genetic variants identified in gene related to the PI3K-AKT pathway, specifically in *RELN*, *CDH5*, *ARAP3* and *KIF11* genes. We identified 6 heterozygous P or LP variants in 6 lymphedema patients. Three genetic variants were missense, resulting in a predicted deleterious amino acidic substitution, while three were insertions or deletions, resulting in termination of the protein and subsequent probable inactivation. All the variants have never been correlated with lymphedema in the literature, apart from the rs387906641 variant in *KIF11*.

DISCUSSION

Lymphedema is a chronic condition characterized by the accumulation of protein-rich interstitial fluid due to lymphatic system insufficiency, leading to swelling, weight gain, tension, and pain in the limbs (2,3,12). While the genetic underpinnings of lymphedema are not completely understood, current research suggests that known diagnostic genes associated with the condition can account for only about one-third of cases (2,13). To enhance the diagnostic sensitivity of lymphedema testing and gain a deeper insight into its molecular basis, it is imperative to identify new gene associations through expanded genetic screenings. Our aim was to investigate the presence of variants in genes known to be crucial for the PI3K-AKT pathway, namely *RELN*, *ARAP3*, *CDH5* and *KIF11* (*Fig. 1*). Given the challenging nature of lymphedema

management, early diagnosis and intervention are crucial for effective disease management and the prevention of complications.

The Reelin protein, encoded by the *RELN* gene, is a lymphatic endothelial-specific matrix molecule implicated in central nervous system and lymphatic vascular development. Reelin plays a pivotal role in cellular migration, cellular maturation, and synaptic function (14-16). Upon secretion, Reelin acts as a ligand that binds to its receptors, notably the very low-density lipoprotein receptor and the apolipoprotein E receptor 2, initiating downstream signaling pathways and modulating the cellular cytoskeleton (14,17). Reelin is secreted by lymphatic endothelial cells and mediates communication with adjacent smooth muscle cells (SMCs), promoting their contraction and active lymphatic circulation (18,19). Reelin activates different signaling cascades in target cells, among which phosphatidylinositol 3-kinase (PI3K) pathway. Reelin activates S6K1 through the mTORC1 complex and induces phosphorylation of Akt through mTORC2. Reelin activation of PI3K and AKT is dependent on SFK activity and Dab1 phosphorylation (17). Reelin-deficient mice show abnormal collecting lymphatic vessels characterised by a reduced number of SMCs, abnormal expression of lymphatic capillary marker lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), and impaired lymphatic function (18,19). Homozygous mutations to *RELN* have been correlated to lissencephaly with or without congenital lymphedema (OMIM 600514). Houriane et al reports a case of distinctive cerebral and

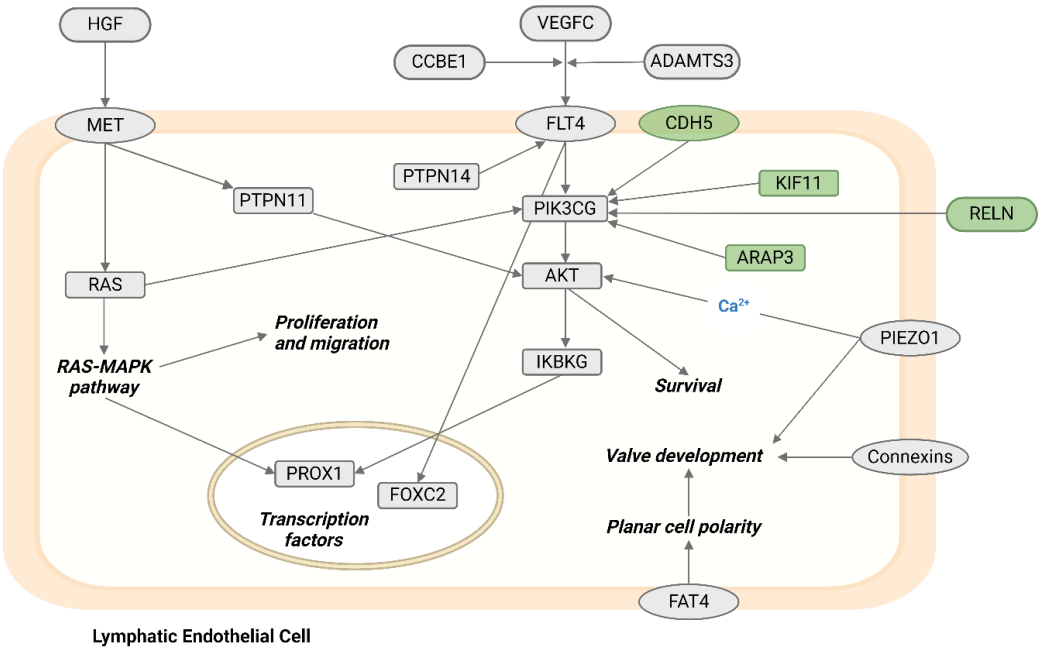

Fig. 1. *Schematic representation of the PI3K-AKT molecular pathway and of related genes involved in lymphedema. Proteins encoded by the genes in which genetic variants were identified are reported in green. Rectangle show cytoplasmic proteins and transcription factors, rounder rectangles show extracellular proteins, ovals show membrane proteins. Created with https://www.biorender.com/.*

cerebellar malformations and congenital lymphedema in lower and upper limbs caused by homozygous mutations to *RELN* (20). The two identified *RELN* genetic variants in lymphedema patients were heterozygous, suggesting a different inheritance for lymphedema and lissencephaly correlated to *RELN* gene. Patients didn't report any main neurologic symptoms.

The *CDH5* gene, also known as vascular endothelial cadherin or VE-cadherin, belongs to the cadherin superfamily (13,21). Cadherins are fundamental for intercellular adhesion and interactions, and they participate in the structural and functional organization of cells across various tissues (13,22). *CDH5* expression is exclusive to endothelial cells and plays a vital role in cell adhesion and the maintenance of endothelial barrier integrity, significantly contributing to the establishment and stability of endothelial junctions (23). Furthermore, *CDH5* interacts with VEGFR2 and VEGFR3, mediating AKT cell signaling. In both blood and lymphatic vessels, CDH5 serves as a critical adhesion molecule, facilitating vascular structure formation and junctional stability (13,24). Studies have underscored the indispensable role of VE-cadherin in vascular development, with its absence leading to significant impairments. Notably, during embryonic development, *CDH5* deletion in lymphatic endothelial cells has been associated with compromised lymphangiogenesis and subsequent embryonic lethality (13,24). Hence, *CDH5* emerges as a pivotal regulator in vascular morphogenesis, essential for both blood and lymphatic vessel development, and holds promise as a therapeutic target in vascular-related disorders. Additionally, *CDH5* plays a role in the maturation of lymphatic valves and is crucial for maintaining forward lymph flow throughout life. It regulates lymphatic valve development by binding to β-catenin, which is necessary for proper lymphatic valve formation during embryogenesis (13,21-23). In this study, we identified one heterozygous pathogenic variant in *CDH5* in one lymphedema patient. This variant has never been described in literature.

The *ARAP3* gene encodes the GTPase-activating protein ARAP3, comprising 35 exons and exhibiting a multidomain structure. This structure encompasses ArfGAP (GTPase-activating protein for ADP ribosylation factor) and RhoGAP (GTPase-activating protein for Rho family proteins) domains, ankyrin repeats, and the pleckstrin homology domain 3 (PH3). The collaborative action of the ArfGAP and RhoGAP domains regulates the cell cytoskeleton, while the PH3 domain facilitates cell signaling by binding to specific phosphoinositides (25-27). *ARAP3* activity is modulated by PI3K and RAP-GTP, which regulate its catalytic activity, cellular localization, and RhoGAP activity (24,25,27). Upon activation by PI3K, *ARAP3* translocates to the cell membrane, where its substrates RhoA-GTP and ARF6-GTP are located. Depletion of *ARAP3* impedes the activation of its substrate GTPases, leading to alterations in endothelial cell shape (27-29). Furthermore, *ARAP3* plays a crucial role in normal lymphatic system development, contributing to lymphatic vessel organogenesis and modulating cell adhesion and migration. The expression and function of *ARAP3* have been studied also in mice models (25,28). However, *ARAP3*-null mice show impaired blood vessel development as well as lymphatic system malformations and die prematurely. *ARAP3* dysregulation has also been reported in a mouse model of lymphatic diseases (25,28). These findings provide evidence that *ARAP3* is necessary for normal lymphangiogenesis during embryo development in mouse models (25). In this study, we identified one heterozygous likely pathogenic variant in *ARAP3* in one lymphedema patient. This variant has never been described in literature.

The *KIF11* gene encodes the kinesin family member 11 protein, also known as EG5 (2,30). This protein plays a crucial role in various cellular processes, particularly in establishing a bipolar spindle during mitosis to ensure proper chromosome positioning and centrosome separation thereby ensuring accurate cell division. Dysregulation of the *KIF11* gene can disrupt these processes, leading to abnormal cell division. Moreover, such dysregulation may impact the function-

ality of lymphatic vessels, potentially contributing to the onset or progression of lymphedema (30). *In vivo*, since *Kif11*+/– mice are phenotypically normal and *Kif11*–/– mice die prior to implantation (31,32). *KIF11* has been correlated to the PI3K/AKT pathway (2,32), and heterozygous mutations in the *KIF11* gene have been associated with a specific syndrome known as MCLMR (microcephaly with or without chorioretinopathy, lymphedema, or mental retardation; OMIM: 152950). Individuals with this syndrome often present with lower limb lymphedema of variable expressivity, along with other associated features such as microcephaly, chorioretinopathy, or mental retardation (2,3,30). Interestingly, *KIF11* heterozygous variants have already been reported to be correlated with non-syndromic primary lymphedema (12).

Although this study lacks functional studies on the identified genetic variants, we restricted our study only on pathogenic or likely pathogenic variants, and only to genes which have already been correlated to lymphedema or lymphatic defects. Moreover, the gene panel analyzed in this study should not be viewed as exhaustive, as ongoing research may unveil other potentially significant genes and pathways implicated in lymphedema, or other genes could be involved in the PI3K-AKT pathway. This is the first study that attempts to correlate several genes involved in the PI3K-AKT pathway to lymphedema development, and we have reported five new genetic variants in the *RELN*, *ARAP3*, *CDH5* and *KIF11* genes.

CONCLUSION

In this study, we identified six genetic variants in genes associated with the PI3K-AKT signaling pathway, supporting their potential role in the development of lymphedema. Our findings support the significance of the PI3K-AKT pathway in the pathogenesis of lymphatic disorders. A comprehensive understanding of this pathway and its associated genes holds promise for the discovery of novel diagnostic and therapeutic strategies for managing lymphedema.

ETHICAL CONSIDERATIONS

The patients were informed about the significance of genetic testing, and we obtained written informed consent from all of them, in accordance with the Declaration of Helsinki. Ethical approval and clearance were received from the Ethical Committee of Azienda Sanitaria dell'Alto Adige, Italy (Approval No. 132-2020). The approval covers the publication for research purposes of anonymized and aggregated genetic and clinical data once the diagnostic work for genetic and rare diseases has been completed.

CONFLICT OF INTEREST

All affiliations of the authors with private companies have been declared to make clear the position regarding the interests of these companies. The authors are affiliated with private companies for which there could be a possible conflict of interest. The authors of this article are reported to be patents inventors.

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