Lymphology 58 (2025) 46-55

GENETIC VARIANTS IN *LZTR1*, *MAP2K1* AND *RAF1*: INSIGHTS INTO THE ROLE OF RAS-MAPK PATHWAY IN PRIMARY LYMPHEDEMA

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ABSTRACT

The lymphatic system, a complex physiological network of lymphatic organs and vessels, is essential for maintaining fluid homeostasis. Dysfunction of lymphatic system can lead to lymphedema, a pathology characterized by the accumulation of interstitial fluid in peripheral tissues. This study aimed to identify novel genetic variants in genes within the RAS/MAPK pathway and assess their potential association with lymphedema onset. We conducted a retrospective analysis of the genetic and clinical data from 408 patients diagnosed with primary lymphedema. These patients were previously tested using a next-generation sequencing panel that included 28 diagnostic genes and 71 candidate genes. The analysis revealed five genetic variants in the genes LZTR1, RAF1 *and* MAP2K1. *Among the identified variants, four of them have never been reported in the literature. In silico analysis and molecular modelling supported the possible pathogenicity of one missense variant in* RAF1 *(c.1344T>G; p.Ile448Met), which could affect protein activation by phosphorylation. The results of this study highlight the genes involved in the RAS/MAPK signaling pathway as potential diagnostic targets for primary lymphedema.*

Keywords: LZTR1, MAP2K1, primary lymphedema, RAF1, RAS/MAPK pathway

INTRODUCTION

Lymphatic vasculature, critical for lipid absorption and inflammatory responses, main-

ly regulates fluid homeostasis by absorbing interstitial fluid in peripheral tissues. Altered lymphatic function results in accumulation of interstitial fluid, resulting in a chronic condition known as lymphedema (1-3). Lymphedema is marked by persistent swelling resulting from a buildup of protein-rich fluid, which under normal circumstances would be cleared by the lymphatic system. While it most frequently appears in the arms or legs, it can also affect areas such as the abdomen, neck, or genitals. (3,4). Women are affected more often than men and this condition is usually unilateral and affects the distal part of the limbs (5). Primary lymphedema arises from genetic defects that disrupt normal lymphatic system development, and it generally manifests during infancy, childhood, or adolescence (3). Its occurrence is rare, with an estimated prevalence of 1 in 100,000 individuals (2,3).

Over thirty genes and loci have been clinically implicated in lymphangiodysplasia or lymphangiogenesis (6,7). Lymphangiogenesis refers to the formation of lymphatic vessels through the sprouting of lymphatic endothelial cells (LECs) from existing vessels. This process is primarily controlled by signaling pathways, with the VEGFs/VEGFRs pathway playing a central role (8). The mitogen-activated protein kinase (MAPK) signaling pathway (RAS/RAF/MEK/ERK pathway) is one of the most commonly expressed signal transduction cascades (9,10). RAS signaling comprises the cascade of RAS-RAF (rapidly accelerated fibrosarcoma)-MEK (mitogen-activated protein kinase kinase)-ERK kinases, involving in each step several isoforms. The main function of the RAS/MAPK pathway is to transduce extracellular input, usually growth factors and small molecules that bind to their receptors, into the intracellular environment (10). Activation of the pathway begins when a growth factor binds to a protein tyrosine kinase receptor (RTK) to promote cell growth, migration, and proliferation (10,11). Activation of RTK leads to autophosphorylation and interaction with GRB2 (Growth Factor Receptor-Bound Protein 2). GRB2 acts as an adaptor protein between the EGF receptor and SOS (Son of Sevenless) protein (10,12,13). SOS proteins function as guanine nucleotide exchange factors, facilitating the exchange of GDP for GTP on RAS proteins, which in turn elevates the concentration of the active, GTP-bound form of RAS (12-14). The first targets of activated RTKs are the three RAS proteins—H-RAS, N-RAS, and K-RAS—which cooperatively regulate LECs proliferation and lymphatic vessel growth (1,11). RAS proteins are small guanosine nucleotide-bound GTPases which comprise a critical signalling network within the cell switching between an active GTP-bound form and an inactive GDP-form (8,10). Active GTP-bound RAS activates RAF, MEK and ERK cascades (8,10).

As the RAS/MAPK signaling pathway plays an essential role in regulating cell cycle, growth, differentiation and survival, its dysregulation leads to severe consequences. Several genes were identified to participate in the RAS/MAPK pathway, such as *LZTR1* (Leucine Zipper-Like Transcriptional Regulator 1, OMIM 600574), *RAF1* (Raf-1 Proto-Oncogene Serine/Threonine Kinase, OMIM 164760) and *MAP2K1* (Mitogen Activated Protein Kinase Kinase 1, OMIM 176872). Furthermore, Research has highlighted the critical role of RAS signaling pathways in development, uncovering specific mutations associated with a group of developmental disorders known as "RASopathies." These conditions result from germline mutations in key regulators of the RAS/MAPK pathway, and include Noonan syndrome (NA, OMIM 163950), Cranio-facio-cutaneous syndrome (OMIM 115150), and LEOPARD syndrome (OMIM 151100) (8,14,15). RASopathies have overlapping clinical features, but each syndrome has its unique characteristics. Most of these syndromes are marked by short stature, facial dysmorphism, congenital heart defects, lymphatic dysfunction, and intellectual disability (15).

The main purpose of this study is to identify in patients affected by primary lymphedema novel genetic variants in genes associated with the RAS/MAPK pathway detected. Although numerous studies have explored the genetic basis of lymphedema, molecular mechanisms underlying this condition remain largely unresolved. A deeper understanding of the

molecular and cellular mechanisms involved in the pathogenesis of lymphedema is pivotal to identify new diagnostic and therapeutic strategies.

MATERIALS AND METHODS

Subjects and Samples collection

We retrospectively analyzed the genetic and clinical data of a cohort of Italian patients diagnosed with primary lymphedema. Clinical evaluation didn't distinguish between syndromic and non-syndromic lymphedema, and only the lymphological context was discussed. Prior to sample collection of peripheral blood or saliva, all participants underwent pre-test counseling, during which detailed clinical data, including personal and family medical histories, were obtained. Patients were informed about the relevance of the genetic test and we obtained written informed consent from all patients in accordance with the Declaration of Helsinki. Ethical approval was granted from the Ethical Committee of Azienda Sanitaria dell'Alto Adige, Italy (Approval No. 132-2020). This approval authorizes the publication of anonymized and aggregated genetic and clinical data for research purposes after the completion of diagnostic work for genetic and rare diseases. Genomic DNA was extracted from peripheral blood and saliva samples with the SaMag Blood DNA Extraction Kit (Sacace Biotechnologies, Como, Italy) following the manufacturer's protocol.

Designing the Gene Panel and NGS Sequencing

Patients were sequenced using a NGS panel comprising 99 genes (28 diagnostic and 71 candidate), among which are genes correlated to RAS/MAPK signalling pathway. Of the analyzed genes, this retrospective study identified genetic variants in 3 candidate genes, namely *LZTR1*, *MAP2K1* and *RAF1*. More information regarding the gene panel and the diagnostic and candidate genes correlation with lymphedema can be found in Bonetti et al. (7). The analysis utilized oligonucleotide-based target capture techniques, employing the Illumina Nextera Rapid Capture Custom Assay and the Twist Custom Panel EF Workflow. Sequencing was conducted using 150 bp paired-end reads on the Illumina MiSeq platform (Illumina, San Diego, CA, USA), adhering to the protocols specified by the manufacturer. Details regarding PCR reaction, primer sequences and sequencing conditions are available on request.

Bioinformatic Analysis and Variant Classification

Bioinformatics procedures and details of workflow can be found in Donato et al. (16). Following sequencing, raw data were obtained in the form of Fastq files. Fastq files were produced and aligned to a reference genome with Burrow-Wheeler Aligner software (version 0.7.17-r1188). For clearer analysis, duplicate reads were eliminated with SAMBAMBA (version 0.6.7) and MarkDuplicates GATK (version 4.0.0.0). BAM alignment files were refined with GATK's RealignerTargetCreator and IndelRealigner tools. Minor allele frequencies (MAF) were obtained from the Genome Aggregation Database (GnomAD). VarSome was utilized for *in silico* predictions of the deleterious effects of nucleotide changes. Finally, variants were categorized following the standards and guidelines for the interpretation of sequence variants of the American College of Medical Genetics (ACMG) into five classes: pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign, or benign. Further subclassification of VUS variants into Hot, Middle, and Cold categories was performed based on an internal algorithm developed by Cristofoli et al. (17). Ultimately, only pathogenic, likely pathogenic, and hot VUS variants were considered for further studies.

In silico analysis of missense variants

VEP software (Available online: https://www.ensembl.org/info/docs/tools/vep/index.html) was used to calculate SIFT and Polyphen scores. Thresholds of SIFT < 0.05

TABLE 1
Characteristics of genetic variants identified in genes involved in the RAS/MAPK signaling pathway

Gene	RefSeq	Nucleotide Change	Amino Acid change	dbSNP	ACMG	Zygosity	Frequency in GnomAD	Reference
LZTR1	NM_006767	c.1149+1G>T	NA	rs767191322	P	Het	0.00000822	19
LZTR1	NM_006767	c.1616 G>A	p.Gly539Asp	rs746896119	LP	Het	0.00000137	NA
RAF1	NM_002880	c.1016 G>A	p.Ser339Asn	rs1057518084	VUS/hot	Het	NA	NA
RAF1	NM_002880	c.1344 T>G	p.Ile448Met	NA	LP	Het	0.000000684	NA
MAP2K1	NM_002755	c.251 A>G	p.Lys84Arg	rs774932586	VUS/hot	Het	0.00000616	NA

(P = Pathogenic; LP = Likely Pathogenic; VUS = Uncertain significance; NA = Not Available).

and Polyphen > 0.8 were used to select the variants to model. Modeling and evaluation of genetic variants on protein structure was performed using VENUS Software, part of MICHELANGLO suite (Available online: https://michelanglo.sgc.ox.ac.uk/michelanglo).

RESULTS

We retrospectively analyzed the genetic and clinical data of 408 patients diagnosed with primary lymphedema using a targeted NGS panel. The cohort of analyzed patients has been defined in Dundar et al. (18). Following sequencing and bioinformatics analysis, we identified 5 genetic variants in candidate genes correlated to the RAS/MAPK pathway in 5 unrelated patients diagnosed with primary lymphedema. *Table 1* reports the characteristics of genetic variants identified in *LZTR1*, *RAF1* and *MAP2K1* genes. We identified 1 pathogenic, 2 likely pathogenic, and 2 variants with uncertain significance, subclassified as hot. Among the identified variants, 4 variants were missense, resulting in a different amino acid incorporated into the structure of protein. One genetic variant was reported as an intronic splice site that occurs within the non-coding regions of a gene.

The clinical data of the patients in which genetic variants were identified are reported in *Table 2*. The average age of the probands analyzed for this study was 49 ± 19, ranging from 31 to 71 years old. All patients in our study were females and had lymphedema localized to the upper limbs, lower limbs or combined. According to literature, only one genetic variant in the *LZTR1* gene (rs767191322) has been previously described (19).

In silico analysis of missense variants showed deleterious SIFT and PolyPhen values in only p. (Ile448Met) variant in *RAF1* (*Table 3*). Consequently, molecular modelling and evaluation was performed on the selected variant. *Figure 1* shows the proximity of the mutated variant to phosphorylation sites in RAF1.

DISCUSSION

Recent studies have revealed that

TABLE 2
Clinical features of the probands analyzed for this study

Characteristic		Case Subjects (n=5)
Age	Mean	49 ± 19
	Median	43
Gender	Females	5 (100%)
Period of onset	Childhood (1-10 years)	2 (40%)
	Adult (>18 years)	3 (60%)
Age of onset	Mean	24 ± 16
	Median	29
Familiarity	Sporadic	3 (60%)
	Familiar	1 (20%)
	UNKNOWN	1 (20%)
Phenotype	Lower limbs	3 (60%)
	Upper limbs	1 (20%)
	Combined	1 (20%)

TABLE 3
Results of the *in silico* analysis of the identified genetic variants

Gene	Amino Acid Change	SIFT	PolyPhen	Estimated ΔΔG (with - without backbone movement allowed)	Predicted Effect
LZTR1	p.(Gly539Asp)	0.16	0.985	-	-
RAF1	p.(Ile448Met)	0	0.997	1 kcal/mol – 4 kcal/mol	Structurally neutral
RAF1	p.Ser339Asn	0.29	0.821	-	-
MAP2K1	p.Lys84Arg	0.07	0.277	-	-

SIFT and PolyPhen are from VEP software. Predicted effect is from VENUS software. ΔΔG was reported as calculated by VENUS software, which reports ΔΔG values with and without backbone movement allowed.

dysregulation of RAS/MAPK signaling pathway is a common molecular basis for a group of rare genetic disorders called RASopathies, most notably Noonan syndrome (NS). Clinical features of NS include lymphatic anomalies that may arise in more than 20% of NS cases, but are poorly documented (20,21-23). The lymphatic disorders related to RASopathies and NS have a characteristic pattern with bilateral lower limb lymphedema, genital swelling, and frequent systemic involvement. Lymphedema associated with NS usually presents at birth, although it is seen at all ages (23,24). In our study, we reported 5 novel genetic variants in candidate genes possibly correlated to primary lymphedema, namely

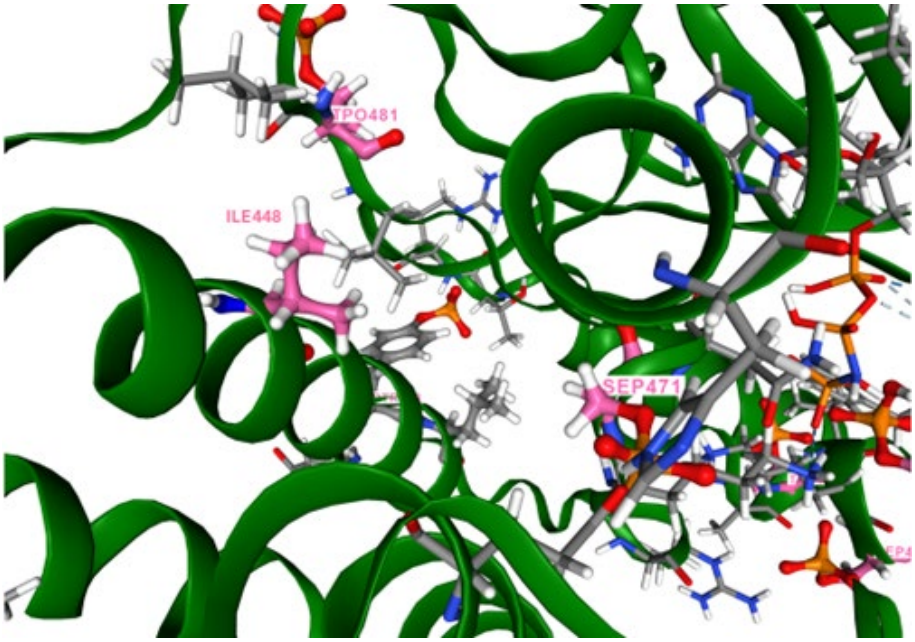

Fig. 1. *Proximity of Ile448 to phosphorylation residues in RAF1. Ile448, Phosphoserine (SEP)471, and Phosphothreonine (TPO)481 are highlighted in pink with phosphorus atoms in orange. Nearby residues are depicted in grey. The RAF1 backbone is in green.*

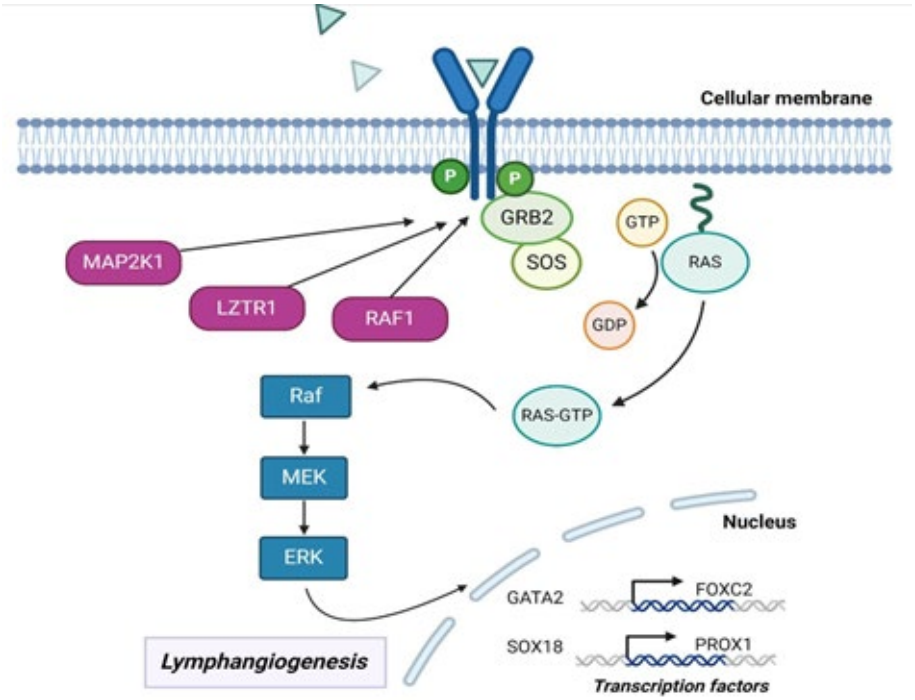

Fig. 2. *Schematic representation of the RAS/MAPK signaling pathway highlighting genes correlated to primary lymphedema. Candidate genes in which genetic variants were identified in this study are marked in purple.*

LZTR1, *RAF1*, *MAP2K1*, all associated with RAS/MAPK signaling pathway (*Figure 2*).

LZTR1 is a tumor suppressor gene that encodes a protein classified within the BTB-Kelch superfamily. The exact function of *LZTR1* is still poorly understood (25,26). It was initially described as a substrate effector for cullin 3 (CUL3)-RING ubiquitin ligase (CRL3) complexes (26,27). The protein is broadly expressed and is localized to the cytoplasmic surface of the Golgi network through specific protein-protein interactions facilitated by the BTB/POZ domain (25,26,28). In 2018, Steklov et al. demonstrated that RAS ubiquitination affects RAS/MAPK signaling and loss of *LZTR1* resulted in enhanced RAS activity and phosphorylation of MEK1/MEK2 and ERK1/ERK2 in all tested mutants (27). Several studies have linked to *LZTR1* as a causative gene in RASopathies (26,28,29). Analysis of *LZTR1* variants associated with NS suggests that this gene is functionally connected to the RAS/MAPK pathway, playing a role in negatively regulating RAS protein levels and MAPK signaling (26,29).

In the current study, we identified two heterozygous variants in the gene *LZTR1,* one of which (rs767191322) in a 71-year-old patient diagnosed with lower limb lymphedema. This variant has been already described in compound heterozygosity in a patient with an autosomal recessive form of NS, but without any clinical symptoms of lymphedema (19). Nevertheless, *LZTR1* has been proposed to be involved with lymphedema and lymphatic anomalies by two main studies (23,28), and this is the first study to report the two variants rs767191322 and rs746896119 as correlated to lymphedema.

RAF1 is a mitogen-activated protein kinase that is a part of the RAS/RAF/MEK/ERK signaling pathway regulating cell migration, apoptosis and differentiation (30,31). RAF1 is typically activated by the binding of growth factors or other extracellular signals to activated RTKs on the cell surface. Upon activation, RAF1 phosphorylates and activates MEK1/2 (MAPK/ERK kinase 1/2). MEK1/2 then phosphorylates and activates ERK1/2 (extracellular signal-regulated kinases 1/2), which in turn translocate to the nucleus to regulate gene expression and mediate cellular responses (21). *RAF1* mutations are linked to NS and related disorders, which can present with lymphatic dysplasia due to the abnormal development and function of the lymphatic system influenced by MAPK/ERK signaling (22). Nevertheless, *RAF1* is correlated in OMIM with LEOPARD Syndrome 2 (OMIM 611554) and Noonan syndrome 5 (OMIM 6115533), and published studies do not describe *RAF1* genetic variants as being correlated with syndromic or non-syndromic lymphedema. In our study, we identified two heterozygous missense variants in *RAF1* in patients diagnosed with primary lymphedema, suggesting its possible association with lymphatic defects. *In silico* analysis of the identified *RAF1* variants selected p.(Ile448Met) as the most promising for further studies. One variant of uncertain significance in this residue p.(Ile448Val) is associated in ClinVar to pathological cardiovascular phenotypes and RASopathy (Available online: https://www.ncbi.nlm.nih.gov/clinvar/variation/1770388/). Molecular modelling of p.(Ile448Met) variant shows the proximity of the mutated residue to two phosphorylation sites in RAF1, Ser471 and Trp481 (*Figure 1*). Phosphorylation on Ser471 is critical for Raf-1 kinase activity, and the mutated residue may disrupt the nearby a phosphorylation sites (32). Further studies should evaluate more in detail the effects of this variant on RAF1 activity.

MAP2K1, also known as MAPK/ERK kinase 1 (MEK1), is another gene involved in the disruption of signal transduction through the RAS/MAPK pathway. This dual-specificity kinase is essential in the ERK pathway, where it activates ERK1 and ERK2 (33). This gene encodes for MEK1 protein, an important component of the MAPK/ERK cascade. Despite its known role in activating ERK1/2, the precise functions of the *MAP2K1* gene and its broader effects on the RAS/MAPK signaling pathway remain not completely understood (34). Nevertheless, *MAP2K1* has already been proposed as being correlated to Noonan syndrome with lymphatic symptoms (35). In this article, we report a genetic variant in

MAP2K1 which has never been correlated to lymphedema in scientific literature. Although further *in silico*, *in vitro* and *in vivo* studies could support the effect of the identified variants in *LZTR1*, *RAF1* and *MAP2K1* on the mutated proteins' activity, this is one of the first studies that evaluate RAS/MAPK pathway for its correlation specifically to lymphatic defects and primary lymphedema, and could possibly help in improving diagnostic practice for patients with lymphedema.

CONCLUSION

In our study we reported five genetic variants in *LZTR1*, *RAF1* and *MAP2K1* genes, all implicated in the RAS/MAPK signaling pathway. Notably, all of these variants have not been previously reported in the literature as being associated with primary lymphedema. *In silico* analysis evaluating the deleteriousness of the identified variants showed no promising results, apart from the c.1344T>G variant in RAF1. Molecular modelling further revealed c.1344T>G possible effect on RAF1 phosphorylation. These findings suggest the importance of considering genes related to the RAS/MAPK pathway in genetic analysis for primary lymphedema, while additional studies are needed to confirm the correlation of the identified variants with the pathological phenotype.

ACKNOWLEDGMENTS

This research was funded by Provincia Autonoma di Trento in the scope of LP 6/2023.

ETHICAL CONSIDERATIONS

The patients were informed about the significance of genetic testing and informed consent was obtained from all subjects involved in the study. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethical Committee of Azienda Sanitaria dell'Alto Adige, Italy (Approval No. 132-2020).

CONFLICT OF INTEREST

All authors with affiliations to private companies have been declared to clearly note the potential interests of these companies. Some authors of this article are also reporting to be patent inventors.

AUTHORSHIP CONFIRMATION/ CONTRIBUTION STATEMENT

M.B.: Conceptualization; G.B., J.K., M.B.: Methodology; I.B., V.G., G.B., J.K., A.M., M.C.M.: Formal analysis; G.G., M.R., M.C., B.A., F.B., G.D.F., Silvia M., Serena M., Sandro M.: Investigation; M.R., M.C., B.A., F.B., G.D.F., Silvia M., Serena M., S.C., M.B., Sandro M.: Resources; G.B., J.K.: Data curation; I.B., G.B., J.K.: Writing – Original Draft; D.V., R.K., L.F., K.D., G.G., S.M., J.M., P.E.M.: Writing – Review & Editing; I.B., J.K.: Visualization; G.B., D.V.: Supervision; S.M., M.B.: Project administration; M.B.: Funding acquisition.

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