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GENETIC STUDY OF HGF-MET SIGNALING PATHWAY IN PRIMARY LYMPHEDEMA PATIENTS: SUPPORTING EVIDENCE FOR LOSS OF FUNCTION VARIANTS IN HGF

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ABSTRACT

The lymphatic system is composed of lymphatic vessels that connect lymphatic organs and play a critical role in draining lymph fluid from the intracellular environment. Disruptions in this system can lead to defects in lymphatic development and lymphedema which is characterized by chronic inflammation and fluid accumulation in tissues, finally resulting in swelling, pain, and fibrosis. If caused by genetic variants, this condition is referred to as primary lymphedema. This study explores the genetic basis of primary lymphedema in an Italian cohort retrospectively analyzing sequencing data and focusing specifically on genes involved in the HGF/MET signaling pathway. This pathway is crucial for lymphangiogenesis and lymphatic function, but the involved genes miss a clear association with lymphedema in clinical practice. The analysis identified 8 variants in three key genes, namely HGF, MET, *and* CBL, *among 8 unrelated patients. Seven of these variants have never been reported in literature as associated with lymphedema. We performed molecular modeling studies to evaluate the effect of the three identified missense variants in* MET, *supporting the pathogenicity of two of them. The identification of these variants supports the significance of alterations in HGF/MET signaling pathway as possible causes of primary lymphedema.*

Keywords: Primary Lymphedema, HGF-MET pathway, genetic variants, NGS, CBL

INTRODUCTION

The lymphatic system comprises lymphatic organs such as lymph nodes, tonsils,

thymus, and spleen interconnected by a network of lymphatic vessels. It plays a critical role in maintaining interstitial homeostasis by draining lymph fluid from the interstitial spaces between cells (1). These vessels, running parallel to the venous circulation throughout the human body, facilitate the transport of lipids, proteins, and immune cells (2,3). Disruptions in lymphatic system function can lead to the accumulation of lymph in peripheral tissues, resulting in a condition known as lymphedema. Lymphedema is a chronic inflammatory disorder characterized by the buildup of protein-rich fluid in interstitial spaces, leading to edema, inflammation, adipose tissue hypertrophy, and fibrosis. Consequently, affected individuals may experience restricted movement, impaired function, and physical disfigurement due to swelling and subsequent hardening of the affected area (2,3).

Lymphedema predominantly affects the lower extremities, but it can also involve the upper limbs, torso, genitals, and face (4). It is classified based on its underlying cause into primary lymphedema, resulting from inherited genetic disorders, and secondary lymphedema, triggered by external factors such as infection, trauma, or surgery (3,5). Around 140–250 million people are affected by lymphedema worldwide, although this number can vary due to interchangeable diagnoses of obesity or lipedema (6,7). The prevalence of primary lymphedema is 1:100 000 and it occurs twice more frequently in women compared to men (4). Secondary lymphedema is the most common type of lymphedema, with a prevalence of approximately 1 in 1000. The risk of developing secondary lymphedema can be heightened by obesity or by cancer therapy, depending on the type of chemotherapy or surgical procedures employed, and on the number of lymph nodes removed (2,8). Additionally, genetic predisposition can increase the risk of secondary lymphedema (6,9). According to the literature, several variants in specific genes (e.g., *ADAMTS3, FLT4, FOXC2, SOX18, VEGFR3*) are associated with disorders in lymphatic development and lymphedema (10–12). These genes play critical roles in various molecular signaling pathways crucial for lymphatic development. For instance, the hepatocyte growth factor (*HGF*) and its high-affinity receptor (*HGFR or MET*) are involved in the HGF/MET signaling pathway, which induces diverse cellular responses including proliferation, angiogenesis, tissue regeneration, and lymphangiogenesis (13). MET functions as a tyrosine kinase receptor and is activated by binding HGF. Genetic variants in genes such as *CBL* or *PTPN11*, involved in the HGF/MET pathway, could lead to decreased activation of the MET receptor or a reduced signaling cascade. This reduction in signaling can result in an altered lymphatic phenotype, contributing to the pathogenesis of lymphedema. There is growing evidence that *HGF* and *MET* are associated with lymphatic disorders (10,11). Variants in these genes may serve as crucial diagnostic genetic markers for primary lymphedema.

This study aims to identify pathogenic and likely pathogenic genetic variants in genes associated with lymphatic disorders and involved in the HGF/MET signaling pathway in an Italian cohort of patients affected by primary lymphedema. Through comprehensive genetic analysis, our objective is to contribute to the understanding of the molecular mechanisms involved in the pathogenesis of primary lymphedema, identifying novel genetic biomarkers that could potentially serve as diagnostic and therapeutic targets.

MATERIAL AND METHODS

Sample collection and DNA extraction

This study retrospectively analyzed clinical and genetic data of a cohort of Italian patients diagnosed with primary lymphedema. Clinical evaluation did not distinguish between syndromic and non-syndromic lymphedema, and only the lymphatic phenotype was considered. The diagnosis of lymphedema was performed by clinicians from different Italian hospitals as previously reported (13). 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 significance of genetic testing and written informed consent was obtained from each participant, in accordance with the principles outlined in the Declaration of Helsinki. Ethical approval and clearance for the study were obtained 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 using the SaMag Blood DNA Extraction Kit (Sacace Biotechnologies, Como, Italy) following the manufacturer's protocol.

Gene panel targeting HGF-MET pathway genes and NGS Sequencing

Samples were analyzed with a Next-Generation Sequencing (NGS) panel consisting of 99 genes associated with lymphatic malformations and lymphedema. More details about the gene panel can be found in Bonetti et al (2). This study focused on genes involved in the HGF/MET signaling pathway, specifically on *HGF, MET* and *CBL* (2,12). Prior to sequencing, DNA samples underwent standard processing procedures as outlined previously (2). Sequencing was performed by oligonucleotide-based target capture (Twist Custom Panel EF Workflow and Illumina Nextera Rapid Capture Custom Assay) followed by 150 bp paired-end reads sequencing using a MiSeq personal sequencer (Illumina, San Diego, CA, USA). Primer sequences, PCR and sequencing conditions are available after request.

Bioinformatic analysis

Fastq files (both forward and reverse) were acquired subsequent to sequencing. These reads underwent mapping to the genome utilizing Burrow-Wheeler Aligner software (version 0.7.17-r1188). To ensure accuracy, duplicate reads were eliminated using SAMBAMBA (version 0.6.7) and MarkDuplicates GATK (version 4.0.0.0). The resulting BAM alignment files underwent refinement through local realignment and recalibration of base quality scores, employing the RealignerTargetCreator and IndelRealigner GATK tools. Minor allele frequencies (MAF) were sourced from the Genome Aggregation Database (GnomAD). VarSome was utilized for *in silico* prediction of nucleotide changes' deleteriousness. Variants were categorized based on the standards and guidelines for the interpretation of sequence variants of the American College of Medical Genetics (ACMG), with classifications including pathogenic, likely pathogenic, variant of unknown significance (VUS), likely benign, or benign (Available online: https://www.acmg.net/acmg/medical-genetics-practice-resources/practice-guidelines.aspx; (13). Further subclassification of VUS variants into Hot, Middle, and Cold categories was performed based on their proximity to upper (likely pathogenic and pathogenic) or lower (likely benign and benign) classes, as detailed (14). Ultimately, only pathogenic, or likely pathogenic variants were reported.

In silico analysis of missense variants

SIFT and Polyphen scores were calculated using the VEP software (Available online: https://www.ensembl.org/info/docs/tools/vep/index.html). The structures of CBL and MET were modelled in order to overlay the mutations over the three-dimensional structures of the proteins. For CBL the X-ray solved structure with PDB entry ID 1FBV (15) was used, while for MET, SWISS-MODEL (Available online: https://swissmodel.expasy.org/) was used. The input sequence was taken from Uniprot (Available online: https://www.uniprot.org/) entry with ID P08581-2 (16), corresponding to the second isoform of MET (in which an arginine is present, instead of a lysine at position 1132), and template structure was taken from the structure with SMTL ID 8a2d.1 (17). Additionally, the PDB entry 4EEV, corresponding to the MET isoform 1 kinase domain, was used. The variants were then modelled into the structures by using

TABLE 1
Characterization of Study Subjects Analyzed According to Average Age, Gender Distribution, Period of Disease Onset, Average Age of Disease Onset, Familiarity, and Location of Edema.

Characteristic		Case Subjects (n=8)
Age	Mean ± Standard deviation	49 ± 14
	Median	49
Gender	Females/Males	7 / 1 (87.5% /22.5%)
Age of disease onset	Mean ± Standard deviation	26 ± 14
	Median	17
	UNKNOWN	1
Period of disease onset	Adolescent (11-17 years)	4 (50%)
	Adult (>18 years)	3 (37.5%)
	UNKNOWN	1 (12.5%)
Familiarity	Sporadic	3 (37.5%)
	Familial	4 (50%)
	UNKNOWN	1 (12.5%)
Location	Unilateral lower limb	3 (37.5%)
	Bilateral lower limbs	3 (37.5%)
	UNKNOWN	2 (25%)

PyMOL's (Available online: https://www.pymol.org/) mutagenesis functionality. Additionally, mCSM (Available online: https://biosig.lab.uq.edu.au/mcsm/stability) was used to check the predicted stability change, representing the effect of the variants in the folding of the protein. Finally, Mutation Taster (Available online: https://mutationtaster.org/) was used to predict disease potential of genetic variants.

RESULTS

We retrospectively analyzed genetic and clinical data of 408 patients diagnosed with primary lymphedema (18), and we identified 8 genetic variants in genes correlated with the HGF/MET pathway (5,19,20) in 8 unrelated patients. The characterization and clinical features of the subjects in which genetic variants were identified are reported in *Table 1*. The average age of the selected probands was 49 ± 14. The age of disease onset varied from 12 to 45 years. All 8 patients in our study had lymphedema localized to the lower limbs, either unilateral or bilateral. The genetic variants identified in genes *CBL*, *HGF* and *MET* are reported in *Table 2*. We identified 2 pathogenic variants, 3 likely pathogenic variants, and 3 hot-VUS variants, the majority being in *HGF*. All variants were in the heterozygous state. Five genetic variants were classified as nonsense, which due to insertion or deletion could lead to protein termination and loss of function. We identified 3 missense genetic variants leading to predicted severe substitution of amino acids. In our set of reported variants, one variant in *HGF,* p.(Arg630*), was already associated with lymphedema in existing literature (10).

We performed *in silico* analysis of three identified missense variants found in the *CBL* and *MET* genes to predict their potential functional and structural impact on the proteins, as reported in *Table 3*. All identified missense variants had a SIFT score of less than 0.05 and PolyPhen score greater than 0.8, indicating a strong likelihood of functional disruption. The mentioned scores reflected the po-

TABLE 2
Genetic Variants Identified in Genes Involved in HGF/MET Signaling Pathway. (P = Pathogenic; LP = Likely Pathogenic, VUS – Uncertain Significance). Segregation Analysis has been Performed for Variants Reported in Bold.

Gene	RefSeq	Nucleotide Change	Amino Acid Change	dbSNP	Classification	Frequency in GnomAD	Lymphedema Reference
CBL	NM_005188.4	c.1112A>C	p.(Tyr371Ser)	rs387906666	P	0.00000069	-
HGF	**NM_000601.6**	**c.1888C>T**	**p.(Arg630*)**	**rs769585774**	**P**	**0.00000205**	**(10)**
HGF	NM_000601.6	c.252C>A	p.(Cys84*)	-	LP	-	-
HGF	NM_000601.6	c.1939C>T	p.(Arg647*)	rs545249104	LP	0.0000024	-
HGF	**NM_000601.6**	**c.1048C>T**	**p.(Arg350*)**	**rs373442319**	**LP**	**0.000000684**	**-**
MET	NM_001127500	c.3394A>G	p.(Arg1132Gly)	-	VUS-hot	-	-
MET	NM_001127500	c.3824C>G	p.(Thr1275Ser)	rs2117067315	VUS-hot	-	-
MET	NM_001127500	c.79A>T	p.(Lys27*)	rs2116579656	VUS-hot	-	-

TABLE 3
In Silico Analysis of Identified Missense Variants in CBL and MET Genes. (DC = disease causing).

Gene	Amino Acid Change	SIFT	PolyPhen	Predicted Stability Change (ΔΔG)	Stability Effect	Mutation taster
CBL	p.(Tyr371Ser)	0	0.997	-2.64 kcal/mol	Highly destabilizing	DC
MET	p.(Arg1132Gly)	0.02	0.969	-0.15 kcal/mol	Destabilizing	DC
MET	p.(Thr1275Ser)	0.01	0.836	-0.28 kcal/mol	Destabilizing	DC

tential damaging effect of identified missense variants on protein function. Additionally, the predicted stability change (ΔΔG) values provide insights into how these amino acid substitutions influence protein stability. The ΔΔG score less than -3 kcal/mol indicated that the presence of mutations can highly destabi-lize the protein structure, which may result in significant functional alterations.

We modeled the identified missense variants, as illustrated in *Figs. 1 and 2*. *Figure 1b* p.(Tyr371Ser) introduces in CBL structure an amino acid with a smaller side chain. Serine creates hydrogen bonds with backbone atoms inside the helix. The RING domain shown in pink, and the linker domain shown in blue, pack extensively with the CBL TKB domain. The linker sequence integrates into the struc-

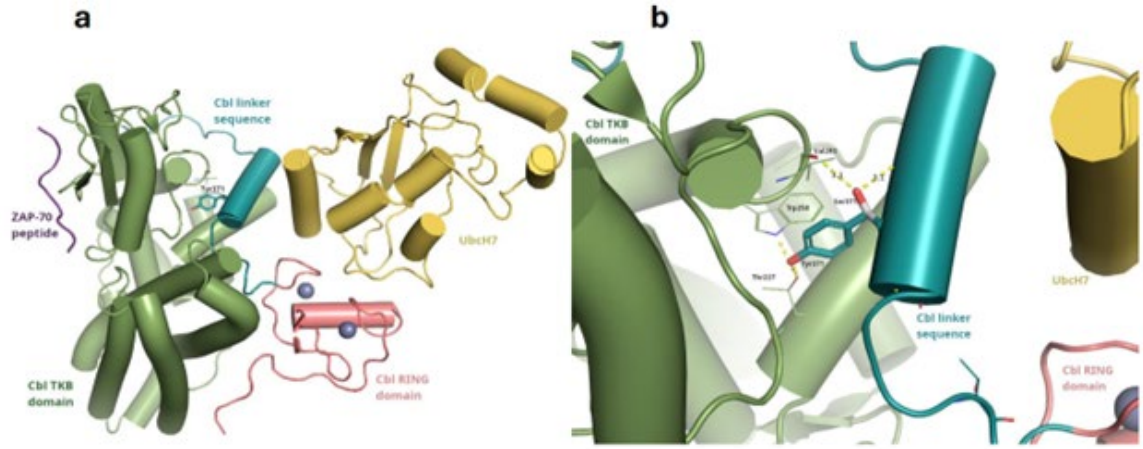

Fig. 1. *a) Ribbon diagram of the CBL-UbcH7-Zap70 complex. The Cbl linker sequence, where the variant is found, is shown in cyan, the TKB domain in green, the Cbl RING domain in salmon pink, and the ubiquitin-conjugating enzyme E2 L3 (UbcH7) is shown in yellow. b) Tyrosine371 (wildtype) and serine371 (mutant) residues are shown as sticks. Nearby residues, involved in interactions with Tyr371, are shown as lines. Dashed yellow lines represent hydrogen bonds. The two gray spheres represent Zn ions.*

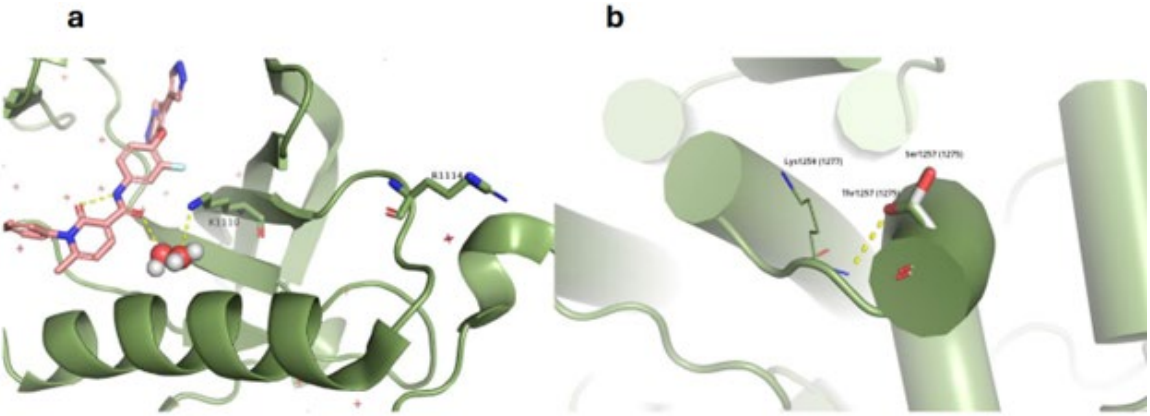

Fig. 2. *Ribbon diagrams of the missense variants in the MET isoform 1, P08581-1. Numbers in bold show the respective positions in the P08581-2 isoform. Hydrogen bonds are shown as dashed yellow lines. a) The active site of the kinase domain, with Arg1132 (Arg1114 in isoform 1, shown here) and Lys1128 (Lys1110 in isoform 1) shown as sticks. Lys1110 interacts with the substrate (inhibitor LY2801653 in this case, shown in pink) through two molecules of water (shown in spheres, red and gray for oxygen and hydrogen, respectively). b) Position 1275 (1257 in isoform 1) is highlighted with the wildtype threonine and mutant serine, shown as green and white sticks, respectively. Thr1275 forms hydrogen bonds with the backbone of Lys1277, shown as line.*

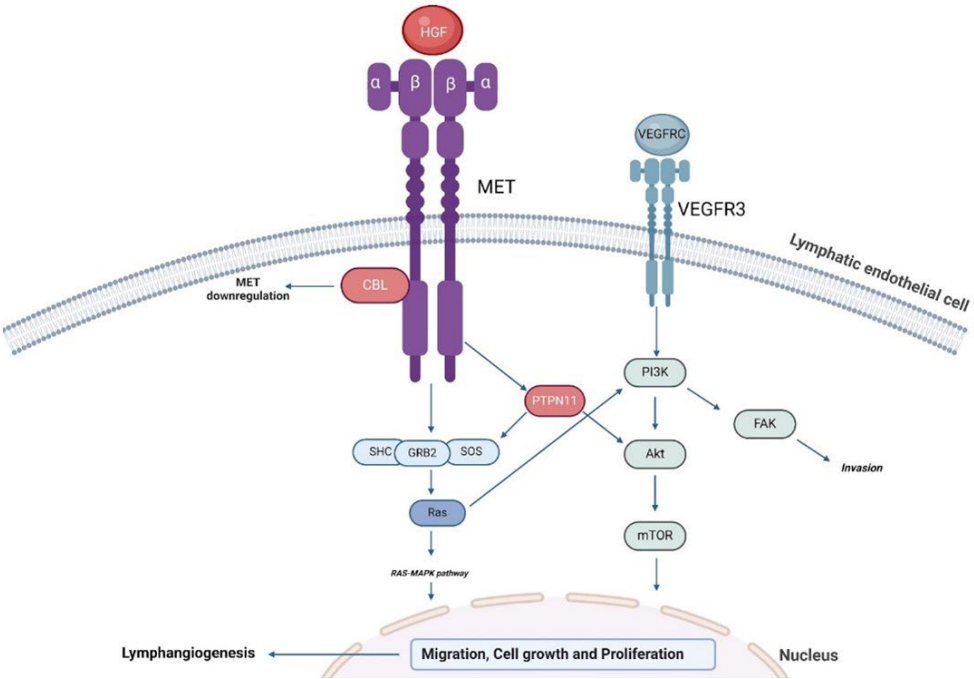

Fig. 3. *Schematic representation of the HGF/MET signaling pathway. Proteins encoded by genes involved in the HGF/MET pathway are reported in red or violet. Proteins involved in the RAS/MAPK pathway are reported in light blue, while proteins involved in either the VEGF/VEGFR3 or the PI3K/AKT pathway are reported in green. Image created with https://www.biorender.com/.*

ture of the TKB domain by forming an ordered loop and an α-helix, making it an important part of the TKB domain. The interactions between the linker and TKB depend on the conserved Tyr368 (not shown) and Tyr371 residues, which are buried in the environment, make hydrogen bonds to Asn259 (not shown) and Thr227, respectively, as well as van der Waals interactions with other buried hydrophobic TKB residues.

In the kinase domain active site, Lys1110 (Lys1128 in the P08581-2 isoform) interacts with the substrate LY2801653 (originally ATP), through two water molecules as shown in *Fig. 2a*, indicating its functional importance. On the other hand, Arg1114 (Arg1132 in P08581-2), which lies in a nearby loop in the C- direction, seems to be important in maintaining a rigid loop structure that could contribute to the interaction with the substrate. Position 1257 in the P08581-2 isoform (*Fig. 2b*) is located in the tyrosine kinase domain. It is occupied by a threonine residue and forms hydrogen bonds with the backbone of Lys1259 (Lys1277 in P08581-2).

DISCUSSION

Activation of the HGF/MET signaling pathway controls several cellular processes, such as proliferation, angiogenesis, regeneration of tissues, and lymphangiogenesis (13). Given the absence in literature of a clear association between genes involved in the HGF/MET pathway with primary lymphedema, our study focused on these genes and retrospectively analyzed the clinical and genetic data of a large cohort of Italian patients diagnosed with primary lymphedema. We identified 8 genetic variants in three key genes involved in the HGF/MET signaling pathway, namely *HGF, MET,* and *CBL.*

HGF stimulates lymphangiogenesis and

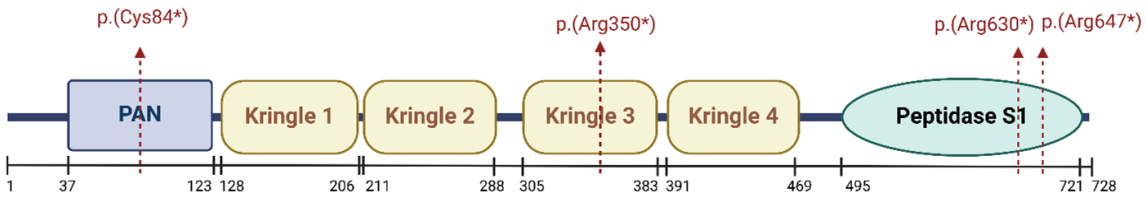

Fig. 4. *Schematic linear representation of HGF gene and its domains. The identified genetic variants in HGF and their locations in the sequence are shown in red. Image was created with https://www.biorender.com/.*

the proliferation of LECs, while the proto-oncogene *MET* regulates cell signaling and cellular function (21). According to the literature, genetic variants in both genes are associated with primary lymphedema development, lymphangiectasia, and an increased risk of developing secondary lymphedema correlated with breast cancer (2,10).

However, *MET* and *HGF* are not correlated with lymphedema or lymphatic malformations in OMIM or other clinical databases (www.omim.org). Indeed, the understanding of primary lymphedema molecular bases is still insufficient and increasing knowledge of variants in these genes could increase evidence of *MET* and *HGF* and their pathway as diagnostic biomarkers (Fig. 3).

HGF (Hepatocyte growth factor; OMIM: 142409) is located at chromosome 7q21.11 and encodes a protein that as the only known ligand binds to the MET receptor (22,23). HGF is secreted by stromal and mesenchymal cells and participates in regulating the growth, morphogenesis, and motility of epithelial and endothelial cells. It also mediates tissue regeneration (23). This protein is also known as a Scatter factor (SF) because of its ability to induce cell dispersal, which is crucial for its role in tissue remodeling and migration (24).

The inactive precursor form pro-HGF is cleaved by a serine protease between Arg494 and Val495 into active HGF in the extracellular environment (25). Structurally, mature HGF is a heterodimer composed of Alpha and Beta chains, linked by a disulfide bond. The binding of HGF to MET is mediated by high affinity and activates its kinase activity. The pro-HGF also binds to MET receptor, but with low affinity that does not trigger any kinase activity (23). According to the literature, the gene *HGF* induces lymphangiogenesis and angiogenesis and can be involved in the pathogenesis of lymphedema (20,23). Previous *in vitro* and *in vivo* studies on mice have reported the importance of HGF binding in lymphangiogenesis stimulation, an essential process in the formation of the lymphatic system. HGF strongly promotes the formation of lymphatic vessels when overexpressed in transgenic mice, while blocking HGFR disrupts lymphatic vessel function (20,26). The study by Saito et al., (27) showed the potential novel and safe approach of HGF gene therapy to managing lymphedema by stimulating the growth of the lymphatic vascular system. HGF treatment increased lymphatic endothelial cell proliferation and migration, and reduced swelling in a mouse model of breast cancer-related lymphedema. Clinical trials have demonstrated the safety of HGF plasmid DNA, and upcoming trials aim to assess its efficacy in managing lymphedema through therapeutic lymphatic vessel growth (27).

Previous studies have reported various missense or loss of function genetic variants in *HGF* that affect the binding domain for the receptor in patients with lower extremity lymphedema and variable age of disease onset. Due to the loss of function variants, patients showed severe phenotypes of primary lymphedema (20). In our study, we identified four heterozygous genetic variants in *HGF*, of which three were likely pathogenic variants, and one pathogenic variant. Their location in the sequence of HGF is shown in *Fig. 4*. Three reported variants have never been described in

literature, while variant rs769585774 (10) has already been identified in correlation with lymphedema in previous studies. The patient with variant rs769585774 (c.1888C>T, p.(Arg630*)) found in the study by Finegold et al., was affected by lymphedema and lymphangiectasia (10). We identified this pathogenic variant associated with unilateral familial lymphedema phenotype.

Interestingly, out of the eight analyzed patients in this cohort, seven were females. This high female-to-male ratio is consistent with previous findings in lymphedema (2,18,28). HGF is a key cytokine in several gender-related pathologies, such as breast and ovarian cancer, where serum HGF has been also proposed as a possible biomarker (29,30). Further studies should investigate the levels of serum HGF in lymphedema patients to explore potential correlations with gender, lymphedema stage and symptoms, and with associated comorbidities.

Notably, all the identified variants in HGF in this study are heterozygous loss-of-function variants. While homozygous variants in *HGF* are linked to Deafness (OMIM: 608265), this study supports previous published results (19) and proposes heterozygous variants in *HGF* as causes of primary lymphedema. Dosage sensitivity of HGF (https://search.clinicalgenome.org/kb/genes/HGNC:4893) may explain phenotypic differences between autosomal dominant and recessive inheritance. Therefore, according to evidence in current literature and to the results of this study, we propose to include *HGF* gene in genetic diagnostic analysis for primary lymphedema, and to make this association definitive and recorded on clinical databases.

MET (Mesenchymal Epithelial Transition; HGFR, hepatocyte growth factor receptor, OMIM: 164860) is a proto-oncogene and receptor tyrosine kinase for HGF, located at chromosome 7q31.2. MET belongs to the MET family and is expressed on various epithelial and endothelial cell surfaces (31,32). The primary function of MET is to regulate cellular processes such as cell differentiation, proliferation, and apoptosis (33). A single chain MET precursor is proteolytically cleaved into a heterodimer consisting of alpha and beta chains linked by disulfide bonds, forming a mature receptor. The beta chain is an extracellular domain of MET encoded by exon 2, which forms a SEMA (semaphorin) domain and PSI (plexin semaphorin integrin) domain. The SEMA domain mediates the binding of HGF to MET, and the activation of the receptor, while the PSI domain stabilizes this interaction (32,33). Somatic mutation in *MET* could correlate with lymphovenous disorders (2).

In our study, we identified three hot-VUS variants in *MET* that have not been previously described in the literature. All these variants were found in patients suffering from familial lymphedema. We reported one variant, rs2116579656 (p.Lys27*), located in exon 2, which is associated with stability and binding to HGF (32). The patient with this variant had the period of disease onset set in adolescence and suffered from lower extremities lymphedema and glaucoma. Other studies identified variants located in the SEMA or the MET domains, which are encoded in exon 2 of *MET* as being associated with lymphedema (10). In the study by Obst-Sander et al., (2022), one variant p.(Thr1217Serfs*5) was identified as correlated with the formation of a protein that lacks the MET tyrosine kinase domain causing lymphedema (17). We identified two missense variants in the *MET* gene p.(Arg1132Gly), and p.(Thr1275Ser) for which we performed molecular modeling. *Fig. 2a* shows the p.(Arg1132Gly) variant. The mutation is located in the intracellular kinase domain of MET, and glycine is introduced in place of arginine. The presence of glycine might allow for additional flexibility, making the loop more mobile, compared to the loop in the presence of arginine, which can form hydrogen bonds with nearby amino acids or water molecules, potentially contributing to maintaining a more rigid loop structure. The substitution of arginine would allow the loop to adopt more diverse conformations. Additionally, the loss of the positive charge could alter the electrostatic environment of this region. Finally, since the loop is involved in substrate binding, increased flexibility could affect its function (https://www.uniprot.org/uniprotkb/P08581/publications). Additionally, *Fig. 2b*

illustrates the missense variant p.(Thr1275Ser). The mentioned variant constitutes a change into an amino acid with similar physicochemical properties, from threonine to serine, capable of maintaining the hydrogen bond and increasing flexibility.

CBL (Casitas B-lineage lymphoma; OMIM 165360) is a proto-oncogene that encodes E3 ubiquitin protein ligase (34). The CBL protein family includes 3 homologues: CBL, CBL-b, CBL-c. All of them consist of a conservative RING finger domain, which is essential for the enzymatic activity of CBL and mediates the ubiquitination (35–37). CBL works as a negative regulator that causes ubiquitination and degradation of receptor/non-receptor tyrosine kinases implicated in transferring the signal from immune cell receptors (e.g., T-cell/B-cell receptors). In the physiological state, the RING finger domain of CLB transfers the ubiquitin from ubiquitin E2 to some specific substrates (36). The main functions of the CBL protein are related to various cell pathways (including cell survival, migration, and cell proliferation, hematopoietic development), involved in physiologic and pathologic processes. *CBL* is involved in the HGF-MET pathway, where it participates in the regulation of MET internalization through tagging MET receptors with ubiquitin, down-regulating its signaling (2). The presence of genetic variants in *CBL* (mostly in exons 8 and 9) affects the RING domain, leading to disruption of the E3 ubiquitin ligase activity. This loss of function is associated with myeloid malignancies and disorders involved in the RAS-MAPK signaling pathway, which is related to the development of primary lymphedema (34,36). According to the literature, heterozygous germline mutations in *CBL* have been correlated with Noonan syndrome (OMIM: 613563) and Juvenile myelomonocytic leukemia (OMIM: 607785), also presenting primary lymphedema symptoms (2,34,36).

In our study, we identified a heterozygous pathogenic variant in the gene *CBL* (c.1112A>C, p.(Tyr371Ser), rs38790666) in a 14-year old patient suffering from unilateral lower extremity lymphedema. In literature, the presence of this variant in heterozygous state was associated with Noonan syndrome (38), Juvenile myelomonocytic leukemia (39), and Rasopathies (40), while it has never been described as associated with only primary lymphedema. This missense variant is located in the 8th exon correlated with loss function of E3 activity. From the model of the variant (*Fig. 1*), it can be seen that tyrosine contributes to a network of hydrogen bond interactions involving Trp258 and Thr227. The substitution of tyrosine with serine at position 371, results in a loss of the h-bond between Tyr371 and Thr227. While the introduction of serine creates an additional hydrogen bond with the backbone of Val263, it may not compensate properly. Tryptophan is an amino acid often seen in functional sites, due to its particular physicochemical properties. Replacement or loss of stability of this amino acid could lead to adverse effects on the protein structure. The loss of the hydrogen bond between Tyr371 and Thr227 could lead to an unsteady tryptophan, preventing the proper packing of the TKB domain (15).

The primary limitation of this study is the reliance on *in silico* predictors, molecular dynamics simulations, and literature review to assess the pathogenicity of the variants, without performing functional *in vitro* or *in vivo* studies to confirm their impact on protein function and their direct role in lymphedema. Moreover, the custom NGS panel targeted 99 genes associated with primary lymphedema, potentially missing other relevant genes. Finally, the absence of longitudinal data limits insights into the condition's natural history and the long-term effects of the identified variants. Nevertheless, we selected a significant cohort of patients, and we analyzed several genes correlated to lymphedema in literature, providing an initial insight into the genetic basis of lymphedema and highlighting the importance of the HGF/MET signaling pathway by identifying 8 genetic variants, seven of which have never been associated to lymphedema.

CONCLUSIONS

Although the HGF/MET pathway has

been proposed to be correlated with primary lymphedema, previous studies have not provided sufficient evidence to confirm the causative role of genes involved in this pathway in the onset of lymphedema. In our study we reported 8 genetic variants in genes involved in the HGF/MET signaling pathway in patients with primary lymphedema. Notably, seven of them have not been previously reported in the literature as being associated with primary lymphedema. Moreover, molecular modeling showed two of our identified variants in *MET* to affect protein structure. Our findings suggest the involvement of *HGF* in primary lymphedema, supporting its addition to future diagnostic strategies. Given that the understanding of primary lymphedema onset remains limited, our study proposes a candidate molecular pathway that may guide the development of more effective diagnostic approaches.

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None.

ETHICAL CONSIDERATIONS

All patients involved in this study were informed about the significance of genetic testing and signed informed consent 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).

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 patent inventors.

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