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GENETIC SCREENING IN A LARGE COHORT OF ITALIAN PATIENTS AFFECTED BY PRIMARY LYMPHEDEMA USING A NEXT GENERATION SEQUENCING (NGS) APPROACH

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ABSTRACT

*Primary lymphedema is a rare inherited condition characterized by swelling of body tissues caused by accumulation of fluid, especially in the lower limbs. In many patients, primary lymphedema has been associated with variations in a number of genes involved in the development and maintenance of the lymphatic system. In this study, we performed a genetic screening in patients affected by primary lymphedema using a next generation sequencing (NGS) approach. With this technology, based on a custom-made oligonucleotide probe library, we were able to analyze simultaneously in each patient all the coding exons of 10 genes (*FLT4, FOXC2, CCBE1, GJC2, MET, HGF, GATA2, SOX18, VEGFC, KIF11*) associated with primary lymphedema. In the study population, composed of 45 familial and 71 sporadic cases, we identified the presence of rare variants with a potential pathogenic effect in 33% of subjects. Overall, we found a total of 36 different rare nucleotidic alterations, 30 of which had not been previously described. Among these, we identified 23 mutations that we considered most likely to be disease causing. Patients with an* FLT4 *or* FOXC2 *alteration accounted for the largest percentage of the sample, followed by* MET, HGF, KIK11, GJC2 *and* GATA2. *No alterations were identified in* SOX18, VEGFC, *and* CCBE1 *genes. In conclusion, we showed that NGS technology can be successfully applied to perform molecular screening of lymphedema-associated genes in large cohort of patients with a reasonable effort in terms of cost, work, and time.*

Keywords: Primary lymphedema, lymphedema genes, genetic screening, next generation sequencing, genetic variations, lymphoscintigraphy, genotype-phenotype correlations

Primary lymphedema is a pathologic condition characterized by lymphatic anomalies resulting from defects in the development, maturation, or function of the lymphatic system. Patients with lymphedema show abnormal accumulation of interstitial fluid due to inefficient uptake or occlusion of lymphatic drainage, leading to abnormal swelling of one or more extremities (1).

Lymphedema can be classified as primary or secondary, according to the pattern of inheritance and age of onset.

Primary lymphedema is generally described as a congenital disorder with autosomal dominant transmission characterized by reduced penetrance and variable expressivity (2). Secondary lymphedema (the most common form of lymphatic dysfunction) is in contrast a sporadic pathology with a variable age of onset, triggered by an external event such as filariasis, mechanical trauma, radiation therapy, or tumors (3-5). Nevertheless, recent findings suggest that secondary lymphedema cannot be exclusively attributed to environmental insult, and there may be genetic susceptibility (6). In the last few years, an increasing number of genes such as *FLT4* (7) (encoding VEGFR3), *GJC2* (8), *FOXC2* (9), *SOX18* (10), *HGF* (11), *MET* (11), *CCBE1* (12), *KIF11* (13), *VEGFC* (14) and *GATA2* (15) have been linked to inherited forms of lymphedema.

In 1998, Ferrell and colleagues identified in *FLT4* (fms-related tyrosine kinase 4) the first pathogenic variant in a family with primary lymphedema (16). Successively, Karkkainen et al confirmed the association between this gene and lymphatic disorders, discovering several variants in *FLT4* in families with Milroy disease (MD) (7). Milroy Disease (also described as hereditary lymphedema type I) is an autosomal dominant form of congenital lymphedema of the lower limbs (17) often associated with hydrocele in males, cellulitis, prominent veins, upslanting toenails, and papillomas. According to the LOVD database (www.lovd.nl/flt4), at least 68 different *FLT4* variants have been identified in MD patients. Interestingly, all these variants are localized in exons 17 to 26 that encode the highly conserved tyrosine-kinase (TK) domain (residues 843-943 and 1,009-1,165) (18,19). A single homozygous variant located outside the TK domains of the VEGFR3 receptor was recently identified in a family in which the lymphedema phenotype segregates with an autosomal recessive pattern of inheritance (20).

FOXC2, also known as *MFH-1*, is a transcription factor gene characterized by a distinct DNA-binding forkhead domain (FHD). Variations in *FOXC2* (forkhead box C2) have been identified in patients with hereditary lymphedema-distichiasis (LD) syndrome, a non-congenital autosomal dominant form of primary lymphedema associated with the development of extra eyelashes (distichiasis) (9). Additional features of *FOXC2* mutated patients can include cardiac defects, cleft palate and extradural cysts (21). Most *FOXC2* nucleotidic alterations are frameshift or nonsense variants that reduce *FOXC2* transcriptional activation, inducing the formation of inactive truncated proteins (22,23). Interestingly, functional studies also demonstrate that p.(Arg121His) and p.(Ser125Leu) missense variants can impair forkhead domain (FHD) DNA-binding ability, supporting the hypothesis that *FOXC2* variations associated with LD, act by a loss-of-function mechanism (24).

Variations in *GJC2* genes have been identified in some families with late-onset autosomal dominant lymphedema classified as type 1C. Sometimes skin infections and cellulitis have been reported in severe cases (25). More recently variations in *GJC2* were also identified in secondary lymphedema patients, supporting the idea that even common forms of lymphedema can be influenced by genetic factors (6). The relationship between lymphedema phenotype and variation in *GJC2* is somewhat unexpected, since this gene is mainly expressed in the central nervous system (26). However, through in vitro assays, Finegold provided evidence that p.(Pro384Ser) and p.(His412Tyr) *GJC2* variants identified in patients with lymphedema are dysfunctional and act with a gain-of-function mechanism (6). Interestingly, loss-of-function *GJC2* variants have been found in patients with autosomal recessive Pelizaeus-Merzbacher-like disease (OMIM 608804) and spastic paraplegia type 44 (SPG44) (MIM# 613206), two neurological disorders that are not associated with lymphatic defects.

Ostergaard et al, performing whole-exome sequencing analysis in patients with MLCRD syndrome (microcephaly, primary lymphedema and chorioretinal dysplasia) (OMIM 152950), identified 10 different variations in the *KIF11* gene (13). In this syndrome, inherited with an autosomal dominant pattern of transmission, microcephaly is associated with primary lymphedema that is typically confined to the lower limbs and chorioretinal dysplasia. Variations in *KIF11* have also been identified in patients with CDMMR syndrome, characterized by chorioretinal dysplasia, microcephaly and mental retardation, suggesting that these disorders should be considered a single entity with variable clinical features (13).

In 2008, Finegold identified six alterations in hepatocyte growth factor (*HGF*) and proto-oncogene *MET* in 154 subjects with familial lymphedema and 21 patients diagnosed with intestinal lymphangiectasia (11). MET is a single-pass tyrosine kinase receptor essential for many development processes; it is activated after direct interaction with its specific ligand HGF. Activation of the HGF/MET signaling pathway has been shown to lead to a wide array of cell responses including proliferation, angiogenesis, tissue regeneration and lymphogenesis (27,28). So far, only one study has established an association of *HGF* and *MET* with lymphedema. Further analysis in other cohorts of patients are needed to confirm these findings.

Three variants in the transcription factor gene *SOX18* have been identified in patients with hypotrichosis-lymphedema-telangiectasia syndrome (OMIM 607823), which is characterized by lymphedema associated with reduced body hair and localized cutaneous telangiectasias (abnormal dilation of blood vessels leading to focal red lesions). Lymphedema does not occur solely in the legs of patients but can be more generalized. Hypotrichosis that includes absence of eyebrows and eyelashes may be present at birth or develop in infancy. This syndrome may be inherited with autosomal dominant or recessive transmission (10). In mice, loss of *SOX18* function induces defects in blood vessel maturation, resulting in vessel enlargement associated with hemorrhage. This suggests that *SOX18* acts as a transcriptional activator of genes required for vascular structural integrity and is essential for developing blood vessels (29).

Variations in *GATA2* (GATA-binding protein 2) have been linked to several disorders. These disorders include Emberger syndrome (15) and combined immunodeficiencies known as DCML (30), as well as MonoMAC (31). Emberger syndrome segregates as an autosomal dominant trait with incomplete penetrance and is characterized by primary lymphedema, associated with deafness and immune dysfunctions. Onset of Emberger syndrome is usually in childhood followed by progression to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (32). Most of the variations described in patients with Emberger syndrome are gene deletions or frameshift variants but also missense mutations, predicted to result in a loss of function of one *GATA2* allele, have been identified. *GATA2* is localized selectively in lymphatic vessels, especially in the endothelial cells that compose the valves of lymphatic vessels. In vitro functional studies have shown a role of *GATA2* in lymphatic vascular development and function, particularly in regulating expression of genes important for valve morphogenesis (32).

Hennekam syndrome (HS) is an autosomal recessive form of primary lymphedema characterized by generalized lymphatic dysplasia associated with intestinal lymphangiectasias, typical facial features, mental retardation and hydrops fetalis. In seven subjects with HS, Alders and colleagues identified homozygous and compound heterozygous mutations in the *CCBE1* gene (12). *CCBE1* encodes collagen and calcium-binding EGF domain-1, a secreted protein that enhances the lymphangiogenic effects of *VEGFC* (33) and is required for embryonic

lymphangiogenesis in zebrafish (34). More recently, whole-exome sequencing in two families with hereditary lymphedema identified variations in *VEGFC* (vascular endothelial growth factor C), the major ligand of VEGFR3. In vitro functional cell expression assays and studies in zebrafish showed that the variant (c.571insTT) induces formation of a stable truncated protein that is not secreted efficiently. This variant resulted in loss of function without a dominant-negative effect, consistent with haploinsufficiency (14, 35).

Primary lymphedema is a disease with high genetic and clinical heterogeneity where a clear genotype/phenotype correlation in affected patients has not been established. The aim of the present study was to estimate the variation frequency of 10 genes in a large cohort of patients with sporadic and familial primary lymphedema, using a next generation sequencing (NGS) approach with a custom-designed multi-gene panel.

MATERIALS AND METHODS

Study Subjects

Patients underwent a series of clinical investigations leading to the diagnosis of primary lymphedema and exclusion of secondary causes of the disease. Briefly, the diagnosis of lymphedema was confirmed by three-phase lymphoscintigraphy according to the protocol of Bourgeois (36); color Doppler echography and magnetic resonance lymphangiography were also performed in specific cases. All clinical assessments were conducted at San Giovanni Battista Hospital ACISMOM (Rome, Italy). The staging system established by The International Society of Lymphology (ISL) was used to determine disease severity (37).

All 116 patients received genetic counseling to explain the risks and benefits of genetic testing. Each blood sample was accompanied by written informed consent to genetic testing by the patients. The informed consent forms include consent to use anonymized genetic results for research. In this report, we used the data of patients who consented to use of their anonymized data for research. The blood samples were sent to MAGI Non-Profit Human Medical Genetics Institute (Rovereto, Italy) for genetic testing.

Custom Panel Design

A custom-made oligonucleotide probe library was designed to capture all coding exons and flanking exon/intron boundaries (+/- 100 bp) of 114 genes known to be associated with a large group of cardiovascular and lymphatic diseases from the literature or databases [Human Gene Mutation Database (HGMD Professional), Online Mendelian Inheritance in Man (OMIM), Orphanet, NCBI GeneReviews, NCBI PubMed and specific database]. The 10 genes known to be involved in primary lymphedema diseases (*Table 1*) were included in the panel. The DNA probe set, complementary to the target regions (GRCh37/hg19), was designed using the online tool, Illumina DesigStudio (Nextera Rapid Capture Custom Assay Technology; http://designstudio.illumina.com/Home/SelectAssay/), resulting in generation of 5106 capture probes over 2010 targets, 586 bp in size.

Library Preparation, Targeted Capture and Sequencing

In-solution target enrichment was performed according to the manufacturer's protocol using the Nextera Rapid Capture Enrichment kit (Illumina). Briefly, 5 ng of genomic DNA was simultaneously fragmented and tagged by Nextera transposon-based shearing technology. Limited cycle PCR was carried out to incorporate specific index adaptors to each sample library. 500 ng of each indexed DNA library was combined with the 12-plex library pool and then hybridized with target-specific biotinylated probes. The libraries were subsequently

TABLE 1
List of Primary Lymphedema Genes Analyzed in this Study

Gene	RefSeq number	OMIM
CCBE1	NM_133459	235510
FLT4	NM_182925, NM_002020	153100
FOXC2	NM_005251	153400
GATA2	NM_001145662, NM_032638, NM_001145661	614038
GJC2	NM_020435	613480
HGF	NM_001010934, NM_000601, NM_001010931, NM_001010933 NM_001010932	142409
MET	NM_000245, NM_001127500	164860
SOX18	NM_018419	607823
VEGFC	NM_005429	615907
KIF11	NM_004523	152950

For each gene the relative RefSeq and OMIM accession number were reported.

captured using streptavidin magnetic beads and underwent a second round of hybridization, capture, PCR amplification and PCR clean-up. The final enriched pooled libraries, with sizes mainly distributed between 500 and 600 bp, were quantified using the Qubit method (Invitrogen, Carlsbad, CA, USA) and sample quality was verified using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA). 150 bp paired-end reads sequencing was performed on MiSeq personal sequencer (Illumina, San Diego, CA) according to the manufacturer's instructions.

Data Analysis

The raw read data in fastq format, generated by the Ilumina MiSeq reporter software (version 2.5), was analyzed to generate the final set of sequence variants using an in-house pipeline that includes following modules: mapping, duplicate read removal, indel realignment, quality calibration, coverage analysis, variant calling, and annotation. In brief, the sequencing reads were mapped to the genome build hg19 using the Burrows-Wheeler Aligner (BWA version 0.7.5a-r405) (38) with default settings. Next, duplicate fragments were marked and eliminated with the *MarkDuplicates* GATK tool (version v2.5-2-gf57256b) (39,40). The BAM alignment files generated were refined by local realignment and base quality score recalibration using the *RealignerTargetCreator* and *IndelRealigner* GATK tools. Statistical and coverage analysis of final BAM files was performed using SAMTools and BEDTools (41). Reads aligned to the designed target regions (coding exons and 15 bp flanking of gene-disease subpanel) were collected for variant calling and subsequent analysis. The following data per sample was generated by coverage analysis: average read depth, low coverage target regions (<10X); % of target bases with coverage ≥ 10X. Sequence variant calling was performed using three SNP and genotype calling tools: GATK *UnifiedGenotyper*, *Varscan* (version v2.3) (42) and *Bcftools* of SAMTools (version 0.1.19-44428cd). The output data from the three variant callers was joined and converted to a standard vcf file using a custom-script. Called variants were annotated using *Annovar* software (43) with the aid of

information from publicly available databases [database for allele frequency data: 1000 Genomes Project (http://www.1000genomes.org/), dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and Exome Variant Server (evs.gs.washington.edu/EVS) databases; variant-disease association databases: Human Gene Mutation Database (HGMD), HumsVar (http://omictools.com/humsavar-tool) and LOVD (Leiden Open Variation Database)]. The potential deleterious effect of missense variants was determined by using various *in silico* prediction algorithms [SIFT (Sorting Intolerant From Tolerant, http://sift.jcvi.org/www/SIFT_enst_submit.html), PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/index.shtml) and Mutation Taster (http://www.mutationtaster. org/)].

Variant Filtering and Prioritization

Variants were selected for subsequent study on the basis of the following criteria: a) previously reported in HGMD and HumsVar database; b) present in dbSNP, EVS, and 1000 Genome Project with allelic frequency ≤ 0.03. The following criteria were applied to evaluate the pathogenic nature of the variant set selected: 1) known variant; nonsense, frameshift, essential splice site (affecting conserved consensus motif) and start- or stop-loss variants were considered most likely to be disease causing; 2) missense variants having an allelic frequency less than 0.01 in dbSNP and with deleterious effects predicted by at least 2/3 *in silico* pathogenicity prediction tools (SIFT, PolyPhen-2 and Mutation Taster) were considered potentially pathogenic variants. All candidate variants were carefully checked for previous description in the literature and databases.

Sanger Validation and Sequencing of Poorly Covered Target Regions

Target region coverage of less than 10 reads was further analyzed by Sanger sequencing according to the manufacturer's protocols (CEQ8800 Sequencer, Beckman Coulter). Each predicted pathogenic variants was confirmed by conventional Sanger sequencing using genomic DNA from different aliquots of blood sample.

RESULTS

Genetic analysis was performed on a group of 116 unrelated patients with clinical findings of primary lymphedema. In this study, we performed NGS analysis on a panel of 114 genes encompassing multiple cardiovascular and lymphatic disorders including primary lymphedema. However, data analysis was limited to ten known primary lymphedema-related genes (*FLT4, FOXC2, CCBE1, GJC2, MET, HGF, GATA2, SOX18, VEGFC, KIF11*, see *Table 1*). The average number of mappable reads per sample was 1.3M, resulting in a mean coverage of targeted bases of 370x per sample. On average, 94% of all bases were covered at least 10x.

The analysis of the entire sample of patients led us to identify a total of 36 rare nucleotidic alterations with an allelic frequency less than 0.01. Among these variations we selected a subgroup of variants that we considered most likely to be disease causing. In this group we included nonsense and frameshift variants, essential splice site and start- or stop-loss variants. In addition, only missense variants that were considered "deleterious" by at least 2 out 3 in silico pathogenicity prediction tools, were considered potentially pathogenic.

With this approach, in 26 affected subjects we identified 23 alterations that we considered to be causative (*Tables 2 and 3*) while thirteen of these variants did not completely fulfill the criteria we applied in this study for the identification of putative pathological mutations. However, being unable to completely exclude the possibility that they are causative for this phenotype, we reported the list of these rare variants in *Table 4*.

TABLE 2
Pattern of Transmission, Age of Onset, and Molecular Diagnosis of 26 Patients with Primary Lymphedema

Patient ID (gender)	Familiarity	Age of onset	Gene	Affected district	Lymphedema Stage	Variant	Reference
R682 (M)	sporadic	congenital	*FLT4*	right lower limb	II	c.1430G>A; p.(Arg477Gln)	This study
R218 (F)	familial	congenital	*FLT4*	both feet	III	c.2560G>A; p.(Gly854Ser)	45
R683 (F)	familial	congenital	*FLT4*	both feet	I	c.2740G>C; p.(Gly914Arg)	45
R441 (F)	sporadic	19 years	*FLT4*	left lower limb	II	c.2860C>T; p.(Pro954Ser)	This study
R155 (F)	sporadic	36 years	*FLT4*	right leg	II	c.2860C>T; p.(Pro954Ser)	This study
R684 (F)	sporadic	14 years	*FLT4*	left foot	I	c.3460G>A; p.(Gly1154Arg)	This study
R685 (M)	sporadic	19 years	*FOXC2*	left foot	II	c.238C>T; p.(Leu80Phe)	This study
R686 (M)	familial	12 years	*FOXC2*	both ankles	II	c.327C>A; p.(Tyr109*)	This study
R100 (M)	sporadic	17 years	*FOXC2*	legs	II	c.374C>T; p.(Ser125Leu)	23
R033 (F)	familial	27 years	*FOXC2*	right foot,	III	c.595dup; p.(His199Profs*264)	46
R101 (F)	familial	12 years	*FOXC2*	both feet	II	c.638delT; p.(Ile213Thrfs*1)	This study
R687 (F)	sporadic	congenital	*FOXC2*	both feet	I	c.682G>A; p.(Val228Met)	This study
R688 (F)	familial	3 years	*MET*	both lower limbs	II	c.2568T>G; p.(Asn856Lys)	This study
R030 (F)	familial	40 years	*MET*	both feet	II	c.3290A>G; p.(His1097Arg)	This study
R099 (F)	familiar	25 years	*MET*	left foot and ankle	II	c.3650_3651del; p.(Thr1217Serfs*5)	This study
R689 (F)	sporadic	14 years	*MET*	both feet	II	c.4066T>C; p.(Tyr1356His)	This study
R690 (F)	sporadic	24 years	*GATA2*	right foot	I	c.1215G>T; p.(Lys405Asn)	This study
R017 (F)	sporadic	55 years	*GJC2*	both feet	II	c.1150C>T; p.(Pro384Ser)	6
R691 (F)	sporadic	15 years	*GJC2*	both lower limbs	II	c.1234C>T; p.(His412Tyr)	6
R227 (M)	familial	congenital	*KIF11*	right foot	II	c.77+5G>C	This study
R439 (F)	familial	11 years	*KIF11*	both feet	III	c.77+5G>C	This study
R692 (F)	familial	20 years	*KIF11*	both thighs	II	c.77+5G>C	This study
R693 (F)	sporadic	congenital	*HGF*	both lower limbs	II	c.532C>T; p.(Arg178*)	This study
R032 (M)	familial	12 years	*HGF*	both lower limbs	II	c.1270C>T; p.(Arg424Cys)	This study
R139 (F)	familial	9 years	*HGF*	both lower limbs	II	c.1351T>C; p.(Trp451Arg)	This study
R694 (M)	familial	27 years	HGF	both feet	I	c.1473delA ; p.(Lys491Asnfs*6)	This study

TABLE 3
Description, Exon Position and Evaluation of Pathogenicity of Identified Variants

Patient ID	Gene	Nucleotide substitution	Protein substitution	Exon	SIFT	Polyphen2	Mutation Taster	dbSNP acc. Number	MAF%
R682	*FLT4*	c.1430G>A	p.(Arg477Gln)	11	T	PoD	DC	rs755736057	NA
R218	*FLT4*	c.2560G>A	p.(Gly854Ser)	18	D	PrD	DC	NA	NA
R683	*FLT4*	c.2740G>C	p.(Gly914Arg)	19	D	PrD	DC	NA	NA
R441	*FLT4*	c.2860C>T	p.(Pro954Ser)	21	D	B	DC	rs34255532	0.49%
R155	*FLT4*	c.2860C>T	p.(Pro954Ser)	21	D	B	DC	rs34255532	0.49%
R684	*FLT4*	c.3460G>A	p.(Gly1154Arg)	26	D	PrD	DC	rs75614493	0.02%
R685	*FOXC2*	c.238C>T	p.(Leu80Phe)	1	D	PrD	DC	NA	NA
R686	*FOXC2*	c.327C>A	p.(Tyr109*)	1	-	-	-	NA	NA
R100	*FOXC2*	c.374C>T	p.(Ser125Leu)	1	D	PrD	DC	rs121909106	NA
R033	*FOXC2*	c.595dup	p.(His199Profs*264)	1	-	-	-	NA	NA
R101	*FOXC2*	c.638delT	p.(Ile213Thrfs*1)	1	-	-	-	NA	NA
R687	*FOXC2*	c.682G>A	p.(Val228Met)	1	T	PrD	DC	NA	NA
R688	*MET*	c.2568T>G	p.(Asn856Lys)	11	D	B	DC	NA	NA
R030	*MET*	c.3290A>G	p.(His1097Arg)	15	D	PrD	DC	NA	NA
R099	*MET*	c.3650_3651del	p.(Thr1217Serfs*5)	18	-	-	-	NA	NA
R689	*MET*	c.4066T>C	p.(Tyr1356His)	21	D	B	DC	NA	NA
R690	*GATA2*	c.1215G>T	p.(Lys405Asn)	6	D	PrD	DC	NA	NA
R017	*GJC2*	c.1150C>T	p.(Pro384Ser)	2	T	B	P	rs535282333	NA
R691	*GJC2*	c.1234C>T	p.(His412Tyr)	2	D	B	P	rs200334298	NA
R227	*KIF11*	c.77+5G>C	-	1/2	-	-	-	rs200188195	0.04%
R439	*KIF11*	c.77+5G>C	-	1/2	-	-	-	rs200188195	0.04%
R692	*KIF11*	c.77+5G>C	-	1/2	-	-	-	rs200188195	0.04%
R693	HGF	c.532C>T	p.(Arg178*)	5	-	-	-	NA	NA
R032	HGF	c.1270C>T	p.(Arg424Cys)	10	D	PrD	DC	NA	NA
R139	HGF	c.1351T>C	p.(Trp451Arg)	10	D	PrD	DC	NA	NA
R694	HGF	c.1473delA	p.(Lys491Asnfs*6)	13	-	-	-	NA	NA

SIFT score: tolerated (T), damaging (D); Polyphen 2 score: benign (B), possibly damaging (PoD, less confident prediction), probably damaging (PrD, more confident prediction); Mutation Taster score: polymorphism (P), disease-causing (DC); MAF%, minor allele frequency in percent in European American population from Exome Variant Server.

TABLE 4
List of the 13 Nucleotidic Alterations with a MAF < 1% that were Not Considered Pathogenic According to the Criteria of Variant Selection Applied in this Study

Patient ID	Gene	Nucleotide substitution	Protein substitution	Exon	SIFT	Polyphen2	Mutation Taster	dbSNP acc. Number	MAF%
R695	FLT4	c.14C>G	p.(Ala5Gly)	1	T	B	P	NA	NA
R697	FLT4	c.361G>A	p.(Glu121Lys)	3	T	B	DC	rs371804364	0.0%
R698	FLT4	c.619G>A	p.(Glu207Lys)	5	T	B	DC	NA	NA
R117	FLT4	c.3908G>C	p.(Gly1303Ala)	30	T	B	P	rs146806202	0.35%
R018	FLT4	c.3971G>C	p.(Arg1324Pro)	30	T	B	P	NA	NA
R016	FOXC2	c.637A>G	p.(Ile213Val)	1	T	B	DC	NA	NA
R447	MET	c.2908C>T	p.(Arg970Cys)	14	T	B	DC	rs34589476	0.47%
R185	GJC2	c.1027G>T	p.(Ala343Ser)	2	T	B	P	NA	NA
R701	GJC2	c. 1193C>T	p.(Thr398Ile)	2	T	B	P	rs140942230	NA
R699	KIF11	c.1359C>G	p.(Asp453Glu)	12	T	B	DC	rs775797014	NA
R696	KIF11	c.2771-6T>A	-	19/20	-	-	-	rs75876570	0.65%
R700	HGF	c.1672G>A	p.(Asp558Asn)	15	T	B	P	rs370757602	0.02%
R212	GATA2	c.121C>G	p.(Pro41Ala)	2	T	B	DC	rs143590990	0.08%

Missense variations were considered causative when classified as "disease causing" by at least two pathogenicity prediction tools among SIFT, PolyPhen and Mutation Taster. SIFT score: tolerated (T), damaging (D); Polyphen 2 score: benign (B), possibly damaging (PoD, less confident prediction), probably damaging (PrD, more confident prediction); Mutation Taster score: polymorphism (P), disease-causing (DC); MAF%, minor allele frequency in percent in European American population from Exome Variant Server.

We discovered variations in *FLT4, FOXC2, MET, KIF11, HGF, GATA2*, and *GJC2* but not in *SOX18, VEGFC*, and *CCBE1* genes. Overall, a variation was found in 22.4% (26/116) of patients. Most patients had lymphedema localized to the feet or lower legs, whereas a minority had swelling of the entire leg. Swelling was more often bilateral rather than unilateral. An overview of age of onset and clinical features of mutant-bearing patients is described in *Table 2.*

Analysis of the *FLT4* gene identified five missense variants in six patients; two variants have already been described by Gordon and colleagues (44) while the other three are new variants. In the conserved tyrosine-kinase (TK) domain of *FLT4* we identified four variants [p.(Gly854Ser), p.(Gly914Arg), p.(Pro954Ser) and p.(Gly1154Arg)] while p.(Arg477Gln) was the first non-kinase domain heterozygous variant identified in association with lymphedema so far. The pathogenic role of p.(Arg477Gln) is supported by several factors: i) the prediction software Polyphen2 and Mutation Taster classified the variant as "possibly damaging" and "disease causing," respectively; ii) the c.1430G>A variation, described in dbSNP as rs755736057, is classified as a very rare variant with a MAF of 0.000065 by ExAC database; iii) the arginine in position 477 of FLT4 is highly conserved in human, chimpanzee, rat, mouse, dog, chicken, frog, and zebrafish. We therefore assumed that the p.(Arg477Gln) variant can be considered pathogenic.

In the *FOXC2* gene, we identified six variants, including two frameshift [p.(His199Profs*264) and p.(Ile213Thrfs*1)] and one nonsense variant [p.(Tyr109*)]. Variants p.(Ser125Leu) and p.(His199Profs*264) have already been identified in patients with primary lymphedema (23, 45) while p.(Leu80Phe), p.(Tyr109*), p.(Ile213Thrfs*1) and p.(Val228Met) were identified for the first time in this study.

In 2008, Finegold and colleagues identified variations in *MET* and *HGF* for the first time in patients with primary lymphedema (11). Interestingly, in our Italian sample, analysis of *MET* and *HGF* transcripts also led to identification in each gene of four variants that had not been previously reported. *MET* variations were identified in important functional domains of the protein, such as the intracellular tyrosine kinase domain [p.(His1097Arg), p.(Thr1217Serfs*5) and p.(Tyr1356His)], the IPT4 domain [p.(Asn856Lys)] and in the juxtamembrane domain [p.(Thr992Ile)]. We also identified in the *HGF* gene, one nonsense variant p.(Arg178*) in kringle domain K1, two missense variants [p.(Arg424Cys) and p.(Trp451Arg)] mapping into the kringle domain K4 and a frameshift variant [p.(Lys491Asnfs*6] that causes the introduction of a premature stop codon in the Serine Proteinase domain.

In the *GJC2* gene, we detected two missense variants causing amino acid changes in the intracellular domain of the protein that the pathogenicity predictor software classified as "tolerated" or "benign." However, p.(Pro384Ser) and p.(His412Tyr) have been classified as pathogenic variants because they have been previously described by Finegold in patients with secondary lymphedema (6).

In *GATA2* gene we identified a missense variant p.(Lys405Asn) that all the pathogenicity predictor software classified as "disease causing." This alteration, is not present in dbSNP but in ExAC database (http://exac.broadinstitute.org/) is classified as a rare variant with an allele frequency in the European population of 1.499e-05.

Finally, in the *KIF11* gene, we identified the intronic c.77+5G>C variant in three unrelated patients affected by familial lymphedema. This alteration, described in dbSNP as rs200188195, is classified as a rare variant with a MAF of 0.0008. Human Splicing Finder (http://www.umd.be/HSF/) predicts that alteration of the wild type c.77+5G nucleotide may affect splicing of *KIF11* mRNA. In addition, the c.77+5G nucleotide is highly conserved in chimpanzee,

rat, mouse, and dog, suggesting that this residue can play a key role during splicing between exon 1 and 2 of *KIF11*. Finally, since we did not find this intronic variation in the ethnically matched control population of our study, we considered the c.77+5G>C alteration a pathogenic variant. Interestingly, all mutant-bearing patients manifested primary lymphedema without the typical features of the MLCRD phenotype (chorio-retinal dysplasia, microcephaly and mental retardation) described to be associated with variations in this gene (13).

DISCUSSION

In this study we performed variant analysis of 10 lymphedema-associated genes in a cohort of 116 Italian patients affected by primary lymphedema. Patients with an *FLT4* or *FOXC2* alteration accounted for the largest percentage of the sample (6/116 patients for each gene; 5.1%), followed by *MET* (4/116 patients; 3.4%), *HGF* (4/116 patients; 3.4%), *KIF11* (3/116 patients; 2.6%), *GJC2* (2/116 patients; 1.7%), and *GATA2* (1/116 patients; 0.86%). Overall, in the whole sample, composed of 45 familial and 71 sporadic cases, a genetic variation was found in 22.4% (26/116) of the patients. The variant detection rate decreased to 17% (12/71) for cases with sporadic or unknown inheritance and reached 31% (14/45) for familial cases.

The phenotype associated with the *FLT4* variants proved to be classical Nonne-Milroy disease as previously reported (46), and *FLT4* variations were found both in familial and sporadic cases. Four variants p.(Gly854Ser), p.(Gly914Arg), p.(Pro954Ser) and p.(Gly1154Arg) induce amino acid alterations in the conserved tyrosine-kinase (TK) domain of FLT4, while variant p.(Arg477Gln), identified in exon 11, induces an amino acid change in the Ig-like domain of the protein. Since we were aware of the difficulty of establishing whether a base change is pathogenic for a disease, the possibility that p.(Arg477Gln) may be non-causative was carefully considered. In line with the pathogenicity prediction of Polyphen2 and Mutation Taster, the high conservation level of Arg477 residue in most vertebrates and the extremely low frequency of the c.1430G>A variant, we considered this alteration to be causative of lymphedema in patient R682. This is the first time that a variant associated with primary lymphedema is identified outside the tyrosine-kinase domain. This finding is not at all surprising since almost all previous variation screening was restricted to exons 17-26 that encode the TK domain of *FLT4*. We therefore think it would be worthwhile extending molecular analysis to non-kinase-domains of *FLT4* in order to detect putative variants in the N-terminal portion of the protein.

In *FOXC2* we identified six variants: three missense, two frameshift and one nonsense. According to the LOVD database, nonsense or frameshift variants occur in 84% of patients with *FOXC2* variations, representing by far the most common class of alterations associated with the disease (http://databases.lovd.nl/shared/genes/FOXC2). In contrast, in our Italian sample, mutant *FOXC2* alleles causing a prematurely truncated protein constituted only 50% of *FOXC2*-related cases (3/6 alleles). These findings are in line with the data reported by Michelini and colleagues (46) in another sample of Italian patients, where frameshift and nonsense variants were identified in only 33% of patients with *FOXC2* variations. So far, at least 77 variants have been identified in *FOXC2*; 56 of them map outside the forkhead domain (FHD) (http://databases.lovd.nl/shared/genes/FOXC2), a DNA-binding motif highly conserved from yeasts to humans. We identified one missense variant p.(Val228Met) outside FHD, whereas p.(Leu80Phe) and p.(Ser125Leu) mapped inside this functional domain. Variant p.(Leu80Phe) was located in the Helix 1 portion of FHD that controls the protein's nuclear localization and its transcriptional regulation and mediates its binding with

DNA. The mutant leucine residue is highly conserved in all nine human *FOX* genes, suggesting that this amino acid residue is important in control of protein activity (24). p.(Ser125Leu) also seems to play an important role in controlling FHD function. It is reported to behave as a loss-of-function variant, impairing the DNA-binding ability of FHD and leading to reduction in transcriptional activity of the gene (24). Interestingly, in this Italian sample, the *FOXC2* variants were not associated with distichiasis in 5/6 probands (83%). A similar finding was recently reported by Michelini (46) and Van Steensel (47) who described an absence of distichiasis in over 80% of lymphedema patients with *FOXC2* variations. These new results seem to be in contrast with the literature, once again confirming the phenotypic variability of *FOXC2*-related variations. In the light of these results, a clear genotype/phenotype correlation cannot be assumed and *FOXC2* variant analysis should therefore also be carried out in patients with lymphedema but without distichiasis as long as careful ocular phenotyping rules out scattered extra eyelashes.

MET and its ligand HGF are essential for many development processes including lymphogenesis (27,28). Variant analysis of these two genes led us to the identification of four variants in *MET* and four in the *HGF* gene. MET variant p.(Thr1217Serfs*5) is a frameshift alteration that introduces a premature stop codon, causing formation of a truncated protein lacking the tyrosine kinase (TK) domain. Deletion of the TK domain prevents auto-phosphorylation of the receptor at specific tyrosine residues, inactivating downstream signal transducers (48). Interestingly, a second variant p.(Tyr1356His) that we identified in the TK domain also down-regulates MET function, removing one of the conserved tyrosine residues (Tyr1356) that control TK activity. In particular, phosphorylation of Tyr1356 activates the multi-substrate signal transducer docking site that is responsible for much of MET-mediated signal transduction. Functional analysis also suggests that tyrosine 1356 is crucial for regulating cell morphogenesis and interactions of MET with SHC, SRC, and GAB1 (49). We therefore believe that p.(Thr1217Serfs*5) and p.(Tyr1356His) can be classified as loss-of-function variants that reduce or abolish MET activity. On the other hand, functional studies are necessary to understand whether p.(Asn856Ly), and p.(His1097Arg) also share this pathogenic mechanism of action with p.(Thr1217Serfs*5) and p.(Tyr1356His). Likewise, for MET and its specific ligand HGF, we were unable to identify a correlation between the phenotype of mutant-bearing patients and their genotype. In patients with *MET* or *HGF* variations, we identified familial and sporadic cases as well as congenital and late-onset lymphedema. Unlike Finegold and colleagues (2008), we never found intestinal lymphangiectasia in patients with *MET* or *HGF* variations (11).

In two patients of our sample, we also discovered two *GJC2* variants that had already been identified in individuals developing secondary lymphedema after treatment for breast cancer (6). Finegold and colleagues used in vitro assays to show that variants p.(Pro384Ser) and p.(His412Tyr) are dysfunctional and act with a gain-of-function mechanism. The identification in our sample of two independent *GJC2* variants, previously reported in secondary lymphedema, clearly confirms that genetic susceptibility is an important risk factor to consider even in the more common forms of lymphedema. We therefore consider it worthwhile to extend genetic analysis of primary lymphedema-causing genes to subjects with risk factors for secondary lymphedema (e.g., in response to mechanical trauma, radiation therapy, chemotherapeutic insult), allowing patients with genetic variations to benefit from early medical observation and therapy before clinical manifestation of the disease.

We identified, in a patient without a family history of lymphedema, a missense variant in *GATA2* gene p.(Lys405Asn).

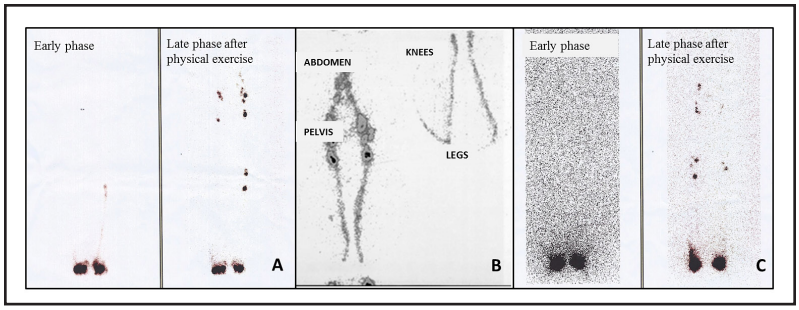

*Fig. 1. Lymphoscintigrams in a family positive in genetic testing. A) Patient R693, heterozygous for the p.(Arg178 *) variant in the HGF gene, is the only subject with clinical manifestations of lymphedema in her family. Lymphoscintigraphy displays bilateral delayed lymphatic drainage with greater delay in the right leg. Visualization of lymphatic ducts and lymph nodes of the right lower limb was absent, even in the late phase after physical exercise; B) Lymphoscintigraphy showing a normal lymphatic circulation and lymph node localization in the proband's daughter who did not inherit the p.(Arg178 *) variant; C) Lymphoscintigraphy of the clinically healthy proband's son carrying the HGF p.(Arg178 *) variant shows delayed lymphatic drainage (small delay on the right with more evident delay on the left), thus revealing a subclinical phenotype.*

Variations in *GATA2* gene have been described as causing lymphedema associated with myelodysplasia or acute myeloid leukemia but patient R690 was diagnosed with classical primary lymphedema with no additional symptoms. This is not completely surprising if we consider that 35% of the *GATA2*-mutated patients affected by Emberger syndrome showed minimal or absent hematological abnormalities as reported by Ostergaard and colleagues (15).

Finally, in three unrelated familial cases, we identified the same splicing variant (c.77+5G>C) in the *KIF11* gene. Although variations in this gene are generally associated with microcephaly, primary lymphedema and chorioretinal dysplasia (MLCRD syndrome; MIM 152950), no MLCRD phenotype was observed in patients with the *KIF11* variant in our sample. This difference may be explained once again by the genetic heterogeneity of primary lymphedema, where no clear genotype-phenotype correlations have been established.

In conclusion, this is the first genetic study in which all major genes known to be causative of primary lymphedema were investigated in a large sample of patients affected by this genetic pathology. However, in our sample, analysis of 10 lymphedema-associated genes by a next-generation-sequencing-based approach explained only a part of familial and sporadic cases of primary lymphedema. Even increasing the variant detection rate by including only familial cases, the majority of familial primary lymphedemas remained unexplained, confirming the likelihood of causative genes that have not yet been identified. In the next few years, it is reasonable to expect that whole-exome sequencing technology will greatly increase the number of known lymphedema-associated genes, making traditional genetic analysis techniques (dHPLC, Sanger sequencing, HRM) obsolete due to cost and analysis time. In our experience, screening of a complete lymphedema gene panel by an integrated NGS approach can be a powerful and useful strategy for discovering the genetic causes of primary lymphedema at reasonable cost, in terms of work and time, in large samples of patients.

Interestingly, when we extended the clinical and genetic study to relatives of mutated lymphedema probands, we recognized that the presence of the variant in relatives without a clearly recognizable phenotype is a strong predisposition factor towards overt disease.

As proof of principle, we reported the result obtained from the genetic and lymphoscintigraphy analysis we performed in the offspring of patient R693. Lymphoscintigraphy of R693's daughter (genetically wild-type), showed a normal lymphatic circulation, while in the male son that inherited the *HGF* variant from the mother, lymphoscintigraphy showed a marked groin lymphatic hypoplasia, absolutely not detectable through physical examination (*Fig. 1*).

The results of segregation studies on familial cases reported in this paper are currently being further examined.

Moreover, since the pathogenesis of many lymphedema cases remains unclear, we are also focusing our research on somatic variations that could contribute together with a germline variation to determine disease progression and severity. Thus we are currently screening for variants in tissue samples by a custom bionformatic method able to obtain a base to base comparison between germinal and somatic DNA. Identification of somatic causative genes and variations in tissues could provide new important information about mechanisms of lymphedema pathogenesis. Future applications may enable disease-free areas to be traced to reduce the possibility of relapse after surgery or the use of molecular therapeutics targeting mutant tissues.

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