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CLINICAL AND GENETIC STUDY OF 46 ITALIAN PATIENTS WITH PRIMARY LYMPHEDEMA

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ABSTRACT

Primary lymphedema is characterized by altered morphological development of lymphatic vessels causing fluid accumulation in interstitial spaces. In familial forms, it is primarily transmitted as a dominant Mendelian trait with heterozygous mutations in genes involved in lymphangiogenesis. We used PCR and direct sequencing to analyze the region of the fms-related tyrosine kinase 4 (FLT4) gene encoding the "tyrosine-kinase domain" and the single exon of the forkhead box C2 (FOXC2) gene in 46 Italian probands with primary lymphedema, 42 of whom had familial forms. We identified 12 mutations in 12 patients (12/46, 26%), six in the FLT4 gene and six in the FOXC2 gene. Most of the mutations (9/12, 75%) were new, and none were identified in 100 healthy subjects or listed in the NCBI dbSNP. A clear relation emerged between genotype and phenotype because 4/5 (80%) probands with onset at birth showed FLT4 mutations and 4/5 (80%) probands without distichiasis and with FOXC2 mutations had an amino-acid substitution outside the forkhead domain. Besides the allelic heterogeneity shown by unique mutations in each proband, the absence of mutations in almost 75% of familial cases of primary lymphedema also suggests genetic heterogeneity.

Keywords: FOXC2, FLT4 alias VEGFR3, primary lymphedema, distichiasis, forkhead domain, genotype-phenotype relationship

Primary lymphedema is a pathologic condition characterized by altered morphological development of lymphatic vessels followed by occlusion of lymphatic drainage which results in fluid accumulation in interstitial spaces and swelling in the affected body part (1). When the phenotype shows a positive family history, which can occur in varying degrees of severity and/or various clinical signs associated in the different affected members of the family, the cause is usually a simple heterozygous mutation of one of the genes involved in lymphangiogenesis. In familial cases, the condition is generally transmitted in an autosomal dominant manner (2,3).

The two genetic syndromes most often associated with primary lymphedema are Nonne-Milroy syndrome (also described as hereditary lymphedema type I) caused by mutations in the *FLT4* gene (fms-related tyrosine kinase 4, also known as gene

VEGFR3 (3,4); OMIM* 136352) that encodes a receptor for vascular endothelial growth factors C and D (5), and the lymphedema-distichiasis syndrome caused by mutations in the *FOXC2* gene [forkhead box C2 also named Mesenchyme Fork Head-1 (MFH-1); OMIM* 602402] that encodes a transcription factor (2,6). *In vitro* and animal model studies suggest that *FLT4* mutations are associated with hypoplasia of lymphatic vessels, since the growth factor VEGFC no longer activates the homodimeric receptor FLT4 or promotes the tyrosine-kinase cascade that governs lymphangiogenesis (7). With regard to the pathogenesis of the gene FOXC2, evaluation of the dynamics of lymph flow and immuno-histochemical examination of lymphatic tissues of mice with a heterozygous disruption of the FOXC2 gene, have shown a generalized hyperplasia and valvular incompetence of lymphatic vessels with distichiasis and various abnormalities that involve skeletal and cardiovascular systems (8).

Primary lymphedema has also occasionally been associated with defects in the genes *HGF, MET* (9), *FABP4, NRP2, SOX17, VCAM-1* (10) and *GJC2* (11). Other hereditary forms of primary lymphedema, correlated with more accentuated syndromes, have been associated with mutations in various genes including: mutant *SOX18* responsible for the hypotrichosis-lymphedema-teleangiectasia syndrome (12), mutant *GATA2* for Emberger syndrome (primary lymphedema associated with a predisposition for acute myeloid leukemia) (13), and mutant *CCBE1* for Hennekam syndrome (lymphedema-lymphangiectasia) (14).

Nonne-Milroy syndrome (OMIM#153100) has autosomal dominant transmission with incomplete penetrance, variable expression, and variable age of onset. Human Genome Mutation Database (HGMD) and PubMed databases contain 38 mutations of the gene *FLT4* that are all located in the tyrosine-kinase domain of the protein (exons 17 to 26 of the reference sequence) (15). Although adult and adolescent onset cases have been reported, Brice et al (16) observed that onset of FLT4-related lymphedema in about 85-90% of subjects occurs before 3 years of age.

The first study describing subjects with primary lymphedema (4) involved 13 North American families of western European origin (mainly United Kingdom and France). The study of these families, which included more than 100 affected subjects, showed approximately double the frequency in females than males (2.3:1), and incomplete penetrance since among those predicted to be affected, only 80% manifested the disease. In one of these 13 families, the missense mutation p.L1114P was identified.

In a more recent European study, Connell et al (15) enrolled the largest number of affected subjects to date. A mutation in the gene FLT4 was identified in 22 (42%) of 52 English patients with primary lymphedema; 14 (70%) of the 20 different mutations reported had not previously been reported. Complete analysis of the coding region of the gene confirmed the already reported absence of mutations outside the tyrosine-kinase domain of the protein.

In Italy, to our knowledge there have been no studies on primary lymphedema related to FLT4 mutations.

The lymphedema-distichiasis syndrome (OMIM#153400) has autosomal dominant inheritance with variable expression and onset between birth and 40 years of age (17-20). Distichiasis (double row of eyelashes) is encountered in 90-95% of affected subjects (19). Evaluation of the gene *FOXC2* in 86 families (71 of European origin) with lymphedema, characterized by broadly phenotypic heterogeneity on the basis of associated clinical signs, revealed 11 probands (12.8%) with 10 different mutations that determine synthesis of a prematurely truncated protein (18). Brice et al (19) found that no more than 80% of subjects with mutations of the *FOXC2* gene manifested lymphedema before 30 years of age, although all did so by 40 years. The same study also documented an age of onset that varied with gender because half the

males were affected by 11 years of age, while half the females were affected by 20 years of age.

Only one Italian case report describes a patient with lymphedema-distichiasis associated with a new *de novo* frameshift mutation in the gene *FOXC2* (21).

The aim of the present study was to estimate the frequency of molecular defects of the *FLT4* and *FOXC2* genes in a large cohort of Italians with a clinical diagnosis of primary lymphedema and to establish whether a recurrent gene defect with a particular clinical picture was involved. Since *FOXC2* gene defects are associated with broad phenotypic variability, such as early varicose veins (49%), blepharoptosis (31%), palatoschisis (4%), heart abnormalities and venous reflux (100%) (19,22), and at least 10 families with lymphedema but without distichiasis in one or more members have been described (18,23), molecular analysis of the gene was carried out in all patients, irrespective of the presence or absence of this clinical sign.

MATERIALS AND METHODS

Patients and Controls

Patients with suspected primary lymphedema, referred to the network of clinics involved in the project *SOS Linfedema* (participating centers are in the regions of Lombardy, Liguria, Umbria, Latium, Marches, Apulia, Sicily and Sardinia), were evaluated by a team of lymphology specialists and medical genetists (the latter from MAGI Human Medical Genetics Institute, a non-profit organization based in Rovereto, Italy). Patients underwent a series of clinical examinations and tests for clinical diagnosis of primary lymphedema to be eligible for genetic testing. Genetic counseling and specialist examination were also offered to family members of probands when the pathogenic mutation responsible for the disease was identified.

Inclusion criteria included fleeting or absent fovea sign and dermal backflow detected by lymphoscintigraphy. To recognize and exclude patients with secondary lymphedema, specific investigations and/or biochemical tests were performed on the basis of medical history and physical examination (the main secondary causes of lymphedema are tumors, surgery, internal post-traumatic scarring, deep venous thrombosis, tropical infections and iatrogenic causes related to radiation therapy).

When the molecular origin of primary lymphedema was identified, patients were staged by International Society of Lymphology criteria (24) (*Table 1*). Patients and family members enrolled in the study signed two informed consents, one for the genetic test and a second to make the clinical and genetic data available for research purposes. To determine whether new mutations causing amino acid changes were innocuous polymorphisms or pathogenic mutations, 100 healthy subjects were recruited from the same geographical areas as the patients.

Genetic Analysis

Approximately 5 ml of peripheral blood from 46 probands of Caucasian race were sent to MAGI Laboratory for DNA extraction using a commercial kit (Blood DNA kit E.N.Z.A., Omega Bio-tek, Inc. Doraville, GA, USA). PCR and direct sequencing of amplified fragments (Thermo PX2 thermo-cycler, Beckmann Coulter CEQ 8000 sequencer) were used to analyze exons coding for the tyrosine-kinase domain of FLT4 protein (exons 17-26, Ref. Seq. NG_011536.1), the only exon encoding the *FOXC2* gene (Ref. Seq. NG_012025.1), and the respective portions of the intron regions adjacent the exons studied. Primer sequences, PCR reaction conditions and sequencing conditions are available on request.

In Silico Analysis and Evaluation of Pathogenicity of the New Mutations

TABLE 1
Age of Onset, Stage and Molecular Diagnosis of 12 Probands With Primary Lymphedema

ID Proband† [gender]	Age of onset	Stage*	Mutant allele FLT4	Mutant allele FOXC2	Reference genotypes§
P1-F1 [F]	18 years	II	c.2777T>C p.Ile926Thr	-	This study (JQ664719)
P2-F2 [F]	14 years	II	-	c.1109 G>C p.Ser370Thr	This study (JQ664716)
P3 [M]	congenital	II	c.2560G>A p.Gly854Ser	-	28
P4-F4¥ [M]	congenital	II	c.2740 G>T p.Gly914Trp	-	This study (JQ664718)
P5-F5 [M]	congenital	II	c.2575 G>A p.Val859Met	-	This study (JQ664717)
P6¶ [M]	12 years	II	-	c.826_827del2 Met276Aspfs*186	This study (JQ664711)
P7-F7 [F]	3 years	II	3122G>A p.Arg1041Gln	-	27
P8-F8 [F]	congenital	II	c.2632G>A p.Val878Met	-	28
P9 [M]	congenital	II	-	c.8 C>G p.Ala3Gly	This study (JQ664713)
P22 [F]	26 years	II	-	c.1460 T>C p.Leu487Pro	This study (JQ664714)
P32-F11 [M]	26 years	III	-	c.1475C>T p.Ala492Val	This study (JQ664715)
P12-F12 [F]	20 years	II	-	c.1258C>T p.Gln420*	This study (JQ664712)

† Patients from families with at least one other affected member are identified by codes containing the capital letter F
*Severity classes according to Consensus Document guidelines of the International Society of Lymphology (2009) indicated by Roman numbers from I to III for phenotypes of increasing severity.
¥ The proband also showed patent foramen ovale.
¶The proband also showed distichiasis and prominent veins.
§For new mutations the GenBank accession number is indicated.

Nucleotide peaks of all amplified fragments were compared with the respective reference sequences by a bioinformatic tool known as blast 2 sequences (http://blast.ncbi.nlm.nih.gov). All fragments containing sequence variations were then analysed with the nucleotide blast and blastx algorithm (http://blast.ncbi.nlm.nih.gov), and the mutations determining an effect on the protein were sought in the Single Nucleotide

Polymorphism Database (dbSNP) database of the NCBI (http://www.ncbi.nlm.nih.gov/snp) and in the HGMD database (http://www.hgmd.cf.ac.uk) to see whether they were entered as polymorphisms or known mutations, respectively .

To establish the possible impact of the new missense mutations, these were evaluated with the tool UniProt (http://www.uniprot.org/) to determine the degree of conservation in the course of evolution, and by means of physicochemical parameters to compare wild-type and mutant amino acids (25). In addition, the new sequence variations were screened in 200 chromosomes of Caucasian origin, and they were classified as polymorphisms if found in at least one other DNA or otherwise as pathogenic mutations.

RESULTS

The number of primary lymphedema patients enrolled was 46. Forty-two were classified as familial forms and 4 with forms presumed to be sporadic because family involvement could not be investigated. The mean age of onset was 20 years, and the female:male ratio was 3.3:1.

Analysis of *FLT4* and *FOXC2* revealed 12 different mutations in 12 (26%) affected patients. Four patients were classified as sporadic cases while the other eight had at least one other affected family member. Except for one patient who was assigned to stage III, the other 11 had stage II phenotypes (24). The genetic defects involved *FLT4* in 6 cases (13%) and *FOXC2* in the other six, but only one proband with a defect in the latter gene manifested distichiasis as a clinical sign associated with primary lymphedema (*Table 1*). Four (80%) of the five probands with onset at birth had *FLT4* mutations. Four (80%) of the five probands with *FOXC2* mutations not associated with distichiasis had a single amino-acid substitution outside the highly conserved region known as the forkhead domain (26) (*Tables 1 and 2*). The mean age of onset of the disease was 3.5 and 16.3 years in subjects with *FLT4* and *FOXC2* defects, respectively. Nine (75%) of the 12 different mutations (three in *FLT4* and six in FOXC2) are novel and in three families at least one other family member was affected and carried the familial mutation *(Table 2*). Three of the VEGFR3 mutations were not novel (27,28).

None of the new mutations were identified in 200 alleles of healthy subjects or in the dbSNP database of NCBI. Among them, two determined the synthesis of a prematurely truncated protein, and thus it is plausible that the FOXC2 protein encoded is inactive. The other seven missense mutations were classified in three distinct categories (*Table 2*) on the basis of their different impact on the encoded protein:

- a major deficit is likely for mutations p.I926T and p.G914W in the gene *FLT4*. Their respective amino-acid residues are conserved in four classes of vertebrates, and there is a marked difference in polarity between wild-type and mutant amino acids;
- a less important deficit is likely for the other two missense mutations, p.V859M (FLT4 gene) and p.A3G (*FOXC2* gene), because the concomitant conservation of the respective amino-acid residue in four classes of vertebrates is not associated with a sharp difference in polarity of the amino acids involved;
- a minor deficit is likely for the remaining *FOXC2* mutations because the conservation of the respective amino-acid residue only in two (p.L487P e p.A492V) or one (p.S370T) class of vertebrates is associated with minor or no difference in polarity between wild-type and mutant amino acids.

DISCUSSION

The pathogenic role of the *FLT4* and *FOXC2* genes in primary lymphedema has largely been unclarified in Italian patients.

TABLE 2
Characteristics of the New Mutations, Evaluation of Degree of Possible Impact on the Protein and Segregation in Probands' Families

ID patient	Gene involved	Nucleotide change (effect on protein)	Regions of protein involved	Class* of vertebrates in which amino acid is conserved	Polarity	Predicted impact of the mutations on protein functionality†	Affected family members with the same genotype
P6	FOXC2	c.826_827del2 (Met276Aspfs*186)	C-terminal to forkhead domain	-	//	X	sporadic ?
P12-F12	FOXC2	c.1258C>T (p.Gln420*)	C-terminal to forkhead domain	-	//	X	son
P1-F1	VEGFR3	c.2777T>C (Ile926Thr)	Tyrosine kinase domain	M, B, R, F	Apolar/Polar	+++	DNA of the daughter was not available
P4-F4	VEGFR3	c.2740G>T (Gly914Trp)	Tyrosine kinase domain	M, B, R, F	Apolar/Polar	+++	Great-aunt and her two children
P5-F5	VEGFR3	c.2575G>A (p.Val859Met)	Tyrosine kinase domain	M, B, R, F	Apolar/Apolar	0	DNA of the paternal cousin was not available
P9	FOXC2	c.8C>G (p.Ala3Gly)	N-terminal to forkhead domain	M, B, A, F	Apolar/Apolar	0	sporadic ?
P22	FOXC2	c.1460T>C (p.Leu487Pro)	C-terminal to forkhead domain	M, B	Apolar/Apolar	0	sporadic ?
P32-F11	FOXC2	c.1475C>T (p.Ala492Val)	C-terminal to forkhead domain	M, B	Apolar/Apolar	0	DNA of the father and of the paternal grandmother were not available
P2-F2	FOXC2	c.1109G>C (p.Ser370Thr)	C-terminal to forkhead domain	M	Polar/ Polar	0	father‡

*M=mammals; B=birds; R=reptiles; A=amphibians; F=fish; †Predicted impact of the mutations on protein functionality =
X [absence of activity]: null allele plausibly associated with absence of activity; +++[severe dysfunction] : maximum degree of conservation for specific residue in vertebrate classes and relevant differences in polarity of the amino acids involved; ++[mild to moderate dysfunction]: maximum degree of conservation for specific residue in vertebrate classes without relevant differences in polarity of the amino acids involved; +[mild dysfunction]: residue-specific conservation limited to mammalian class (or mammalian and bird classes) without relevant differences in polarity of the amino acids involved.
‡The subject has a latent condition defined as stage 0 (ISL Consensus Document from Lymphology, 2009).

The only study published in Italy reports the molecular characterization of a *FOXC2* defect in a single patient (21). It was therefore necessary to understand the frequency and type of *FLT4* and *FOXC2* gene defects in a number of subjects representative of the Italian patient population.

In the present study, we assessed whether 46 Italian probands (with the clinical diagnosis of primary lymphedema) carried point mutations in the *FOXC2* gene or in the *FLT4* exons from 17 to 26 that encode the highly conserved tyrosine-kinase domain (residues 843-943 and 1,009-1,165) (29); among them, more than 25% (12/46) of patients had a pathogenic allele identified with the same frequency between *FLT4* and *FOXC2* genes (*Table 1*).

As reported in other populations (15,19,30), the disease also shows high allelic heterogeneity among Italian patients. In our study, in fact, this phenomenon manifested as failure to characterize a recurrent mutation and identification of nine (75%) new mutations (*Table 2*). Among the latter, two mutations are associated with absence of activity because they determine a prematurely truncated protein, while for the remaining 7 missense mutations, three different degrees of impact on the function of the encoded protein were predicted by a combination of two strategies (*Table 2*). It was only possible to verify the presence of the mutation in other affected family members in 3 of the 9 families with new mutations. Affected relatives of three other probands were not evaluated because DNA was not available (*Table 2*). At the same time, no clear relation was found between severity of the genotype and the parameters age of onset or lymphedema stage. As previously reported (16), the *FLT4* defect was also often (4/5 cases) associated with neonatal onset of lymphedema in our cohort (*Table 1*). However, unlike in other studies where mutant *FOXC2* alleles causing a prematurely truncated protein represented fractions from 80-100% of patients (18,19,29), our population found this type of allele in only 33% of FOXC2-related cases (2/6 alleles). None of the remaining fraction of mutant FOXC2 alleles (all determining an amino-acid change) involved the typical forkhead domain of FOX transcription factors (*Table 2*). Originally, three missense FOXC2 mutations that cause "loss of function" were mapped (26) to the forkhead domain, a DNA-binding motif typical of metazoa, which can be identified from yeasts to humans.

The four missense mutations identified by us can be added to the five recently described by van Steensel et al (23). Four of these share the same region, outside the forkhead domain, that were demonstrated to be univocally associated with a "gain in function" of the mutant protein.

In contrast to what has commonly been presented in the literature, and in confirmation of the phenotypic variability of FOXC2-related mutations, in our population the FOXC2 gene defect was not associated with distichiasis in 5 (83%) out of 6 probands (*Table 1*). A similar finding was recently reported by van Steensel et al (23) who described an absence of distichiasis in 9 (81%) out of 11 lymphedema patients with FOXC2 mutations. According to Finegold et al (18), the absence of this typical clinical FOXC2-related sign had already been reported, albeit in only one (9%) of the 11 families carrying mutations, and it was related to a premature stop codon.

A close genotype-phenotype relationship was also evident since the absent distichiasis is often observed in subjects whose mutant FOXC2 allele is a missense mutation that does not map to the forkhead domain of the FOXC2 protein (4/5 [80%] of our patients and 4/9 [44%] of the patients from van Steensel et al) (23) (*Table 2*).

Comparison of the frequency of the *FLT4* molecular defect in our cohort (13%) to the American population (42-75% if family history is considered an inclusion criterion) (15) showed a sharp difference in involvement of the gene in the two populations. However,

considering the high allelic heterogeneity of the disease, evaluation of gene regions not coding for the tyrosine-kinase domain could increase the detection rate.

Our prevalence of *FOXC2* gene mutations (13%) was similar to that reported by Finegold et al (18) in 86 patients (12.8%), more than 80% of whom were of European origin, but was almost 3.5 times greater than that described by van Steensel et al (23) in a population of 288 patients (3.81%). The limited prevalence recorded in the latter study could be due to less stringent inclusion criteria because the authors did not look for lymphoscintigraphic evidence of dermal backflow or identify and exclude patients with secondary lymphedema.

Almost 75% (thirty-four) of our patients did not show any *FLT4* or *FOXC2* gene defect. Considering their positive family history and the highly heterogeneous nature of the phenotypic manifestation of lymphedema also described within individual families (20,31), it is reasonable to hold that phenotype may be correlated with defects in other genes, such as *HGF, MET* (9), *FABP4, NRP2, SOX17, VCAM-1* (10) and *GJC2* (11) which have only occasionally been reported with mutations in primary lymphedema patients.

In conclusion, mutations in the coding regions of the *FLT4* and *FOXC2* genes (only the tyrosine-kinase domain was evaluated in the former) may explain the defect in more than 25% of the Italian cohort of primary lymphedema patients. While the prevalence of *FLT4* mutations is not comparable to that reported in an American study (13% vs 42-75%), the prevalence of FOXC2 mutations was similar to that reported in a European study (13% vs 12.8%). As expected in cases with onset at birth, we often found a *FLT4* mutation (80%) while, in line with the trend recently reported by van Steensel et al (23), the same frequency was also observed in patients without distichiasis that carry a missense mutation of FOXC2 from outside the forkhead domain. As reported by other studies, genetic defects generally represent unique mutations, and indeed 75% of the variants identified (9/12) are not described in the literature. The possible involvement of other genes is suggested by the many (almost 75%) probands negative for the test, by a constantly positive family history and by the heterogeneous nature of the primary lymphedema phenotype. Though not completely exhaustive, our study shows that the chosen genetic tests used in this study are useful to ensure molecular diagnosis in more than 25% of Italian patients with a clinical diagnosis of primary lymphedema. This could enable informed reproductive choices through reproductive risk assessment by prenatal genetic services, and in the middle to long term could offer patients FLT4-related therapy with recombinant proteins. This therapy has already been reported to produce good results in animal models (32). Considering the potential morbidity of hereditary lymphedema, the possibility of molecular diagnosis may also facilitate legal recognition of rare disease status.

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