Clinical heterogeneity in lymphoedema-distichiasis with *FOXC2* truncating mutations

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Abstract

***Background*—Hereditary lymphoedema-distichiasis (LD) is an autosomal dominant disorder that classically presents as lymphoedema of the limbs, with variable age of onset, and extra aberrant growth of eyelashes from the Meibomian gland (distichiasis). Other major reported complications include cardiac defects, cleft palate, and extradural cysts. Photophobia, exotropia, ptosis, congenital ectropion, and congenital cataracts are additional eye findings. Recently, we reported that truncating mutations in the forkhead transcription family member *FOXC2* resulted in LD in two families.**

***Methods*—The clinical findings in seven additional families with LD, including the original family described by Falls and Kertesz, were determined and mutational analyses were performed.**

***Results*—Distichiasis was the most common clinical feature followed by age dependent lymphoedema. There is a wide variation of associated secondary features including tetralogy of Fallot and cleft palate. The mutational analyses identified truncating mutations in all of the families studied (two nonsense, one deletion, three insertion, and one insertion-deletion), which most likely result in haploinsufficiency of *FOXC2*.**

***Conclusions*—*FOXC2* mutations are highly penetrant with variable expressivity which is not explicable by the pattern of mutations.**

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Keywords: lymphoedema; distichiasis; forkhead gene; clinical heterogeneity

Lymphoedema is oedema or tissue swelling resulting from failure of the lymphatic system to transport excess capillary filtrate containing liquid, macromolecules, and mobile cells back to the blood. It is usually secondary to filariasis, surgery, trauma, or infection. Primary lymphoedema is thought to have a strong genetic component and arises from faulty development or function of the lymphatic system. It can be sporadic or inherited. Congenital lymphoedema can be found associated with known syndromes including Noonan and Turner syndromes. Hereditary lymphoedema is heterogeneous with more than 10 Mendelian forms that usually occur as autosomal dominant traits. Three commonly recognised forms of hereditary lymphoedema are Milroy disease, Meige disease, and lymphoedema-distichiasis (LD). Among the inherited forms of lymphoedema, LD has most frequently been associated with other congenital abnormalities, including cardiac defects, cleft palate, photophobia, and spinal extradural cysts. This can be understood by examination of the function of genes responsible for the disorders. Milroy disease can be caused by mutations in the vascular endothelial growth factor receptor-3 gene (*VEGFR-3* or *FLT4*),[1–3] while the gene causing Meige disease remains unknown. Lymphoedema-distichiasis can be caused by mutations in the *FOXC2* forkhead transcription factor, a gene expressed in multiple tissues during development.

Recently, we reported that truncating mutations in the *FOXC2* gene resulted in LD in two families and hypothesised a position effect as the cause of LD in a third translocation subject.[4] We now describe mutations and clinical findings in an additional seven families with LD, including the original family described by Falls and Kertesz.[5] Distichiasis was the most common clinical feature followed by age dependent lymphoedema. In addition, there is a large variation in secondary features that affect a number of tissues in which the *FOXC2* gene is likely to play a key developmental role.

Methods

Informed consent for both participation and collection of tissue samples was obtained from all family members, in accordance with the procedures of the Institutional Review Boards at the University of Arizona and University of Michigan Medical Schools.

FAMILIES

Families 1 and 2 were previously described by Fang *et al*.[4] Family 3 is the original family which initially delineated the LD syndrome and was possibly mentioned by Neel and Schull[6] but described in detail by Falls and Kertesz.[5] Thirty-one subjects from three generations of this family gave informed consent to clinical examination and blood sampling. They were designated affected if they had, at minimum, distichiasis on examination. Thirteen of the 31 family members exhibited clinical features associated with LD. The oldest living affected member of this family is an 82 year old female who has distichiasis as her only feature of LD. She has three children, of whom one daughter has distichiasis, neck webbing, and photophobia. Her grandson also has

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distichiasis and neck webbing. A niece exhibits distichiasis and lymphoedema with cellulitis. Another affected niece has distichiasis and developed lymphoedema at the age of 12. She has three children and, of these, one son has lymphoedema, distichiasis, and cleft palate. An affected nephew has had lymphoedema since the age of 12 and also exhibits distichiasis, neck webbing, ectropion, photophobia, torus mandibularis, and blepharospasm. He has six children and, of these, two males are affected. One of the affected sons has distichiasis, while the other has mild lymphoedema, distichiasis, and torus mandibularis. A second affected nephew has had severe lymphoedema since puberty, resulting in elephantiasis, and has distichiasis, neck webbing, and ectropion. A third affected nephew has had distichiasis, neck webbing, and lymphoedema since the age of 10. This nephew had three sons and, of these, only the twins have lymphoedema and distichiasis.

Affected subjects from family 4 have a spectrum of clinical features associated with LD. The 41 year old father of this family has distichiasis and developed lymphoedema at the age of 12. In addition, he exhibits a ventriculoseptal defect with infundibular stenosis of the pulmonary artery. He has four children, of whom three are affected. His oldest child is a 13 year old male who exhibits distichiasis, lymphoedema since the age of 12, ptosis, and nasal speech. His second child is an 11 year old female who is healthy. His third child is an 8 year old male who has distichiasis, ptosis, and velopharyngeal insufficiency, but has not yet exhibited lymphoedema. His youngest child is a 4 year old female who developed neonatal lymphoedema and periorbital oedema, and also suffers from distichiasis and tetralogy of Fallot.

Family 5 consists of a mother and child, both of whom have distichiasis and cleft palate, but no lymphoedema. No other family members exhibit any clinical features associated with LD. Upon examination, the 26 year old mother showed distichiasis and repaired cleft palate but did not have lymphoedema. Her 3 month old daughter has distichiasis and a cleft palate to about the middle third of the hard palate.

In family 6, there is a four generation pedigree with LD. The proband is an 81 year old woman with distichiasis since birth. She has had repeated corneal irritations and now has cataracts, in addition to photophobia. She has severe, pubertal onset, bilateral, lower extremity lymphoedema, which is predominantly below the knee with lymph leakage (fistula), which was treated by a Thompson debulking procedure. In addition, she suffers from narcolepsy. Her son exhibited distichiasis at birth and then pubertal onset, bilateral, lower extremity lymphoedema. He died at 38 years from tetralogy of Fallot, for which he had undergone two operations. The proband's mother also had distichiasis since birth, photophobia, and severe, pubertal onset, bilateral, lower extremity lymphoedema with multiple infections which were treated surgically. She died at the age of 93 from heart disease. Her maternal grandfather had severe elephantiasis, but it is not known whether he exhibited distichiasis. The proband's maternal aunt had mild, bilateral, lower extremity lymphoedema. It is not known whether the maternal aunt had distichiasis; however, her daughter had distichiasis with mild, pubertal onset, bilateral, lower extremity lymphoedema. In addition, the maternal aunt's grandson has had distichiasis since birth with pubertal onset, bilateral, lower extremity lymphoedema.

Family 7 represents a sporadic case of LD. A male child was examined at 6 weeks of age because of a very posterior cleft of the soft palate. At that time, he had slight puffiness of the dorsum of the feet bilaterally. No eye problems were noted. There was redundancy of the nuchal skin with a very short neck. During early childhood, the puffiness of the feet progressed and lymphoedema rising nearly to the knee occurred. By the age of 10, bilateral, lower extremity lymphoedema was evident. Distichiasis also became apparent and electrolytic removal of eyelashes projecting into the cornea is required annually. He is currently 14½ years old with lymphoedema that remains to the level of the knees.

The proband in family 8 is a 6 year old boy. A total of 16 family members in four generations have distichiasis, while lymphoedema is known in three and not known in the other 13 affected relatives. There was no report of other birth defects. The proband has distichiasis and underwent electroepilation of all four lids at the age of 6. His Meibomian gland spacing was normal in the upper lids and decreased in the lower lids. He has a history of congenital third nerve palsy in the right eye, exotropia in the left eye, and bilateral optic nerve hypoplasia. His mother also has distichiasis in all four lids and reports onset of lymphoedema during pregnancy. Neither mother nor son have findings of congenital ptosis, cataracts, or other birth defects. DNA of the proband's father and his mother's first child (the proband's half brother) was also included in the mutational analysis.

Family 9 consists of a mother and daughter with LD. The mother had onset of bilateral, lower extremity lymphoedema at puberty. She removes irritating eyelashes with tweezers. Her daughter has more severe distichiasis, having had surgical removal of portions of her eyelids at the age of 3 and continues to have electrolysis of some follicles. Her lymphoedema is milder and had its onset at the age of 22.

LABORATORY PROCEDURES

DNA was extracted from either venous blood or buccal swabs according to standard procedures. As described by Fang *et al*,[4] mutation analysis of family members initially referred to the laboratory was performed by sequencing PCR products of the coding region of *FOXC2* from one or more affected family members. Mutation analysis for additional family members was performed in some cases by PCR analysis followed by restriction enzyme digestion of novel restriction sites created by the mutation.

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Table 1 FOXC2 mutations

Family No	FOXC2 mutation	Effect on FOXC2 protein
1*	297C→G	Y99X
2*	1093^1094dupGGCC	PTC + 98aa†
3	602-681insACAAAdel79nt	PTC + 235aa†
4	792^793insA	PTC + 197aa†
5	951C→A	C317X
6	298C→T	Q100X
7	914-921delACGCCGCC	PTC + 154aa†
8	683^684insG	PTC + 233aa†
9	818^819insG	PTC + 188aa†

*From Fang *et al*.[4]
†PTC + #aa = premature termination codon + number of novel amino acids.

Results

Tables 1 and 2 summarise the *FOXC2* mutations and their phenotypic effects for all LD families we analysed. *FOXC2* mutations in families 1 and 2 were previously described by Fang *et al*[4] and have a nonsense mutation and a 4 bp insertion, respectively.

Affected subjects from family 3 have a 5 nucleotide (nt) insertion followed by a nt79 deletion in the mutant allele of *FOXC2*, which results in a frameshift after codon 201 that leads to a stop at codon 436. The mutation was verified by subcloning PCR products from both mutant and wild type alleles into a TA cloning vector (Invitrogen). PCR products were generated using primers that flanked the mutation (5'-TCACCTTGAACGGCATC TAC-3' and 5'-GCCCTGCAGCGCGCTC TCGG-3'). Both mutant and wild type clones from one affected subject were sequenced. Mutation analysis of other family members was by 2.0% agarose gel electrophoresis of PCR products generated with the flanking primers described above, in order to separate the normal (439 bp) and mutant (365 bp) PCR products.

In family 4, there is an insertion of an adenine after nt792 in the mutant allele, which

Table 2 Phenotypic effects

Family No	Sex	Age	Lymphoedema (age onset in years)	Distichiasis	TOF/ heart defect	Cleft palate	Webbed neck	Other clinical features
1	M*	42	+ (12)	+	−	−	−	−
	M*	14	−	−	−	−	−	Healthy, no mutation
	M*	13	+	+	−	−	−	−
	F*	a	+					Hydrops fetalis, cystic hygroma
	F*	a						Hydrops fetalis
2	M	d	+	+	−	−	−	−
	F*	55	+ (30)	+	−	−	−	−
	F*	34	+ (17)	+	−	−	−	−
	F	14		+	−	−	−	−
	M*	6		+	+	+	−	Photophobia, cystic hygroma, arachnoid cysts
3	F†	82	−	+	−	−	−	−
	F	39	−	+	−	−	+	Photophobia
	M*	19	−	+	−	−	+	
	F†	69	+	+	−	−	−	Cellulitis
	F†	57	+ (12)	+	−	−	−	−
	M*	28	+	+	−	+	−	−
	M*†	56	+ (12)	+	−	−	+	Ectropion, photophobia, torus mandibularis, blepharospasm
	M*	33		+	−	−	−	−
	M*	24	+	+	−	−	−	Torus mandibularis
	M*†	50	+ (12)	+	−	−	+	Ectropion, elephantiasis
	M†	50	+ (10)	+	−	−	+	−
	M*	23	+	+	−	−	−	−
	M*	23	+	+	−	−	−	−
4	M*	41	+ (12)	+	+	−	−	−
	F*	35	−	−	−	−	−	Healthy, no mutation
	M*	13	+ (12)	+	−	−	−	Ptosis, nasal speech
	F*	11	−	−	−	−	−	Healthy, no mutation
	M*	8		+	−	−	−	Ptosis, velopharyngeal insufficiency
	F*	4	+ (neonatal)	+	+	−	−	Periorbital oedema, velopharyngeal insufficiency
5	F*	26		+	−	+	−	−
	F*	3 mth		+	−	+	−	−
6	M	d	+	?	−	−	−	Elephantiasis
	F	d (93)	+ (puberty)	+	−	−	−	Photophobia
	F	d	+ (puberty)	?	−	−	−	−
	F*	81	+ (puberty)	+	−	−	−	Photophobia, narcolepsy
	M	d (38)	+	+	+	−	−	−
	F	d	+ (puberty)	+	−	−	−	−
	M		+ (puberty)	+	−	−	−	−
7	M*	14	+ (6 wk)	+	−	+	+	−
8	F*	28	+ (16)	+	−	−	−	−
	M*	12	−	−	−	−	−	Healthy, no mutation
	M*	28	−	−	−	−	−	Healthy, no mutation
	M*	6	−	+	−	−	−	Exotropia, bilateral optic nerve hypoplasia
9	F	61	+ (puberty)	+	−	−	−	−
	F	28	+ (23)	+	−	−	−	−

*Mutation analysis performed.
†Initially described by Falls and Kertesz.[5]
TOF = tetralogy of Fallot, a = abortion, d = dead.

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results in a frameshift after codon 264 that leads to a stop at codon 461. This mutation leads to elimination of a *Bgl*I restriction site in the affected allele. Therefore, mutation analysis of additional affected family members and two unaffected family members was by *Bgl*I restriction digestion of PCR products generated using primers (5'-AGGTGGTGATCAAGA GCGAG-3' and 5'-GCGAGGTTGAGAGC GCTCAGG-3') that flank the mutation and 2.0% agarose gel electrophoresis. All affected family members tested had the mutation, while neither of the unaffected family members had the mutation.

Affected subjects from family 5 have a transition of a cytosine to an adenine at nt951 in their mutant allele which leads to a stop at codon 317. The 951C→A transition leads to the creation of a novel *Sac*I site. Mutation analysis was verified by *Sac*I digestion of PCR products generated using flanking primers (5'-AGGTGGTGATCAAGAGCGAG-3' and 5'-GCGAGGTTGAGAGCGCTCAGG-3') and 2.0% agarose gel electrophoresis.

DNA from only one of the affected subjects from family 6 was available for *FOXC2* mutation analysis. The mutant allele in this subject has a transition of a cytosine to a thymine at nt298 that leads to a stop at codon 100. Because the mutation creates a novel *Bfa*I site, it was verified by restriction analysis of PCR products generated using primers flanking the mutation (5'-TCTCTCGCGCTCTCTCG CTC-3' and 5'-TGCCAGCCCTGCTTGTT CTCC). Interestingly, the location of this mutation in the forkhead domain is right next to the mutation identified in family 1, which is a 297C→G transversion.

Only the singleton in family 7 was analysed for a mutation in *FOXC2*. This subject has an 8nt (ACGCCGCC) deletion from nt914 to nt921 in his mutant allele, which results in a frameshift after codon 304 that leads to a stop at codon 458. Interestingly, this same mutation was identified in a family described by Bell *et al*.[7]

Of those analysed for *FOXC2* mutations in family 8, the two affected subjects had an insertion of a guanine after nt683 in the mutant allele, which results in a frameshift after codon 228 that leads to a stop at codon 461. No restriction sites were created or eliminated with this mutation, so mutation analysis sequencing was used for all family members tested. The two unaffected family members did not have the mutation.

Only DNA from one member of family 9 was analysed. This subject has an insertion of a guanine after nt818 in the mutant allele, which results in a frameshift after codon 273 that leads to a stop at codon 461. Because no restriction sites were created or eliminated with this mutation, verification of this mutation was by reverse sequencing of the PCR product.

Discussion

Lymphoedema-distichiasis was first described as an autosomal dominant disorder presenting in a large pedigree as lymphoedema of the limbs with variable age of onset, double rows of eyelashes (distichiasis), and variable expression of photophobia, ectropion, and torus mandibularis (it is now not certain if these are the same family).[5 6] Later reports added other variable features, including cardiac defects, cleft palate, and spinal extradural cysts,[8–11] which suggested a defect in a gene with pleiotropic effects acting during development. We initially reported mutations in the *FOXC2* gene in two families, and a probable translocation position effect in a third subject.[4] Our further analysis of *FOXC2* mutations in seven additional families with lymphoedema-distichiasis discloses the finding that truncating mutations can be associated with a variety of phenotypes (table 2). A variety of mutations were found in these families, including two nonsense mutations, three single nt insertions, an 8 bp deletion, and a large insertion/deletion. All insertions and deletions led to frameshifts and all mutations are predicted to truncate the FOXC2 protein. There was no apparent correlation of phenotype with mutant alleles. These results are consistent with our earlier interpretation that haploinsufficiency for *FOXC2* is responsible for the phenotype.[4] There has only been one missense mutation in *FOXC2* among 33 LD families[4 7 12] (this report), a marked contrast to the findings with *FOXC1* mutations and Axenfeld-Rieger anomaly.[13]

As previously mentioned, distichiasis is the most constant finding in affected subjects, with probable 100% penetrance in our cohort. Lymphoedema is also highly penetrant, occurring in all families, but with age dependence. Lymphoedema occurs most often by puberty, but in family 5 the 26 year old mother does not yet have lymphoedema and in family 3 there are three adults over 19 years of age with no clinical lymphoedema. However, an underlying lymphangiodysplasia and lymphatic insufficiency may nonetheless be present and visible on imaging studies even where lymphoedema is not clinically manifest. On the other hand, lymphoedema has sometimes been so severe as to result in therapeutic abortion for hydrops fetalis; the hydrops fetalis was so severe that it was thought to be the result of Turner syndrome in family 1 (the karyotype was normal in one of the two hydropic fetuses).[4] Cleft palate has been quite frequent in these families and tetralogy of Fallot has been the most common congenital heart anomaly (see table 2 for summary of findings), both occurring in approximately 10% of affected subjects.

Heterozygosity for *FOXC2* mutations leads to a variably expressed multiple malformation syndrome in which we find distichiasis to be the most common feature. Finegold *et al*[12] and Bell *et al*[7] have also presented recent analyses of lymphoedema-distichiasis families. Our results are in general agreement with these findings, with the exception that they can be contrasted to the report by Finegold *et al*,[12] in which lymphoedema rather than distichiasis was the constant feature in families. They reported one family in which only lymphoedema and not distichiasis was found. This led the authors[12] to implicate *FOXC2* mutations in a large number

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of lymphoedema syndromes and to emphasise the heterogeneity of previously separated lymphoedema syndromes. However, this was one small family of the 11 studied by Finegold *et al*,[12] and of the 33 total from the combined studies and is, thus, not typical. This discrepancy may represent the method of ascertainment in that our families were ascertained for lymphoedema and distichiasis, while lymphoedema alone was the main criterion in the study by Finegold *et al*.[12]

The *FOXC2* mutation phenotypes can be interpreted in light of expression studies of *Foxc2* (*Mfh*-1) in embryonic mice.[14–16] *Foxc2* was found to be highly expressed first in non-notochordal mesoderm and later in areas of mesenchymal condensation in the head, trunk, and limbs in developing embryos. *Foxc2* transcripts were detected in somites, mesoderm, head, and endocardium at day 8.5.[16] At embryonic day 10.5, expression of *Foxc2* was detected in cartilaginous tissue, metanephros, arch arteries, and dorsal aorta.[15 16] In addition, expression in the head region was particularly strong in cartilaginous condensation around the optic vesicle and the skull underlying the midbrain.[15] This mesenchyme gives rise to palatal processes and the outer optic vesicle. The distal region of the mandibular component of the first branchial arch also showed strong expression of *Foxc2* at this embryonic time point.[15] During development, *Foxc2* expression becomes stronger and is restricted to cartilaginous tissues, kidney, and the dorsal aorta.[14 15]

The role of *Foxc2* in development was further explored by Lida *et al*[15] and Winnier *et al*[17] in knockouts using embryonic stem cell technology. Some *Foxc2*$^{-/-}$ embryos die in utero and others at birth with heart defects.[15 17] Almost all of the *Foxc2*$^{-/-}$ embryos had interruptions, coarctation, or tubular hypoplasia of the aortic arch, and all newborns had complete cleft secondary palate. There were abnormalities of the skull, including abnormal formation of the optic canal, absence of the posterior wall of the foramen ovale, and fusion of the malleus and incus, primarily defects of neural crest origin.[15] Axial skeletal anomalies with short vertebral bodies, spina bifida, and spina bifida occulta were also found. These defects in homozygous deficient mice affect structures that are variably abnormal in heterozygous humans, including the heart, palate, and vertebra, and include a number of additional features.

Foxc2 heterozygous mice were found to be either normal[15] or to exhibit segmental eye defects similar to those in Axenfeld-Rieger anomaly.[18] Such ocular defects have not yet been noted in lymphoedema-distichiasis patients. The photophobia reported by some patients could be reflective of diminished iris development, or it could stem from corneal abrasions caused by distichiasis. Lymphoedema has not been described in heterozygous mice, nor has it been carefully studied.

The finding that presumed 50% levels of *FOXC2* frequently leads to childhood onset oedema extends our knowledge of factors involved in lymphangiogenesis. In contrast to "blood vasculogenesis" and "haemangiogenesis", the molecular and cellular control of lymphatic vasculogenesis and "lymphangiogenesis" remain poorly understood. Beyond the important VEGF-C,[19–22] a key "lymphatic growth factor", a sequential cascade of growth factors, such as laid out for the blood vascular system, has not yet been postulated.[19] Support for the theory that VEGF-C exerts the same driving force for lymphatic development as VEGF-1 for blood vessels arises from a transgenic model overexpressing VEGF-C in keratinocytes,[23] which displays lymphatic, but not blood, vascular dilatation and lymphatic endothelial proliferation in the skin. Recent investigations have shown a related result for VEGF-D.[24] In addition, expression of soluble *VEGFR-3* in keratinocytes, one of two receptors for VEGF-C and VEGF-D, inhibits lymphangiogenesis in surrounding tissues.[25] Mutations in *VEGFR-3* cause Milroy hereditary lymphoedema in some pedigrees.[1–3]

Additional genes that influence lymphatic development in the mouse are *Prox1* and *Ang2*. *Prox1* deficient mice fail to develop lymphatic vasculature, in addition to other abnormalities.[26] *Ang2* knockout mice display an absence or paucity of lymphatic vessels and nodes associated with a "peculiar lymph accumulation in the abdomen" observed in the newborn mice representing chylous ascites and a profound, yet survivable, lymphatic truncal maldevelopment/arrest.[27] These results point to a vital role for *Ang2* in the formation of the vast and pervasive network of lymphatic channels and regional lymph nodes and reminiscent of an array of human lymphoedema-angiodysplasia syndromes.[28] Our previous results[4] and those reported herein strongly implicate *FOXC2* as another key gene involved in lymphangiogenesis.

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