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LYMPHEDEMA-DISTICHIASIS SYNDROME WITHOUT *FOXC2* MUTATION: EVIDENCE FOR CHROMOSOME 16 DUPLICATION UPSTREAM OF *FOXC2*

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ABSTRACT

A patient with the classical phenotype of Lymphedema-Distichiasis syndrome (OMIM 153400) is described who showed no mutations in the sequence of FOXC2. Accordingly, a Gene Chip 250k array analysis was undertaken with dense SNP genotyping of the genomic region surrounding the FOXC2 locus on Chromosome 16 followed by copy number evaluation by real time PCR. The latter assay showed evidence of a duplicated region 5' of FOXC2 that could be causative for the patient's striking phenotype, which included both distichiasis and a hyperplastic refluxing lymphatic vascular and lymph node phenotype associated with pubertal onset lymphedema, scoliosis and strabismus.

Keywords: lymphedema, distichiasis, *Foxc2*, chromosome copy number variation, chromosome 16 duplication, *FOXC2*

In 2000, the *FOXC2* gene on Chromosome 16 was shown to be associated with Lymphedema-Distichiasis (LD) syndrome (OMIM 153400) (1,2). The clue to the specific locus rested on a rearrangement found in a male infant with lymphedema that was near this locus. Since that time, many reports have confirmed a variety of *FOXC2* mutations in patients with LD (3-7). The spectrum of clinical phenotypes has also been expanded to include superficial varicose veins and deep venous insufficiency and some patients with lymphedema without documented distichiasis (3-10).

Interestingly, we have shown that *Foxc2* haploinsufficient mice also exhibit distichiasis and other ocular abnormalities in addition to a hyperplastic lymphatic system (both increased lymphatic vessels and lymph nodes) with lymph reflux from valve incompetence (11). This distinctive lymphatic phenotype is strikingly similar to human LD as originally described on oily contrast lymphograms by Kinmonth in 1972 (12) and Dale (13). Although not originally recognized to have an LD phenotype, *Foxc2* knockout mice also typically exhibit the remaining features of the full LD clinical spectrum including cardiovascular abnormalities consisting of aortic arch and interventricular septal defect resembling Tetralogy of Fallot, ocular anomalies other than distichiasis, spinal and other bony abnormalities as well as cleft palate, and rarely survive embryonic life (14); defects in lymphatic valve formation have also been described (15). On the other hand, *Foxc2* overexpressing mice (using an

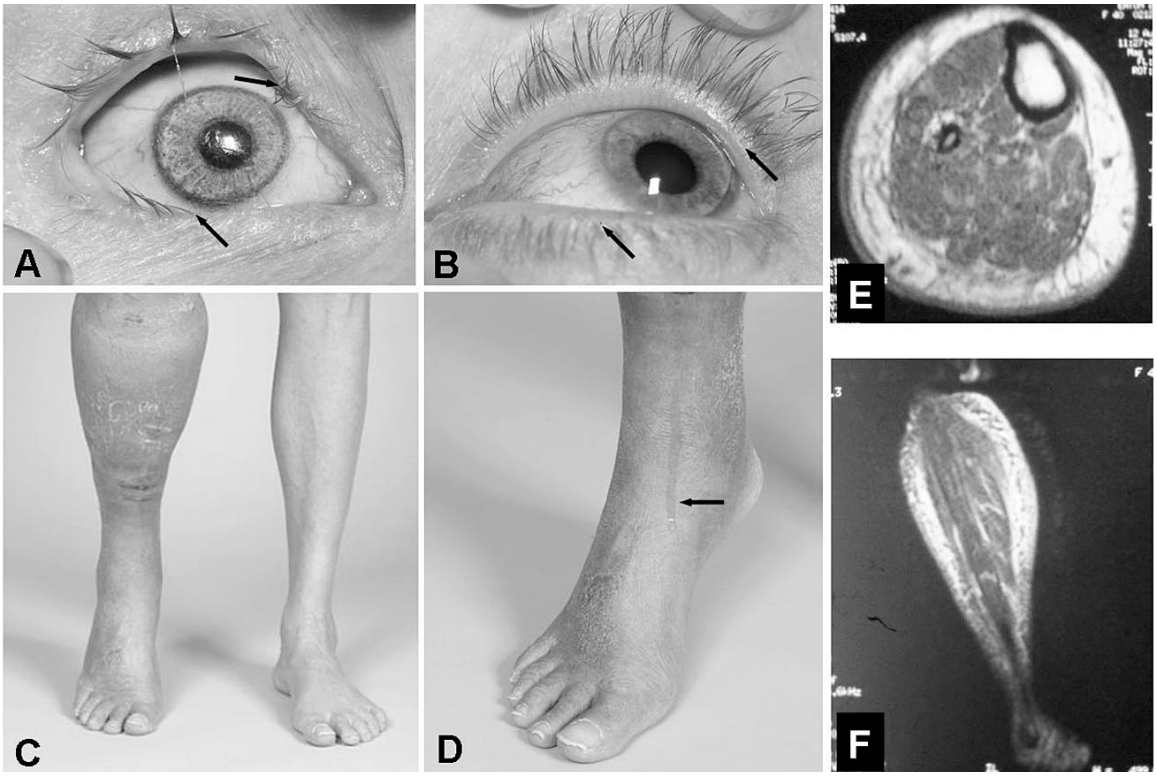

Fig. 1. (A,B) Bilateral distichiasis. Arrows point to extra eyelashes inside the gray zone. (C) Brawny right leg lymphedema below the knee. The left leg is edema free. Higher magnification displays clear yellow fluid (arrow) weeping from the pre-tibial area (lymphorrhea) (D). Magnetic Resonance Imaging of the lower right leg (E, transverse; F, coronal) demonstrates prominent edema in subcutaneous tissue space with characteristic honeycomb pattern and absence of edema in the muscular compartment.

adipocyte aP2 promoter) not only exhibit abnormalities in brown-white fat distribution with a lean phenotype, but also display a hyperplastic lymphatic system phenotype (16,17). This distinctive finding without eye abnormalities supports a Foxc2 dose/imbalance effect at least regarding the lymphatic abnormalities, a suggestion also made regarding human gain-of-function *FOXC2* mutations outside the forkhead domain (7).

Previously, only one other report has documented the existence of LD patients without mutations in the coding region of *FOXC2* (8), and no other associated gene mutations or chromosomal rearrangements have yet been identified in LD patients or families. This is in contrast to multiple chromosomal copy number variants near *FOXC1* on 6p associated with Axenfeld-Rieger syndrome (18-23). Here we report on a patient with a striking LD phenotype without mutations in the coding region of *FOXC2* in whom we found evidence of a chromosome 16 duplication upstream of *FOXC2*.

CASE REPORT

A 41 year old white woman, who was adopted and knew only sketchy details of her birth family history, was first seen at the age of 21 in 1982 at University of Arizona Medical Center (UMC) for corneal abrasions and infections of her right eye associated with distichiasis and also swelling of her right

lower leg since age 17. She gave a history of amblyopia and strabismus as a child, which was surgically corrected at age 9, and also of lower thoracic dextroscoliosis. Bipedal contrast lymphography at that time revealed a hyperplastic lymphatic system with areas of increased number of lymphatic vessels and lymph nodes in the peripheral and central lymphatic system and was interpreted as a "congenital hyperplastic lymphedema" according to the Kinmonth criteria (11). Contrast venogram and repeated Doppler ultrasound venograms of the lower extremities were both normal. The corneal infections and abrasions became so severe that enucleation of the right eye was carried out in 1998 with placement of a glass eye prosthesis. When she returned to UMC in 2002, the lymphedema and repeated infections in the right leg had progressively worsened, particularly after a right knee injury 6 years earlier. Severe, intractable right leg pain and tenderness had led to serious disability as well as difficulty with physical treatment of the lymphedema. On physical examination, the right glass eye was noted along with bilateral distichiasis (*Fig. 1A*), scoliosis, and a brawny erythematous swollen right leg below the knee draining clear yellow fluid from the pre-tibial area (*Fig. 1B*). The left leg and both arms were normal in appearance without swelling. MRI showed edema only in the subcutaneous compartment of the right lower leg with the subfascial and muscular compartments free of edema (*Fig. 1C*), a characteristic picture of lymphatic rather than venous edema. Doppler ultrasound venogram of the lower extremities was again noted to be normal without evidence of valvular incompetence or deep venous thrombosis. Four limb whole body ^{99m}Tc sulfur colloid lymphangioscintigraphy (*Fig. 2*) showed hyperplastic lymphatic vessels and lymph nodes with tracer reflux in both the right and, to a lesser extent, also the non-swollen left extremity as well as in the upper limbs. Efforts to treat the lymphedema with combined physical therapy including compression bandaging, manual lymph drainage, and compression garments along with antibiotics were ineffective in part related to patient non-compliance from persistent severe leg pain and tenderness. She did not contact our office again until 2003 when she informed us that an above-the-knee amputation of the right leg was planned that week at another hospital. At the time of surgery, Evans blue dye was injected intradermally in the foot of the amputated right limb and showed disorganized lymphatics with multiple areas of splotchy blue dye deposit in the subcutaneous tissue throughout the lower leg but without clear lymphatic streamers. Histopathology (*Fig. 3*) of the skin showed acute and chronic inflammation with an area of ulceration and granulation tissue formation in the pretibial area. Away from the ulcer, prominent dilated lymphatics were noted to be irregularly distributed and unassociated with smooth muscle actin. Some of the lymphatics appeared increased in complexity with multiple intraluminal extensions containing proliferating endothelial cells and resembling valves arising from the endothelial lining. Immunohistochemistry of a popliteal node from the amputated limb showed further details of these supernumerary lymphatic valves and complex endothelial walls of dilated D2-40 and CD31 positive lymphatics. Post-operatively, after fitting with a right limb above-the-knee prosthesis, her quality of life was greatly improved. Subsequently, she called to inform us that her left leg had become swollen and painful. A left leg Doppler venous ultrasound at that time again showed no evidence of venous reflux or deep vein thrombosis but did visualize a 3.1 cm lymph node with a fatty hilus in the left proximal thigh. MRI of the lower left leg showed subcutaneous edema characteristic of lymphedema and cystic bone infarcts in the femoral and tibial condyles and calcaneum.

Genetic Studies

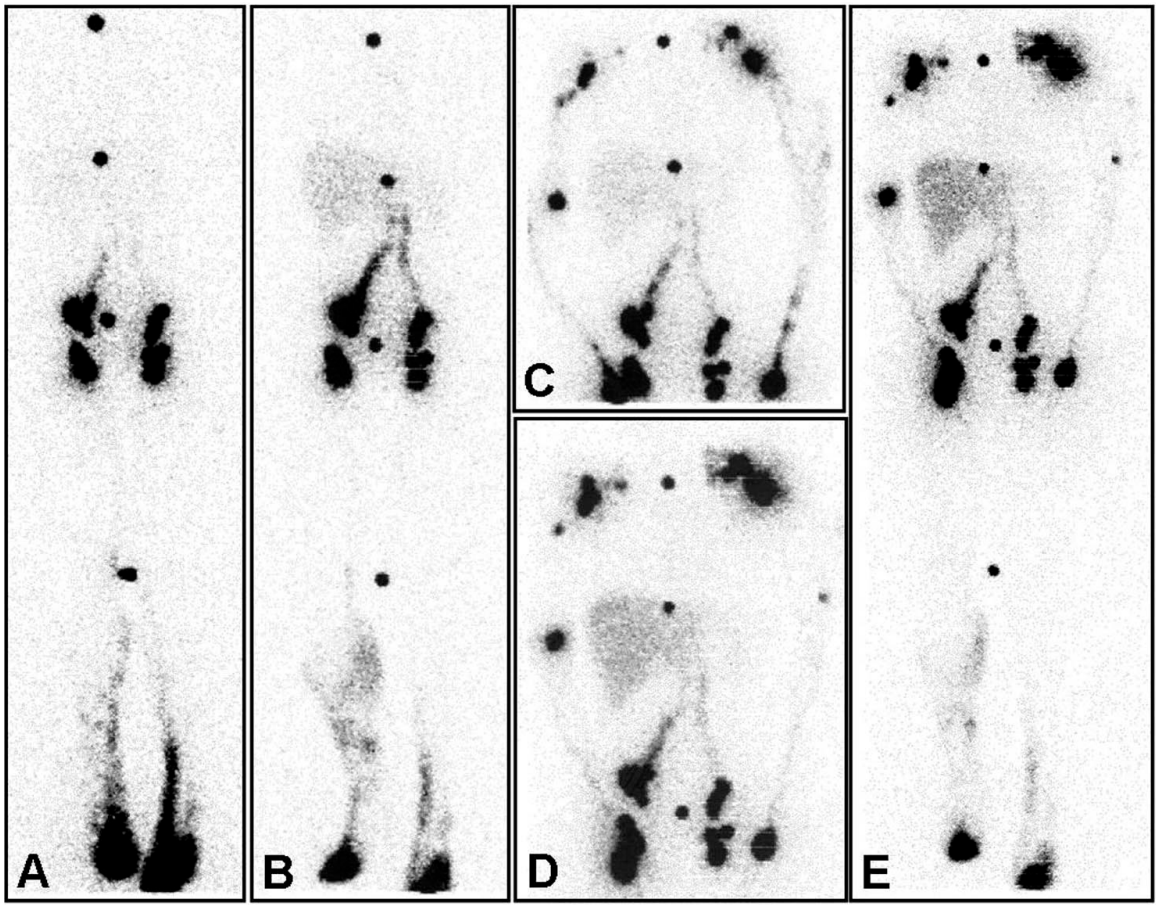

Fig. 2. ^{99m}Tc sulfur colloid lymphangioscintigrams. Early (A, 1 hr) and late (B) images of the lower limbs display areas of increased lymphatics on the right lower leg and an increased number of small discrete lymph nodes (bunches of grapes appearance) in inguinal/pelvic region. Early (C, 20 min) and late (D, 2.5 hr) images of the upper limbs displays areas of increased lymphatics on the left arm and an increased number of small discrete lymph nodes in the supraclavicular/ epitrochlear area. (Hyperplastic lower extremity and pelvic findings (both lymphatics and lymph nodes) were also described in the patient's 1982 direct contrast lymphogram report 20 years earlier). A later combined image (E, 2.5 hr arms, 8 hr legs) demonstrates dermal diffusion from reflux into superficial lymphatics in the right lower leg which was also noted in the edema-free lower left leg and right elbow area. Edema appeared in the left leg for the first time several years later. Central dots are knee/pelvis/pubic/xiphoid/and sternal notch markers.

After obtaining informed consent under an approved Institutional Review Board protocol, genetic studies were undertaken to determine whether *FOXC2* had mutations and/or surrounding genomic regions were deleted or rearranged.

Sequencing of *FOXC2* from -100 to +95, including all of the coding exon, disclosed no mutations, both in DNA from blood and from the affected leg [since somatic mutations often occur (24)]. We then used the GeneChip 250K Nsp array (Affymetrix, Inc, Santa Clara, CA) to look for regions of homozygosity in or near *FOXC2*. Dense single nucleotide polymorphism (SNP) genotyping of the genomic region, including and surrounding the *FOXC2* locus on chromosome 16, identified a region of homozygosity for twenty-four SNPs. These spanned from bp 85,041,967 (rs9924288) to

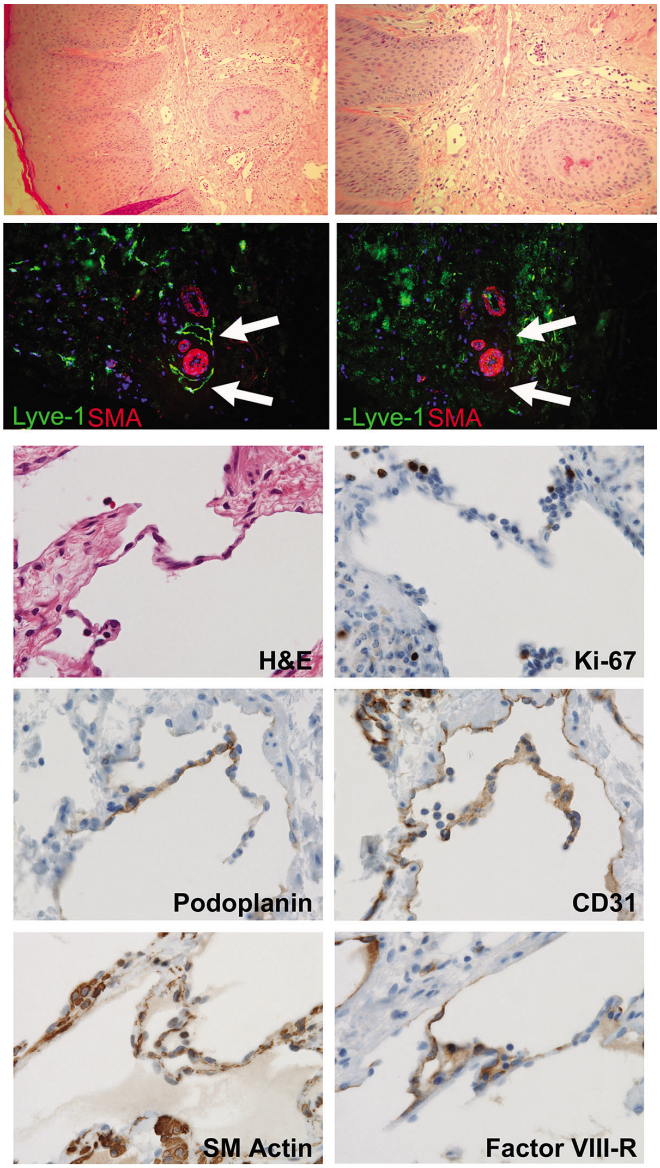

Fig. 3. Histopathology and immunohistochemistry (IHC) from above-knee amputated right leg. Skin section (Top row): H&E shows edema, stratum corneum thickening, collagenosis, acute and chronic inflammation, and increased number of dilated lymphatics (left) with prominent intraluminal endothelial-lined projections (right) (Original magnifications 10X and 20X respectively). IHC (2nd row) with alpha smooth muscle actin (SMA) (red) with and without Lyve-1 (green) demonstrates lack of colocalization in the subcutaneous Lyve-1 positive lymphatics of the right lower leg. SMA is seen lining the Lyve-1 negative blood vessel wall. (Original magnification 20X) Right popliteal lymph node (3rd, 4th and bottom row): H&E and IHC with Ki-67, podoplanin, CD-31, SMA and Factor VIII-related antigen (R) demonstrates an increased number of abnormal and dilated lymphatics along with chronic and acute inflammation. A remarkable increase in endothelial-lined, aberrant (ectatic and cellular) valves (illustrated here by 3 separate valves within a single lymphatic channel in serial sections) is also noted within the lymphatics (podoplanin, Factor VIII R, CD-31 positive; CD-34 negative) and less prominently in venous (Factor VIII-R, CD-31 positive; podoplanin negative) vessels, and these are proliferative (Ki-67positive) as well as invested with SMA, which is also associated with the intraluminal valve structures. (Original magnification 40X)

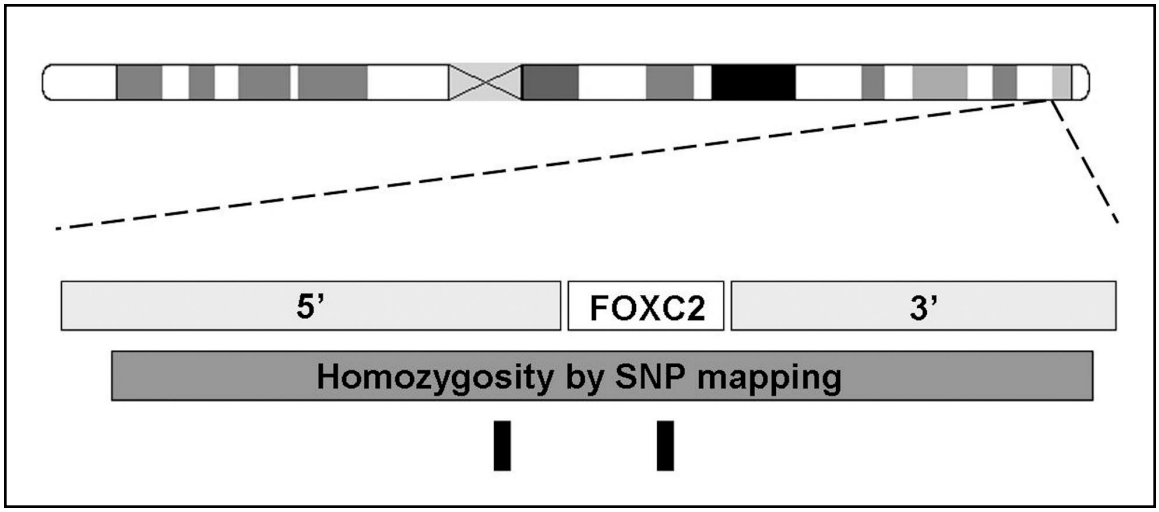

Fig. 4. Diagram of regions used for analysis. (Top) ideogram of chromosome 16 displaying location of 16q24.1 region. The FOXC2 *gene (85,158,199 to 85,160,633) is shown flanked by 5' and 3' regions (light gray boxes). The region of homozygosity determined by SNP mapping (85,041,967 to 85,177,692) is shown (dark gray box) as well as the locations for RT-PCR (black bars at 85,147,842 to 85,148,093 and 85,159,759 to 85,159,898).*

TABLE 1
Primers for Real Time PCR in or near FOXC2 and Control

	Primer	Sequence	Location	Tm	%GC
In *FOXC2*	forward	gacccaaccagacaattaag	1560-1579 mRNA	60	53%
	reverse	gaattaaagttacctgcgct	1699-1680 mRNA	60	
5' upstream of *FOXC2*	forward	tggaagtgtgtgcaggattc	85,147,842-23, ch 16	60	52%
	reverse	tcaagtgatcctctgcctca	85,148,074-93, ch 16	60	
APOB-100	forward	acccctgcttttgtttgatg	76512-76493 gene	58	38%
	reverse	gaccagcaacagatcccatt	76406-76425 gene	60	

bp 85,177,692 (rs7189414) on chromosome 16. *FOXC2* is located from bp 85,158,199 to bp 85,160,633 *(Fig. 4)*. However, this analysis cannot distinguish between homozygosity and hemizygosity with a deletion of one chromosome, and runs of homozygosity measuring up to 4 Mb are common in demonstrably outbred individuals (25).

In order to quantitate the copy number of *FOXC2*, we used real time PCR. A number of different primers were designed but the ones successfully used are shown in *Table 1*. Copy number was analyzed using the $2^{\Delta\Delta CT}$ (delta delta CT) method of comparing fold difference to the endogenous control (26). The ratio for copy number (the measured fold difference between *FOXC2* and the control *APOB* gene) was one, i.e., two copies, in our patient and an unrelated control (*Table 2*). However, when the region

TABLE 2
Fold Differences* In FOXC2 and 5' Upstream Sequence

	FOXC2	*5' of FOXC2*
Patient	1.01	1.57, 1.65
Unrelated Control	1.00	1.00, 1.00

*Ratio of experimental to control (APOB)

18,000 bp 5' of the gene (around rs7189970 on chromosome 16 at 85,147,931) was quantitated, an approximate 1.5 fold increase from our patient but not the unrelated control, was found (*Table 2*). This unexpected result was replicated.

DISCUSSION

In LD families with *FOXC2* mutations as well as in *Foxc2* deficient mice, the close association of *FOXC2* deficiency with the defining and fully expressed LD phenotype comprises strong evidence for a specific cause-effect connection. Our patient, however, exhibited the classical feature of LD syndrome – including both distichiasis and hyperplastic refluxing pubertal onset lymphedema as well as scoliosis and strabismus – but without documented LD family history and without missense or nonsense mutations in *FOXC2*. Interestingly, she did not show superficial venous varicosities or venous valvular incompetence, reported as a typical finding in LD patients (9,10), and she exhibited a strikingly increased number of lymphatic valve structures where these are usually difficult to find in normal subjects on routine tissue cross-sections. Lymphatic vessels and lymph nodes were increased in number (on lymphogram, lymphangioscintigram, and histopathologic examination), and lymphatic endothelial mitotic rate (Ki67) was high. Contrary to findings by Petrova et al (17), smooth muscle actin was not detected in lymphatic capillary walls (*Fig. 3*) but did invest larger lymphatic collecting channels.

The mechanism underlying the distinctive hyperplastic lymphatic system phenotype and a connection to the supernumerary eyelashes remain unclear. Of interest, throughout the animal kingdom from yeast through *Drosophila* to mammals, the forkhead transcription factor family is known to exert control over cellular proliferation rate and cell and organ size, including in ocular development (28,29). Distichiasis and the spectrum of ocular growth anomalies seen in human LD and mimicked in *Foxc2* deficient mice as well as the hyperplastic lymphatic system may reflect a disturbance in regulation of cell proliferation and fate. Absence or deficiency of valves have been described in *Foxc2* knockout mice (17), although not clearly demonstrated or differentiated from valve incompetence in *Foxc2* haploinsufficient mice or in LD patients. On the other hand, the apparently excessive number of lymphatic valves described in our patient would support the notion of *Foxc2/FOXC2's* influence on proliferation rate and endpoints and provide a common mechanism for the ocular and lymphatic abnormalities.

Dosage alterations near genes frequently alter their expression, e.g., *FOXC1* (18-23). The long stretch of homozygosity in and around *FOXC2* initially suggested the possibility of a hemizygous deletion in the gene. However, careful gene dosage studies

showed normal dosage in the gene but increased dosage in a region 5' to the gene. It is possible that a chromosomal duplication/ deletion event with the deletion being 3' to *FOXC2* occurred. It is also possible that this is a previously uncharacterized long stretch of homozygosity, which are frequently encountered in the human genome (25). The evidence for a chromosome 16 duplication upstream of the *FOXC2* gene locus in our patient is consistent with a direct role of *FOXC2* in LD and a dosage effect in the pleiotrophic pathway leading to the characteristic phenotype.

Nonetheless, there is a discrepancy between the finding of a run of homozygosity in and around *FOXC2* and the finding of normal copy number of the gene and an apparent copy number increase (equivalent to 3 copies of the upstream sequence) approximately 18 kb upstream. At issue is that the increased dosage is within the region of homozygosity. It is possible that a complex duplication causing a copy number increase followed by a partial deletion of one copy of the duplicated region has occurred. It is of interest that, as discussed above, both increased and decreased dosage of *Foxc2* in mice cause an abnormal hyperplastic lymphatic vessel and lymph node phenotype (11,16). Moreover, gain-of-function *FOXC2* mutations outside the forkhead domain may increase *FOXC2* transcriptional activity yet cause a clinical lymphedema phenotype with or without associated distichiasis (7). Nonetheless, the basis for this patient's clear-cut LD phenotype is not completely conclusive and will require further investigation and/or evaluation in multiple individuals containing the demonstrated or similar alterations.

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