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A NOVEL MISSENSE MUTATION AND TWO MICROREARRANGEMENTS IN THE *FOXC2* GENE OF THREE FAMILIES WITH LYMPHEDEMA-DISTICHIASIS SYNDROME

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ABSTRACT

Lymphedema-distichiasis (LD) syndrome is a rare autosomal dominant disorder of the FOXC2 gene, which codes for a forkhead transcription factor. Most of the mutations described in this gene to date are deletions or insertions, suggesting a mechanism of haploinsufficiency. We studied three independent families with LD presenting with both lymphedema and distichiasis. Two microrearrangements (one 8-bp deletion and one 7-bp duplication) occurring in a GC-rich genomic region (c.893-930) known to be prone to mutations were identified. A new missense mutation (p.Lys132Glu) located in a highly conserved sequence, the forkhead domain, was also identified. Mutations in this domain have been previously shown to impair FOXC2 transactivation ability. At a genetic level, this study confirms the heterogeneity of mutations responsible for LD and is consistent with a mechanism of haploinsufficiency. At a clinical level, it reinforces the importance of genetic testing in subjects with familial lymphedema or distichiasis, since measures can be taken at an early stage to prevent complications and to reduce the progression of lymphedema or delay its occurrence.

Keywords: lymphedema-distichiasis syndrome, *FOXC2* gene, familial, mutation, haploinsufficiency

Lymphedema-distichiasis syndrome (LD; OMIM 153400) is a rare, highly penetrant, autosomal dominant disorder caused by mutations in the *Forkhead Box C2* (*FOXC2*) gene (1,2). Distichiasis, or aberrant eyelashes arising from the meibomian glands, is observed at birth, whereas lymphedema occurs later at puberty and typically involves the limbs (3). Other clinical abnormalities are often associated including congenital heart malformations (tetralogy of Fallot, ventricular septal defect), ptosis, neck webbing, varicose veins, cleft palate and spinal extradural cysts.

FOXC2 or *MFH-1* (cDNA *GenBank* accession number: NM_005251.1) is a gene for regulatory transcription factor, localized on chromosome 16q24.3. *FOXC2* contains a highly conserved DNA-binding motif termed the forkhead domain (FHD) and the flanking terminal regions of this gene are essential for transcriptional activation (4). *FOXC2* is involved in embryogenesis, particularly in lymphatic and blood vascular development (5), but its target genes have not been identified.

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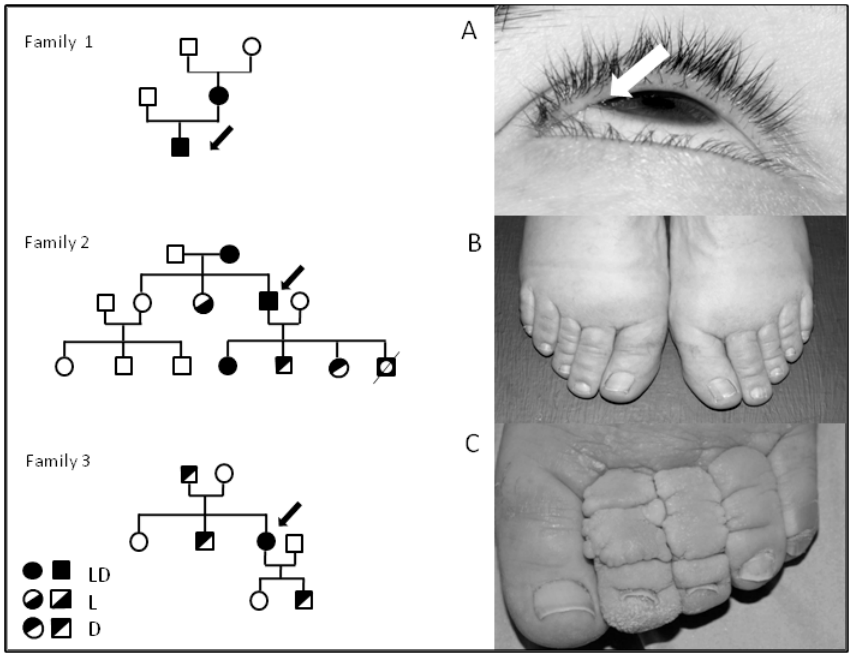

Fig. 1. Distichiasis, lymphedema, papillomatosis and genealogical trees of the three families studied. A. Distichiasis observed in the index case of family 1. B. Lymphedema of the feet observed in the index case of family 2. C. Papillomatosis of the toes observed in the index case of family 3.

This study was carried out in three unrelated families with LD, presenting with both lymphedema and distichiasis, to find and identify mutations in the *FOXC2* gene.

PATIENTS AND METHODS

Patients

Three Caucasian families in which LD was diagnosed on the basis of lymphedema and distichiasis in the index case were studied. These families were selected if at least one first-degree relative presented lymphedema and/or distichiasis. All subjects gave their informed consent for genetic testing.

Genetic analysis

Peripheral blood samples were drawn in EDTA tubes. Genomic DNA was collected after digestion by proteinase K and saline extraction.

The *FOXC2* gene (the single exon and 500-bp upstream and downstream) was amplified using eight pairs of primers. We added DMSO and/or betaine for GC-rich regions amplification. Genetic testing was performed by direct sequencing using sequencing agents and kits from Applied Biosystems and run on a 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA). DNA sequences were analyzed using Sequencher software (Gene Codes Corporation). Mutations were detected with both sense and reverse primers. Independent direct sequencing was carried out as a control.

RESULTS

Family 1 consisted of a 9-year-old boy and his mother who both presented with distichiasis (*Fig. 1A*) and unilateral lower

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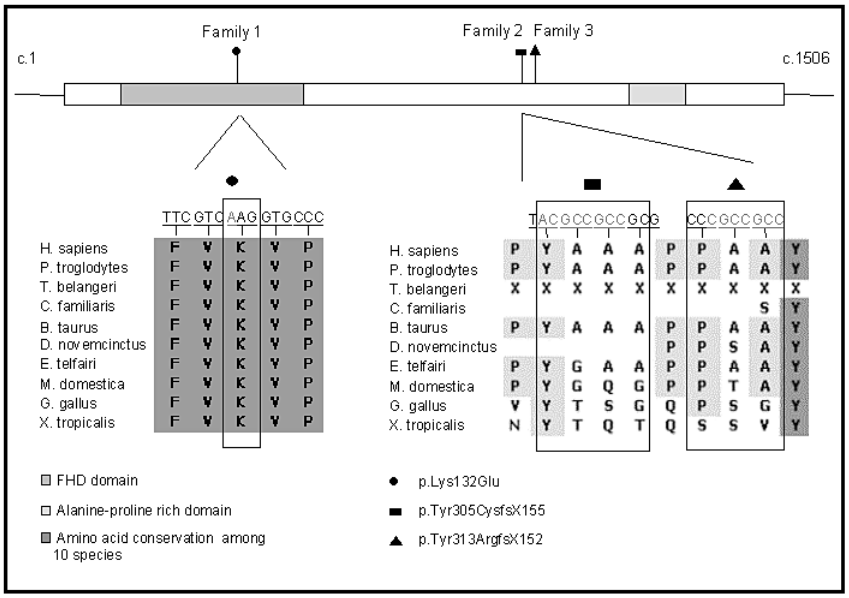

Fig. 2. Location of detected mutations in FOXC2 and its protein in the three families (top). Alignment of the forkhead domain of humans and nine other species (bottom). Family 1: c.394A>G, p.Lys132Glu; Family 2: c.914_921delACGCCGCC, p.Tyr305CysfsX155; Family 3: c.930_936dupCGCCGCC, p.Tyr313ArgfsX152

limb lymphedema. The presence of severe venous insufficiency, recurrent erysipelas and venous ulcers in the maternal grandfather suggested vertical transmission. In family 2, the proband was a 40-year-old male who presented with distichiasis and bilateral lymphedema (*Fig. 1B*) of the feet like his mother and one of his daughters. The two other children had only distichiasis. One of his sisters had bilateral lymphedema, while the second sister and her children did not have LD. In family 3, the proband was a 61-year-old female who had distichiasis, neck webbing and bilateral lymphedema with toe papillomatosis (*Fig. 1C*). Her father, brother and son also had distichiasis and significant neck webbing.

Three different mutations were observed in the three families (*Fig. 2*). Family 1 had a heterozygous missense mutation c.394 A>G, p.Lys132Glu (K132E), which has not been described previously. Families 2 and 3 had a heterozygous frameshift mutation that introduced a premature termination codon in the gene: an 8-nucleotide deletion c.914_921del, p.Tyr305CysfsX155 previously described by Bell et al (6), and a 7-nucleotide duplication c.930_936dup, p.Tyr313ArgfsX152 reported by Finegold et al (7).

DISCUSSION

This report describes three different *FOXC2* mutations in subjects with typical LD, confirming the heterogeneity of *FOXC2* mutations and the role of this gene in the development of the lymphatic system (5,8). Approximately 55 *FOXC2* mutations are listed in the HGMD Database (www.hgmd.cf.ac.uk). Unlike mutations in other *FOX* genes, the majority of these mutations are deletions or insertions, with only a minority of missense mutations (20%). To our knowledge, the missense mutation K132E is reported for the first time. Its segregation with the disease, conservation of the lysine residue at position 132 (*Fig. 2*), and change of charge from a basic amino acid

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(lysine) to an acidic amino acid (glutamate) are arguments for the functional significance of this mutation. In addition, lysine 132 is located in the FHD ß1 strand, a domain where a conserved and intact structure is required to preserve DNA binding and transcription of target genes (7,9). It is likely that the missense mutation K132E modifies the FHD conformation and impairs FOXC2 protein-DNA binding or FOXC2 transactivation ability, leading to LD. The two other mutations were frameshift mutations caused by either deletion or duplication of a short stretch of DNA sequence. Both are located behind the DNA-binding domain and lead to a truncated protein (6,7). As described by Finegold et al (7), these mutations lead to the elimination of key α-helical domains required for *FOXC2*-transcription complex interaction.

A relatively large variety of mutations has been described in the *FOXC2* gene (2). This variety is favored by the presence of GC-rich regions, which predispose to a high deletion frequency, especially at the mutation hotspot containing an incomplete GCCGCCGC repeat from nucleotides 893 to 930 (10,11). Despite this heterogeneity at the DNA level, it is likely that the different mutations act in the same manner through a mechanism of haploinsufficiency. This is usually the case for frameshift mutations that are predicted to truncate and thus inactivate one allele of *FOXC2* (1,7,9). Haploinsufficiency rather than a dominant negative effect is also probably involved with our missense K132E mutation. Indeed, Berry et al (9) showed that missense mutations in the FHD lead to mutated proteins that are expressed at similar levels to wild-type FOXC2 but that lack any functional activity. Recently, lymphedema-distichiasis syndrome was reported without a detectable *FOXC2* mutation (12).

The phenotypic heterogeneity of LD is reflected by the presence of mutations in patients presenting with either lymphedema or distichiasis (7,13). From a clinical point of view, an early diagnosis of LD is extremely important in families with this disease. Like other authors (7), we propose that analysis of the *FOXC2* gene should always be performed in familial lymphedema or distichiasis. Even if the lymphedema is not life-threatening, it is nevertheless a chronic condition which frequently causes major physical and aesthetic discomfort and predisposes sufferers to recurrent infectious complications (14). Severe lymphedema affects the quality of life, social relationships and body image, and can be detrimental to a patient's professional career. Thus, early genetic counseling is imperative. Physical examination and paraclinical tests will allow the detection of any possible associated cardiovascular abnormalities. Early treatment of distichiasis (electrotherapy, surgery, or epilation) prevents ophthalmic complications that can threaten visual function (15), and early initiation of compression therapy and education on the prevention of cellulitis are required to avoid the progression of lymphedema.

As a conclusion, we report here a new *FOXC2* missense mutation and confirm the heterogeneity of mutations in LD which seem to lead to disease by the same haploinsufficiency mechanism. Molecular analysis is required for adequate genetic counseling, and an early diagnosis with intervention should minimize LD complications for the patient.

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