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## Lymphoedema-distichiasis and *FOXC2*: unreported mutations, de novo mutation estimate, families without coding mutations

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**Abstract** Lymphoedema-distichiasis (LD) is a syndromic form of primary lymphoedema, where mutations in the gene for the developmental transcription factor *FOXC2* have been shown to be causative. The disorder has been considered very rare, but our group has now ascertained 34 families and 11 sporadic cases in the UK. Two families with LD have no mutation in the coding region of *FOXC2*, although both are consistent with linkage to the *FOXC2* locus. A deletion has been ruled out as a possible cause of LD in these families, leaving promoter mutations as the most likely cause. Sixteen previously unpublished mutations are reported, plus an estimate of the frequency of new mutations in this disorder.

### Introduction

Primary lymphoedema shows an autosomal dominant pattern of inheritance with varying degrees of penetrance, and is characterised by chronic swelling of the limbs, most often of the legs (Mortimer 1995). There are three major forms of dominantly inherited primary lymphoedema: Milroy disease (MD) [MIM 153200], lymphoedema-distichiasis (LD) [MIM 153400] and Meige disease (MGD) [MIM 153200]. Of these, the causative genes are identified as *VEGFR3* for at least a subset of MD patients (Ferrell et al. 1998, Irrthum et al. 2000), *FOXC2* for LD (Mangion et al. 1999; Fang et al. 2000; Bell et al. 2001), but as yet no gene locus has been identified for MGD.

Patients with LD usually show signs of lymphoedema (late childhood or later onset) associated with distichiasis, which is the development of aberrant eyelashes arising from the meibomian glands of the eyelid (Dale et al. 1985; Robinow et al. 1970). There has been a report that some families with MGD disease have mutations in *FOXC2* (Finogold et al. 2001). Our experience is that this is not the case, and that stringent phenotyping will show some of the syndromic features of LD in at least some family members. It is possible that in isolated cases lymphoedema could be the only manifestation of LD, as the expression of the clinical signs is highly variable. Any case where a mutation is found in *FOXC2* should, in our opinion, be classed as LD, not MGD, lymphoedema with ptosis, or any other form of lymphoedema.

We have previously reported 18 families with LD, and six isolated cases. All but one family had a mutation in *FOXC2* (Bell et al. 2001; Brice et al. 2002). This family was consistent with linkage to a marker near the LD locus (D16S520), but was not tested at that time with markers in the *FOXC2* gene itself. In this paper we show that markers 5' and 3' to *FOXC2* support the linkage for the previously described family with no mutation, report 16 novel mutations in *FOXC2* (including two missense

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changes), another family with no coding *FOXC2* mutation, and provide an estimate of new mutations.

## Materials and methods

### DNA extraction

DNA was extracted from venous blood using the Nucleon extraction and purification kit (Amersham Life Sciences). The protocol supplied by the manufacturer was followed.

### Mutation analysis

The *FOXC2* gene was screened for mutations using sequence analysis. DNA sequencing was carried out on an ABI 3100 DNA analyser, using Bigdye chemistry (Applied Biosystems, Foster City, Calif., USA). The *FOXC2* gene is composed of a 1.5-kb single exon, which was divided into four overlapping regions that were amplified and sequenced. This enabled the entire coding region to be analysed for mutations. In cases where no mutations were identified in the coding region of *FOXC2*, the 5' and 3' regions of the *FOXC2* gene were also sequenced and analysed. The primers and their conditions are listed in Table 1. Those for the coding region of *FOXC2* have been previously described (Bell et al. 2001), as have those in the 5' region and 3' region (Kovacs et al. 2003). A second set of primers for the 3' region was designed for this study. Cycling involved an initial denaturation step of 95 °C for 5 min, followed by 35 cycles each of 95 °C for 1 min, various annealing temperatures (see Table 1) for 1 min and 72 °C for 1 min, and a final extension of 72 °C for 10 min.

### Haploid cell line

Haploid cell lines were made of chromosome 16, using circulating lymphocytes from an affected LD patient showing no *FOXC2* mutation (GMP Genetics, USA). DNA was provided by GMP Genetics from cell hybrids containing single copies of the human chromosome 16 s, which were confirmed by microsatellite analysis using D16S520, and the polymorphisms 5' and 3' to *FOXC2* used in the current study. The entire *FOXC2* gene of both haploid cell lines was amplified, including 639 bp upstream and 303 bp downstream of the coding region.

## Results

Eleven of the mutations are deletions, two are duplications, one is nonsense, and two are missense (Table 2). This brings to four the number of missense mutations reported by our group, S125L and R121H being those previously identified (Bell et al. 2001; Brice et al. 2002). We are not aware of any other missense mutations in *FOXC2* being reported as causative for LD, with those previously published being either frame shift or nonsense mutations (Fang et al. 2000; Finegold et al. 2001; Erickson et al. 2001; Bell et al. 2001; Brice et al. 2002; Traboulsi et al. 2002; Brooks et al. 2003; Yildirim-Toruner et al. 2004).

We previously reported a pedigree with LD but no *FOXC2* mutation (Brice et al. 2002), which showed linkage to the microsatellite D16S520, which is about 100 kb from the *FOXC2* gene. We have used polymorphisms in the *FOXC2* gene to confirm that this linkage applies to the gene itself (Fig. 1a, lodscore of one using the haplotype as a single marker). A second pedigree (Fig. 1b) has now been ascertained, where the clinical signs are completely consistent with LD, but there is no

**Table 1** Sequences of primers used to amplify the *FOXC2* gene and its upstream and downstream regions

Name of primer	Sequence (5'-3')	Annealing temp (°C)	Product size (bp)	DMSO %	MgCl <sub>2</sub> mM
FOXC2-1F <sup>a</sup>	TCTGGCTCTCTCGCGCTCT	58	476	6	1.5
FKHL14-2R	AGTAACTGCCCTTGCCGG				
FOXC2-2F <sup>a</sup>	ACCGCTTCCCCTTCTACCGG	60	519	10	1.5
FOXC2-2R	TCATGATGTTCTCCACGCTGAA				
FKHL14-4F <sup>a</sup>	GAAGGTGGTGATCAAGAGCG	60	496	6	1.5
FOXC2-3R	GAGGTTGAGAGCGCTCAGGG				
FOXC2-4F <sup>a</sup>	CTGGACGAGGCCCTCTCGGAC	61	464	10	1.5
FOXC2-4R	GGAGGTCCCGGGACACGTCA				
FOX_5P_1F <sup>b</sup>	GCCGACGGATTCTGCGCTC	61	378	10	1.5
FOX_5P_1R	CCGCTCCTCGCTGGCTCCA				
FOX_5P_2F <sup>b</sup>	CCGATTGCTGGGGGCTTGGAG	61	607	6	1.5
FOX_5P_2R	GCGGGCTGGTGGTGGTGGTAGG				
FOX_3P_1F <sup>b</sup>	CAACGTGCGGGAGATGTCAAC	61	464	10	1.5
FOX_3P_1R	CACAGCACAGCCCTCCTGGTAG				
FOX_3P_2F <sup>c</sup>	TACTGACGTGTCCCGGGACC	61	468	6	1.5
FOX_3P_2R	CCACACATTTGTACAGCACGGTTG				

<sup>a</sup>Primer pairs from Bell et al. (2001)

<sup>b</sup>Primer pairs from Kovacs et al. (2003)

<sup>c</sup>Primer set designed for this study

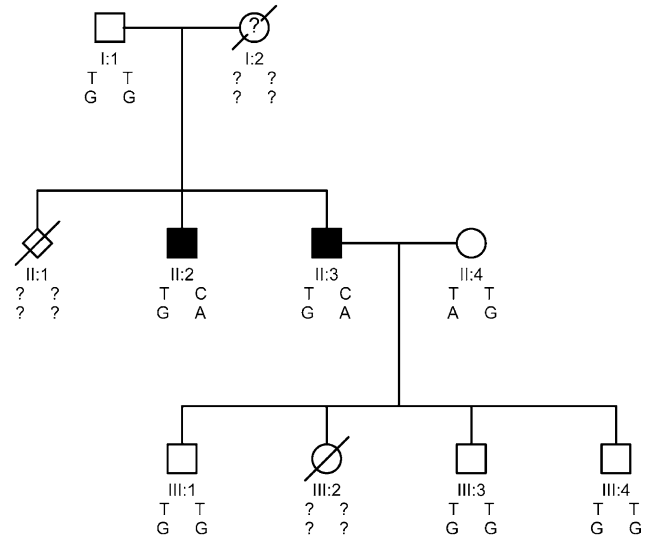
**Table 2** List of novel mutations and effect on protein (if produced)

Mutation	Effect
<i>Missense</i>	
346T > A	Trp116Arg
704G > T	Ser235Ile
<i>Nonsense</i>	
438G > A	Trp146STOP
<i>Frameshift</i>	
nt333delG	88 novel amino acids, then STOP
nt474 del C	42 novel amino acids, then STOP
nt747delC	23 novel amino acids, then STOP
nt854delG	2 novel amino acids, then STOP
nt902-920dupTggcgctgcctacgcgc	29 novel amino acids, then STOP
nt893-915 dup GCCGCCGCtggcgctgcctac	35 novel amino acids, then STOP
nt1024delG	27 novel amino acids, then STOP
nt1140delC	51 novel amino acids, then STOP
nt1229-1253del CGACCCGCAGC-CCGGGGCCGCCCGC	14 novel amino acids, then STOP
nt1298delA	38 novel amino acids, then STOP
nt1331delA	27 novel amino acids, then STOP
nt1418delG	29 novel amino acids, then STOP
nt1420-1426delAATGCCA	26 novel amino acids, then STOP

mutation in the coding region of *FOXC2*. Polymorphisms in *FOXC2* were used to investigate the possibility of a gene deletion in either family A or B. The 5' polymorphism (-512C > T) has previously been reported (Ridderstrale et al. 2002), while that in the 3' region (1761G > A) is newly identified in this study. This 1761G > A polymorphism has a frequency of 18.8% in our control population (Caucasians from the UK). The heterozygosity for both polymorphisms is shown in Fig. 1. The same polymorphisms have been utilised to show that family B is also consistent with linkage to *FOXC2*, and also to demonstrate that there is no large-scale deletion of the *FOXC2* gene in either family. To ensure that there was no small deletion between the polymorphisms, we had haploid cell lines made from lymphocytes from an affected individual in family A. The PCR of a 2,445-bp fragment using primers that were 5' and 3' to the two polymorphisms showed that the same sized fragment was produced for both chromosomes (data not shown). Amplification of DNA from an affected individual in each family with the same primers also produced only a single fragment. There were no deletions identified in either case. Sequencing of the 2,445-bp fragment in the haploid lines and the DNA from the individual from whom the lines originated showed that there were no small deletions outside the coding region, and that the -512 and 1761 polymor-

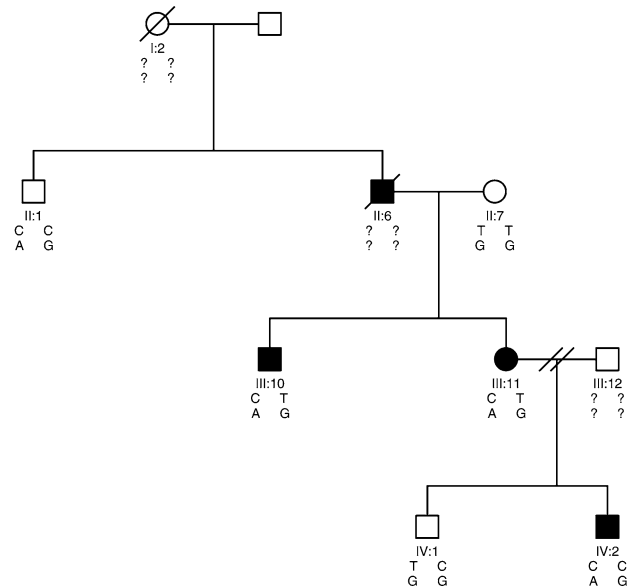
**A** Marker 1 = -512C>T

Marker 2 = 1761G>A



**B** Marker 1 = -512C>T

Marker 2 = 1761G>A



**Fig. 1** The two pedigrees of familial LD where no mutations have been identified within the *FOXC2* gene. The 5' (C-512T) and 3' (G1761A) SNPs are shown below each individual tested, with the 5' at the top. **a** Marker 1 = -512C > T; marker 2 = 1761G > A. **b** Marker 1 = -512C > T; marker 2 = 1761G > A

phisms had the C and A, and G and T alleles in *cis*, as predicted from the linkage studies (see Fig. 1).

From our families and sporadic cases, we were able to calculate an approximate value for the frequency of new mutations in LD. Excluding the two families where we have not found a mutation, we have 32 families and 11 sporadic cases with LD. Of these, one apparently sporadic case was adopted and the parental phenotype was

not available. Seven others were demonstrated to be new mutations by sequencing DNA from the probands and both parents, but in the other three cases it was not possible to get DNA from both parents. There were eight examples of only a parent and child apparently being affected, and of these one was definitely a new mutation and one probably so, as the affected father had four unaffected siblings and there was no clinical suggestion that the deceased grandparents had been affected. Only the definite new mutation was scored as such. The remaining six affected parent/child pairs had insufficient information to determine whether the parent was a new mutation. In none of the more extended familial cases was a new mutation suggested, based on DNA analysis coupled with clinical family history. The seven definite de novo mutations we identified are given in Table 3.

## Discussion

We have shown, along with other groups, that LD is mainly produced as a result of nonsense mutations, and insertions or deletions that produce frameshifts (Bell et al. 2001; Finegold et al. 2001; Erickson et al. 2001; Brice et al. 2002). Although the mutations are scattered throughout the gene, there is a hotspot for recombination in the region 893–930 bp, where we have found seven mutations, none of them identical. There are three reports of the 914-921delACGCCGCC mutations in the literature (Bell et al. 2001; Erickson et al. 2001; Bahau et al. 2002). The presence of a GCCGCC element either side of a 14-bp sequence, plus an additional GCCGCC in this region, might explain how misalignment and recombination could produce deletions and insertions.

We have also now found four missense mutations that co-segregate with the condition. Of these, there is compelling evidence that those previously reported, S125L and R121H, are definitely causative, since analogous changes in *FOXC1* (S131L and R127H) have been shown by computer modelling and cell studies to have severe effects on protein function (Saleem et al. 2003). S131L does not transactivate at all in a luciferase re-

**Table 3** List of definite de novo mutations identified after sequencing of parental and proband DNA

nt 201-202insCT
C298T
nt818-819insA
nt866-867insC
nt914-921delACGCCGCC
nt922-929delGCGCCGCC
nt1048-1051dupGTCC

porter assay (Saleem et al. 2003), and is considered by that research group as a completely inactive protein in this respect (M. Walter, personal communication). R127H shows greatly reduced nuclear localisation compared to wild type, and transactivation in the luciferase reporter assay was 16% of wild-type levels. Computer modelling for R127H shows that the mutation alters the electrostatic charge on the DNA binding surface of the fork head domain, which presumably affects the ability of the mutant protein to attach to its DNA target.

The two-missense changes reported in this paper do not have an analogy with any known *FOXC1* mutations, and it is therefore difficult to be sure of their effect on the protein. Both are absent from 200 control chromosomes. W116R is in the fork head domain and exhibits an R group change in both size and charge. The tryptophan is part of a motif (including R121) that is conserved across all mouse *Fox* genes (Carlson et al. 2002) and other species (Fig. 2). The likelihood is that this is a causative mutation. For S235I the position is less certain. It is outside the fork head domain of *FOXC2*, and the function of this region of the protein is unclear. The residue is either conserved in homologues from a BLAST search (Fig. 2), or is replaced by a glycine (e.g. in mouse *Foxc2*). Clearly a change to isoleucine could produce more profound effects, but with so little information on the function of this part of the protein it is impossible to be definite as to the effect in this case. If it is causative, future studies using this in a construct could help elucidate the function of the protein C-terminal to the fork head domain in *FOXC2*.

Our data on the number of new mutations in LD is not robust, but it does give some idea of the frequency of

**Fig. 2** *FOXC2* protein alignment of nine different species and one homologous protein to human *FOXC2*. The letters in *bold* are the equivalent amino acids to those altered by the SNPs. The alignments were obtained using the computer program ClustalW (<http://www.ebi.ac.uk/clustalw/>)

zebrafish	VKPPYSYIALITMAIQNSPDKKVTNLNGIYQFIMERFPFYRDNKQGWQNSIRHNLNLNECF	132
Tetraodon	VKPPYSYIALITMAIQNSPDKKVTNLNGIYQFIMERFPFYRDNKQGWQNSIRHNLNLNECF	132
FOXC1_Human	VKPPYSYIALITMAIQNAPDKKVTNLNGIYQFIMDRFPFYRDNKQGWQNSIRHNLNLNECF	136
human	VKPPYSYIALITMAIQNAPEKKITLNGIYQFIMDRFPFYRENKQGWQNSIRHNLNLNECF	130
mouse	VKPPYSYIALITMAIQNAPEKKITLNGIYQFIMDRFPFYRENKQGWQNSIRHNLNLNECF	129
frog	VKPPYSYIALITMAIQNAPDKKVTNLNGIYQFIMDRFPFYRENKQGWQNSIRHNLNLNECF	130
chicken	VKPPYSYIALITMAIQNAPDKKVTNLNGIYQFIMDRFPFYRENKQGWQNSIRHNLNLNECF	130
honey bee	VKPPYSYIALITMAIQNAPDKKVTNLNGIYQFIMERFPFYRENKQGWQNSIRHNLNLNECF	1134
Anopheles	VKPPYSYIALITMA ILQS POKKLTLSGICEFIMTRFPFYKKEKFPQWQNSIRHNLNLNDCF	190
Drosophila	AKPPYSYISLITMAIQNNPTRLMLTLSEIYQFIMDLFPFYRQNRQQRWQNSIRHSLSFNDCF	268
zebrafish	PKI ESPDSSSS--MSSGS---PHSIPSTRSLSDSAG-EQHH-QAPAQ-----	275
Tetraodon	PKI ESPDSSSS--MSSGS---PHSIPSNRSIGMDSSEHHQHGGQASAQ-----	273
FOXC1_Human	PKI ESPDSSSSSLSSGSSPPGSLPSARPLSLDGADSA PPPPAPSAPP-----HS	305
human	TKVETLSPEE--ALQGS--PRSAASTPAGSPDGSLPEHHAAAPNGLP-----	268
mouse	TKVETLSPEG--ALQAS--PRSAASTPAGSPDGSLPEHHAAAPNGLP-----	267
frog	TKVENLSPDGGGAMQDS--PRSVASTPSVSTDNSIPDQHPASN-----	257
chicken	TKVETLSPGSGGGLRDS---PRSGRIASRRLP-RRFAARAAPGGNGLP-----	275
honey bee	AKYGLHSPIDQTKTAVVTTTAVAVAGQSVIQTALGHQVHQAHQVHQDAGIQDVMGLDP	1307
Anopheles	AFPGVFAPFAPFWIRKP-----	254
Drosophila	DHHQHHKEAGGASIAQVNVLSAAHSKDAEALAMLANAELCLSQPQ-----	397

new mutations in LD. Of the 43 probands in our analysis, 11 were apparently sporadic, but one was adopted and so removed from the analysis. All the other seven cases were de novo *FOXC2* mutations, as was one from families where only a parent and child were affected. From our 42 cases, we therefore have 19% new mutations at the lowest possible estimate, with a top limit of 43% in the unlikely event of all six parent/child couplets being new mutations. With the one highly probable case included plus the three other sporadics, and one from the six parent/child pedigrees assumed to be a new mutation, a figure of 30% emerges as a reasonable estimate of new mutations in LD.

The seven definite new mutations are a mixture of insertions deletions and a missense, with no obvious clustering (Table 3).

The two families with no mutation in the coding region of *FOXC2* are clinically indistinguishable from those where mutations have been identified. In view of haploinsufficiency as a postulated mechanism underlying LD (Fang et al. 2000; Bell et al. 2001; Erickson et al. 2001; Finegold et al. 2001; Brice et al. 2002), we had expected that gene deletions might be involved in these cases, but polymorphism studies, and haploid cell line analysis showed that this is not the case. Both pedigrees are consistent with linkage to the *FOXC2* gene using 5' and 3' polymorphic markers, but the lod scores are only 1 and 0.75 due to the sizes of the families. The disorder in these families could be due to mutations in another gene, but the linkage data are more in favour of *FOXC2* being the gene affected than an alternative locus. Promoter mutations could well be involved in these cases, but there is very little information on the promoter sites of *FOXC2*. Analysis of possible sites, and upstream sequencing, needs to be conducted to see if any changes can be identified.

Our present data suggest that LD is more common than was supposed in the pubertal onset lymphoedema population, is most likely to be genetically homogeneous, and has a new mutation rate of at least 20%. The nature of the promoter region of *FOXC2*, and the downstream targets of the gene, are currently unknown, and will be the subject of further work.

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