Identification of novel mutations in FOXL2 associated with premature ovarian failure

S.E.Harris1, A.L.Chand1, I.M.Winship2, K.Gersak3, K.Aittomäki4 and A.N.Shelling1

1Department of Obstetrics and Gynaecology, National Women’s Hospital, Auckland, 2Department of Molecular Medicine, University of Auckland, New Zealand, 3Department of Obstetrics and Gynaecology, University Medical Centre, Ljubljana, Slovenia and 4Department of Clinical Genetics, Helsinki University Central Hospital, Finland

Premature ovarian failure (POF) affects ~1% of women and is known to be caused by sex chromosome abnormalities, iatrogenic agents and autoimmune diseases, but in the majority of cases the cause is unknown. However, several families have been identified as having an inherited predisposition to POF, suggesting a genetic component to the condition in these cases. The FOXL2 gene of 70 POF patients from New Zealand and Slovenia was screened for mutations. In a Slovenian POF patient, a novel 30 bp deletion was identified that was predicted to remove 10 out of 14 alanines (A221_A230del), from the polyalanine tract downstream of the winged helix/forkhead domain of the FOXL2 protein. A novel single nucleotide substitution, 772 (1009)T>A, which is predicted to change amino acid 258 from tyrosine to asparagine (Y258N), was identified in a New Zealand POF patient. Neither mutation was identified in 200 normal control chromosomes from 100 control samples. Three previously unreported single nucleotide substitutions, considered to be non-functional polymorphisms, were also identified.

Key words: forkhead/FOXL2/inertility/ovarian failure/winged helix

Introduction

Premature ovarian failure (POF) is defined as the cessation of ovarian function under the age of 40 years and is characterized by amenorrhoea, hypoestrogenism and elevated serum gonadotrophin concentrations. It affects 1% of all women and occurs in 0.1% before the age of 30 years (Coulam et al., 1986). The major problems associated with POF are the loss of fertility at an early age and the psychological problems associated with this, along with the possible physiological effects of reduced estrogen, including an increased risk of osteoporosis and cardiovascular disease. Interestingly, 5–10% of women spontaneously conceive after diagnosis (Conway, 1997; Kalantaridou et al., 1998). It is believed that POF may be caused by either a decreased number of follicles forming during ovarian development, or by an increased rate of follicle loss. Several causes of POF have been identified, including various X chromosome aberrations (Shelling, 2000). Turner syndrome with a 45,X karyotype causes a depletion of the ovarian follicles before puberty, resulting in primary amenorrhoea. Iatrogenic factors, such as chemotherapy and radiotherapy can also reduce follicle numbers. While conditions such as autoimmune diseases or diabetes mellitus are also associated with cases of POF, in the majority of cases the cause is unknown. However, many women with POF have a family history of the condition, suggesting an inherited predisposition to POF (Conway, 1997; Shelling, 2000). To date, mutations associated with POF have been identified in a small number of genes, including those encoding inhibin α (INHA), the FSH receptor and the LH/choriogonadotrophin receptor (Aittomäki et al., 1995; Latronico et al., 1996; Beau et al., 1998; Touraine et al., 1999; Shelling et al., 2000). As each mutation has been associated with <10% of POF cases, it can therefore be assumed that POF may be caused by mutations at many different loci. FOXL2 is a member of the winged helix/forkhead transcription factor family and is known to be expressed in the human and mouse adult ovary and the developing eyelid of the mouse, with little or no expression in other tissues that have been examined (Crisponi et al., 2001). The winged helix/forkhead family is a large and diverse family that is defined by the presence of a conserved 101 aa DNA binding domain composed of a winged helix structure (Gajiwala and Burley, 2000). Members of this family are expressed in a wide range of tissues, are involved in a variety of developmental processes and are thought to play an important role in mediating transforming growth factor (TGF)-β superfamily signals by binding to members of the Smad family of proteins (Attisano et al., 2001). At present, the exact function and binding partners of FOXL2 are unknown and it shares no conservation with any other family members outside of the winged helix/forkhead domain.

Mutations in FOXL2 have recently been associated with blepharophimosis/ptosis/epicanthus inversus syndrome (BPES), which affects eyelid development and in the case of BPES type I, also causes POF. To date, 21 different mutations in FOXL2 have been identified in BPES types I and II. Mutations predicted to result in a truncated protein lead to BPES type I and those leading to an expanded protein cause BPES type II (Crisponi et al., 2001; De Baere et al., 2001). However, it should be noted that De Baere et al. also reported menstrual abnormalities and fertility problems in two BPES type II families, suggesting an overlap between the phenotypes of the two disorders (De Baere et al., 2001). The initial association of FOXL2 mutations and BPES type I raised the question of whether or not mutations in FOXL2 could lead to isolated POF, without eyelid defects (Prueitt and Zinn, 2001). Therefore, De Baere et al. also screened 30 unrelated patients with isolated POF, but found no causal mutations in FOXL2 (De Baere et al., 2001).

We hypothesized that other mutations in FOXL2 could lead to POF...
without eyelid defects and 70 POF patients from New Zealand and Slovenia were screened, by direct sequencing of PCR products, for mutations in their FOXL2 genes. Our results indicate that mutations in FOXL2 may lead to diverse forms of POF without eyelid defects.

Materials and methods

Mutation nomenclature
In accordance with current mutation nomenclature recommendations (den Dunnen and Antonarakis, 2000), the A of the ATG of the initiator Methionine codon of FOXL2 is denoted nucleotide +1. Nucleotide numbers in parentheses refer to GenBank accession no. AF301906.

Patient information
Forty New Zealand and 30 Slovenian women, with POF, were recruited for this study by the Departments of Obstetrics and Gynaecology in Auckland, New Zealand and Ljubljana, Slovenia. POF was defined as cessation of menses for a duration of ≥6 months before the age of 40 years, along with a FSH concentration of >40 IU/l. A complete medical and gynaecological history was taken from each patient as previously described (Shelling et al., 2000). Fifty normal control samples were obtained from each of the general populations of New Zealand and Slovenia.

PCR
Genomic DNA was extracted from 10 ml samples of blood as previously described (Shelling et al., 2000) and 100 ng was used as a template in a PCR. Primers were designed to flank the coding region of the single exon gene, FOXL2, using the Primer Select module in the DNASTar computer program from Lasergene 1994 (DNASTAR Inc., Madison, WI, USA) and were as follows. FOXL2F: 5'-GGCGGACTTGCGGCCAAAAGAACCT-3' (nucleotides 152–172, accession no. AF301906); FOXL2R: 5'-AGGCGGGCCCAAGGTTGTA-3' (nucleotides 1484–1464, accession no. AF301906). Reaction conditions were 0.4 µmol/l of each primer, 0.2 µmol/l of each dNTP, 0.625 U Taq polymerase, 1×Q solution and 1×PCR buffer (Qiagen GmbH, Hilden, Germany) in a 25 µl total volume. Thirty cycles of PCR were performed, consisting of 1 min at 94°C, 1 min at 62°C and 1 min at 72°C, with a final 10 min extension at 72°C.

DNA sequencing
PCR products to be sequenced were purified using Roche’s High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). Sequencing reactions were performed using the ABI prism big dye Terminator Sequencing Kit under standard conditions and then separated either on an ABI prism 377 DNA Sequencer XL machine or an ABI prism 3100 Genetic Analyzer (PE Biosystems, Foster City, CA, USA). All sequencing reactions were performed at the Centre for Gene Technology, University of Auckland. Primers used for sequencing were FOXL2F, FOXL2R (described above) and primer C: 5'-CAGGCCAACAAGGATGTCCTCA-3' (De Baere et al., 2001).

Restriction fragment length polymorphism (RFLP) and forced RFLP (FRFLP) analysis
To confirm the sequencing data and to rapidly screen 200 control chromosomess, for variant 1 [501(738)C>T] and variant 2 [536(773)C>G], a combined RFLP and FRFLP assay was devised. Primers were designed to amplify the region of FOXL2 containing the variants, such that an AccI restriction site was introduced, by the reverse primer, into samples without the 772(1009)T transversion found in a New Zealand POF patient (variant 4). The primers used were FOXFRFLP: 5’-CTGTGGGCGCTACCCGACGCTTCCTACCC-3’ and FOXFRFLPR: 5’-GGGCGGACCGCCAAGGCTACGCTCAGCGTGTAT-3’.

PCR conditions were as described above, with an annealing temperature of 62°C. In the absence of 772(1009)T>A, a 198 bp PCR product was reduced to 172 bp when digested with AccI. The undigested and digested PCR products were reduced by 30 bp, to 168 and 142 bp respectively in the presence of the 661_690(898_927)del found in a Slovenian POF patient (variant 3).

PCR products were visualized by ethidium bromide staining after electrophoresis in an 8% (w/v) polyacrylamide gel at a constant voltage of 170V for 1 h.

Results
Forty New Zealand and 30 Slovenian POF patients were screened for mutations in the FOXL2 gene by PCR amplification and direct sequencing. Five variants were identified. Variant 1 consisted of a silent substitution at nucleotide 501(738)C>T (Figure 1a) and 5/70 patients were heterozygous for this variant. A second variant, 536(773)C>G (Figure 1a), which is predicted to cause a conservative amino acid change at position 179 from alanine to glycine (A179G), was also identified in these same five patients, suggesting that the two variants were inherited co-ordinately. One hundred New Zealand and 100 Slovenian control chromosomes and the parents of one of the patients carrying the two variants were screened for variant 1 by a rapid RFLP screen. Seven controls and the normal mother of the patient were identified as heterozygous for variant 1, and one control was shown to be homozygous (Table I). A second RFLP screen confirmed that samples heterozygous for variant 1 were also heterozygous for variant 2, and the control sample homozygous for variant 1 was homozygous for variant 2 (data not shown), supporting the theory that the two variants are in linkage disequilibrium.
Menopause was normal until age 36 years when menopausal symptoms were noted. In this patient, menarche occurred at age 14 years and menstruation was ceased at age 38 years by FSH measurement. The normal mother and sister of this patient were both shown not to carry this variant, but the status of her father could not be determined as he was deceased.

The coding region of the FOXL2 gene, in 70 POF patients, was screened for mutations by the direct sequencing of PCR products. A silent substitution was identified in a New Zealand sporadic POF patient (Figure 1b). In this patient, menarche occurred at age 14 years and menstruation was normal until age 36 years when menopausal symptoms were noted. Menopause was confirmed at age 38 years by FSH measurement. 772(1009)T>A is predicted to cause a non-conservative amino acid change (Y258N). This New Zealand patient was also heterozygous for variants 1 and 2. Her parents were screened for 772(1009)T>A and her mother was found to be heterozygous (as she was for variants 1 and 2).

One hundred normal New Zealand and 100 normal Slovenian control chromosomes were screened for variant 3 [661_690(898_927)del] and variant 4 [772(1009)T>A] by RFLP (Figure 3). Neither mutation was identified in any of the 200 control chromosomes (Table I).

Finally, a silent substitution at nucleotide 858(1095)T>G (variant 5) was identified in a further three patients, all of whom were heterozygous for this variant (Figure 1c and Table I).

**Discussion**

The coding region of the FOXL2 gene, in 70 POF patients, was screened for mutations by the direct sequencing of PCR products. A silent substitution and a substitution leading to a conservative amino acid change were identified in the same five patients (3.6% of POF chromosomes) and also shown to be present in the normal population at a similar frequency (4.5% of normal chromosomes). These two variants appear to be in linkage disequilibrium. The conservative amino acid change, A179G, is unlikely to have a functional effect on the protein, particularly as the mouse protein contains a proline at this position. A second silent substitution was identified in a further three patients. These variants were all considered to be normal polymorphisms, with no functional consequences, although functional tests are needed to confirm this.

Figure 3. RFLP analysis of FOXL2 772(1009)T>A and 661_690(898_927)del variants, using AccI. Undigested control DNA gives a single band of 198 bp and digested control DNA gives a single band of 172 bp. DNA samples heterozygous for 772(1009)T>A yield two bands of 198 bp and 172 bp when digested with AccI. DNA samples heterozygous for 661_690(898_927)del give two bands of 198 and 168 bp when undigested, due to a 30 bp deletion, and these are reduced to 172 and 142 bp respectively when digested. Nucleotide numbers in parentheses refer to GenBank accession no. AF301906.
to familial POF, otherwise it would be rapidly lost from the population. The previously identified INHA variant (Shelling et al., 2000) was also identified in a POF patient and her normal mother. Therefore, data from this study, the INHA study and a report that the dominant transmission of POF occurs with 79.1% penetrance (Vegetti et al., 1998) all suggest that incomplete penetrance may be a common mechanism for the inheritance of mutations leading to POF. Heterozygous mutations, leading to haploinsufficiency, can only reduce the final protein product to a maximum of 50%. Therefore, environmental and other genetic factors may play an important role in determining the final phenotype and thus explain the incomplete penetrance observed in numerous inherited conditions including familial POF. It has been suggested that patients with mutations in the TGF-β superfamily member, bone morphogenetic protein receptor II, may only develop primary pulmonary hypertension when exposed to a certain environmental stimulus or if they carry a second mutation in a disease-modifying gene (Loscalzo, 2001; Newman et al., 2001; Trembath et al., 2001).

The deletion of 10 of 14 alanines from the FOXL2 protein of a Slovenian POF patient is intriguing. As far as we are aware, this is the first report of a deletion within a polyalanine tract being associated with a disease phenotype, although polyalanine expansions are known to be causative in several conditions. In fact the polyalanine tract of FOXL2 has been shown to be expanded in some families with BPES type II, including one with fertility problems (Crisponi et al., 2001; De Baere et al., 2001), and expanded polyalanine tracts in HOXA13 (Goodman et al., 2000) and HOXD13 (Muragaki et al., 1996) cause hand–foot–genital syndrome and polysyndactyly respectively. The exact function of polyalanine tracts is unknown, although they are associated with transcriptional repression domains (Licht et al., 2001). This would lead them to cause a dominant negative effect over the wild-type protein. It is possible that a deletion in a polyalanine tract could lead to similar phenomena. Alternatively, the deletion may affect the flexible spacer elements between functional domains (Karlin and Burge, 1996) or that they are directly involved in protein–protein interactions (Han and Manley, 1993b; Licht et al., 1994). Goodman and Scambler suggested that polyalanine expansions may prevent normal protein–protein interactions from occurring and possibly even cause novel interactions, whilst still allowing the transcription factor to bind their normal DNA targets (Goodman and Scambler, 2001). This would lead them to cause a dominant negative effect over the wild-type protein. It is possible that a deletion in a polyalanine tract could lead to similar phenomena. Alternatively, the deletion may cause loss of DNA binding or transactivation from the mutant allele, leading to haploinsufficiency. Alanine-rich domains have also been associated with transcriptional repression domains (Licht et al., 1990, 1994; Han and Manley, 1993a,b) and it is possible that normal transcriptional repression activities are lost by the deletion. To identify the functional implications of the 661_690(898_927)del and 772(1009)T>A FOXL2 variants, we intend to investigate the DNA and protein binding and transcriptional activation/repression activities of both normal and variant recombinant proteins.

Results from this study support the hypothesis that FOXL2 is involved in the aetiology of POF. The data are further supported by indirect evidence for the role of FOXL2 in the inhibin/activin signalling pathway. The phenotype of the INHBB knockout mouse includes eyelid defects, specifically an apparent failure of eyelid fusion during development, as well as impaired reproductive ability in females (Schrewe et al., 1994; Vassalli et al., 1994). The main symptoms of BPES are eyelid defects and in many cases, POF or other fertility problems. This would suggest that FOXL2 may be involved in an inhibin/activin pathway(s) responsible for the correct fusion of the eyelids during development and the regulation of female fertility. Previous studies from our group identified a mutation in the INHA gene in 7% of POF patients (Shelling et al., 2000). The identification of FOXL2 mutations in only 2/70 POF patients is, therefore, not surprising. It is likely that the entire inhibin/activin-responsive pathway is important in the development of POF and that mutations in individual molecules within the pathway give rise to POF. We therefore intend to screen POF patients for mutations in other members of this pathway.

In summary, we have identified two different and rare variations in the FOXL2 gene that may cause different forms of POF, one with early onset and another with later onset and incomplete penetrance. Neither mutation was identified in 200 normal control chromosomes, nor has been reported in any case of BPES. We suggest that FOXL2 is an important transcription factor that regulates ovarian function and eyelid development via the inhibin/activin signalling pathway.

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References


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