Functional evidence implicating FOXL2 in non-syndromic premature ovarian failure and in the regulation of the transcription factor OSR2

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ABSTRACT

Background: FOXL2 encodes a forkhead transcription factor whose mutations are responsible for the blepharophimosis-ptosis-epicanthus inversus syndrome (BPES), involving craniofacial/palpebral abnormalities often associated with premature ovarian failure (POF).

Results: We describe a FOXL2 variant (p.Gly187Asp) in a case of POF without BPES. The subcellular localisation of FOXL2-G187D was normal but its transactivation capacity tested on two reporter promoters, one of which should be relevant to the ovary, was significantly lower than that of normal FOXL2. However, FOXL2-G187D was able to activate strongly a reporter construct driven by the promoter of Osr2 (odd-skipped related 2 transcription factor), which we have suggested to be a crucial target of FOXL2 in the craniofacial region. This is compatible with the absence of BPES in our patient.

Conclusions: Our data provide evidence in favour of the implication of FOXL2 variants in non-syndromic POF and confirm the regulatory interaction between FOXL2 and OSR2 whose perturbation might contribute to the palpebral abnormalities observed in BPES patients.

Figure 1 FOXL2 sequence alignment around Gly187. The alignment involves sequences from vertebrates ranging from amphibian and fishes to mammals. The relevant Gly187 residue is highlighted within a frame. The immediately previous highly conserved Tyr/Y residue is predicted to be phosphorylatable. The negative charge of aspartate in FOXL2-Gly187Asp might interfere with the post-translational modification process or mimic the modification itself.
presence of the heterozygous variant c.560G>A, inherited from her father, leading to the amino acid substitution p.Gly187Asp (FOXL2-G187D) in a highly conserved segment, C-terminal to the forkhead DNA binding domain (fig 1). This variant was absent in 110 control chromosomes. Interestingly, we have previously detected this variant in an XX male but its link with this condition, if any, is unclear. To assess the potential deleterious effect of the amino acid change we used the SIFT software (http://blocks.fncrc.org/sift/SIFT.html). SIFT uses protein sequence conservation data to calculate the probability of a substitution being deleterious. Scores <0.05 suggest a potential pathogenicity. The score of p.Gly187Asp, obtained using the alignment shown in fig 1, was 0. The substitution p.Gly187Asp implies an important change in polarity. Glycine is a small neutral amino acid whereas aspartic acid is acidic and negatively charged at pH 7. Interestingly, Tyr186 is predicted to be phosphorylatable (http://www.cbs.dtu.dk/services/NetPhosK/). Therefore, we hypothesise that the negative charge of the side chain of aspartate might interfere with the post-translational modification process or mimic the modification itself, perturbing ovarian specific protein interactions and/or regulations.

To assess subcellular localisation and transcriptional activity of FOXL2-G187D, we performed transfection experiments and luciferase reporter assays as previously described. The FOXL2-G187D variant fused to the green fluorescent protein displayed a typical nuclear staining, indistinguishable from that of normal FOXL2. From a functional perspective, FOXL2-G187D was found to activate the promoter of FoxL2 itself and the FOXL2 specific artificial promoter 2xFLRE-luc (containing 2 FOXL2 response elements upstream of a minimal CMV promoter), though significantly less strongly than the normal protein. The p.Ile84Ser mutant, responsible for a BPES associated with POF (that is, type I BPES), was used as a negative control and was, as expected, unable to transactivate our luciferase reporters. We have recently suggested that a positive feedback loop of FOXL2 (which activates its own promoter) might be important in response to oxidative stress in the ovary. Thus, we hypothesise that a lowered transactivation capacity of FOXL2-G187D on ovarian targets might explain the ovarian phenotype in our patient (fig 2A,B). However, the effects of this variant is expected to be promoter dependent, as previously shown for other mutants and as shown below for the third reporter promoter that we tested.

We have previously suggested that, in the craniofacial region, the encoding odd-skipped related 2 transcription factor (OSR2) should be a crucial target of FOXL2. Osr2 is, as Foxl2, highly expressed in the murine periocular mesenchyma. Moreover, as described for Fox2+/− mice, Osr2+/− mutant neonates exhibit open eyelids with similar abnormalities. Unfortunately, no data concerning the ovarian phenotype of Osr2+/− mice are available yet. In agreement with our hypothesis and with the absence of an eyelid phenotype in our patient (and in her mutation carrier father), we found that FOXL2-G187D was able to activate strongly a reporter construct driven by the Osr2 promoter (pOSR2) described by Kawai et al 2005. Coherently, the FOXL2-184S mutant found in a BPES I patient failed to transactivate pOsr2 (fig 2C). However, since no second BPES locus has been reported thus far, it seems unlikely that mutations in OSR2 itself might contribute directly to BPES.

**DISCUSSION**

The fact that FOXL2-G187D may be either normal or hypomorph suggests that it can behave as a susceptibility variant whose activity might be shifted up or down by modifier genes, leading to normal ovarian function or POF, respectively. Given our functional evidence, it would be interesting to assess the association between p.Gly187Asp and POF formally.
A previous mutation screening has detected a 30 bp deletion removing 10 out of 14 alanines of the FOXL2 polyAlanine tract (that is, FOXL2-Ala4) and the substitution p.Tyr258Asn in two POF patients.13 14 Interestingly, neither mutation was identified in 200 control chromosomes. However, the fact that the p.Tyr258Asn variant was maternally inherited and that the patient carrying the FOXL2-Ala4 variant spontaneously conceived and delivered two babies did not allow a formal causal implication of these alterations in the absence of functional assays at the time of these studies. Both variants might display incomplete penetrance or lead, at least, to partial fertility recovery after appropriate treatments. Thus, it would be interesting to assess the functional impact for both alterations.

Taken together, our results provide functional evidence in favour of the implication of FOXL2 variants in non-syndromic POF and confirm the regulatory interaction between FOXL2 and OSR2, whose perturbation might contribute to the palpebral phenotype in BPES. In our opinion, a more systematic genetic screening of FOXL2 mutations is of interest in non-syndromic POF, to improve genetic counselling, and to understand better the molecular aetiology of this frequent pathology.

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