Reproductive BioMedicine Online (2012) **24**, 339–341

www.sciencedirect.com
www.rbmonline.com

SHORT COMMUNICATION

Screening for mutations of the *FOXO4* gene in premature ovarian failure patients

Dora Janeth Fonseca [a,b], Eliana Garzón [a], Besma Lakhal [c], Rim Braham [d], Diego Ojeda [a], Hatem Elghezal [c], Ali Saâd [c], Carlos Martín Restrepo [a,b], Paul Laissue [a,b,*]

[a] *Unidad de Genética, Escuela de Medicina y Ciencias de la Salud, Universidad del Rosario, Carrera 24 N° 63C-69, Bogotá, Colombia;* [b] *Genética Molecular de Colombia, Departamento de Fertilidad Humana, Bogotá, Colombia;* [c] *Department of Cytogenetics and Reproductive Biology, Farhat Hached University Teaching Hospital, Sousse, Tunisia;* [d] *Department of Endocrinology, Farhat Hached University Teaching Hospital, Sousse, Tunisia*

[*] Corresponding author. *E-mail address:* paul.laissue@urosario.edu.co (P Laissue).

Professor Paul Laissue attained the Medical Doctor title at the Universidad del Rosario (Bogotá, Colombia). Then, he obtained a Master degree on Human Genetics (Paris 7 University), a Master degree on Physiopathology of Reproduction (Paris 5 University) and a PhD on Human Genetics (Paris 7 University). At present he is the scientific director of the Genetics Unit of the Universidad del Rosario in Bogotá. The aim of his scientific work is related to understand the molecular basis of human infertility and other complex diseases.

Abstract *FOXO4* constitutes a coherent candidate gene associated with premature ovarian failure (POF) pathogenesis. This study sequenced the coding and exon-flanking regions of this gene in a panel of 116 POF patients and 143 controls of Tunisian origin. In both groups, the IVS2 + 41T > G sequence variant was identified. It is concluded that coding mutations of *FOXO4* should not be a common cause of the disease in women from the Tunisian population. However, this study cannot exclude that *FOXO4* dysfunctions, originated from open reading frame or promoter sequence variations, might be associated with the pathogenesis of the disease in other ethnical groups.

KEYWORDS: female infertility, FOXO4, premature ovarian failure

Introduction

Premature ovarian failure (POF) is a frequent pathology which affects 1% of women. Clinically, POF is defined as at least 6 months of amenorrhoea occurring under 40 years old associated with elevated plasma concentrations of FSH (>40 IU/l). Up to now, several aetiologies have been related with POF pathogenesis such as infectious agents, autoimmune conditions and iatrogenic injuries. However, in the majority of cases, POF is considered as idiopathic, indicating a potential involvement of genetic factors. Chromosomal abnormalities (e.g. Turner's syndrome), as well as

1472-6483/$ - see front matter
doi:10.1016/j.rbmo.2011.11.017

mutations in autosomal and X-linked genes, have been identified as causative of the phenotype. In recent years, the study of genes that encode proteins which belong to the forkhead (fkh) family of transcription factors has demonstrated its crucial role in several mice and human physiological processes, including gonadal development and fertility (Benayoun et al., 2011). Fkh factors share a highly conserved ~100 amino acid region, the forkhead domain, responsible of the identification and binding to specific DNA sequences located on the promoter regions of target genes. In the ovarian context, *FOXL2*, *FOXE1*, and members of the *FOXO* subfamily have been implicated in normal and pathological conditions. Mutations in *FOXL2* have been related to the aetiology of an autosomal dominant disease: the blepharophimosis, ptosis, epicantus-invesrus syndrome (BPES; De Baere et al., 2009). BPES is characterized by eyelid abnormalities associated (BPES type I) or not (BPES type II) with POF. In addition, *FOXL2* mutations have been reported in non-syndromic POF cases (Harris et al., 2002). These findings are consistent with those observed in *Foxl2*$^{-/-}$ mice models, in which morphological granulosa cell impairment and premature massive follicle atresia were observed (Schmidt et al., 2004; Uda et al., 2004). Some evidences pinpoint *FOXE1* as a potential genetic factor related to POF origin, since length variation of its polyalanine tract seems to predispose to the disease (Watkins et al., 2006a). Additional studies suggest that a subset of ROS (reactive oxygen species) genes is regulated by members of the *FOXO* subfamily (Tothova and Gilliland, 2007). It has been proposed that these molecules may have a regulatory role during oocyte maturation and folliculogenesis (Agarwal et al., 2005). Among members of the *FOXO* subfamily, *FOXO3A* and *FOXO1* have been particularly well studied in mammalian reproduction, since they have been related to ovulation and oocyte maturation in mice and humans. *Foxo3a*$^{-/-}$ female mice showed massive follicular activation leading to premature depletion of follicles and infertility (Castrillon et al., 2003). *FOXO3A and FOXO1* screening for mutations led to the identification of sequence variants potentially related to POF aetiology (Wang et al., 2010; Watkins et al., 2006b).

Concerning *FOXO4*, it has been shown that it is expressed in the granulosa cells of the immature mice, as well as in human luteinized mural granulosa cells (Pisarska et al., 2009; Richards et al., 2002). At molecular level, conserved Foxo4 serine/threonine residues are directly phosphorylated by the PI3K/Akt pathway, which leads to its release from DNA and its retention within the cytoplasm (Tran et al., 2003). In this environment, Foxo4 transcriptional effects are silenced, suppressing the expression of target genes such as *Cdkn1b* (also known as *p27*), a negative regulator of the cell cycle. Interestingly, the expressional maintenance of *Cdkn1b* in a mouse model, constitutively expressing *Foxo3a* in oocytes, is related to infertility due to a follicular growth delay (Liu et al., 2007). Conversely, *p27*$^{-/-}$ animals show a premature follicular stock overactivation leading to ovarian dysfunction (Rajareddy et al., 2007). Recently, Ojeda et al. (2011) identified the first *CDKN1B* sequence variation, which could explain some cases of idiopathic POF. In this context, *FOXO4* sequence variations could alter its transcriptional activity on key target ovarian genes, conducing to premature ageing of the ovary and POF.

Materials and methods

In order to establish whether *FOXO4* sequence variants are a common cause of idiopathic POF, this study analysed its complete open reading frame in a panel of 116 POF patients who attended the Farhat Hached University Teaching Hospital, (Sousse, Tunisia). Inclusion criteria were primary or secondary amenorrhoea occurring before the age of 40, normal 46,XX karyotype and elevated plasma concentrations of FSH (>40 IU/l). Patients presenting clinical antecedents of pelvic surgery, autoimmune diseases and anticancer treatments were excluded. The control group population was composed of 143 women over 50 years old, having at least one child and lacking antecedents of reproductive dysfunctions. These individuals were of Tunisian origin and displayed regular menstrual cycles ranging from 28 to 32 days. The study was approved by the local Review Board of each participant institution (El Rosario University at Bogotá, Colombia and the Farhat Hached University Teaching Hospital at Sousse, Tunisia). Genomic DNA from patients and controls was obtained from whole blood samples using the standard phenol/chloroform procedure. The complete coding region of the gene (three exons) was amplified by PCR using exon-flanking primers (primer sequences and PCR conditions are shown in Supplementary Tables 1 and 2, available online only). Each amplicon was purified using exonuclease I and shrimp alkaline phosphatase and subsequently sequenced with internal primers. Sequences were aligned and compared with that of the *FOXO4* wild-type version (NM_005938.3). Human Splicing Finder software (www.umd.be/HSF/) was used to predict potential splicing alterations. No further molecular genetic studies were performed in the POF patients.

Results

Since this study amplified and sequenced the coding region of the gene using exon-flanking oligonucleotides, it was able to identify the IVS2 + 41T > G sequence variant located on the second intron of the gene. Allelic frequencies did not significantly differ between POF and control group patients (0.65 versus 0.65 of the G allele and 0.35 versus 0.35 of the T allele, respectively) (**Table 1**). This nucleotide change was previously reported in public databases of SNPs (rs5980742). The NCBI-SNP database (www.ncbi.nlm.nih.gov/SNP) shows dissimilar allele frequencies of this variant in samples from European, Asian and Mexican origin. Individuals from ethnic groups similar to those described in our study have not been reported. *In silico* analysis of this variant did not reveal splicing changes. No further *FOXO4* sequence variants were found in these POF and control populations.

Discussion

The present work constitutes the first screening of mutations of the *FOXO4* gene in women affected by ovarian dysfunction. The strong expression of this gene in mice and human granulosa cells, as well as its role into PI3K/Akt/Cdkn1b molecular pathway, suggests a functional

Table 1 Sequencing analysis of the *FOXO4* gene: allele frequencies of the IVS2+41T>G sequence variant.

Sequence variation	*Allele frequencies*			
	POF patients (n = *116*)		*Controls* (n = *143*)	
	G	*T*	*G*	*T*
IVS2+41T>G	0.65 (151 alleles)	0.35 (81 alleles)	0.65 (185 alleles)	0.35 (101 alleles)

role during ovarian physiology (Pisarska et al., 2009; Rajareddy et al., 2007; Richards et al., 2002; Tran et al., 2003). The implication of FOXO factors during ageing processes, via ROS regulation, underlies the potential role of *FOXO4* during ovarian dysfunction and POF. However, the intronic IVS2 + 41T > G variant described here is not related to the aetiology of the disease, since it was identified in both POF and control groups at similar allele frequencies. Furthermore, in-silico analysis does not suggest a potential deleterious effect.

Thus, in conclusion, mutations in the coding region of *FOXO4* should not be a common cause of POF in women of Tunisian origin. However, this study cannot exclude that *FOXO4* dysfunctions, originating from open reading frame or promoter sequence variations, might be associated to the pathogenesis of the disease in other ethnical groups.

Acknowledgements

This study was supported by the Universidad del Rosario (grant CS/Genetics).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2011.11.017.

References

Agarwal, A., Gupta, S., Sharma, R.K., 2005. Role of oxidative stress in female reproduction. Reprod. Biol. Endocrinol. 14, 3—28.

Benayoun, B.A., Caburet, S., Veitia, R.A., 2011. Forkhead transcription factors: key players in health and disease. Trends Genet. 27, 224—232.

Castrillon, D.H., Miao, L., Kollipara, R., Horner, J.W., DePinho, R.A., 2003. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science 301, 215—218.

De Baere, E., Fellous, M., Veitia, R.A., 2009. The transcription factor FOXL2 in ovarian function and dysfunction. Folia Histochem. Cytobiol. 47, S43—S49.

Harris, S.E., Chand, A.L., Winship, I.M., Gersak, K., Aittomäki, K., Shelling, A.N., 2002. Identification of novel mutations in FOXL2 associated with premature ovarian failure. Hum. Reprod. 8, 729—733.

Liu, L., Rajareddy, S., Reddy, P., Du, C., Jagarlamudi, K., Shen, Y., Gunnarsson, D., Selstam, G., Boman, K., Liu, K., 2007. Infertility caused by retardation of follicular development in mice with oocyte-specific expression of Foxo3a. Development 134, 199—209.

Ojeda, D., Lakhal, B., Fonseca, D.J., Braham, R., Landolsi, H., Mateus, H.E., Restrepo, C.M., Elghezal, H., Saâd, A., Laissue, P., 2011. Sequence analysis of the CDKN1B gene in patients with premature ovarian failure reveals a novel mutation potentially related to the phenotype. Fertil. Steril. 95, 2658—2660.

Pisarska, M.D., Kuo, F.T., Tang, D., Zarrini, P., Khan, S., Ketefian, A., 2009. Expression of forkhead transcription factors in human granulosa cells. Fertil. Steril. 91, 1392—1394.

Rajareddy, S., Reddy, P., Du, C., Liu, L., Jagarlamudi, K., Tang, W., Shen, Y., Berthet, C., Peng, S.L., Kaldis, P., Liu, K., 2007. P27kip1 (cyclin-dependent kinase inhibitor 1B) controls ovarian development by suppressing follicle endowment and activation and promoting follicle atresia in mice. Mol. Endocrinol. 21, 2189—2202.

Richards, J.S., Sharma, S.C., Falender, A.E., Lo, Y.H., 2002. Expression of FKHR, FKHRL1, and AFX genes in the rodent ovary: evidence for regulation by IGF-I, estrogen, and the gonadotropins. Mol. Endocrinol. 16, 580—599.

Schmidt, D., Ovitt, C.E., Anlag, K., Fehsenfeld, S., Gredsted, L., Treierm, A.C., Treier, M., 2004. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. Development 131, 933—942.

Tothova, Z., Gilliland, G., 2007. FoxO transcriptions factors and stem cell homeostasis: insights from the hematopoietic system. Cell Stem Cell 1, 140—152.

Tran, H., Brunet, A., Griffith, E.C., Greenberg, M.E., 2003. The many forks in FOXO's road. Sci. STKE 172, RE5.

Uda, M., Ottolenghi, C., Crisponim, L., Garciam, J.E., Deianam, M., Kimberm, W., Foraboscom, A., Cao, A., Schlessinger, D., Pilia, G., 2004. Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. Hum. Mol. Genet. 13, 1171—1181.

Wang, B., Mu, Y., Ni, F., Zhou, S., Wang, J., Cao, Y., Ma, X., 2010. Analysis of FOXO3 mutation in 114 Chinese women with premature ovarian failure. Reprod. Biomed. Online 20, 499—503.

Watkins, W.J., Harris, S.E., Craven, M.J., Vincent, A.L., Winship, I.M., Gersak, K., Shelling, A.N., 2006a. An investigation into FOXE1 polyalanine tract length in premature ovarian failure. Mol. Hum. Reprod. 12, 145—149.

Watkins, W.J., Umbers, A.J., Woad, K.J., Harris, S.E., Winship, I.M., Gersak, K., Shelling, A.N., 2006b. Mutational screening of FOXO3A and FOXO1A in women with premature ovarian failure. Fertil. Steril. 86, 1518—1521.

Declaration: The authors report no financial or commercial conflicts of interest.

Received 13 July 2011; refereed 17 November 2011; accepted 17 November 2011.