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Short Communication

Mutational screening of *SF1* and *WNT4* in Tunisian women with premature ovarian failure

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ABSTRACT

Background: *WNT4* and *SF1* genes play an important role in ovarian development. They constitute coherent candidate genes associated with premature ovarian failure (POF) pathogenesis.
Methods: We sequenced the coding region of *WNT4* and *SF1* in 55 Tunisian women with POF and 100 healthy controls.
Results: We identified a synonymous variation in *WNT4* (c.99G>A, p.Ser33Ser) and a substitution (c.G437C) in *SF1* gene inducing G146 to Ala (GGG–GCG) missense mutation. *WNT4* (c.99G>A, p.Ser33Ser) was not associated with POF pathology. However, a positive association of SF1 Gly146Ala polymorphism was noted. Gly146Ala minor allele frequency was significantly higher ($p = 0.029$) in POF patients versus controls and Ala allele containing genotypes ($p = 0.005$) were positively associated with POF pathology. The carriage of 146Ala allele was also associated with a significant reduction in estradiol plasma levels.
Conclusions: SF1 Gly146Ala polymorphism seems to be associated with POF pathology in the Tunisian population likely by reducing estradiol levels.

1. Introduction

Premature ovarian failure (POF) (MIM 311360) is a frequent pathology, leading to infertility. It is estimated that ~1% of women under 40 years old are affected by this disease (Conway, 2000; Coulam et al., 1986). Clinically, POF is defined as at least 6 months of amenorrhea and elevated plasmatic FSH (follicle stimulating hormone) levels (>40 IU/l). The majority of POF cases are classified as idiopathic. However, diverse aetiologies have been described: autoimmune diseases, iatrogenic injuries (i.e. chemotherapy, pelvic surgery), infectious conditions, environmental and genetic factors (Conway, 2000; Hoek et al., 1997; Perez et al., 1997; Santoro, 2003). The latter have been associated with syndromic (i.e. Turner, fragile X and BPES syndromes) (Bodega et al., 2006; Christin-Maitre et al., 2002; Crisponi et al., 2001) and non-syndromic forms of POF. Several autosomal [i.e. *FSHR*, *LHR* (luteinizing hormone receptor), *GDF9*, *INHA*, *GALT*, *NOBOX*, *FIGLA*] and X linked (e.g. *BMP15*) genes have been linked with non-syndromic POF (Laissue et al., 2008; Lakhal et al., 2009; Qin et al., 2000; Zhao et al., 2008). Recently, attention has been paid to the contribution of *WNT4* (wingless-type MMTV integration site family, member 4) and *SF1* (steroidogenic factor 1) genes in POF. Both are expressed in the early stage of ovarian development and play a major role in ovarian function.

SF1, also termed NR5A1, is a nuclear receptor and a key transcriptional regulator of genes involved in the hypothalamic–pituitary–steroidogenic axis (Lin and Achermann, 2008; Lavorgna et al., 1991; Luo et al., 1994). It regulates the transcription of key genes involved in sexual development and reproduction, including *STAR* (encoding steroidogenic acute regulatory protein), *CYP17A1* (encoding 17-alphahydroxylase), *CYP11A1* (encoding cytochrome P-450 cholesterol side-chain cleavage), *LHB* (encoding the beta subunit of luteinizing hormone), *AMH* (encoding anti Müllerian hormone), *CYP19A1* (encoding aromatase), and *INHA* (encoding inhibin alpha subunit) (Gurates et al., 2003; Hanley et al., 2001; Keri and Nilson, 1996; Mendelson and Kamat, 2007; Shen et al., 1994; Sugawara et al., 1997; Weck and Mayo, 2006). *SF1* is expressed in multiple cell types in

Abbreviations: AMH, anti Müllerian hormone; B.M.I., body mass index; BMP15, bone morphogenetic protein 15; CYP17A1, cytochrome P450, family 17, subfamily A, polypeptide 1; FIGLA, factor in the germline alpha; FMR1, fragile X mental retardation 1; FSH, follicle stimulating hormone; FSHR, follicle stimulating hormone receptor; GALT, galactosidase-1-phosphate uridyl transferase; GDF9, growth differentiation factor 9; INHA, inhibin alpha; LHB, luteinizing hormone beta subunit; LHR, luteinizing hormone receptor; NOBOX, newborn ovary homeobox; POF, premature ovarian Failure; SF1, steroidogenic factor 1; SNP, single nucleotide polymorphism; STAR, steroidogenic acute regulatory protein; WNT4, wingless-type MMTV integration site family member 4.

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the foetal, postnatal, prepubertal and mature ovary (Jeyasuria et al., 2004; Lavorgna et al., 1991; Luo et al., 1994). The inactivation of *SF1* specifically in mouse granulosa cells causes infertility associated with hypoplastic ovaries. *SF1*−/− ovaries have follicles but lack corpora lutea, a finding that indicates impaired ovulation (Jeyasuria et al., 2004). Recently, Lourenço et al. (2009) revealed that SF1 mutations were associated with familial and isolated POF cases. They demonstrated that mutated forms of *SF1*show quantitative impairment in the transactivation of *CYP11A1* and *CYP19A1*.

WNT4 is a key regulator of gonadal determination and differentiation. Its mRNA and protein are present in human ovaries during foetal development and at different stages of follicular development in the adult ovaries (Jaaskelainen et al., 2010). Functional analyses revealed that deficiencies in the *WNT4* gene might affect the development of ovaries and follicles (Jaaskelainen et al., 2010; Vainio et al., 1999). Indeed, the ovaries of Wnt4 mutant female mice showed only a few degenerating oocytes (Vainio et al., 1999) and a markedly enhanced rate of oocyte apoptosis (Jaaskelainen et al., 2010). Moreover, germ cells were completely absent in the ovarian cortex of Wnt4 and Fst null gonads mice supporting its critical role in maintaining germ cell survival in the ovary (Yao et al., 2004).

Taken together, we are focused on *WNT4* and *SF1* to identify potential mutations that could explain the POF phenotype in a panel of 55 Tunisian patients.

2. Materials and methods

2.1. Study subjects

POF women were recruited from Hopital Farhat Hached, Sousse, Tunisia. Inclusion criteria were primary or secondary amenorrhea for more than 6 months, occurring before the age of 40 and FSH serum levels higher than 40 mUI/l. Women with clinical signs of Turner's syndrome were excluded from the study as well as patients with personal history of autoimmune disease or with clinical antecedents of pelvic surgery. A karyotype and a screening of *FMR1* gene premutation were assessed for all patients. Indeed, 55 POF cases with 46, XX karyotype and normal *FMR1* gene were included in the study.

The following clinical data of all patients were gathered: age at diagnosis, clinical presentation, family history of POF, hormonal evaluation including the measurement of FSH, LH, estradiol (E2), testosterone (T) and pelvic ultrasonography.

Control population (n = 100) consisted of healthy women with regular menstrual cycles, normal FSH levels, having at least one child and denied any history of a medical disease related to ovarian function. Control individuals have a similar ethnic origin to POF patients.

Informed consent was obtained from all subjects.

Table 1
SF1 primer sequences.

SF1	Primer sequence (5′ 3′)	
Exon 1	No coding	
Exon 2/3	Forward primer	gggcacagagaggggatta
	Reverse primer	gaaggccaatggtactatcc
Exon 4	Forward primer	atggaaggggcttagagag
	Reverse primer	ggcgggaggagagactca
Exon 5	Forward primer	gtgcctcttccttcca
	Reverse primer	aggcctgggtcctcctct
Exon 6	Forward primer	gacccacgtcctctgactgt
	Reverse primer	ctggctgtctccacctctct
Exon 7	Forward primer	gtgaccgagaacctcctttt
	Reverse primer	tgggcatcagaaaatgaacc

Table 2
WNT4 primer sequences.

WNT4	Primer sequence (5′ 3′)	
Exon 1	Forward primer	caccATGAGTCCCCGCTCGT
	Reverse primer	ctcggccccggccagacttac
Exon 2	Forward primer	ccaggaaagatgaggaggtg
	Reverse primer	aatagtcccgttgctcacga
Exon 3/4	Forward primer	atatgcccctcccccctctac′
	Reverse primer	ctatccctacccgctcttg
Exon 5	Forward primer	tcccttgccatctcctgat
	Reverse primer	GGTAGGTGGTGGGAGACTGT

2.2. DNA analysis

Genomic DNA from patients and controls was obtained from whole blood samples using the salting-out method (Miller et al., 1998).

The complete coding regions of *SF1* and *WNT4* were amplified by polymerase chain reaction using exon-flanking primers (primer sequences are shown in Tables 1 and 2). Each amplicon was purified using exonuclease I and shrimp alkaline phosphatase and subsequently sequenced. These products were then sequenced on an ABI 377 automated DNA sequencer (Applied Biosystems Division) using the Big Dye terminator V1.1 cycle sequencing kit. Sequences were aligned and compared with that of the SF1 and WNT4 wild-type version.

2.3. Statistical analysis

Statistical analysis was performed on SPSS v. 17.0 software (SPSS, Chicago, IL, USA). Data were expressed as mean ± SD (continuous variables), or as percentages of total (categorical variables). Allele frequencies were calculated using NCSS V. 2000 software. Pearson's chi square or Fisher's exact test was used to assess inter-group significance and Student's *t*-test was used to determine differences in means.

For study power calculation, we used the Rollin power calculator (http://www.stat.ubc.ca/~rollin/stats/ssize/caco.html.). At $\alpha = 0.05$, the studied sample size (55 POF/100 controls) provided an overall power of 63%.

3. Results

3.1. Study subjects

The characteristics of study participants are presented in Table 3. POF patients were comparable to controls regarding B.M.I. ($p = 0.564$) and testosterone levels ($p = 0.398$). Elevated levels of FSH, LH and a low level of oestrogen are two inclusion criteria of POF therefore significant differences were seen in the factors between patients and controls (<0.001). Significant differences were also seen in age and menarche between the two groups.

Table 3
Characteristics of study participants.

	Cases (n = 55)	Controls (n = 100)	p[a]
Mean age ± SD (years)	26.13 ± 7.4	32.79 ± 3.36	<0.001
B.M.I. ± SD (kg/m^2)	21.93 ± 5.47	21.57 ± 2.35	0.564
Mean menarche ± SD (years)	14.02 ± 1.40	12.8 ± 1.39	<0.001
FSH ± SD (mUI/ml)	73.58 ± 39.45	16.40 ± 4.53	<0.001
	68 (32–190)[b]	15 (9–27)[b]	
LH ± SD (mUI/ml)	29.62 ± 10.67	7.72 ± 3.57	<0.001
	28 (11–50.8)[b]	7.10 (0.8–15)[b]	
Estradiol ± SD (pg/ml)	28.51 ± 6.58	128.58 ± 47.20	<0.001
	27.66 (15–45)[b]	112.5 (38–241)[b]	
Testosterone ± SD (ng/ml)	0.51 ± 0.23	0.48 ± 0.23	0.398

[a] Student's *t* test (2-sided) for continuous variables, Pearson's chi square test for categorical variables.
[b] Median (min–max).