Reproductive BioMedicine Online (2013) **26**, 157–163

www.sciencedirect.com
www.rbmonline.com

ARTICLE

Quantitative analysis of *follistatin* (*FST*) promoter methylation in peripheral blood of patients with polycystic ovary syndrome

Qing Sang [a,b,1], Shaozhen Zhang [d,1], Shien Zou [e,1], Huan Wang [a,b], Ruizhi Feng [a,b], Qiaoli Li [b], Li Jin [a], Lin He [b,c], Qinghe Xing [b,c,*], Lei Wang [a,b,c,*]

[a] *State Key Laboratory of Genetic Engineering and MOE Key Laboratory of Contemporary Anthropology, School of Life Sciences, Fudan University, Shanghai, China;* [b] *Institutes of Biomedical Science, Fudan University, No. 138, Yixueyuan Road, Shanghai, China;* [c] *Bio-X Center, Key Laboratory for the Genetics of Developmental and Neuropsychiatric Disorders, Ministry of Education, Shanghai Jiao Tong University, Shanghai, China;* [d] *Shanghai the Ninth Hospital, No. 639, ZhiZaoJu Road, Shanghai, China;* [e] *Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China*

[*] Corresponding authors. *E-mail addresses:* xingqinghe@hotmail.com (Q Xing), wanglei2004sjtu@gmail.com (L Wang). [1] Qing Sang, Shaozhen Zhang and Shien Zou contributed equally to the paper.

Qing Sang completed his graduate study in Sun Yat-sen Unviersity. Now he is a PhD student in the Institutes of Biomedical Sciences, Fudan University. His fields of special interest and research are genetics and epigenetics of polycystic ovary syndrome and also genetics of birth defects.

Abstract Epigenetic mechanisms may contribute to polycystic ovary syndrome (PCOS). To date, however, no studies have associated CpG methylation levels of any candidate gene with PCOS susceptibility. Follistatin (FST), an activin-binding protein, is expressed in numerous tissues and is shown to have linkage with PCOS. However, results from case–control association analyses between this gene and PCOS are inconsistent. Thus, this study investigated possible association of methylation levels in the promoter and 5′-untranscribed region (UTR) of the *FST* gene with PCOS incidence in peripheral blood leukocytes and endometrial tissue. Using mass array quantitative methylation analysis, first the 5′-UTR methylation in *FST* was analysed in 130 PCOS patients and 120 controls. The methylation level of the *FST* gene was further studied in endometrium from 24 controls and 24 PCOS patients. This study demonstrates that methylation levels of CpG sites in the *FST* promoter and 5′-UTR are not associated with PCOS. Nonetheless, this was the first study to quantitatively evaluate the methylation levels of a candidate gene in association with PCOS. Further studies should be performed to examine methylation in other candidate genes. Understanding the epigenetic mechanisms involved in PCOS may yield new insights into the pathophysiology of the disorder.

1472-6483/$ - see front matter © 2012, Reproductive Healthcare Ltd. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.rbmo.2012.10.011

KEYWORDS: follistatin, FST, PCOS, quantitative methylation analysis

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder among women, affecting approximately 5–7% of those of reproductive age (Carmina and Lobo, 1999). It is characterized by hyperandrogenism, chronic anovulation and polycystic ovaries. Genetic factors are widely believed to contribute to PCOS (Goodarzi et al., 2011). To date, several candidate genes have been identified in association with PCOS. luteinizing hormone/choriogonadotropin receptor, thyroid adenoma associated and DENN/MADD domain containing 1A have been confirmed by recent genome-wide association studies to confer PCOS susceptibility (Chen et al., 2011).

Additionally, mice exposed to androgens *in utero* have a higher risk of being affected by a PCOS-like disease (Sullivan and Moenter, 2004), demonstrating that environmental factors may play a role in PCOS pathogenesis. Environmental factors can modify DNA methylation and cause irregular gene expression, thereby predisposing individuals to developing PCOS (Li and Huang, 2008). Furthermore, loss of methylation in the LHR gene in the ovaries of a mouse model of PCOS shows that epigenetic mechanisms may play an important role in the pathophysiology of PCOS (Zhu et al., 2010). Recently, although a pilot study comparing global DNA methylation patterns between patients with PCOS and controls found no significant differences, investigators suggest that further investigation on methylation in specific gene regions should be pursued (Xu et al., 2010).

Follistatin (FST), an activin-binding protein, is expressed in numerous tissues and shows strong evidence of linkage with PCOS (Urbanek et al., 1999; Sakamoto et al., 1996). The main function of follistatin is to regulate follicular development through binding and neutralizing activins (Bilezikjian et al., 2004). Overexpression of follistatin results in infertility in mice (Guo et al., 1998). Furthermore, the concentration of serum-circulating follistatin is higher in patients with PCOS than in controls (Norman et al., 2001; Eldar-Geva et al., 2001).

This study addressed the question of whether *FST* methylation level is associated with PCOS. First, *FST* methylation in peripheral blood was analysed for a large sample of PCOS patients and normal individuals ($n = 250$). Then CpG site methylation differences in endometrial samples from individuals with PCOS and normal controls ($n = 48$) were analysed. Finally, the expression of the *FST* gene was quantitatively analysed using real-time PCR.

Materials and methods

Sample collection

A total of 250 PCOS patients and controls were recruited from the outpatient clinic of the Xi'an Fourth Hospital and the Shaanxi Hospital for Women and Children. The first cohort consisted of 40 PCOS patients and 40 controls, while the second cohort consisted of 90 PCOS patients and 80 controls. All patients met the revised Rotterdam diagnostic criteria for PCOS (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Women who met at least two of the following criteria were defined as having PCOS: (i) oligo-ovulation and/or anovulation; (ii) clinical and/or biochemical signs of hyperandrogenism; and (iii) polycystic ovaries. None of the patients had taken hormonal medications, including oral contraceptives, for at least 3 months prior to participation in the study. Standardized initial screening was performed on a random day between 09:00 and 11:00 hours and overnight fasting blood was drawn on the third day of the subject's follicular phase. Serum LH, follicle stimulating hormone, prolactin, testosterone and progesterone concentrations were measured according to standard protocols. Control subjects are healthy and nondiabetic female individuals with regular menstrual cycles. All patients and controls were Han Chinese women from the same geographical area.

In addition, endometrial tissue samples from 24 PCOS patients and 24 controls were collected. The endometrium samples were collected in the early proliferative phase for the following reasons: (i) the histological morphology of PCOS is similar to that of the endometrium of normal cycling women in this phase (Maliqueo et al., 2003); and (ii) relatively constant circulating concentrations of 17β-oestradiol resulting from persistent anovulation in patients with PCOS are comparable to those of regularly cycling women in this phase (Giudice, 2006). This study was approved by the Fudan University Ethics Review Committee (approval document reference No. 55, 2 March 2012). Informed consent was obtained from all participants.

DNA extraction and bisulphite conversion

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Subsequent bisulphite treatment of genomic DNA was performed using the EZ DNA CT Conversion Reagent (Sequenom, Inc; Zymo Rsearch) following the manufacturer's protocols.

Mass array quantitative methylation analysis

Five overlapping primer pairs spanning predicted CpG islands in the promoter region of the *FST* gene were designed (www.epidesigner.com/). Primer pairs are listed in **Table 1**. PCR reactions were conducted using PCR polymerase in a volume of 5 μl containing 1 μl (10 ng) bisulphite-converted genomic DNA (Sequenom). The procedure included denaturing at 95°C for 4 min, followed by 45 cycles at 95°C for 20 s, 56°C for 30 s and 72°C for 1 min, and finally incubation at 72°C for 3 min. The PCR products were then treated with shrimp alkaline phosphatase (Promega, USA) and 2 μl were used as templates for in-vitro transcription and RNaseA Cleavage in the T-reverse reaction according to the manufacturer's instructions (Sequenom). The samples were desalted and spotted onto a 384-pad SpectroCHIP (Sequenom) using a MassArray Nanodispenser (Samsung). They were analysed by spectral acquisition on a MassArray Analyser Compact MALDI-TOF MS (Sequenom). Methylation