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

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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.
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., 2011). 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 Research) 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
Quantitative analysis by quantitative real-time PCR

Total RNA was extracted from endometrial tissue using TRizol reagent (Invitrogen). Then, 1 µg RNA was reverse transcribed to cDNA using the Superscript III First-Strand Synthesis System (Invitrogen). Real-time quantitative PCR was performed using the ABI 7900 Sequence Detection System (Invitrogen). Real-time quantitative PCR analysis was performed using the ABI 7900 Sequence Detection System (Invitrogen). Quantitative analysis by quantitative real-time PCR was performed using the ABI 7900 Sequence Detection System (Invitrogen). Real-time quantitative PCR was performed using the ABI 7900 Sequence Detection System (Invitrogen).

Statistical analysis

Percentage methylation at each CpG site was expressed as mean ± SEM. The data were analysed using Student’s t-test. P < 0.05 was considered statistically significant.

Results

Clinical characteristics of study subjects

In total, 130 PCOS patients (mean age 28.00 ± 0.12 years) and 120 controls (mean age 28.04 ± 0.18 years) were recruited for peripheral blood collection. The clinical data of all participants are listed in Table 2. The body mass index of PCOS patients was significantly higher than that of the control group (patients 22.25 ± 0.31, controls 20.77 ± 0.23; P < 0.001). LH/FSH concentrations and total T concentration in PCOS patients were also significantly higher compared with controls (P < 0.001). Prolactin and progesterone concentrations were not different between groups.

In addition, endometrial samples were obtained from 24 PCOS patients and 24 controls. The clinical data of these participants are also listed in Table 2.

CpG islands in the promoter and 5'-UTR of the FST gene

Using the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway), this study analysed known and predicted CpG islands in the promoter and 5'-untranscribed region (UTR) of the FST gene. As shown in Figure 1, the 5'-UTR and part of the promoter of the FST gene were found to contain dinucleotide CpG-rich regions. Altogether, there are 140 CpG sites in the 5'-UTR of the FST gene. Epityper software was used to design five pairs of primers for this region, as shown in Figure 1 and Table 1. Eighty-eight of the 140 CpG sites were considered informative and suitable for analysis.
Mass array quantitative analysis of CpG methylation in the promoter and 5'-UTR of the FST gene in peripheral blood and endometrial samples

First, 5'-UTR methylation in FST of 40 PCOS patients and 40 controls was analysed using mass array (Figure 2). Since the density of CpG sites covered by primers F3/T7R3 was very high, a successful primer pair could not be designed for this region and the CpG sites could not be detected by this method. Generally, there were no differences observed in the methylation levels of CpG sites between PCOS patients and controls. However, amplification by primer pair 1 showed that the percentage methylation of CpG 22,23 in amplicon 1 was higher in PCOS patients than in the control group (patients 12.3 ± 0.004%, controls 11.3 ± 0.003%; P < 0.001; Figure 2A).

To confirm this difference, the methylation analysis was repeated using a second sample consisting of 90 PCOS patients and 80 controls. The methylation level at the previously detected site was similarly elevated in this sample of PCOS patients compared with controls (patients 11.0 ± 0.003%, controls 8.5 ± 0.003%; P < 0.001) (Figure 3).

Because DNA methylation occurs in tissue-specific patterns, this study also compared methylation level of the CpG site in endometrial tissues of PCOS and normal individuals. No significant difference was found in the methylation levels of this site in endometrial tissue sampled from PCOS patients and control subjects (Figure 4).

Figure 1 A schematic diagram of CpG sites in the FST promoter and 5'-UTR region. CpG sites are depicted by lollipop markers. Primer-binding sites targeted in this study are shown below the diagram. → Represents forward primers and ← represents reverse primers. The number of lollipop markers is not indicative of detected CpG sites: 140 CpG sites were identified in the FST promoter and 5'-UTR region.

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Figure 2 Comparison of methylation at each CpG site in the FST promoter and 5'-UTR region of peripheral blood from 40 PCOS patients and 40 controls. (A, B, C, D) Four different amplicons in the promoter and 5'-UTR regions of the FST gene. Values are mean ± SE. *P < 0.001.
The expression of the FST gene in endometrial tissue was also assessed using quantitative real-time PCR. Eight PCOS patients and eight healthy controls were compared. The expression of FST was similar in endometrial tissues of PCOS patients and controls (Figure 5).

Discussion

This study analysed methylation in the promoter and 5'-UTR of the FST gene in peripheral blood and endometrial samples from PCOS patients and healthy controls. Although the methylation level of the CpG 22, 23 site in amplicon 1 was associated with PCOS in peripheral samples, the lack of differences between cases and controls in methylation at other CpG sites and FST expression in endometrial tissues demonstrated that the methylation level of the FST gene in the promoter and 5'-UTR region is not responsible for PCOS.

PCOS is a complex disorder with unknown aetiology. Evidence demonstrates that both genetic and environmental factors contribute to the origin and development of this disease (Franks et al., 1997; Escobar-Morreale et al., 2005). Environmental determinants of PCOS may alter clinical presentation via epigenetic modifications (Hickey et al., 2006). Recently, Xu et al. performed a genome-wide methylation analysis to identify early epigenetic alterations of prenatally androgenized infant and adult Rhesus monkeys. The results suggest that epigenomic methylation perturbations may contribute to the aetiology of PCOS (Xu et al., 2011). To date, however, changes in methylation of candidate genes have not been associated with PCOS.

FST encodes follistatin, an activin-binding protein that antagonizes activin activity in vitro. It can bind to heparin sulphate proteoglycans and present activin proteins to their receptors in vivo. Circulating follistatin concentrations are elevated in patients with PCOS and follistatin mRNA expression is absent in granulose cells in PCOS, indicating that FST may play an important role in the aetiology of PCOS (Nor- man et al., 2001; Eldar-Geva et al., 2001; Roberts et al., 1994). However, the results from studies of the relationship between FST and PCOS are inconsistent. Urbanek et al. provided evidence for linkage between PCOS and follistatin in 150 families. Further, Jones et al. (2007) identified a SNP in FST that was associated with androgenic markers of the disease. Conversely, Calvo et al., 2001; Liao et al. (2000) have independently reported that polymorphisms of the FST gene do not appear to be related to PCOS.

DNA methylation, which does not alter DNA sequence, is the most well-known epigenetic modification mechanism. Methylation of promoter regions can silence genes, which can lead to certain diseases (Bird and Wolffe, 1999). DNA methylation usually occurs in CpG islands, and hypermethylation is often associated with transcriptional inactivation (Sasaki et al., 2001; Yamada et al., 2009). Thus, the current
study quantified methylation levels of the FST promoter in peripheral blood and endometrial samples from a large sample of PCOS patients and controls.

Sequenom's EpiTYPER assay has created the opportunity for high-throughput quantitative analysis of DNA methylation status using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and MassCLEAVETM reagent (Sequenom), which is based on base-specific (C/T) cleavage reactions (Ehrich et al., 2005; Stanssens et al., 2004). The approach can be adapted to discovery of methylation in large stretches of genomic DNA with a single cleavage reaction, where fractions with at least 5% methylated DNA can be detected in mixtures. It can also be adapted to other needs in DNA methylation analysis, including semiquantitative analysis of the relative methylation and methylation pattern analysis. The robustness of this approach for quantifying methylated and unmethylated DNA has been previously demonstrated by other investigators (Rapdour et al., 2008, 2009; Bellido et al., 2010; Zhao et al., 2011).

The CpG island is defined as regions of DNA greater than 200 bp with a G+C content >0.5 and observed/expected presence of CpG ≥0.6 (Gardiner-Garden and Frommer, 1987). Evidence demonstrated that there is a close relationship between methylation level of CpG islands and corresponding gene expression (Deaton and Bird, 2011). Thus, although in the current study the methylation level of CpG 22,23 site in amplicon 1 was significantly higher in peripheral blood of PCOS, there are no consecutive CpG sites (CpG islands) associated with the disease, which demonstrates that methylation of the FST gene may not be responsible for PCOS.

Considering that methylation occurs in a tissue-specific pattern, the current study further investigated methylation level in endometrial samples. The endometrium is a unique organ that undergoes rapid cycling of cell proliferation, differentiation and apoptosis under the influence of ovarian hormones (Talbi et al., 2006). Dysregulated molecular phenotypes have been identified in the endometria of patients with PCOS (Kim et al., 2009; Illingworth et al., 2008). The methylation levels of the CpG site in FST in endometrial tissue were further examined sampled from 24 PCOS patients and 24 controls. No significant difference in methylation of the CpG site was identified in PCOS patients compared with controls. Similarly, we found no significant difference in FST mRNA expression in endometrial tissue from cases and controls.

In conclusion, as far as is known, this is the first study to quantitatively evaluate methylation levels of a PCOS candidate gene. The methylation levels in the promoter and 5'-UTR of the FST gene were determined in peripheral blood and endometrial samples from patients with PCOS and controls. Methylation levels of CpG 22,23 were significantly increased in the peripheral blood of patients with PCOS in comparison with controls. However, the lack of differences between cases and controls in methylation at other CpG sites demonstrate that abnormal methylation in the promoter and 5'-UTR region of the FST gene is not responsible for PCOS. Because aberrant DNA methylation patterns can serve as epigenetic biomarkers for early detection of PCOS, further studies should be performed to examine methylation of other candidate genes. Ultimately, understanding the epigenetic mechanisms involved in PCOS may yield new insights into the pathophysiology of the disorder.

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