DNA methylation of the androgen receptor gene promoter in the granulosa cells of polycystic ovary syndrome patients

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Abstract. Polycystic ovary syndrome (PCOS) is an endocrine disorder in women that is characterized by hyperandrogenism. The androgen effect is mediated by the androgen receptor (AR) expression levels. Epigenetic mechanisms that regulate AR expression and its activities in granulosa cells in the ovaries may promote the progression of PCOS. The aim of this study was to determine the DNA methylation status of the AR gene promoter in the granulosa cells of PCOS patients. In this cross-sectional study, the degree of DNA methylation of the AR promoter in granulosa cells obtained from 10 women who underwent PCOS was compared to that of eight women without PCOS as controls. DNA from the samples was isolated and sodium bisulfite-converted. Methylation-specific polymerase chain reaction (MSP) was used to amplify the DNA. The MSP products were subjected to gel electrophoresis. The band intensities of the samples and positive controls were measured using ImageJ software. Statistical analysis was performed using the independent t-test with significance at P < 0.05. The DNA methylation level of the AR gene promoter was lower in women with PCOS, as compared with the controls (P = 0.01). The AR promoter was hypomethylated in the granulosa cells of PCOS patients, which may increase AR levels leading to hyperandrogenism in women with PCOS.

1. Introduction
Polycystic ovary syndrome (PCOS) is a complex hormonal disorder occurring in about 6%–15% of young women and is characterized by hyperandrogenism, oligomenorrhea, chronic anovulation, and hyperinsulinemia [1,2]. About 75% of the PCOS cases result in anovulatory infertility [1,3]. In Indonesia, PCOS accounts for 8%–10% of cases of infertility [4]. The etiology and pathogenesis of PCOS and related metabolic abnormalities are not entirely understood [5]. Many factors contribute to the development of PCOS, including genetic predisposition as well as environmental and lifestyle factors [9]. Emerging evidence suggests that epigenetic modification or regulation contributes to the development of PCOS and accumulating data strongly suggest that epigenetic factors are critical to the development of PCOS [5,6]. The clinical presentation of PCOS is highly variable, with androgenic...
effects thought to be the most common mechanism responsible for the phenotype of PCOS. Excess androgen production by the ovaries and in the circulation can cause the characteristic features of PCOS of hirsutism, acne, poly follicular ovarian morphology, and menstrual cycle irregularities resulting in anovulation, oligomenorrhea, and infertility [7,8]. The normal development of primary follicles can be impaired by excessive androgen production, with a marked increase in preantral and antral growth, as well as atresia of follicular development [9]. The activities and effects of androgens are facilitated by the androgen receptor (AR) [7], which is a nuclear transcription factor encoded by the AR gene, a member of the steroid receptor superfamily that mediates the cellular functions of testosterone and dihydrotestosterone (DHT) [7]. The AR gene is located on chromosome Xq12 and is composed of eight exons. AR is characterized by a modular structure that includes four functional domains: an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a COOH terminal domain (CTD) [7,10]. AR expression in increased in granulosa cells in PCOS [11].

Epigenetic mechanisms such as methylation of CpG dinucleotide-rich clusters (CpG islands), usually along the promoter regions of genes, can change the transcriptional activity of the AR gene [12]. DNA methylation is a tempting biomarker for epigenetic modification of the genome; it typically inhibits gene expression and has potential diagnostic utility [5,6]. Many studies have reported epigenetic changes as potential mechanisms of PCOS development [6]. The aim of this study was to identify the methylation status of the AR gene in granulosa cells in PCOS patients who underwent in vitro fertilization (IVF), as compared to women without PCOS. Identification of specific epigenetic changes of the AR gene in granulosa cells may be useful to define the etiology of PCOS.

2. Materials and Methods
Granulosa cells were collected from all participants after signing an informed consent form to participate in this study. Samples were collected from 10 women aged 20–40 years that were diagnosed with PCOS according to the Rotterdam criteria [2] and who underwent IVF treatment. As controls, granulosa cells were collected from eight women without PCOS who underwent IVF treatment due to infertility of their partners. Granulosa cells of the patients and controls were carefully collected by the ovum pick-up method and stored in RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA). The granulosa cells were separated from the RNAlater solution by centrifugation. DNA was extracted from the granulosa cells using the Wizard® Genomic Purification Kit (Promega Corporation, Madison, WI, USA) and quantified using a micro-volume spectrophotometer (Maestrogen, Inc., Hsinchu City, Taiwan).

The DNA from granulosa cells was converted by sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen, Hilden, China). For DNA methylation profiling, 20 ng of bisulfite-converted DNA was amplified by the methylation-specific polymerase chain reaction (MSP) using the KAPA Hifi Hotstat Uracil+ReadyMix kit (Kapa Biosystems, Inc., Wilmington, MA, USA). Methylated and non-methylated primers for MSP were designed using the MethPrimer tool (www.urogene.org/methprimer/) following the methylation primer MSP were (Forward-M) 5’ TTATAAGTTCCGGAGTATTGGACGA-3’ and (Reverse-M) 5’ AACGTAAAAATAAAAAACGACGAA-3’. The unmethylation-primer were (Forward-U) 5’ TTATAAGTTCCGGAGTATTGGATGA-3’ and (Reverse-U) 5’ AACATACAAAAATAAAAACACACAAAT-3’. MSP products were visualized by gel electrophoresis with 2.4% agarose gel containing ethidium bromide under ultraviolet light. The band intensities of the MSP products from patients and control samples as well as positive controls were determined using ImageJ software (https://imagej.nih.gov/ij/). The DNA methylation level of the AR gene was calculated as the ratio of the band intensity of the samples to that of the positive controls. Statistical analyses were conducted using the independent t-test. A probability (P) value of <0.05 was considered statistically significant.

3. Results and Discussion
DNA methylation is an important epigenetic modification for the regulation of gene expression. DNA hypomethylation and hypermethylation are alternative mechanisms for gene activation and silencing, respectively [14]. Findings from the electrophoresis of the MSP products of the AR gene of women diagnosed with PCOS are presented in Figure 1a. As shown, there was one non-methylated band in column 5. Recent studies have found that samples from healthy woman have methylated bands (Figure 1b).

**Figure 1.** (a) Electrophoresis of MSP products of the AR gene from women with PCOS. (b) Electrophoresis of MSP products of the AR gene from healthy women. Gel electrophoresis of MSP products of the AR gene with methylated primer (115bp) and unmethylated primer (116 bp) from granulosa cells collected from (a) women with PCOS (1–5) and (b) healthy controls (6-10). m, DNA Marker, M, methylated; U, unmethylated; (+), positive control; (−), no template control.

There was a significant difference in the DNA methylation levels of the AR gene, calculated as the ratio of the band intensities of the samples to that of the positive control (39.7% in women with PCOS vs. 82.2% in healthy women, \( p = 0.001 \), Figure 2) and as determined with ImageJ software.

**Figure 2.** Methylation level of the AR gene from granulosa cells in women with PCOS and in healthy controls. DNA methylation level of AR gene in women with PCOS decreased significantly statistically compared to control (*\( p = 0.001 \)).

PCOS is a heterogeneous disease for which there is no clear consensus regarding diagnosis and pathogenesis. Many factors contribute to the pathogenesis of PCOS, including genetic predisposition, hyperandrogenism, and various environmental and lifestyle factors [9,15]. Hyperandrogenism is the
most consistent feature of PCOS based on evidence that excess androgen production can induce reproductive, metabolic, and endocrine characteristic of PCOS in rodent, sheep, and primate models. Androgen activities are mediated by the AR, and classical AR activities are critical for normal ovarian function [1,10]. A prior animal study found that coupling of androgen with the AR can augment the development of the preantral follicles of mice and rats in a culture system, and that androgen induces apoptosis of granulosa cells. AR expression in rodents and primates regulates follicular development [16,17], while AR expression is regulated by epigenetic factors, including DNA methylation, which could be responsible for the PCOS phenotype. DNA methylation of ovarian granulosa cells is associated with gene expression in PCOS [6]. Epigenetic alterations to the genome such as hypomethylation of the AR promoter could induce excess androgen production [5].

Genomic studies conducted by Xu et al., Yu et al., and Wang et al. have reported wide DNA methylation in the granulosa cells and peripheral blood leucocytes of women with PCOS. In the present study, the AR methylation patterns were assessed using ImageJ software. This study is the first to report hypomethylation of the AR gene promoter in granulosa cells of women with PCOS as compared to healthy controls. Hypomethylation of the AR promoter could result in the binding of the transcription factor to the promoter with subsequent excess production of AR. This assumption is in accordance with the findings of Jonard et al. who showed that the AR mRNA levels were significantly higher in PCOS patients than in controls, especially in the small follicles, and that excessive AR expression in PCOS patients is reportedly a consequence of hyperandrogenism [11].

AR is a steroid transcription factor required for the cellular activities of the androgens testosterone and DHT [10]. The protein and mRNA levels of AR have been monitored in the granulosa cells of the ovaries. Hypomethylation of the AR promoter may contribute to the phenotype of PCOS by impairment of the AR activity. Small amounts of androgen in the ovaries promote follicular growth in the early stages of ovarian follicle development [17] and excess androgen in PCOS, which is mediated by abundant AR expression, could have a negative impact on follicular development, causing atresia and inhibition of meiotic maturation [9]. AR promoter hypomethylation has been suggested to promote hirsutism in women with PCOS. Hirsutism is the presence of excess terminal hair resembling patterns common to those of males in androgen-dependent areas of the female body [19]. Due to an abundance of the AR in the dermal papilla, excessive testosterone and DHT bind to the AR with high affinity and promote the development of hair on the upper lip, chin, chest, arms, upper and lower abdomen, upper and lower back, and thighs. In the sebaceous glands, AR is thought to promote acne in women with PCOS.

4. Conclusion
The AR promoter in granulosa cells in PCOS patients is hypomethylated, which increases AR levels, resulting in hyperandrogenism in women with PCOS. The action of androgen via AR may contribute to the phenotype of PCOS.

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