DNA methylation analysis of anti-mullerian hormone gene in ovarian granulosa cells in PCOS patients

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Abstract. Polycystic ovary syndrome (PCOS) is defined as a complex hormonal disorder that is commonly found in reproductive-age women. The pathogenesis and etiology of PCOS have not been fully understood. It is strongly believed that PCOS is caused by the interaction of numerous complex factors, both environmental and genetic. One factor that is known to play a role in the pathogenesis of PCOS is an increase in anti-Mullerian hormone (AMH). In this study, we analyzed the methylation levels of the AMH gene as it relates to elevation of AMH levels in PCOS patients. In this cross-sectional study we used MSP-PCR method to amplify the DNA samples, which are obtained from the granulosa cells of 13 women with PCOS and 9 women without PCOS. The methylation levels then measured using ImageJ software. We found that there was a statistically significant difference between the methylation percentage of DNA from patients in the PCOS group compared to the control group, (p = 0.001). The PCOS group had a lower methylation percentage compared with the normal group. Our results suggest that a decreased methylation level of the AMH gene may cause an increase in AMH concentration in ovarian follicles and has a correlation with the pathogenesis of PCOS. AMH methylation level could therefore be used as a biomarker for diagnosis of PCOS.

1. Introduction
Polycystic ovary syndrome (PCOS) is defined as a complex hormonal disorder that is commonly found in reproductive-age women [1]. Varied criteria are used to determine a diagnosis of PCOS. However, there are no universally-accepted diagnostic criteria for PCOS. This situation results in PCOS having varied prevalence data depending on which diagnostic criteria are used. The European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) estimates that 15-20% of women suffer with this disorder using their diagnostic criteria. However, the National Institute of Health diagnostic estimates that there are 4-8% women with this disorder [2].

The ESHRE/ASRM consensus defines PCOS as a disorder mainly characterized by biochemical or clinical evidence of elevated androgen levels, cysts formed in one or both of the ovaries (observed
using ultrasonography); and menstrual irregularities (oligo-ovulation) [3]. A woman is suspected to have PCOS if she has two of these three characteristics. Besides the three main characteristics, women with PCOS can present with other features such as elevated serum luteinizing hormone (LH) concentration, obesity, insulin resistance and an increased risk of type 2 diabetes. In PCOS patients, not only serum LH concentration can be elevated, but also testosterone and androstenedione serum levels. In addition, follicle-stimulating hormone (FSH) serum may be lower [4].

The National Institute of Health defines PCOS as a disorder characterized by three features: hyperandrogenism, chronic anovulation (ovulatory dysfunction) and the absence of other endocrine disorders. Hyperandrogenism can be clinically- or laboratory-proven. Clinically, a PCOS patient will present with hirsutism (male-pattern hair growth, such as the presence of a moustache), acne, and/or frontal alopecia. Laboratory diagnosis includes the determination of elevated androgen serum levels without signs of other endocrine disorders (e.g., hyperprolactinemia, congenital adrenal hyperplasia or thyroid abnormalities) [5,6].

Regardless of its varied diagnostic criteria and prevalence data, PCOS is considered a serious issue since it is one of the most common endocrinopathies in women and has many complications that can affect a woman’s health. Women with this disorder have been shown to carry a greater risk of developing many complications, both short- and long-term. Short-term complications of PCOS include a greater tendency to be infertile and having obstetric complications (such as preeclampsia, gestational diabetes mellitus, and preterm delivery). Long-term complications include greater cardiovascular risk (hypertension, dyslipidemia, atherosclerosis, coronary heart disease and stroke), metabolic risk (obesity and diabetes mellitus), oncology risk (endometrial cancer, ovarian cancer and breast cancer) and other disorders (reduced quality-of-life and psychological disorders) [5,6,7].

Currently, the pathophysiology of PCOS is not fully understood, since it is very complex and affected by many factors, both environmental and genetic. Genetic studies of PCOS also face many challenges in identifying genes that contribute to the development of this disorder [8]. One of the genes that is suspected to have a strong correlation with PCOS is the anti-Mullerian hormone (AMH) gene. AMH is a member of the transforming growth factor-β superfamily gene and the determinant factor of AMH production. AMH is exclusively secreted by ovarian granulosa cells in antral and preantral follicles and is believed to reflect the number of ovary primordial follicles [9]. AMH has a role in inhibiting the growth of follicles since it decreases the growing follicle’s responsiveness to FSH, causing it to be arrested in a small antral follicle state and unable to develop to the mature state. In addition, AMH has an inhibitory effect in aromatase enzyme activity, which has a role in the conversion of androgen into estradiol, leading to overconcentration of androgen [10].

The AMH gene is further suspected to have a role in the pathogenesis of PCOS since PCOS patients are usually found to have an elevated serum AMH level. It was proved in the study conducted by Villarroel et al. that AMH serum level has a positive correlation with PCOS [11]. Moreover, several studies also suggest that AMH could be used as a tool to diagnose PCOS [12].

Until now, there have been no studies that investigate AMH level directly from the granulose cells of the ovaries. Therefore, this study’s aim is to determine whether an increased level of AMH is caused by an epigenetic factor, i.e., the levels of methylation of the AMH gene in granulosa cells.

2. Materials and Methods

Ovarian large-follicle tissues were collected after informed consent by laparoscopy procedure from 9 normal women (control) and 13 PCOS patients who were due to undergo in vitro fertilization (IVF) procedures. DNA from the sample tissues were extracted by Qiagen DNA Extraction Kit and the concentration was measured by Maestrogen Maestro Nano Spectrophotometer (USA). The DNA from samples then underwent bisulfite conversion procedure by using EpiTect Bisulfite Kit (48) - QIAGEN before being amplified through MSP-PCR (Methylation Specific Polymerase Chain Reaction) using EpiTect MSP Kit - QIAGEN. The primer sets for MSP are shown in Table 1.
Table 1. Primer sets for MSP-PCR

| Primer Name                          | Sequence                  |
|-------------------------------------|---------------------------|
| AMH Unmethylated F (Forward)        | GTTGAGTGTTTTGTATTTATTTTT |
| AMH Unmethylated R (Reverse)        | TCTTCAACAAACACACACATA     |
| AMH Methylated F (Forward)          | CGAGCGTTTCTATTATTTTTC     |
| AMH Methylated R (Reverse)          | TCTTCAACAAACACACACCGTA    |

The MSP product was then run in 2.4% agarose gel containing ethidium bromide. The band intensity appearing in the image was measured using ImageJ Software.

The percentage of methylation level of each DNA sample was determined by comparison of band intensity from MSP with methylated primers to total band intensity from MSP with methylated and unmethylated primers. Methylation percentage data was then statistically analyzed using the Statistical Program for Social Sciences (SPSS) software version 23. The association of methylation percentage with two groups was analyzed by t-test or Mann-Whitney test, depending on the results of the normality test. The result of p value < 0.05 showed that there was a statistically significant difference between the two groups.

3. Results

Gel electrophoresis of the MSP product of the AMH gene from the samples are shown in Figures 1.

![Gel Image](image)

**Figure 1.** Gel electrophoresis of MSP product of AMH

M=MSP Product with methylated primer; U=MSP Product with unmethylated primer; P(1 and 2)=gene from PCOS patient; C(3 and 4)=gene from control patient (normal women).

The mean percentage of methylation of the AMH in samples from the control group was 69.37%, while methylation of AMH in PCOS patients was 39.56%. Statistical analysis using Mann-Whitney Test from methylation data showed there is significant difference between methylation levels of AMH in the granulosa cells of PCOS patient compared to the control group p = 0.001 (Figure 1).
Figure 2. Methylation levels of AMH in granulosa cells of normal control group (69.37%) and PCOS patients (39.56%).

There is significant difference of methylation of AMH in the control group compared to the PCOS group (p = 0.001)

4. Discussion

Since AMH is only expressed by the granulosa cells of the ovaries and only brings effects to the reproductive organs, AMH could take an important role in the diagnosis of PCOS if it is proved to have a correlation with PCOS status [12].

AMH plays a role in inhibiting follicular growth (follicular arrest), and has been shown to reduce the growth of follicles by 40-50% in vitro [13]. Normally, AMH levels increase and reach maximum levels at puberty; then decrease and reach minimum levels after menopause. In PCOS patients, AMH is usually found at elevated levels, often two to three times higher than normal [14]. Since AMH is produced in the granulosa cells of the ovaries, elevated levels of AMH indicate that there is also an increase in the granulosa cell number. This finding is in line with the theory that states that PCOS patients have a higher follicle count compared to normal women. In the study held by Christiansen et al., AMH found to have positive correlation with follicle count [15].

Overproduction of AMH has been found by several studies to have correlation with the severity of PCOS manifestations such as hyperandrogenism, amenorrhea, or polycystic ovarian manifestations [16,17]. A study held by Dewaily et al. also found that this elevation of AMH level can be used as a diagnostic tool that is more specific and sensitive compared with the current diagnostic tool. From this study, Dewaily et al. found that the cut-off serum AMH level for PCOS is >35pmol/L or >5ng/Ml [18]. However, a study conduct by Illiodromity found that the cut off value of AMH is 4.7 ng/Ml [19]. In contrast to these two studies, meta-analysis performed by Wang et al. showed that there was no correlation of AMH variants with the increased risk of PCOS [20].

In our study, we analyzed the methylation level of the AMH gene in granulosa cells from PCOS patients compared to normal women. Methylation has a role in suppressing AMH expression. Thus, if methylation is high, the suppression of AMH expression will also be high, and the AMH level will be lower than normal. In PCOS patients, AMH levels are usually found to be higher than normal which indicates there is an increase in AMH expression. The increase in AMH expression could be caused by a decrease in methylation of its gene. Therefore, in PCOS patients, theoretically there will be a hypomethylation (lower methylation) of AMH compared with normal women.

Different from other previous studies that have mainly used serum as the sample source, we used granulosa cells as our sample. In our study, we found that the mean methylation percentage of AMH in PCOS patient (39.56%) was lower than the methylation percentage of AMH in the normal control
group (69.37%). This result is in line with the theory which states that PCOS patients tend to have hypomethylation of the AMH gene [15]. Our study found that women with PCOS did demonstrate hypomethylation of the AMH (p-value < 0.05).

5. Conclusion
Decrease in methylation level of AMH in granulosa cells may have a correlation with the pathogenesis of PCOS. The methylation level of AMH could be used as a biomarker for the diagnosis of PCOS. Further research with a bigger sample size is needed to establish this.

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References
[1] Sheehan M T 2004 Polycystic ovarian syndrome: diagnosis and management Clin. Med. Res. 2 13-27
[2] Sirman S M and Pate K A 2013 Epidemiology, diagnosis, and management of polycystic ovary syndrome Clin. Epidemiol. 6 1-13.
[3] Ndefo A U, Eaton A, Green M R 2013 Polycystic ovary syndrome: a review of treatment options with a focus on pharmacological approaches P & T 38, 336-355
[4] Hamzeh R and Balen A H 2006 Up-to-date definition of the polycystic ovary and polycystic ovary syndrome Ultrasound 14 142-144
[5] Lakkakula B V, Thangavelu M and Godla UR 2013 Genetic variants associated with insulin signaling and glucose homeostasis in the pathogenesis of insulin resistance in polycystic ovary syndrome: a systematic review J. Assist. Reprod. Genet. 30 883-95.
[6] Palomba S, Santagni S, Falbo A, and Sala G B L 2015 Complications and challenges associated with polycystic ovary syndrome: current perspectives Int. J. Womens. Health. 7 745-763.
[7] Kosova G and Urbanek M 2013 Genetics of the polycystic ovary syndrome. Mol. Cell. Endocrinol. 373 29-38.
[8] Tal R, Seifer D B, Khanimov M, Malter H E, Grazi R V and Leader B 2014 Characterization of women with elevated antimüllerian hormone levels (AMH): correlation of AMH with polycystic ovarian syndrome phenotypes and assisted reproductive technology outcomes Am. J. Obstet. Gynecol. 211 59-e1.
[9] Dumont A, Robin G, Jonard S C and Dewailly D 2015 Role of Anti-Müllerian Hormone in pathophysiology, diagnosis and treatment of Polycystic Ovary Syndrome: a review Reprod. Biol. Endocrinol. 13 137
[10] Villarroel C et al. 2011 Polycystic ovarian morphology in adolescents with regular menstrual cycles is associated with elevated anti-Mullerian hormone Codner. Hum. Reprod. 26 2861-8.
[11] Pigny P, Jonard S, Robert Y and Dewailly D 2006 Serum anti-Mullerian hormone as a surrogate for antral follicle count for definition of the polycystic ovary syndrome J. Clin. Endocrinol Metab. 91 941-5.
[12] Weenen C, et al. 2004 Anti-Müllerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment Themmen. Mol. Hum. Reprod. 10 77-83
[13] Qi X, Pang Y and Qiao J 2016 The role of anti-Müllerian hormone in the pathogenesis and pathophysiological characteristics of polycystic ovary syndrome. Eur. J. Obstet. Gynecol. Reprod. Biol. 199 82-7.
[14] Christiansen S C, Eilertsen T B, Vanky E and Carlsen S M. Does AMH reflect follicle number similarly in women with and without PCOS? PloS. ONE. 11 e0146739.
[15] Garg D and Tal R 2016 The role of AMH in the pathophysiology of polycystic ovarian syndrome. Reprod. Biomed. Online. 33 15-28.
[16] Mahran A 2016 The relationship between Anti-Müllerian hormone and the clinical, biochemical and sonographic parameters in women with polycystic ovarian syndrome *Middle. East. Fertil. Soc. J.* 21 11-5.

[17] Dewailly D, et al. 2011 Diagnosis of polycystic ovary syndrome (PCOS): revisiting the threshold values of follicle count on ultrasound and of the serum AMH level for the definition of polycystic ovaries *Hum. Reprod.* 26 3123-9.

[18] Illiodromiti S, Kelsey T W, Anderson R A and Nelson S M 2013 Can anti-Mullerian hormone predict the diagnosis of polycystic ovary syndrome? A systematic review and meta-analysis of extracted data *J. Clin. Endocrinol. Metab.* 98 3332

[19] F Wang, B Niu, H J Kong, H Y Guo and Sun Y P 2017 The role of AMH and its receptor SNP in the pathogenesis of PCOS *Mol. Cell. Endocrinol.* 439 363-368