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# Association of DNA Methylation of AMHRII and INSR Genes with the Pathogenesis of Polycystic Ovary Syndrome

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## Abstract

Polycystic Ovary Syndrome (PCOS) is a common gynaecologic endocrinopathy characterised by menstrual disorders, continuous ovulation disorders, high androgen syndrome, and polycystic ovaries, but the mechanism underlying PCOS requires further research. Our objectives were to investigate the clinical and biochemical features of PCOS, and the expression and regional distribution of anti-Müllerian receptor II (AMHRII) and Insulin Receptor (INSR), and to determine the methylation levels of AMHRII and INSR genes in patients with PCO. INSR was highly expressed in the endometrium and stromal vascular endothelial cells of patients with PCOS, which may be correlated with hyperplasia of the endometrium and ovarian stroma. Some areas of elevated AMHRII expression were observed in the endometrium of patients with PCOS, indicating that AMHRII also participated in endometrial lesions. Above all, analysis of DNA methylation suggested that methylation of AMHRII and INSR was associated with insulin resistance and the basic clinical characteristics of PCOS. Our results provide evidence that the AMHRII and INSR genes and their methylation levels are intimately associated with the pathogenesis of PCOS.

**Key words:** Anti-Müllerian hormone; Insulin receptor; DNA methylation; Polycystic ovary syndrome

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## Introduction

Polycystic Ovary Syndrome (PCOS), a common endocrine disorder characterised by menstrual disorders, anovulation, polycystic ovaries, hyperandrogenism, and hyperinsulinemia [1], affects 6-8% of adolescent women [2]. PCOS has various clinical manifestations, showing marked heterogeneity and the familial aggregation phenomenon. Although the exact aetiology and pathogenesis of PCOS remain unclear, genetic factors are believed to play important roles. In recent years, many researchers have focused on the two major secretory characteristics of PCOS, hyperandrogenism and hyperinsulinemia [3], and on PCOS-related genes [4], but the epigenetics of PCOS has rarely been studied.

Anti-Müllerian Hormone (AMH), a member of the transforming growth factor-β family, is produced by the granulosa cells of the ovary [5]. AMH plays important roles in ovarian primordial follicle recruitment and follicular growth [6]. Association studies of AMH-induced local pathological changes have concentrated mainly on the abnormal expression and regulation of local ovarian regulatory factors that lead to a series of pathophysiological changes. AMH levels are two to three times higher in patients with PCOS than in women without PCOS, and AMH levels have high sensitivity and specificity for the diagnosis, follow-up, and prognosis of PCOS [7]. Previous research has shown that AMH exerts biological functions through its corresponding receptors (mainly AMH receptor II [AMHRII]) [5]. However, the role of AMHRII in local ovarian and endometrial pathological changes in PCOS has been rarely reported. Additionally, the molecular genetics mechanism of AMHRII in the process of PCOS has not been described.

Hyperinsulinemia is another important pathophysiological feature of PCOS, manifesting mainly as clinical Insulin Resistance (IR). Recent studies have documented IR in the ovaries and endometria of patients with PCOS [8]. The mechanism of IR is thought to be associated with the number and function of insulin receptors (INSRs); thus, INSRs have become an important target for IR. A previous study also suggested that INSR is one of the susceptibility genes in patients with PCOS [9,10].

With the development of molecular genetics, and especially the field of epigenetics, many scholars have tried to identify genes associated with the typical pathological characteristics of PCOS, and have investigated epigenetic changes in PCOS-related genes to explain the high degree of heterogeneity and the familial aggregation phenomenon. In the present study, we analysed the clinical characteristics of PCOS, and investigated the expression and regional distribution of AMHRII and INSR in the ovary and endometrium. Further, we analysed DNA methylation, a common method for epigenetics examination, and determined the DNA methylation levels of PCOS-related genes to explore possible causes of the pathogenesis of PCOS.

## Materials and Methods

### Subjects and phenotyping

A total of 75 women with PCOS aged 20-39 years and hospitalised at the Family Planning Specialised Hospital of Guangdong Province

from March to December 2014 were selected as case study participants. PCOS was diagnosed based on the European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine Rotterdam criteria. All patients enrolled in the study were required to provide comprehensive general information and register their age, blood pressure, height, weight, hair distribution, and other indices. The position and volume of the uterus, antral follicle count, and endometrial, ovarian, and pelvic situation were recorded based on the results of detailed physician inspection of the subjects' uterus and bilateral attachments. No patient had received hormonal therapy for at least 3 months.

Twenty healthy women were enrolled randomly as controls during reproductive health examinations conducted between September and December 2014. These women were screened carefully to rule out other diseases. They had normal reproductive systems, sexual hormone levels, immune antibodies, and chromosomes, and had not received hormonal therapy for at least 3 months prior to enrolment.

All subjects who met the inclusion criteria and agreed to participate in the study provided written informed consent to the use of all clinical data and blood samples for our research, and the study was performed under the approval and supervision of the ethics committee of the Family Planning Specialised Hospital of Guangdong Province.

### Reproductive hormone analysis

Patient blood samples were collected 2-4 days after onset of the menstrual cycle, and serum levels of Oestradiol (E2), Progesterone (P), Testosterone (T), Luteinizing Hormone (LH), and Follicle Stimulating Hormone (FSH) were measured using the enzyme-linked immunosorbent assay (ELISA) method. The FSH/LH ratio was calculated to assess the presence of endocrine diseases, such as PCOS, hyperandrogenism (T > 2.81 nmol/L), and hyperprolactinemia (HPRL; PRL > 560 mIU/L). Serum Triiodothyronine (T3), Thyroxine (T4), Thyroid Stimulating Hormone (TSH), AMH, Fasting Insulin (FINS), and Fasting Plasma Glucose (FPG) levels were assayed to determine the presence of thyroid dysfunction and diabetes. The IR index (HOMA-IR) was calculated as FINS (mU/mL) × FPG (mmol/L) / 22.5, and IR was diagnosed as HOMA-IR > 2.6.

### Haematoxylin and eosin (HE) staining

Twenty paraffin specimens of PCOS ovaries from ovarian wedge resections and 20 paraffin specimens of normal ovarian tissue obtained from the surgical removal of nest cysts and ovarian benign fibromas were examined as the research group and control group, respectively. Twenty paraffin specimens of endometrial tissue from infertile patients with PCOS undergoing hysteroscopy for repeated failure of in vitro fertilisation and embryo transfer were obtained as the research group, and 20 paraffin specimens of normal proliferative endometria were collected as the control group. These paraffin specimens were cut into 4-μm-thick sections, then stained with HE or immunohistochemical stains. Histopathological morphology was analysed and identified by HE staining, and the expression and regional distribution of AMHRII and INSR in the ovary and endometrium were assessed by immunohistochemical staining.

### Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany), and reverse transcription was performed in a total volume of 20 μL with the first-strand cDNA synthesis kit for RT-PCR (Roche Diagnostics, Indianapolis, IN, USA) using RNA from tissues, M-MLV reverse transcriptase, and random primers, following the manufacturer's instructions. The primers were designed by PyroMark Assay Design 2.0 (Qiagen) and synthesised by the Beijing Genomics Institute (Beijing, China). The primer sequences were as follows: AMHRII, forward strand

5′-TGTGTTTCTCCCAGGTAATCCG-3′ and reverse strand

5′-AATGTGGTCGTGCTGTAGGC-3′, with a PCR product of 164 bp; and INSR, forward strand

5′-CCCGCATTCAAAGAGGT-3′ and reverse strand

5′-AGAGCAGTTGGACGAGGA-3′, with a PCR product of 169 bp. The qRT-PCR reaction was amplified using 40 cycles of denaturing for 40 s at 94 °C, annealing for 40 s at 56.2 °C, and extension for 40 s at 72 °C. Relative quantification of AMHRII and INSR gene expression was conducted based on the ratio of each target gene concentration to that of the housekeeping gene, beta-actin.

### Western blot analysis

Tissue samples were homogenised in ice-cold tissue lysate buffer and proteins were extracted according to the manufacturer's instructions (Beyotime, Haimen, China). The total protein concentration of the supernatant was determined using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA). Proteins (100 μg) were loaded onto a gel for sodium dodecyl sulphide polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad Laboratories, Hercules, CA, USA), then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked at 37 °C for 2 h with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20. AMHRII and INSR were detected using primary polyclonal rabbit anti-AMHRII antibody (Abcam, Cambridge, UK) followed by secondary HRP-conjugated goat anti-rabbit IgG (Abcam), and primary monoclonal mouse anti-INSR antibody (NeoMarkers, Fremont, CA, USA) followed by secondary HRP-labelled anti-mouse IgG (Jackson ImmunoResearch Laboratories, Bar Harbor, ME, USA), respectively. The bands were visualised using the ECL Plus detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and band intensities were quantified using image analysis software (Bio-Rad Laboratories).

### Immunohistochemistry of AMHRII and INSR receptors

Ovarian and endometrial specimens were collected from 20 patients with PCOS and 20 control women and then cut into 4-μm-thick sections. The sections were deparaffinised in xylene and rehydrated, then repaired in citric acid antigen repair solution. After blocking for 30 min with 10% normal goat serum, the sections were incubated overnight with the following antibodies: rabbit anti-mouse AMHRII (Abcam) and rabbit anti-INSR (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (DAKO, Glostrup, Denmark) for 30 min, and stained with Diaminobenzidine (DAB) and counterstained with hematoxylin. Semi-quantitative analysis of optical density on immunohistochemical images was used to characterise the position and distribution of AMHRII and INSR in the ovary and endometrium.

### DNA methylation of AMHRII and INSR genes

**Genomic DNA extraction:** Whole blood from 40 patients with PCOS and 20 healthy female donors was used for genomic DNA extraction. The AMHRII and INSR genes, which are associated with the pathogenesis of PCOS, were selected for DNA methylation analysis in all samples. Genomic DNA from whole blood was extracted by the phenol-chloroform method [11], with some modifications due to the different volumes of whole blood used. All DNA extracts were stored at -20 °C until further use.

**Bisulphite treatment:** Each DNA extract was dissolved in bisulphite solution and then diluted with 800 μL RNase-free water. Reaction reagents for bisulphite treatment were prepared in 200-μL thin-walled PCR tubes according to (Table 1). After mixing well, the thin-walled PCR tubes were placed in a PCR instrument (Beijing Dongsheng Innovation Biotech Co., Beijing, China) for bisulphite-mediated DNA conversion.

**PCR of bisulphite-treated DNA:** Primers directed to bisulphite-treated DNA were designed using PyroMark Assay Design 2.0 (Qiagen) and then synthesised by the Beijing Genomics Institute. Bisulphite-treated DNA was amplified with a reaction protocol of 95 ˚C for 3 min followed by 40 cycles of 94 ˚C for 30 s, 54 ˚C for 30 s, and 72 ˚C for 1 min, then 72 ˚C for 7 min, using a 50-μL PCR reaction system containing 10 μL 5× buffer GC, 1 μL 10 mmol/L dNTPs, 1 μL 50 pmol/L of each primer, 2 μL DNA template, 0.2 μL 5U/μL Taq DNA polymerase, and 34.8 μL water (Table 2).

**Pyrosequencing detection:** PCR products (40 μL), 2 μL (10 mg/mL) streptavidin-coated polystyrene beads, and 38 μL binding buffer were added to a 96-well PCR plate for mixing and denaturation. The substrate, enzyme, and four kinds of dNTP mixture (Qiagen) were placed in a reagent cartridge based on the amount calculated by the pyrosequencing software. The pyrosequencing reaction was carried out in a PyroMark Q96 ID pyrosequencer (Qiagen) according to the manufacturer's instructions, and the Pyro Q-CpG software was used to automatically analyse the methylation status of each site.

### Data analysis

SPSS21 software and EpiData 3.0 software were used for statistical analysis and database creation, respectively. Chi-squared tests and t-tests were used for statistical analysis of count data and measurement data, respectively. Simple linear (Pearson) correlation analysis was used to assess the correlation between IR and gene methylation.

## Results

### Clinical characteristics of patients with PCOS

In total, 75 patients with PCOS and 20 healthy controls, aged 20-39 years, were selected as research subjects. Compared with the control group, the 75 patients with PCOS exhibited diverse clinical characteristics, which included 57 (76%) cases of oligomenorrhea, 54 (72%) cases of polycystic ovary changes, 53 (70.7%) cases of hyperandrogenism, and 48 (64%) cases of IR.

Comparison of cases of normal menstruation and oligomenorrhea in 40 patients with PCOS indicated that younger patients and those with IR were more prone to oligomenorrhea (Table 3).

### HE analysis of pathological morphology

The histopathology of ovarian tissues from patients with PCOS revealed expansion of multiple vesicles, decreases in granular cell layers, and a lack of mature luteal and ovarian stromal hyperplasia; control ovarian tissues showed normal follicles at different developmental stages, relatively thick granular cell layers, and the presence of mature luteal cells (Figure 1A and B). The histopathology of endometrial tissues from patients with PCOS revealed endometrial thickening, glandular epithelial cell hyperplasia, glandular distortion, and endometrial stromal hyperplasia; control endometrial tissues showed no obvious hyperplasia (Figure 1C and D).

**Table 1:** Reaction reagents for bisulphite treatment.

| Reagent | Volume (μL) |
|---|---|
| DNA solution (1-500 ng) | Variable* |
| RNase-free water | Variable* |
| Bisulphite solution | 85 |
| DNA protection solution | 15 |
| Total volume | 140 |

* The total volume of DNA solution plus RNase-free water must equal 40 μL.

**Table 2:** Primers directed to bisulphite-treated DNA.

| Primer name | Primer sequence (5' to 3') | 5' Modification |
|---|---|---|
| AMHRII-F | GTTAATTAGGGAGGTAGAGTTTGTAGTGA | |
| AMHRII-R | AATCTAACTCCAAAATCCAAATTCT | 5' Biotin |
| AMHRII-S | GGTAGAGTTTGTAGTGAG | |
| INSR-F | GGTTGGTTTGTTGAAAATTAGAGA | |
| INSR-R | ATATCTCACTCCCCTTTCCTCAATCC | 5' Biotin |
| INSR-S | AGGTGTTATTTTTTTAATTTGAAT | |

**Table 3:** Comparison between normal menstruation and oligomenorrhea in 40 patients with PCOS.

| Normal menstruation | | Oligomenorrhea | | |
|---|---|---|---|---|
| | (*n* = 9) | (*n* = 31) | *t* | *p* |
| Age (years) | 30.89±3.44 | 27.55±3.55 | 2.5 | 0.02* |
| Weight (kg) | 57.94±6.71 | 59.53±10.93 | -0.41 | 0.68 |
| BMI (kg/m$^2$) | 22.23±3.37 | 23.57±3.80 | -0.95 | 0.35 |
| FSH (mIU/mL) | 5.37±1.20 | 5.41±2.23 | -0.05 | 0.96 |
| LH (mIU/mL) | 5.25±1.67 | 7.06±4.16 | -1.29 | 0.21 |
| LH/FSH | 1.03±0.42 | 1.35±0.68 | -1.34 | 0.19 |
| E2 (pmol/mL) | 146.67±31.10 | 135.52±37.68 | 0.81 | 0.42 |
| PRL (mIU/L) | 316.85±126.84 | 343.03±102.75 | -0.64 | 0.52 |
| P (nmol/L) | 0.68±0.21 | 0.75±0.42 | -0.47 | 0.64 |
| T (nmol/L) | 3.31±1.51 | 3.01±1.50 | 0.52 | 0.61 |
| HOMA-IR | 2.59±2.05 | 4.99±2.76 | -2.41 | 0.02* |

* P < 0.05 was considered to be statistically significant.

### mRNA and protein expression analyses

AMHRII gene expression was significantly higher in ovaries from patients with PCOS than in those from the control group (P = 0.002), whereas INSR gene expression was significantly lower in the endometria of patients with PCOS than in those of the control group (P = 0.036; Table 4).

Western blot analyses confirmed an increased amount of AMHRII protein in the ovaries of patients with PCOS compared with those of controls, and a decreased concentration of INSR protein in the endometria of patients with PCOS compared with those of controls (Figure 2 and 3).

### Immunohistochemical analysis of AMHRII and INSR

Immunohistochemical analysis revealed that AMHRII and INSR were expressed in the ovarian and endometrial tissues of patients with PCOS and controls (Figure 4 and 5). In patients with PCOS, INSR was expressed in ovarian granulosa and stromal cells, and AMHRII was expressed more obviously in granular cells, with a small amount of distribution in the ovarian stroma. In the control group, INSR and AMHRII were expressed mainly in immature ovarian granulosa cells, with a small amount of expression in ovarian stroma. Semi-quantitative immunohistochemical analyses showed significantly stronger INSR expression and slightly greater expression of AMHRII in the endometria of patients with PCOS than in those from the control group (Table 5).

### Quantitative analysis of DNA methylation

Methylation analysis of AMHRII and INSR genes DNA methylation of AMHRII and INSR genes was assessed in 40 patients with PCOS and 20 controls, and four methylation sites per gene were selected for DNA methylation analysis. The analysis revealed significant differences at positions 3 and 4 between patients with PCOS and controls (Tables 6 and 7).

### Correlations between methylation status and PCOS clinical groups

The 40 patients with PCOS were divided into four groups (a + b, a + c, b + c, and a + b + c) according to three clinical manifestations:

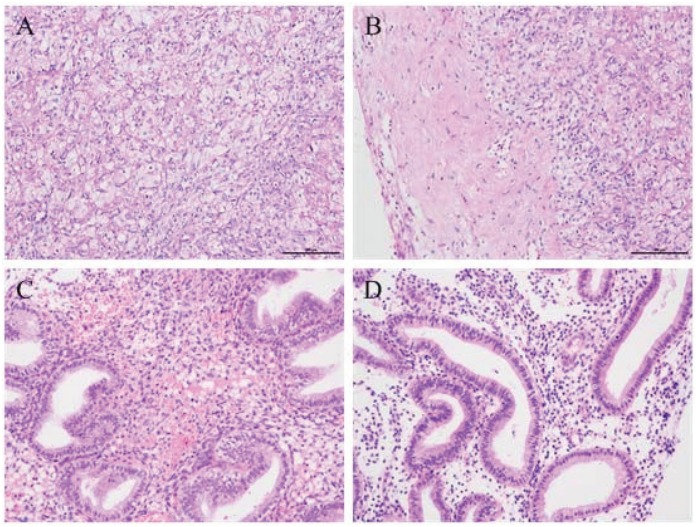


**Figure 1:** Haematoxylin and eosin (HE) staining to assess the pathological morphology of ovarian and endometrial tissues. A) Control ovarian tissue. B) Ovarian tissue of a patient with PCOS, showing thickening of the tunica albuginea and fibre hyperplasia. C) Control endometrial tissue. D) Endometrial tissue of a patient with PCOS, showing simple hyperplasia, inequal gland size, irregular shape, and less obvious polarity of the glandular epithelial cells. Original magnification 200× in all panels.

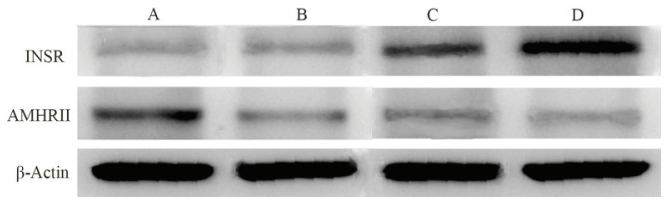


**Figure 2:** Western blot analysis of anti-Müllerian hormone receptor II (AMHRII) and insulin receptor (INSR) proteins. A) PCOS ovary. B) Control ovary. C) PCOS endometrium. D) Control endometrium.

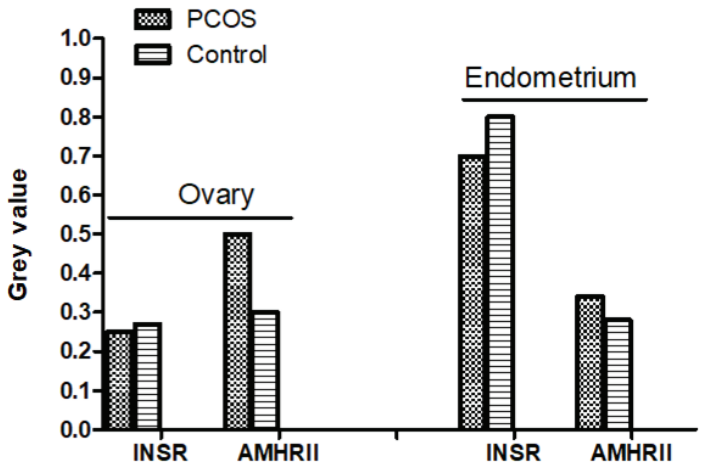


**Figure 3:** Average band intensities of AMHRII and INSR proteins relative to those of β-actin in the endometria and ovaries of patients with PCOS and control subjects.

**Table 4:** Expression of AMHRII and INSR genes in the endometria and ovaries of PCOS and control groups, determined by qRT-PCR (n = 5).

| | AMHRII/β-actin | | | INSR/β-actin | | |
|---|---|---|---|---|---|---|
| | **PCOS** | **Control** | ***P*** | **PCOS** | **Control** | ***P*** |
| Endometrium | 0.370±0.047 | 0.369±0.056 | 0.976 | 0.681±0.151 | 0.892±0.110 | 0.036* |
| Ovary | 0.670±0.091 | 0.415±0.081 | 0.002** | 0.287±0.108 | 0.298±0.019 | 0.828 |

*P < 0.05 and **P < 0.01 (vs. control group) were considered to be statistically significant.

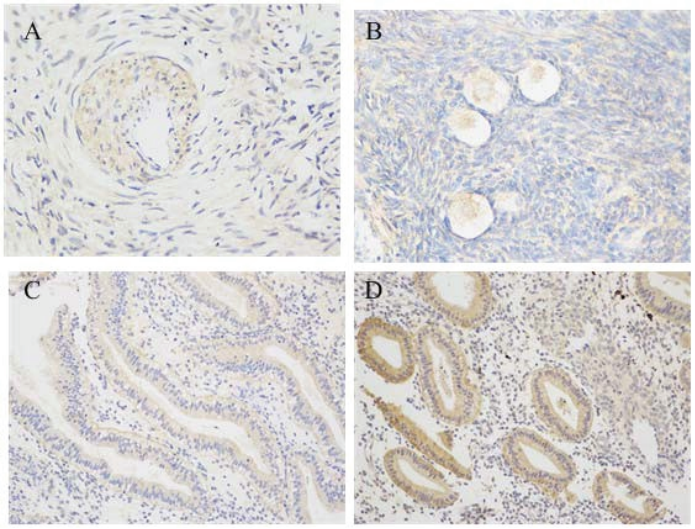


**Figure 4:** Immunohistochemical analysis of AMHRII expression in ovarian and endometrial tissues.

A) Control ovarian tissue (original magnification 400×). B) PCOS ovarian tissue (original magnification 400×). Positive staining was detected in ovarian immature granulosa and stromal cells. C) Control endometrial tissue (original magnification 200×). D) PCOS endometrial tissue (original magnification 200×). Positive expression was found in the stroma and glandular epithelium, and expression was more obvious in the cytoplasm of glandular epithelial cells.

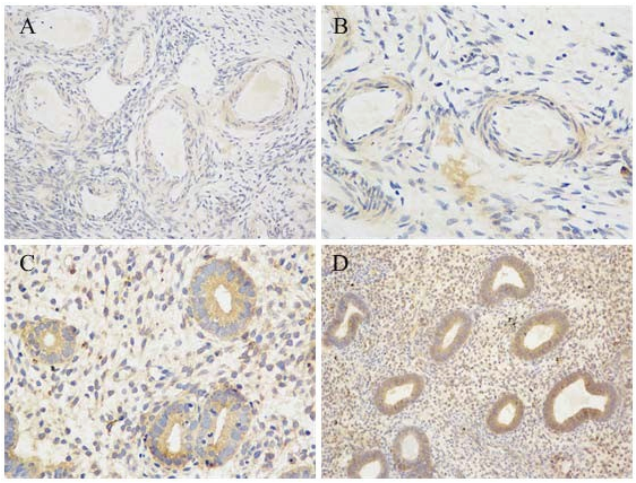


**Figure 5:** Immunohistochemical analysis of INSR expression in ovarian and endometrial tissues. A) Control ovarian tissue (original magnification 400×). B) PCOS ovarian tissue (original magnification 400×). Positive staining was detected in ovarian stromal and vascular epithelial cells. C) Control endometrial tissue (original magnification 200×). D) PCOS endometrial tissue (original magnification 200×). Strong positive expression was found in proliferative endometrium and stromal cells.

**Table 5.** Average optical density values for INSR and AMHRII in different tissues.

| | | INSR | | | AMHRII | |
|---|---|---|---|---|---|---|
| | PCOS | Control | *P* | PCOS | Control | *P* |
| Ovary | 0.252±0.055 | 0.268±0.071 | 0.069 | 0.329±0.026 | 0.317±0.061 | 0.132 |
| Endometrium | 0.657±0.121 | 0.426±0.086 | 0.031* | 0.355±0.113 | 0.304±0.028 | 0.05* |

* P<0.05 was considered to be statistically significant.

**Table 6:** Analysis of four methylation sites of the AMHRII gene.

| Site | Pos. 1 | Pos. 2 | Pos. 3 | Pos. 4 |
|---|---|---|---|---|
| Control | 0.4637±0.0558 | 0.6045±0.1185 | 0.5245±0.2996 | 0.1844±0.0760 |
| PCOS | 0.4951±0.0673 | 0.5615±0.1041 | 0.3664±0.2882 | 0.4076±0.2263 |
| *t* | -1.80 | 1.44 | 1.98 | -5.64 |
| *P* | 0.08 | 0.16 | 0.05* | 0.00* |

*P < 0.05 was considered to be statistically significant.

(a) oligomenorrhea, (b) hyperandrogenism, and (c) polycystic degeneration of one or both ovaries. Then, the methylation sites of the AMHRII and INSR genes were analysed. No significant correlation was observed between DNA methylation status and PCOS clinical groups (data not shown).

### Correlation between methylation and IR

Correlations between methylation of the AMHRII and INSR genes and IR were analysed in the 40 patients with PCOS with different clinical characteristics. Significant correlations were observed between AMHRII methylation and IR (R = 0.532, P = 0.00), and between INSR methylation and IR (R = 0.281, P = 0.03; Figure 6 and 7).

## Discussion

The clinical data on 75 patients with PCOS obtained in this study were consistent with other reports in the literature [2,12], and with the diverse clinical characteristics of PCOS. Comparison between normal menstruation and oligomenorrhea in 40 patients with PCOS showed that those with oligomenorrhea had stronger IR and were younger. These results suggest that younger patients with PCOS and those with IR are particularly prone to oligomenorrhea, which provides a significant reference for the clinical diagnosis and treatment of PCOS.

The clinical characteristics of PCOS are highly heterogeneous, and the pathogenesis of PCOS is very complex. PCOS is recognised as a disease involving endocrine, metabolic, and genetic factors [3]. AMH exerts its biological function mainly through AMHRII and has been considered to be a major factor in the development of polycystic ovarian follicles [5]. Different experimental points in the protein levels of AMHRII and its relationship with PCOS have been established. In our study, no significant difference in ovarian AMHRII expression was observed between patients with PCOS and controls. However, endometrial AMHRII expression was slightly greater in patients with PCOS than in the control group, suggesting that AMHRII is involved in endometrial lesions.

Recent studies have shown that IR is also an important factor leading to a series of pathophysiological changes in patients with PCOS [3,8,13]. We found INSR expression in granulosa cells and ovarian stroma, and especially in the stromal vascular epithelium, suggesting that INSR plays a role in the regulation of ovarian function and the occurrence of polycystic ovarian changes. However, semi-quantitative analysis of immunohistochemical images revealed no significant difference between groups, consistent with a previous report [14]. These findings suggest that the mechanism of IR in patients with PCOS is related to defects in the function of INSR or post-receptors.

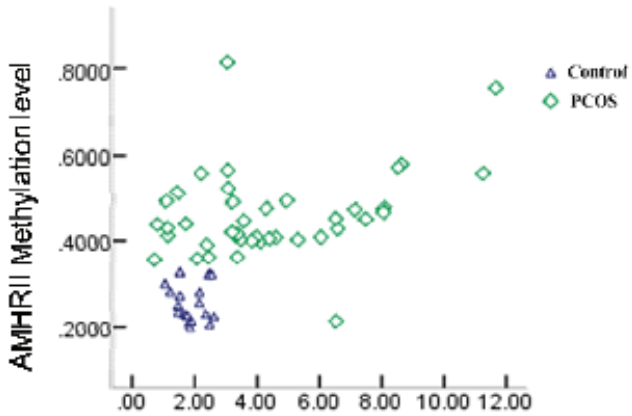


**Figure 6:** Correlation between AMHRII methylation and insulin resistance (IR).

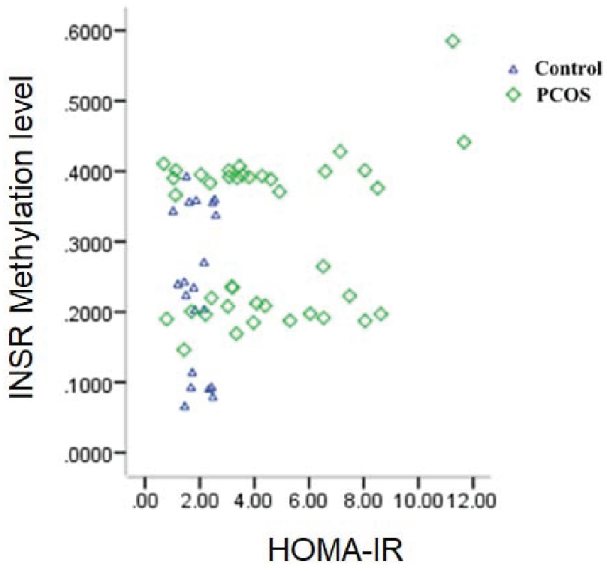


**Figure 7:** Correlation between INSR methylation and IR.

**Table 7:** Analysis of four methylation sites of the INSR gene.

| Site | Pos. 1 | Pos. 2 | Pos. 3 | Pos. 4 |
|---|---|---|---|---|
| Control | 0.2588±0.2035 | 0.7167±0.3966 | 0.1511±0.0369 | 0.5499±0.3689 |
| PCOS | 0.1726±0.0356 | 0.6391±0.3847 | 0.2131±0.0254 | 0.2047±0.1304 |
| *t* | 1.88 | 0.73 | -6.75 | 4.06 |
| *P* | 0.08 | 0.47 | 0.00* | 0.00* |

*P < 0.05 was considered to be statistically significant.

Previous research has shown that the endocrine dysfunction in patients with PCOS not only induces ovarian dysfunction [15], but also corresponds to pathological changes in the endometrium, which manifest mainly as different degrees of endometrial hyperplasia and even endometrial cancer. In this study, different degrees of epithelial cell hyperplasia and a small amount of stromal hyperplasia were observed through HE staining of endometria from patients with PCOS. Therefore, the risk of endometrial cancer in patients with PCOS may be significantly higher than that in women without PCOS. In addition, semi-quantitative analysis of immunohistochemical images showed strongly positive expression of INSR in the endometrial epithelial cells and endometrial stroma of patients with PCOS, significantly higher than in the control group. Our results suggest that INSR is involved in pathological changes of the endometrium, consistent with results from a previous study.

Recent studies have shown that PCOS is associated with the results of common actions of genetic and environmental factors [16]. Epigenetics has generally been regarded as the most likely explanation for PCOS, and DNA methylation has become a hot research focus in epigenetics studies. However, no report has described the relationship between methylation of the AMHRII gene and the pathogenesis of PCOS, and few studies of the methylation of the INSR gene in PCOS have been published. Zhu et al. [17] found abnormal methylation status of the INSR gene in patients with PCOS. In the present study, we assessed four methylation sites of the AMHRII and INSR genes in 40 patients with PCOS and 20 controls, and found no significant difference at positions 1 and 2, but significant differences at positions 3 and 4 of both genes, between patients with PCOS and controls. These findings illustrate a correlation between the clinical characteristics of PCOS and the methylation status of these two genes. We further assessed the correlation between DNA methylation of these two genes and IR in 40 patients with PCOS with different clinical characteristics, and found significant correlations between AMHRII methylation and IR, and between INSR methylation and IR.

Insulin levels and HOMA-IR were higher in patients with PCOS than in the control group. Thus, we hypothesised that the methylation level of the promoter region of the INSR gene in ovaries is involved in the changes in the regulation of expression and functional abnormalities of the INSR gene, which further confirms the results of our previous study [18]. However, we could not assess methylation levels in ovarian and endometrial tissues due to the difficulties of collecting fresh tissues, which was a limitation of this study.

## Conclusion

DNA methylation of AMHRII and INSR genes was correlated with the clinical characteristics of PCOS and IR. INSR was strongly positively expressed in the endometrium and obviously expressed in ovarian stromal vascular epithelium in patients with PCOS, which is likely correlated with endometrial and ovarian stromal hyperplasia. The increased expression of AMHRII in the endometria of patients with PCOS suggests that AMHRII is involved in endometrial lesions. Comparison between patients with PCOS with normal menstruation and oligomenorrhea revealed that younger patients and those with IR may be particularly prone to oligomenorrhea.

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