

# Relationship between abnormal TOX3 gene methylation and polycystic ovarian syndrome

Z. NING, L. JIAYI, R. JIAN, X. WANLI

Shandong University of Traditional Chinese Medicine, Jinan, China

**Abstract. – OBJECTIVE:** The purpose of this work was to examine the relationship between abnormal TOX3 gene methylation and the occurrence of polycystic ovarian syndrome (PCOS).

**PATIENTS AND METHODS:** We selected 30 patients with PCOS and 30 control volunteers. Serum luteinizing hormone, estradiol, testosterone, and thyroid stimulating hormone were detected, methylation, mRNA and protein level of TOX3 were measured.

**RESULTS:** Serum luteinizing hormone, estradiol, testosterone, and thyroid stimulating hormone were significantly higher and follicle stimulating hormone and prolactin were significantly lower in PCOS patients than in the control group. Methylation of the promoter of TOX3 in serum and granular cells was significantly lower in PCOS than in the control group. The mRNA levels of TOX3 in serum were lower in the PCOS group. The levels of TOX3 protein in serum and granular cells was significantly lower in PCOS group than in the control group.

**CONCLUSIONS:** Overall, abnormal TOX3 methylation possibly resulting in changes in TOX3 protein expression is closely related to the occurrence of PCOS and may play a role the development of the pathology.

Key Words

TOX3, Methylation, Polycystic ovarian syndrome.

## Introduction

Polycystic ovarian syndrome (POS) is a reproductive endocrine disease affecting 6-8% of women that has the highest incidence among women in childbearing age<sup>1,2</sup>. The pathogenesis of POS is related to genetic factors and non-genetic factors, among which abnormal DNA methylation is a relevant mechanism that regulates epigenetic control of gene expression<sup>3</sup>. Genome-wide association studies showed that the thyroid adenoma associated (THADA)<sup>4</sup>, Luteinizing hormone/choriogonadotropin receptor (LHCGR)<sup>5</sup>, and Thymocyte selection-associated high mobility group box (TOX3)<sup>6</sup> were under methylated or de-methylated

in PCOS patients. Some studies<sup>7</sup> have attempted to verify these results in blood tests. Here, we further detected the methylation level of TOX3 in serum and granular cells to determine whether this is a valid assay for the diagnosis and treatment of PCOS.

## Patients and Methods

### Patients

We selected 30 patients with PCOS and 30 control volunteers in our clinic. The diagnosis of PCOS was done according to the Rotterdam criteria of 2003. The diagnosis excluded: hyperprolactinemia, thyroid diseases, Cushing's syndrome, application of exogenous androgen, chromosomal abnormalities, diabetes, hypertension, recurrent spontaneous abortion, and immune diseases. Control volunteers presented in the clinic with male (partner) sterility, specific fallopian tube diseases, regular menstrual cycles, normal ovarian reserve and morphology, normal serum testosterone, and scores of hirsutism. All subjects were in non-pregnancy status, did not accept hormone treatment, and did not go on a diet. For this study, we obtained approval of the Ethics Committee of our hospital. Patients and their relatives signed informed consent. The age of the PCOS group ranged from 24 to 36 years old, with an average of  $30.5 \pm 5.5$  years old. The control group ranged from 23 to 37 years old, with an average of  $30.8 \pm 5.7$  years old. We found no statistical differences in age between the two groups.

### Research Methods

Height and weight were measured to calculate body mass index (BMI). The status of uterus, ovary, and accessory organs were evaluated by color Doppler ultrasound. 5 ml of peripheral venous blood was collected on days 2-6 of menstruation. The levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), testosterone (T), prolactin (PRL), and thyroid stimulating hormone (TSH) were detected by chemiluminescence immunoassay

(ZSGB-BIO), and procedures were conducted following the manufacturer's recommendations. All patients accepted conventional treatment for ovulation induction. When the diameter of more than three follicles reached 17 mm, 6,000-10,000 units of human chorionic gonadotropin was injected. 36 h later, ovum was collected through vagina under the guidance of ultrasound. The collection and purification of granular cells were conducted by density gradient centrifugation.

### **DNA Methylation**

DNA was extracted with the QIAamp DNA mini kit (QIAGEN, Dusseldorf, Germany), bisulfate conversion was conducted by applying EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA), and primers were designed with Meth-Primer ([www.urogene.org/methprimer/](http://www.urogene.org/methprimer/)). TOX3: Fw- 5'-TTAGTTTGGTTTtagggTATTTG-3', Rv- 5'-ATAAAACCTCACATCCATACC-3'. PCR amplification reaction: 12.5  $\mu$ l TaKaRa GC Buffer (Takara, Dalian, China), 2  $\mu$ l 2.5 mM dNTP (Takara, Dalian, China), 0.3  $\mu$ l 50  $\mu$ M primer, 7.7  $\mu$ l ddH<sub>2</sub>O and 2.5  $\mu$ l TaKaRa Taq Hot Start (Takara, Dalian, China). We used the touchdown PCR method and implemented hot start at 95°C for 5 min; denaturation at 95°C for 25 sec, annealing at 60°C for 25 sec and extension at 72°C for 30 sec, for 10 cycles; denaturation at 95°C for 25 sec, annealing at 75°C for 25 sec and extension at 72°C for 30 sec, for 35 cycles; reaction ended at 72°C for 5 min. PCR reaction products were separated by 1% agarose gel electrophoresis. A specific band was cut on ultraviolet cutting gel platform and DNA was purified by Axy-Prep DNA Gel Extraction Kit (Axygen, Tewksbury, MA, USA). The DNA fragments were ligated into PMD18-T Simple Vector (TaKaRa, Dalian, China) according to instructions. Independent clones were selected and added into centrifuge tube with 5 ml LB medium containing Amp to culture at 37°C overnight. The bacterial pellet was collected by centrifugation and plasmids were extracted with TIANprep Mini Plasmid Kit (Tiangen Biotech, Beijing, China), according to the manufacturer's recommendations. Bigdye sequencing reaction was conducted by taking the above plasmids as template and taking PMD18-T plasmid universal primer (BioSune Biotechnology, Beijing, China) M13R (-48): AGCGGATAACAATTTTCACACAGGA as sequencing primer. Sequencing was conducted in an ABI 3730x1 96 capillary DNA analyzer (ABI, San Diego, CA, USA) and the sequencing results were interpreted by Sequencher software (Version 4, Gene Codes Corporation, Ann Arbor, MI, USA).

### **Real-time Quantitative PCR**

Total RNA was extracted by RNAiso (TaKaRa, Dalian, China) kit. RNA absorbance was measured by ultraviolet spectrophotometry (NanoDrop Spectrophotometer ND-1000, Thermo Fisher, Waltham, MA, USA) to detect RNA purity and concentration. RNA integrity was detected by agarose gel electrophoresis (Gene-Bio Biotechnology, Beijing, China). Reverse transcription applied PrimeScript First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) to generate cDNA. Primer was obtained from BioSune Biotechnology (Shanghai, China), with the sequence of Fw- 5'-GGTCTCACTCGACTATCACTTGC-3', Rv- 5'-CTCCGGGCTCAATGTATCTCA-3'; internal reference of  $\beta$ -actin Fw-5'-CGACAGOATG-CAGAAGGAG-3, Rv- 5'-ACATCTGCTGGAAG-GTGGGA-3'. Power SYBY Green PCR Master Mix (ABI, San Diego, CA, USA) reaction system included 5  $\mu$ l of Power SYBY Green PGR MasterMix, 0.5  $\mu$ l of forward primer and 0.5  $\mu$ l of reverse primer (0.1  $\mu$ M), 2  $\mu$ l of ddH<sub>2</sub>O and 2  $\mu$ l of cDNA template. Reaction conditions included 95°C 5 min, 95°C for 20 sec, 60°C for 60 sec and 40°C for 20 sec, for a total of 45 cycles, 5°C for 15 sec and 60°C for 60 sec, melting curve, ended with 95°C for 15 sec.

### **Western Blot Analysis**

Total protein was extracted by RIPA lysate (Beyotime Biotechnology, Shanghai, China) and freshly added phenylmethylsulfonyl fluoride. Total protein concentration was measured by BCA (Beyotime Biotechnology, Shanghai, China). Rabbit anti-human TOX3 monoclonal antibody and rabbit anti- $\beta$ -actin antibody (Proteintech, San Diego, CA, USA) were diluted with antibody diluent plus (ProteinSimple, San Diego, CA, USA) 1:50. Other reagents were purchased from Simon-Rabbit (12-180 kDa) Master Kit (ProteinSimple, San Diego, CA, USA). The steps were conducted according to Simon recommended steps (ProteinSimple, San Diego, CA, USA, [www.proteinsimple.com/simon](http://www.proteinsimple.com/simon)). Data analysis was conducted by applying Compass Software from Simon (ProteinSimple, San Diego, CA, USA).

### **Statistical Analysis**

Statistical analysis was conducted by applying SPSS20.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were indicated with mean  $\pm$  standard deviation. The inter-group comparison applied independent sample *t*-test and enumeration data were indicated with the number of cases or (%). The inter-group comparison was verified with  $\chi^2$ -test. *p*<0.05 indicated that the differences were statistically significant.

**Table I.** Levels of BMI and endocrine hormone.

Group	BMI (kg/m <sup>2</sup> )	FSH (IU/L)	LH (IU/L)	E2 (pmol/L)	T (nmol/L)	PRL (μg/L)	TSH (mU/L)
PCOS	23.5 ± 2.6	6.3 ± 1.2	10.3 ± 1.6	224.7 ± 34.5	2.3 ± 0.6	13.6 ± 2.4	13.5 ± 2.3
Control	20.4 ± 2	8.2 ± 1.4	3.5 ± 1	172.3 ± 32.6	1.2 ± 0.3	19.2 ± 2.6	4.2 ± 1.2
<i>t</i>	4.527	4.638	6.527	6.537	5.247	5.539	8.527
<i>p</i>	0.039	0.036	0.012	0.010	0.027	0.023	0.000

Note: BMI, body mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; E2, estradiol; T, testosterone; PRL, prolactin; TSH, thyroid stimulating hormone.

## Results

### **BMI and Levels of Endocrine Hormones**

In the initial examination of the PCO and control groups, we found that the PCOS group had a higher BMI than the control group (Table I). We next analyzed the levels of several endocrine hormones. Serum LH, E2, T, and TSH were significantly higher in PCOS patients than in the control group. In contrast, the levels of FSH and PRL were significantly lower in the PCOS group compared to controls (Table I).

### **TOX3 DNA Methylation Level**

Following on previous reports suggesting a connection between TOX3 methylation and PCO, we determined TOX3 methylation in serum and in granular cells. The DNA methylation rates for TOX3 in serum and granular cells were significantly lower in the PCOS group compared to controls (Table II). These results support previous observations of the link between TOX3 methylation and PCOS.

### **TOX3 mRNA Levels**

To understand the relevance of TOX3 under-methylation, we next determined the expression of TOX3 by detecting TOX3 mRNA levels in serum and granular cells (Table III). We found that the levels of TOX3 mRNA in serum were

significantly lower in the PCOS group compared to controls. When we measured TOX3 mRNA in granular cells, we found the same trend for lower levels in the PCOS group, but the difference was not statistically significant (Table III).

### **TOX3 Protein Levels**

Finally, we wanted to determine if the differences in TOX3 mRNA translated in differences in TOX3 protein. After conducting Western blot, we found that the levels of TOX3 protein were significantly lower in the PCOS group in both serum and granular cells (Table IV).

## Discussion

Epigenetic information consists of non-heritable DNA changes, such as DNA methylation, DNA hydroxymethylation, and histone methylation and acetylation. During DNA methylation, 5-methylcytosine (the fifth base)

**Table II.** Detection of DNA methylation rate [cases (%)].

Group	Cases	Serum	Granular cells
PCOS	30	18 (60.0)	22 (73.3)
Control	30	25 (83.3)	28 (93.3)
$\chi^2$		4.022	4.320
<i>p</i>		0.045	0.038

**Table III.** TOX3 mRNA levels.

Group	Serum	Granular cells
PCOS	0.4652 ± 0.0527	0.5327 ± 0.0429
Control	0.4834 ± 0.0635	0.5646 ± 0.0382
<i>t</i>	0.632	0.527
<i>p</i>	0.349	0.521

**Table IV.** TOX3 protein levels.

Group	Serum	Granular cells
PCOS	0.13±0.04	0.29±0.08
Control	0.47±0.06	0.53±0.10
<i>t</i>	6.238	6.652
<i>p</i>	0.018	0.013

replaces cytosine, with the highest incidence at CpG sites (Cytosine Phosphate Guanine)<sup>8</sup>. Endocrine abnormalities and changes during pregnancy can result in epigenetic changes, including the loss of gene imprinting or abnormal methylation of CpG sites<sup>9</sup>. Abnormal methylation is closely related to the occurrence of several disorders, including diabetes and tumors<sup>10</sup>.

Previous studies<sup>8</sup> showed that the overall methylation level of genomic DNA in blood in the same in PCOS and normal subjects. However, the promoter region of CYP19A1 was hypermethylated in ovarian tissues in PCOS patients, with a consequent decrease in gene expression<sup>11</sup>. Various abnormally methylated genes were detected by MassARRAY, including EPHX1, SRD5A1 and CYP11A1<sup>12</sup>. Other studies proved that TOX3 interacted with LHCGR and follicle-stimulating hormone receptor (FSHR) and directly participated in the secretion and expression of luteinizing hormone, chorionic gonadotropin, and other endocrine hormones<sup>13</sup>. TOX3 is widely expressed in granular cells, follicular theca cells, luteal cells, and interstitial cells. Also, hormone levels also affected the methylation level of TOX3, an important factor affecting gene transcription and expression<sup>14</sup>.

### Conclusions

Our observation that serum levels of several endocrine hormones were altered in PCOS was consistent with the endocrine disorder of PCOS. The TOX3 DNA methylation, TOX3 mRNA levels in serum, and the level of TOX3 protein in serum and granular cells were clearly abnormal in PCOS compared to control subjects. Although the methylation status of TOX3 is closely related to PCOS, it cannot affect transcriptional activity<sup>15</sup> but can affect protein translation and expression level<sup>16</sup>. The methylation status may be an abnormal manifestation of gene transcription or gene transcription is affected by upstream “switches molecule”<sup>17</sup>. The specific mechanism and regulatory pathway of methylation in changing and affecting the occurrence of PCOS need to be further studied.

### Acknowledgements

This work was done with support from the National Natural Science Foundation (No. 81273667).

### Conflict of Interest

The Authors declare that they have no conflict of interests.

### References

- 1) ROJAS J, CHÁVEZ M, OLIVAR L, ROJAS M, MORILLO J, MEJÍAS J, CALVO M, BERMÚDEZ V. Polycystic ovary syndrome, insulin resistance, and obesity: navigating the pathophysiologic labyrinth. *Int J Reprod Med* 2014; 2014: 719050.
- 2) YANG B, SUN ZJ, CHEN B, ZHANG J, ZHAO H, LI CW, CAO YK, CAO F. Statin ameliorates endothelial dysfunction and insulin resistance in Tibet women with polycystic ovary syndrome. *Eur Rev Med Pharmacol Sci* 2016; 20: 1185-1191.
- 3) WANG XX, WEI JZ, JIAO J, JIANG SY, YU DH, LI D. Genome-wide DNA methylation and gene expression patterns provide insight into polycystic ovary syndrome development. *Oncotarget* 2014; 5: 6603-6610.
- 4) GOODARZI MO, JONES MR, LI X, CHUA AK, GARCIA O, CHEN YDI, KRAUSS RM, ROTTER JI, ANKENER W, LEGRO RS, AZZIZ R, STRAUSS JF, DUNAIF A, URBANEK M. Replication of association of DENND1A and THADA variants with polycystic ovary syndrome in European cohorts. *J Med Genet* 2012; 49: 90-95.
- 5) ALMAWI WY, HUBAIL B, AREKAT DZ, AL-FARSI SM, AL-KINDI SK, AREKAT MR, MAHMOOD N, MADAN S. Luteinizing hormone/choriogonadotropin receptor and follicle stimulating hormone receptor gene variants in polycystic ovary syndrome. *J Assist Reprod Genet* 2015; 32: 607-614.
- 6) McALLISTER JM, LEGRO RS, MODI BP, STRAUSS JF. Functional genomics of PCOS: from GWAS to molecular mechanisms. *Trends Endocrinol Metab* 2015; 26: 118-124.
- 7) WELT CK, DURAN JM. The genetics of polycystic ovary syndrome *Semin Reprod Med* 2014; 32: 177-182.
- 8) JONES MR, BROWER MA, XU N, CUI J, MENGESHA E, CHEN YDI, TAYLOR KD, AZZIZ R, GOODARZI MO. Systems genetics reveals the functional context of pcos loci and identifies genetic and molecular mechanisms of disease heterogeneity. *PLoS Genet* 2015; 11: e1005455.
- 9) SANG Q, LI X, WANG H, WANG H, ZHANG S, FENG R, XU Y, LI Q, ZHAO X, XING O, JIN L, HE L, WANG L. Quantitative methylation level of the EPHX1 promoter in peripheral blood DNA is associated with polycystic ovary syndrome. *PLoS One* 2014; 9: e88013.
- 10) FRADIN D, FUR SL, MILLE C, NAQUI N, GROVES C, ZELENKA D, MCCARTHY MI, LATHROP M, BOUGNÈRES P. Association of the CpG methylation pattern of the proximal insulin gene promoter with type 1 diabetes. *PLoS One* 2012; 7: e36278.
- 11) STARLARD-DAVENPORT A, ORLOFF MS, DHAKAL I, PENNEY RB, KADLUBAR SA. Genotypic and allelic variability in CYP19A1 among populations of African and European ancestry. *PLoS One* 2014; 10: e0117347.

- 12) SUN M, YANG X, YE C, XU W, YAO G, CHEN J, LI M. Risk-association of CYP11A1 polymorphisms and breast cancer among Han Chinese women in southern China. *Int J Mol Sci* 2012; 13: 4896-4905.
- 13) XU Y, LI Z, AI F, CHEN J, XING Q, ZHOU P, WEI Z, SHI Y, HE XJ, CAO Y. Systematic evaluation of genetic variants for polycystic ovary syndrome in a Chinese population. *PLoS One* 2015; 10: e0140695.
- 14) BROWER MA, JONES MR, ROTTER JI, KRAUSS RM, LEGROS, AZZIZ R, GOODARZI MO. Further investigation in Europeans of susceptibility variants for polycystic ovary syndrome discovered in genome-wide association studies of Chinese individuals. *J Clin Endocrinol Metab* 2015; 100: E182-E186.
- 15) YU X, LI Z. TOX gene: a novel target for human cancer gene therapy. *Am J Cancer Res* 2015; 5: 3516-3524.
- 16) SØRENSEN AE, WISSING ML, SALØ S, MIKKELSEN ENGLUND AL, TORP DALGAARD L. MicroRNAs related to polycystic ovary syndrome (PCOS). *Genes (Basel)* 2014; 5: 684-708.
- 17) SHEN HR, QIU LH, ZHANG ZQ, QIN YY, CAO C, DI W. Genome-wide methylated DNA immunoprecipitation analysis of patients with polycystic ovary syndrome. *PLoS One* 2015; 8: e64801.