

# Apolipoprotein A-IV as a novel gene associated with polycystic ovary syndrome

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**Abstract.** Polycystic ovary syndrome (PCOS) is a common endocrine-metabolic disorder, affecting 6-10% of women of reproductive age. The etiology remains poorly understood. To investigate the differentially expressed proteins from PCOS patients versus healthy women, the protein expression in follicular fluid was analyzed using two-dimensional electrophoresis (2-DE). Since follicular fluid contains a number of secretory proteins required for oocyte fertilization and follicle maturation, it is possible that follicular fluid can be used as a provisional source for identifying pivotal proteins associated with PCOS. In this study, six overexpressed proteins [kininogen 1, cytokeratin 9, antithrombin, fibrinogen  $\gamma$ -chain, apolipoprotein A-IV (apoA-IV) precursor and  $\alpha$ -1-B-glycoprotein (A1BG)] in follicular fluids from PCOS patients were identified with matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) and nano LC-MS/MS. Western blot analysis confirmed that the protein expression levels of apoA-IV precursor and A1BG were increased in follicular fluid from PCOS patients compared with those from normal controls. The analysis of protein expression for other proteins revealed individual variation. These results facilitate the understanding of the molecular mechanisms of PCOS and provide candidate biomarkers for the development of diagnostic and therapeutic tools.

## Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders, affecting 6-10% of women of

reproductive age, and is diagnosed by combination of polycystic ovaries, hyperandrogenism or oligo-anovulation (1-3). Serum luteinizing hormone (LH) hypersecretion, compensatory hyperinsulinemia from insulin resistance and reduced fecundity are common features of PCOS and at least 50% of PCOS women are insulin resistant when compared with age- and weight-matched controls (4). Genetic studies on this disorder have certain difficulties due to heterogeneity in the phenotype of PCOS patients. Therefore, association studies have been used as a powerful genetic approach to analyze the complex traits in PCOS. Most molecular and genetic analyses have focused on genes involved in folliculogenesis and insulin signaling (5-12).

Follicular fluid, secreted by granulosa cells and by diffusion from the theca capillaries, plays an essential role in the physiology of follicular growth, oocyte maturation and ovulation (13). A number of studies have revealed that follicular fluid inhibits the binding of spermatozoa to the zona pellucida (14), and promotes hyperactivation and acrosome reaction of sperm (15,16), and oocyte maturation and embryo development (17,18). Moreover, follicular fluid contains high levels of gonadotropin and growth factors that are important to oocyte maturation and embryo development (19). Therefore, the chemical composition of fluid from dominant follicles can be used as an indicator of the secretory activities and metabolism of follicular cells, which regulate the follicular quality. Recent proteomic studies demonstrated that most proteins in human follicular fluid are also present in the plasma, suggesting that these proteins can be used as biomarkers for reproductive diseases using both follicular fluid and plasma (20-23). In this study, we performed genetic and proteomic analyses to identify novel PCOS-associated factors in follicular fluid from PCOS patients.

## Materials and methods

**Subjects and biochemical determinations.** Women with PCOS were recruited at the Fertility Center at CHA General Hospital in Seoul, Korea. The criteria for diagnosis of PCOS rely on the combination of clinical symptoms, ultrasonographic examination, and biochemical data based on the revised diagnostic criteria announced in the 2003 ASRM/ESHRE Rotterdam

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Table I. Clinical characteristics of normal and PCOS patients for follicular fluid and blood serum.

	Normal	PCOS
No.	5 (3)	5 (7)
Body mass index (kg/m <sup>2</sup> )	20.94 (19.78-22.38) (20.02-21.36)	23.07 (19.97-26.35) [22.37 (18.02-25.24)]
FSH (IU/l)	6.1 (3.7-8.2) [6.4 (5.0-7.5)]	5.74 (2.9-8.2) [5.8 (3.1-8.5)]
LH (IU/l)	4.98 (2.3-6.7) [2.49 (1.5-4.0)]	10.46 (8.0-13.0) [7.28 (0.4-24.1)]
E <sub>2</sub> (pmol/l)	27.86 (9.3-49.0) [17 (11.76-25.13)]	32.6 (19.0-61.0) [27 (7.81-52.66)]

Numbers in parentheses represent normal and polycystic ovary syndrome (PCOS) patients whose sera were used for western blot analysis.

consensus (24). Healthy female volunteers were enrolled and used as the control group. Their healthy state was determined based on medical history, physical and pelvic examination and complete blood chemistry. Their normal ovulatory state was confirmed by transvaginal ultrasonography and a plasma hormone assay was conducted during the luteal phase of the cycle.

The clinical characteristics of normal and PCOS patients are listed in Table I. Informed written consent was obtained from all participants. Follicular fluid was prepared as previously described (20). To carry out western blot analysis, serum samples were prepared as previously described (20). Venous blood samples were obtained prior to follicular aspiration, and were collected in sterile plastic tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and immediately centrifuged. All samples were stored at -80°C until use.

For genetic analysis, 233 patients with PCOS and 120 healthy women of reproductive age were recruited from the Fertility Center at CHA General Hospital. Blood samples were obtained in tubes containing EDTA, which acts as an anticoagulant. The follicular fluid and blood serum used for proteomic analysis were also used in the genomic study. Genomic DNA was extracted using a DNA purification system kit (Gentra Systems, Minneapolis, MN, USA). Biochemical analysis of various hormones from blood including insulin, DHEA-S, testosterone, DHEA-S, FSH and LH was conducted. Furthermore, the participants underwent a series of physical examinations including weight, height and waist/hip ratio measurements, and the history of the patient and family were recorded (Table I). The human follicular fluid and blood samples obtained from controls and PCOS patients were approved by the CHA General Hospital Institutional Review Board.

*Two-dimensional electrophoresis (2-DE) and image analysis.* Immobililine DryStrips (IPG) pH 3-10 nonlinear 18 cm, and Pharmalytes pH 3-10 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden); Griess reagent, CHAPS, urea, DTT, Tris base, thiourea, glycine, ammonium persulfate and SDS were obtained from Sigma (St. Louis, MO, USA); Coomassie brilliant blue (CBB) G-250 and TEMED were from Bio-Rad (Hercules, CA, USA). All other chemicals were obtained from various commercial sources and were of the highest grade.

Depletion of the six most abundant proteins (i.e., albumin, transferrin, IgG, IgA, haptoglobin and antitrypsin) in follicular

fluid was carried out using a Multiple Affinity Removal Column (MARC) (Agilent Technologies, Inc., Wilmington, DE, USA). Then, 2-DE and image analyses were performed, as previously described (20).

*In-gel digestion and LC-MS/MS.* Excised spots were destained, reduced, alkylated, and then digested with trypsin (Promega, Madison, WI, USA) as previously described (25). For matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) analysis, the tryptic peptides were concentrated using a poros R2 and oligo R3 column (Applied Biosystems, Foster City, CA, USA) and eluted in  $\alpha$ -cyano-4-hydroxycinnamic acid. Spectra were obtained using a 4700 TOF/TOF spectrophotometer (Applied Biosystems). Proteins were identified from the peptide mass maps using MASCOT ([http://www.matrixscience.com/search\\_form\\_select.html](http://www.matrixscience.com/search_form_select.html)), MS-Fit (<http://prospector.ucsf.edu>), and ProFound (<http://prowl.rockefeller.edu/prowl/cgi/profound.exe>) to search for the proteins in the protein database, Swiss-Prot and GenBank. For nano LC-ESI-MS/MS analysis, the peptides digested with trypsin were concentrated using a poros R2 and oligo R3 column (Applied Biosystems).

*Bioinformatics.* Each MS/MS spectrum obtained was searched against the non-redundant protein sequence database using the Spectrum Mill software tool. Sequences of uninterpreted CID spectra were identified by correlation with the peptide sequences present in the protein sequence database (NCBI nr 2012.1) using the Spectrum Mill MS Proteomics Workbench (Rev A.03.00.015; Agilent Technologies, Inc.).

*Western blot analysis.* Human follicular fluid proteins diluted to 1/10 with PBS buffer and human sera were subjected to SDS-PAGE. Albumin was removed from the follicular fluid to detect kininogen 1, cytokeratin 9, antithrombin, fibrinogen  $\gamma$ -chain, apolipoprotein A-IV (apoA-IV) precursor, and  $\alpha$ -1-B-glycoprotein (A1BG) using the Aurum serum protein mini kit (Bio-Rad). The proteins were loaded in each lane of a 7.5% SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were incubated with a blocking solution, containing a 1:200 dilution of anti-kininogen 1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or anti-cytokeratin 9 antibodies (Abcam, Cambridge, UK). In addition, an antithrombin antibody (Abcam) diluted to 1:1,000 with the blocking solution was used. Also, the membranes were incubated with a blocking solution, containing a 1:500 dilution of

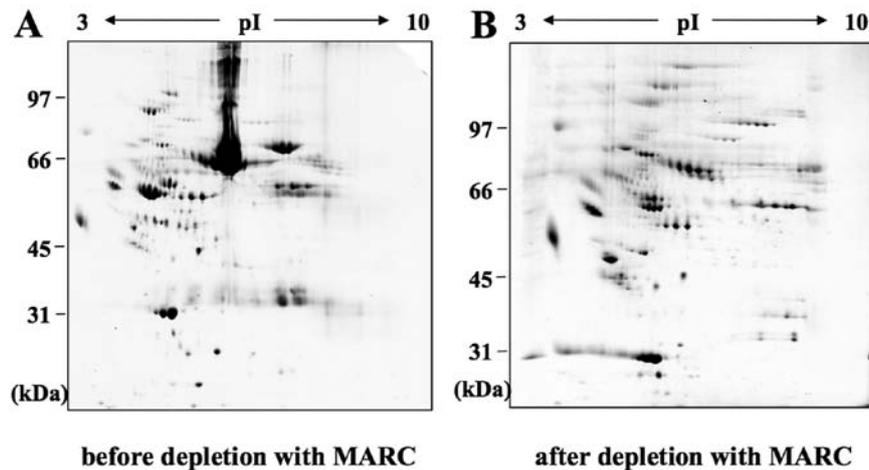


Figure 1. 2-DE images of follicular fluid. The most abundant proteins in follicular fluid derived from a normal patient were depleted using an immunoaffinity column (MARC) and then separated by 2-DE. 2-DE images of follicular fluid (A) before, and (B) after depletion.

anti-fibrinogen  $\lambda$ , anti-apoA-IV (Santa Cruz Biotechnology, Inc.), or anti-A1BG (Aviva Systems Biology, San Diego, CA, USA) antibodies. The membranes were then incubated with a blocking solution containing a 1:5,000 dilution of horseradish peroxidase-conjugated secondary antibodies (KPL, Gaithersburg, MD, USA). An ECL system (Elpis Biotech, Daejeon, Korea) was used to detect signals. Bands from the western blot analysis were scanned and digitized using Fluor-S™ MultiImager (Bio-Rad). Values are expressed as the means  $\pm$  standard error of the mean (SEM).

**Genetic analysis.** Restriction fragment length polymorphism (RFLP) analysis was performed for *apoA-IV* using isolated genomic DNA from the blood samples obtained from all subjects. PCR primers were designed and used to amplify the DNA for the genotyping analysis. To determine the Gln360His variant genotype, the following primers were designed: forward, 5'-CTT CCA GAT GAA GAA GAA CG-3' and reverse, 5'-AGT TTC TGC CTG AGC TGT T-3'. The PCR conditions were as follows: denaturation at 94°C for 30 sec, annealing at 55°C for 10 sec and polymerization at 72°C for 15 sec. Amplified PCR products were purified using a PCR purification kit (AccuPrep®; Bioneer, Daejeon, Korea) and screened by restriction digestion with *Fnu4HI* (New England Biolabs, Beverly, MA, USA) at 37°C for 6 h. In addition, amplified PCR products for each genotype were sequenced in order to confirm the genotyping results from the RFLP analysis.

**Statistical analysis.** For the statistical analysis, the HapAnalyzer (National Genome Research Institute, Seoul, Korea) and SAS ver. 9.1 (2006) were used to analyze the distributions of the *apoA-IV* genotype. P-value <0.05 was considered to indicate a statistically significant difference.

## Results

**2-DE analysis on follicular fluid of PCOS.** A typical 2-DE separation was performed in the pI range of 3-10 on proteins extracted from the follicular fluid of PCOS patients and healthy women as a control to identify proteins that were

aberrantly expressed in PCOS patients. In order to deplete the samples of the most abundant proteins found in follicular fluid, an immunoaffinity column (MARC) was applied to collect flow-through fractions (Fig. 1), as previously described (20).

Follicular fluids were collected from *in vitro* fertilization (IVF) patients suffering from PCOS, and from normal controls who underwent an IVF cycle due to male infertility factors with the treatment of the GnRH agonist for pituitary downregulation. Clinical and biochemical profiles of PCOS patient and normal control groups are shown in Table I. To ascertain differences in protein expression between normal and PCOS patients, we applied the isoelectric focusing technique using differential-display proteomic analysis methods described previously (20). Follicular fluid depleted of the most abundant proteins with an immunoaffinity column revealed better distinguishable spots on 2-DE gels (Fig. 1). The same separation method was then performed on proteins extracted from the follicular fluid of PCOS patients and healthy women as a control to identify proteins that were aberrantly expressed in PCOS patients (Fig. 2A and B).

**Identification of differentially expressed proteins in PCOS patients.** When protein expression levels in the follicular fluid from five PCOS patients with polycystic ovaries, oligo- or amenorrhea and hyperandrogenism, were compared with five normal women as a control, six spots were determined to be overexpressed in the 2-DE analysis based on relative fold increase in volume intensity (Fig. 2C-E). The expression level was determined by examining the ratio of the relative spot volume of a protein in the gel (Fig. 2F). The identification of these different proteins was performed by MALDI-TOF-MS or nano LC-MS/MS. The identified proteins included kininogen 1, cytokeratin 9, antithrombin chain B, fibrinogen  $\gamma$ -chain, apoA-IV precursor and A1BG (Table II).

**Western blot analysis using follicular fluid and plasma.** The follicular fluids were analyzed by western blot analysis using the appropriate antibodies to validate their identities. As shown in Fig. 3A, apoA-IV was detected in the follicular fluid of both the PCOS and the control group. ApoA-IV

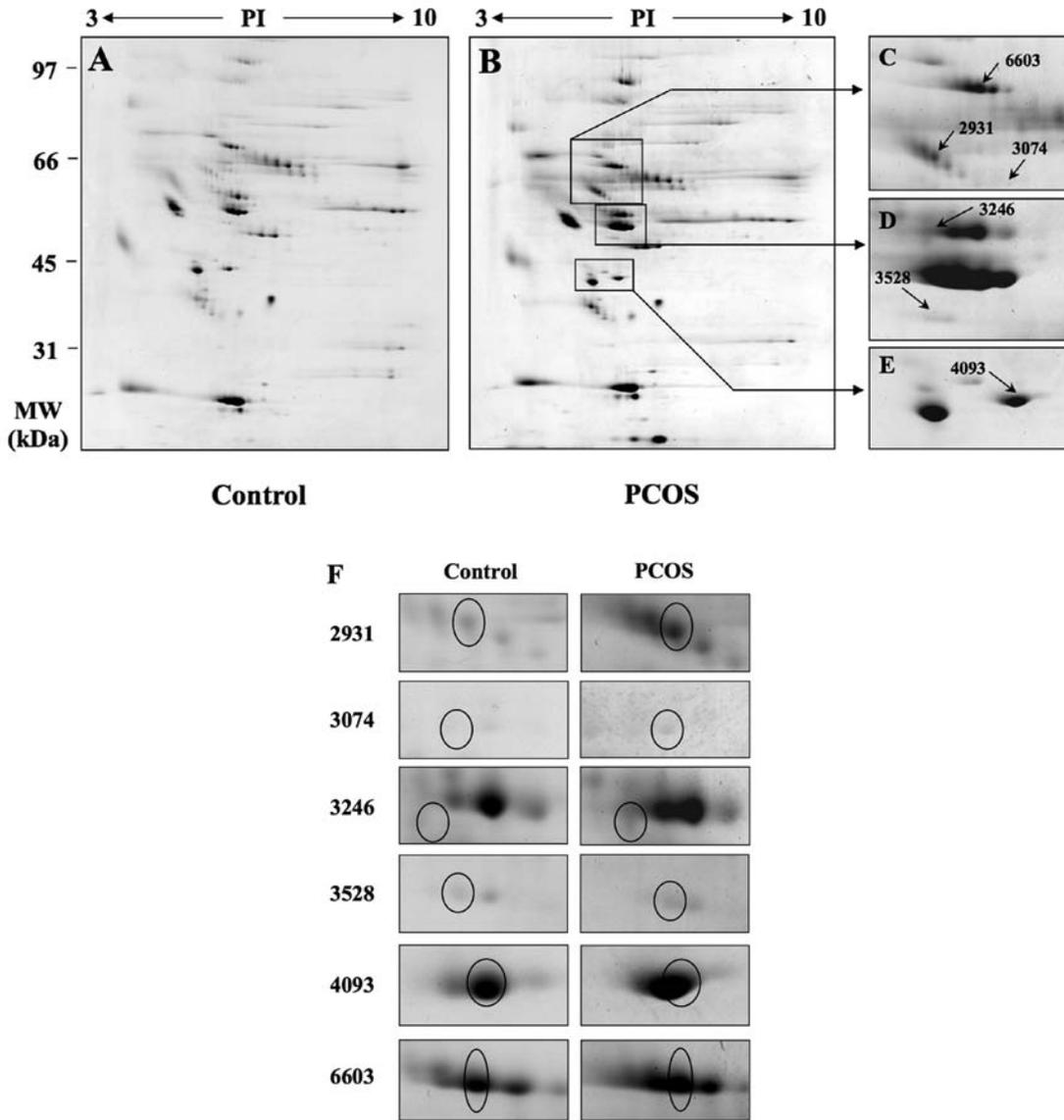


Figure 2. Master images of 2-DE stained with Coomassie blue G-250. One milligram follicular fluid proteins were prepared as described in Materials and methods and separated on an 18 cm pH 3-10 IPG strip and then a 9-16% gradient SDS-PAGE. (A) 2-DE image of follicular fluid from a control (healthy woman), (B) 2-DE image of follicular fluid from a PCOS patient. (C-E) In the follicular fluid from a PCOS patient, 6 spots were determined to be overexpressed in 2-DE analysis based on the relative fold increase in volume intensity. (F) Proteins identified by MALDI-TOF-MS: 2931, kininogen 1; 3074, cytokeratin 9; 3246, chain B, antithrombin; 3528, fibrinogen  $\gamma$ -chain; 4093, apolipoprotein A-IV precursor; and 6603,  $\alpha$ -1-B-glycoprotein.

(45 kDa) expression in PCOS patients was significantly higher than that in controls (1.00 vs.  $3.21 \pm 0.89$ ,  $P < 0.005$ ) (Fig. 3B). Fig. 3C shows the western blot analysis of the A1BG (52 kDa), which was detected in the follicular fluid of controls and PCOS patients. A1BG expression in PCOS patients was also increased by  $\sim 3$ -fold compared with the control (1.00 vs.  $2.70 \pm 0.31$ ,  $P < 0.005$ ) (Fig. 3D). On the other hand, western blot analysis revealed individual variations in the expression of kininogen 1, cytokeratin 9, fibrinogen  $\gamma$  and antithrombin between the PCOS and the control group (Fig. 4A). Fig. 4A shows that the expression level of these proteins varied among normal or PCOS patients.

Since the plasma protein enrichment process occurs during the production of follicular fluid, western blot analysis was conducted using human sera derived from the control and PCOS patients (Fig. 4B). Clinical and biochemical profiles of PCOS patient and normal control groups are shown in paren-

theses in Table I. In human sera from PCOS patients, apoA-IV, A1BG, antithrombin and fibrinogen  $\gamma$  were not significantly altered in their expression levels (Fig. 4B). However, cytokeratin 9, kininogen 1 and actin were not detected at the protein level (data not shown).

*Association study between apoA-IV and PCOS using RFLP analysis.* Several researchers have performed association studies between various genes and PCOS for genetic analysis to identify the etiology of PCOS. Therefore, in this report, an association study for *apoA-IV*, which was highly expressed at the protein level in follicular fluid and sera of PCOS patients, was also conducted. RFLP analysis was performed on the samples collected from 233 PCOS and 120 control patients. All PCOS patients had clinical and biochemical characteristics in accordance with the definition by ASRM/ESHRE (24); 149 patients (63.95%) showed polycystic ovaries and oligo- or

Table II. Protein identities determined by mass analysis.

Spot ID	Accession no.	Protein name	Score <sup>a</sup>	Matched peptides no.	Sequence coverage	Matching sequence	Theoretical MW/pi
2931	gi 4504893	Kininogen 1	89	11/44 (25%)	21%	MKLITLFLCSR, TVGSDTFYFVKYEIK, QVVAGLNFR, VQVVAGKKY FIDFVAR, RPPGFSPFRSSRIGEIKEETTSHLR, GRPKAGAEPAASER	47853/6.29
3074	gi 435476	Cytokeratin 9	83	17/184 (9%)	41%	LSRSGGGGGGLGSGGSRSSYSRFSSSGGR, FSSSSGYGGSSRVCGRGGGSGFGYSYGGGGGGGFSASSLGGGFGGGSR, VQALEEANNNDLENK, NYSPYNTIDDLKQIVDLTVGNKTLDDIDNTRMTLDDFRKIFEMEQLNR, SDLEMQYETLQEELMALKK, TLNDRMQEY EQLIAK, HGVQELEIELQSLSKK, GSGGSGYGGGSGGGYGGGSGSR, GSGGSHGGGSGGSGGSGYGGGEEASGSGGGYGGGSGK	62092/5.29
3246	gi 999514	Chain B, antithrombin	68	17/184 (9%)	53%	HGSPVDICTAKPRDIPMNPICAR, RVWELSKANSRFAITFYQHLADSK, TSDQIHFFFAK, LQPLDFKENAEQSR, SKFS PENTR, VAEGTQVLELPEFKGDDITMVLILPKPEK, VEKELTPEVLQEWLDELEEMMLVHMFRFRIEDGFSLKEQLQDMGLVDFLFSPEK, LPGIVAEGRDDLYVSDAFHKAFLEVNEEGSEAAASTAVVIAGR, ANRPELVFIREVPLNTIIFMGR	48916/5.95
3528	gi 182489	Fibrinogen $\gamma$ -chain	68	16/165 (10%)	45%	NLIFYFALLFLSSTCVAYVATRDNCCILDER, TSEVKQLIKAIQLTYNPDESSKPNMIDAATLK, YEASILTHDSSIRYLQEIYNSNNQK, EKVAQLEAQCQEPCKDTVQIHDIITGK, NWIQYKEGFGHLSPGTTFEFLGNEK, VELEDWNGRTSTADYAMFKVGPPEADKYR, TRWYSMK, TTM KIIPENRLTIGEGQQHHLGG AK	49450/5.61
4093	gi 1773110	Apolipoprotein A-IV precursor	277	25/50 (50%)	50%	SELTQQLNALFQDKLGEVNTYAGDLQKKLVPFATELHER, LLPHANEVSQK, LEPYADQLRTQVSTQAEQLRRQLTPYAQR, ENADSLQASLRPHADELK, IDQNVEELKGRLLTPYADEFKVKIDQTVVEELRRSLAPYAQDTQEKLNHQLEGLTFQMK, LAPLAEDVRGNLR, SLAELGGHLDQQVEEFRRRRVEPYGENFNKALVQQMEQLR, LGPHAGDVEGHLSELEK, DKVNSFFSTFK	45344/5.28
6603	gi 69990	$\alpha$ -1-B-glycoprotein	82	16/135 (12%)	45%	RLETPDFQLFKNGVAQEPVHLDSPAIKHQFLITGDTQQR, SGLSTGWTQLSKLLELTGPK, GVTFLLR, GEKELVPRSSPDRIFFFHLNAVALGDGGHYTCR, LHDNQNQWSGDSAPVELLSDETLPAPEFSPESGR, FALVREDR, LELHVDGPPRRPQLRATWSGAVLAGR, TPGAAANLELIFVGPQHAGNYR, SWVPHTFE SELSDPVLLVAES	51908/5.65

<sup>a</sup>Score is  $-10 \times \text{Log}(P)$ , where  $P$  is the probability that the observed match is a random event; it is based on the NCBInr database using the MASCOT searching program as MS/MS data.

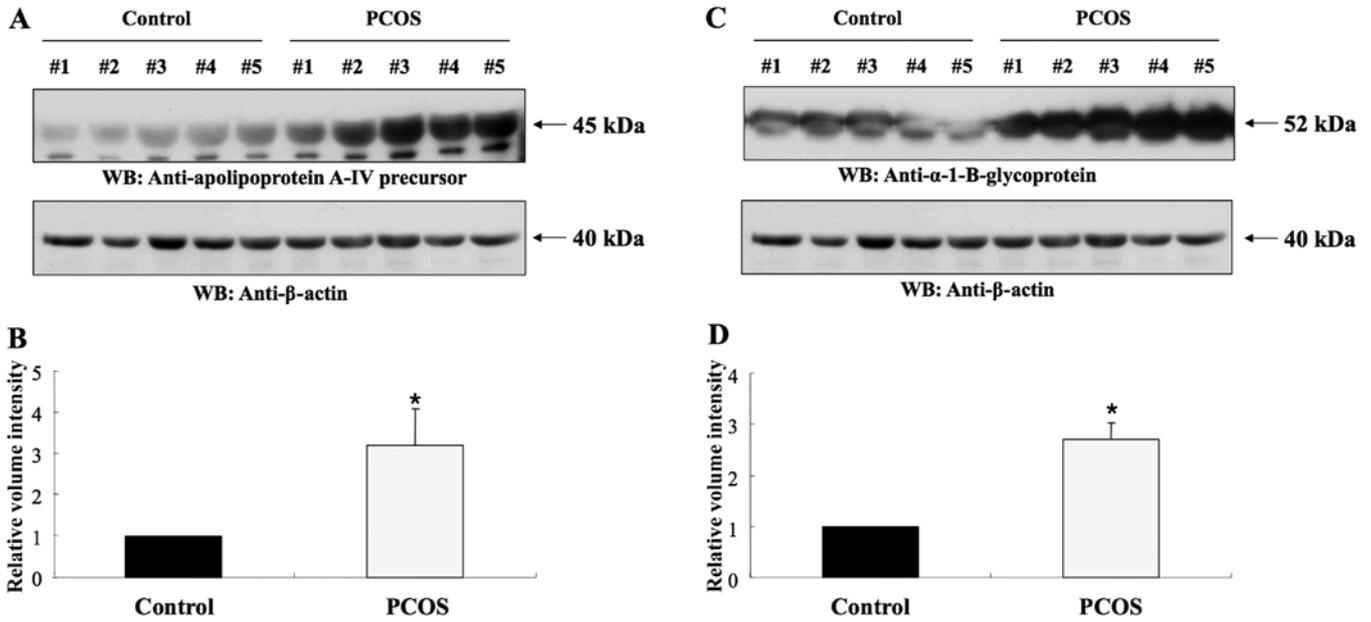


Figure 3. Western blot analysis of apolipoprotein A-IV precursor. Western blot analysis of the follicular fluid proteins from controls and PCOS patients was carried out. (A) Western blot analysis using an anti-apolipoprotein antibody. (B) Quantitative density of gel bands for apolipoprotein A-IV (45 kDa). (C) Western blot analysis using anti- $\alpha$ -1-B-glycoprotein antibody. (D) Quantitative density of gel bands in the  $\alpha$ -1-B-glycoprotein (52 kDa). The bars represent the mean  $\pm$  SEM of the density of the gel bands determined from PCOS patients and controls. Significant difference was assessed by a Student's *t*-test (\* $P$ <0.005).

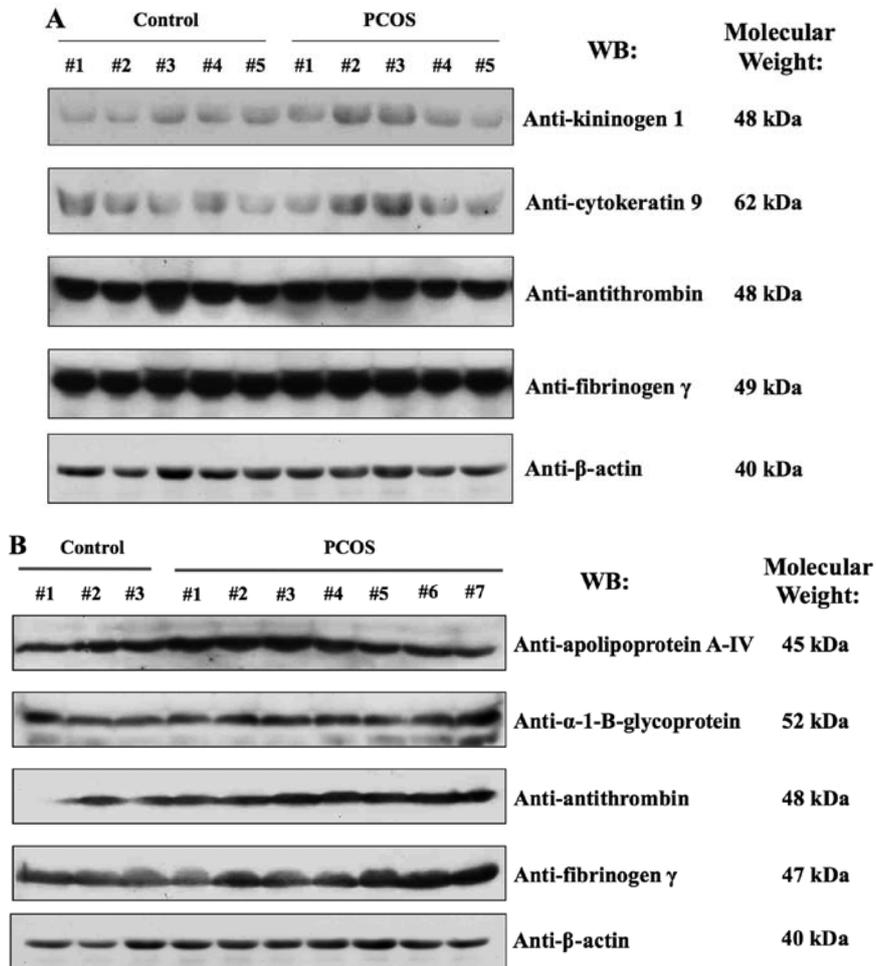


Figure 4. (A) Western blot analysis of kininogen 1 (48 kDa), cytokeratin 9 (62 kDa), antithrombin (48 kDa), and fibrinogen  $\gamma$  (49 kDa) in the follicular fluid of PCOS patients and normal controls. Individual variation of protein expression is shown. (B) Western blot analysis of apolipoprotein A-IV (45 kDa),  $\alpha$ -1-B-glycoprotein (52 kDa), antithrombin (48 kDa), and fibrinogen  $\gamma$  (47 kDa) in human sera of PCOS patients and normal controls. Individual variation of protein expression is shown.

Table III. Clinical and biochemical characteristics of normal controls (n=120) and patients with PCOS (n=233).

Characteristics	Normal controls (n=120)	PCOS patients (n=233)
Age (year)	33.13±3.36 (29-36)	32.41±3.04 (29-35)
BMI (kg/m <sup>2</sup> )	20.88±2.62	22.78±3.76
Waist/hip ratio (WHR)	0.80±0.05	0.82±0.06
Obesity	n=6 (5.45%)	n=44 (18.88%)
Polycystic ovaries and oligo- or amenorrhea	n=0 (0.00%)	n=149 (63.95%)
Polycystic ovaries and hyperandrogenism	n=0 (0.00%)	n=27 (11.59%)
Oligo- or amenorrhea and hyperandrogenism	n=0 (0.00%)	n=31 (13.30%)
Polycystic ovaries, oligo- or amenorrhea, and hyperandrogenism	n=0 (0.00%)	n=26 (11.16%)
FSH levels (mIU/ml)	6.13±2.23 (3.20-8.49)	6.61±7.17 (3.00-11.50)
LH levels (mIU/ml) <sup>a</sup>	3.32±3.06 (1.00-17.02)	6.63±4.78 (1.00-7.10)
E <sub>2</sub> levels (pg/ml)	71.49±298.60 (9.10-81.40)	52.92±128.92 (5.00-219.90)
Prolactin levels (ng/ml)	13.79±10.47 (2.30-20.90)	14.59±13.44 (4.10-46.40)
TSH levels (μIU/ml)	2.98±6.57 (0.46-5.47)	2.59±5.08 (0.03-4.06)
DHEA-S levels (μg/dl) <sup>a</sup>	168.10±70.35 (45.30-377.20)	194.86±77.93 (67.20-257.40)
Testosterone (ng/ml) <sup>a</sup>	0.23±0.14 (0.06-0.86)	0.38±0.22 (0.01-0.54)

<sup>a</sup>Indicates P-value <0.05 statistically. PCOS, polycystic ovary syndrome.

Table IV. Allele frequencies of the Gln360His *ApoA-IV* gene.

	PCOS patient group	Control group
Genotype		
GG	138 (59.2%)	117 (97.5%)
GT	45 (19.3%)	3 (2.5%)
TT	50 (21.5%)	0 (0.0%)
Co-dominant	OR (95% CI) = 15.68 (5.14-47.89), P-value <0.001	
Dominant	OR (95% CI) = 28.71 (8.83-93.33), P-value <0.001	
Recessive	OR (95% CI) = N/A, P-value =0.997	
Total	OR (95% CI) = 37.33 (11.74-118.70), P-value <0.001	

PCOS, polycystic ovary syndrome.

amenorrhea, 31 patients (13.3%) had oligo- or amenorrhea and hyperandrogenism, 27 patients (11.59%) had polycystic ovaries and hyperandrogenism, and 26 patients (11.16%) exhibited polycystic ovaries, oligo- or amenorrhea, and hyperandrogenism. The level of LH, DHEA-S and testosterone were significantly high in the PCOS group (Table III).

In the RFLP analysis, the association of *apoA-IV* and PCOS was confirmed by the G→T substitution, which led to a change from glutamine to histidine, by restriction enzyme *Fnu4HI* (Fig. 5). While restriction enzyme *Fnu4HI* cleaved normal DNA fragments into 101 and 26 bp (Fig. 5A), variant DNA fragments by the G→T substitution were not cleaved (Fig. 5B).

The homozygote of the variant allele (TT) was not detected in the healthy control group and heterozygote genotype (GT) was only observed in three control patients. Of note, 50 PCOS patients (21.5%) had the TT variant homozygote genotype, and 45 PCOS patients (19.3%) had the GT variant heterozygote

genotype (Table IV). Statistical analysis using HapAnalyzer and SAS programs identified that the Gln360His variant was significantly associated with PCOS in dominant (P<0.05) and co-dominant (P<0.05) (Table IV). However, the genotype of PCOS patients used for proteomic analysis only displayed the GG homozygote.

## Discussion

PCOS is a common endocrinopathy affecting 6-10% of women of reproductive age (1). There is clear evidence of the underlying genetic cause for PCOS based on familial clustering of cases. Most studies have been consistent with an autosomal dominant pattern of inheritance. However, it has been difficult to draw solid conclusions from several of these studies due to the small sample sizes, errors in statistical analysis, and differences in diagnostic criteria, an inevitable consequence of PCOS being

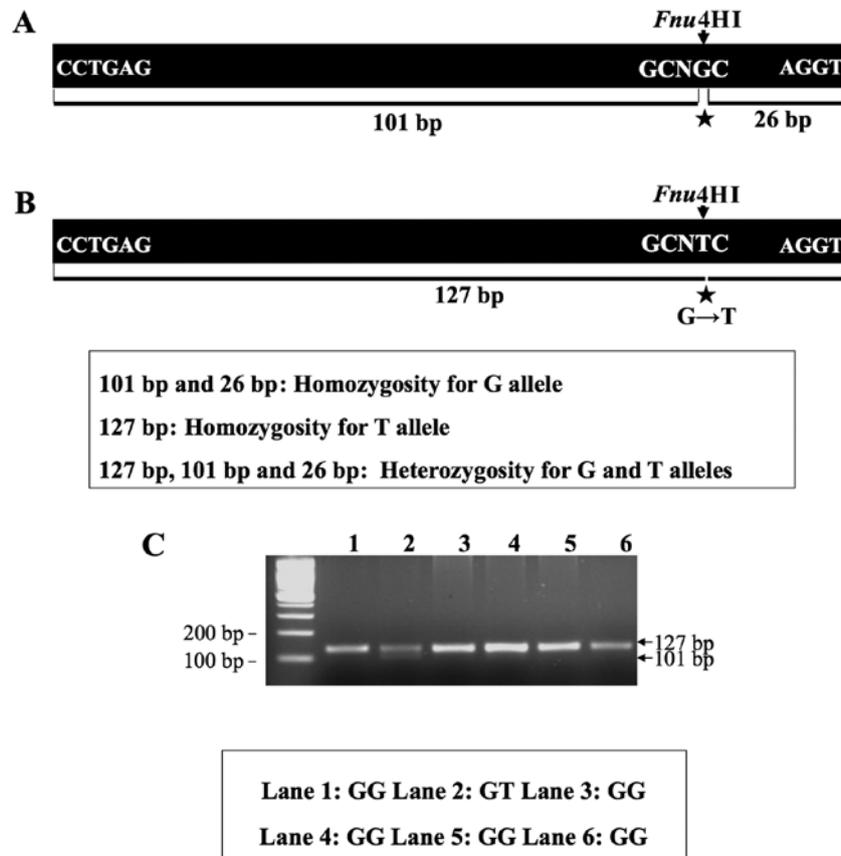


Figure 5. Structure of the *apoA-IV* gene. (A) The 127 bp fragment was amplified by PCR. The major allele is the G allele, which is recognized by the restriction enzyme *Fnu*4HI. (B) If the G allele was modified to the T allele, then the restriction enzyme *Fnu*4HI does not recognize the site. (C) The G/T polymorphism in the *apoA-IV* gene (Gln360His) by RFLP analysis.

a heterogeneous disorder. It has been suggested that PCOS develops as a consequence of a primary genetic abnormality in ovarian androgen production, interacting with environmental factors or other factors causing hyperinsulinemia.

Body fluids such as follicular fluid, amniotic fluid and cervical-vaginal fluid have been used to investigate the pathogenesis for pregnancy and birth including recurrent pregnancy loss (RPL), preeclampsia, intra-amniotic infection (20-23,25,26). In order to investigate novel etiology factors of PCOS, the proteins expressed in the follicular fluid of PCOS patients were investigated using 2-DE analysis, and six proteins were found to be aberrantly expressed. These proteins were kininogen 1, cytokeratin 9, antithrombin chain B, fibrinogen  $\gamma$ , apoA-IV precursor and A1BG (Fig. 3). However, it is unclear if these proteins were induced due to the ovarian hyperstimulation caused by IVF therapy even though control samples did not show upregulation of these proteins.

ApoA-IV is a 45 kDa glycoprotein triglyceride-rich glycoprotein (27). It is expressed in the small intestine and its synthesis and secretion increases in response to fatty meals (28). ApoA-IV has several proposed roles, including lipid transport, lipoprotein metabolism (29), control of food intake (30) and gastric function (31). apoA-IV has been shown to be antiatherogenic (32), and there is also evidence supporting apoA-IV antioxidant activity (32-34). Two mechanisms have been suggested for apoA-IV's antiatherogenic action: enhancement of cellular lipid efflux (i.e., 'reverse cholesterol transport') (35) and antioxidant activity (33,34).

We showed that the expression of apoA-IV was significantly increased in the follicular fluid from PCOS patients. One of the possibilities is that this may result in aberration of lipoprotein metabolism and transport, causing the generation of polycystic ovaries. Identifying the etiology of PCOS is extremely difficult as it is caused by a combination of hormonal, genetic and environmental factors. For these reasons, a combination of proteomic and genetic analyses has been used to identify new factors associated with these diseases (35-37). Several researchers have investigated a diverse range of genes that may be associated with PCOS to identify the etiology of this disease, and apoA-IV has also been studied in numerous diseases, including obesity, using genetic analysis (38). Gln360His and Thr347Ser are well-studied functional polymorphisms of apoA-IV. It has been demonstrated that these affect obesity and lipid/lipoprotein metabolism (39,40). In particular, Gln360His polymorphism is related with type 1 diabetes mellitus (T1DM) and obesity (41,42).

In women with PCOS, insulin resistance or deficiency is one of the main characteristics of patients with obesity (43,44). Therefore, we performed a single nucleotide polymorphism (SNP) study for Gln360His polymorphism of apoA-IV to investigate its association with PCOS. In these experiments, this polymorphism was shown to be highly related to PCOS. The homozygote of the variant allele was not detected in the control group. This suggests that the polymorphism affects the development of PCOS by regulating apoA-IV protein expression or by impacting the function of the protein. However, the

relationship between the aberrant expression of apoA-IV in follicular fluid and Gln360His polymorphism and their role in the generation of PCOS remains unclear. The study had certain challenges due to the fact that PCOS is an endocrine disorder influenced by diverse factors.

We also determined the expression level of  $\alpha$ -1-B-glycoprotein (A1BG) by western blot analysis, and found that the expression of this protein is increased in PCOS patients relative to healthy women. A1BG is a known plasma protein with unknown function and a member of the immunoglobulin superfamily. Little is known about the biological functions of A1BG with regard to infertility diseases. Therefore, further studies are needed to explain why the A1BG protein is increased in PCOS patients.

Although we showed that the expression of kininogen 1 and cytokeratin 9 was increased in the follicular fluids from PCOS patients by 2-DE analysis, western blot analysis showed individual variation between PCOS and normal patients. However, the expression level also varied among normal or PCOS patients, suggesting that these proteins may be involved in the etiology of PCOS in certain cases. Therefore, continuous investigation with 2-DE analysis is required for the identification of proteins associated with the pathogenesis of PCOS.

In addition, we showed that the expression of antithrombin and fibrinogen  $\gamma$  was increased in the follicular fluids from PCOS patients by 2-DE analysis. Western blot analysis, however, showed individual variation between PCOS and normal patients. In the case of antithrombin, Tsanadis *et al* (45) reported that the concentration difference was not statistically significant between women with PCOS and the control women, which is in agreement with our data. There are several studies in the literature regarding the concentration of fibrinogen in females with PCOS. Atiomo *et al* (46) reported a statistically higher concentration of fibrinogen in women with PCOS than in normal control women. Dahlgren *et al* (47) found that the concentration of fibrinogen was lower in females with PCOS. Kelly *et al* (48) did not find statistically significant differences between these two groups. Considering the discrepancies in the literature with regard to the expression level of fibrinogen in PCOS patients, further studies are required in order to more accurately understand the function of fibrinogen in PCOS patients.

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