

A single nucleotide polymorphism in exon 7 of *sorbin and SH3-domain-containing-1* (*SORBS1*) in Korean PCOS patients

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Received October 19, 2007; Accepted November 20, 2007

Abstract. Patients with polycystic ovarian syndrome (PCOS) are characterized by high levels of androgens, irregular or no menstrual cycle and increased hair growth. In addition, insulin resistance and glucose tolerance are caused in patients with PCOS. It was recently reported that PCOS in women is associated with a single nucleotide polymorphism (SNP) of genes, including the *sorbin and SH3-domain-containing-1* (*SORBS1*) gene involved in insulin resistance and glucose uptake. *SORBS1*, which belongs to the sorbin homology (SoHo) family of proteins, becomes protein-protein interaction through three SH3 domains. In addition, *SORBS1* is related to c-Cbl-associated protein (CAP) that has interacted with the downstream signaling components of glucose uptake. We therefore studied the association between the SNP of the *SORBS1* gene and PCOS in a Korean population. *SORBS1* raises glucose uptake and insulin resistance. The frequency of G allele was slightly higher in the patient group (n=233) than in the control group (n=105). A statistically significant relationship was found between the SNP in exon 7 of the *SORBS1* gene and PCOS in a Korean population group.

Introduction

Polycystic ovarian syndrome (PCOS) is a heterogeneous hormonal disorder that affects a woman's menstrual cycle, fertility, hormone production, insulin resistance and obesity (1-3). Women with PCOS have aberrant glucose tolerance, hyperandrogenism and diabetes mellitus (3,4). They are characterized by high levels of androgens, irregular or no menstrual cycle and hirsutism, as well as by significant metabolic abnormalities such as glucose-stimulated hyperinsulinemia, abnormality of expenditure energy and dyslipidemia

(3). It has been reported that approximately 4.9% of female college students in Korea have exhibited PCOS (5). In women of other ethnic groups, that number has been reported as 4-12% (1,6,7).

Recently, various single nucleotide polymorphisms (SNPs) have been investigated by means of linkage and association studies in women with PCOS. A number of SNPs associated with PCOS have been reported (8-20), as have cases of PCOS related to the target genes of glucose uptake and insulin resistance. Insulin receptor substrate-1 (IRS-1) and -2 (IRS-2) play key roles in the pathogenesis of PCOS (10,11). In addition, it has been reported that IRS-1 (Gly⁹⁷²Arg) and -2 (Gly¹⁰⁵⁷Asp) influence type-2 diabetes; patients with these polymorphisms revealed phenotypic features of PCOS (12). A recent study indicated that the *insulin* and *insulin receptor* gene is a genetic factor, as women with PCOS have increased type-2 diabetes (8). Other research has indicated that *CYP17*, encoding for the enzyme P450c17, is associated with the pathogenesis of PCOS (13-17). Previous reports have shown that the Pro12Ala variant of the *PPARγ* gene, a gene associated with insulin resistance and glucose tolerance in Caucasian woman with PCOS (18,19), is a key component of the pathogenesis of PCOS (18,19). Consequently, insulin resistance is of importance in various disorders including obesity, diabetes mellitus, glucose tolerance and hypertension, and an investigation of the SNPs of genes involved in glucose uptake and insulin resistance will aid in the clinical therapy of patients with PCOS.

It was recently suggested that clinical and biochemical variability in PCOS patients is caused by environmental and genetic factors, including glucose uptake and insulin resistance (20,21). Previous reports suggest that the candidate genes of women with PCOS are involved in insulin-mediated glucose transport, obesity and type-2 diabetes (20-26). We therefore hypothesized that downstream signaling proteins, such as insulin-mediated glucose transport, obesity and type-2 diabetes, were involved in this disorder.

The binding of insulin to its receptor activates the phosphorylation of insulin receptor and downstream signaling molecules. One of these downstream signaling components is c-Cbl, which is involved in glucose uptake and interacts with c-Cbl-associated protein (CAP), encoded by the *sorbin and SH3-domain-containing-1* (*SORBS1*) gene (20). The *SORBS1* gene, also called *CAP*, *SH3P12* or *Ponsin*, is one of the candidate genes for the pathogenesis of PCOS and is expressed

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Key words: insulin resistance, polycystic ovary syndrome, single nucleotide polymorphism, *sorbin and SH3-domain-containing-1*

in sensitive tissues such as the adipose tissues, skeletal muscles and liver (21-23). *SORBS1* belongs to the sorbin homology (SoHo) family of proteins and is involved in protein-protein interaction through three SH3 domains (20-23). Following the insulin stimulation and phosphorylation of Cbl, CAP-Cbl complexes are translocated from the insulin receptor to lipid rafts and interact with flotillin. It has been reported that the N-terminal region of CAP in 3T3-L1 adipocytes blocked glucose uptake by insulin, therefore a feature of the CAP-Cbl complex is a crucial protein for the stimulation of glucose uptake (24).

SORBS1 is located at chromosome 10q23.3-24.1 and consists of 34 exons (20,21). Of 14 SNPs identified in the *SORBS1* gene, two affect amino acid sequences encoding missense variants at R74W and T228A (26). However, one of the missense variants - R74W polymorphism - is not associated with obesity or diabetes. In contrast, T228A polymorphism in exon 7 of *SORBS1* is a protective factor for obesity and diabetes (26). T228A results from a nucleotide substitution at 682, changing ACG (Thr) to GCG (Ala) (26). It is implied that human *SORBS1* plays a key role in the pathogenesis of disorders with insulin resistance, type-2 diabetes and obesity (21,26). Previously, it was reported that the polymorphism T228A was associated with obesity and diabetes in a Chinese adult group (21). In the present study, we investigated, by means of a case-control study, whether *SORBS1* is associated with PCOS in a Korean population.

Materials and methods

Subjects. All subjects were recruited from the CHA General Hospital Fertility Center in Seoul, Korea. The patient group included 233 Korean patients with PCOS, based on the revised diagnostic criteria announced in 2003 by the American Society for Reproductive Medicine/European Society for Human Reproduction and Embryology (ASRM/ESHRE) Rotterdam consensus (27,28), and the control group another 105 healthy Korean women. The clinical and biochemical characteristics of the women with PCOS and the controls are presented in Table I. Their state of health was determined by medical history, physical and pelvic examination and complete blood chemistry. For the diagnostic criteria of PCOS, oligomenorrhea was defined as a reduction in the frequency of menses with intervals of 40 days to 6 months, and hyperandrogenism as serum testosterone (T) >0.6 ng/ml and/or serum DHEAS ≥ 300 μ g/dl (29,30).

Written informed consent was obtained from all of the participating women. Blood samples for molecular genetic studies were collected in tubes containing EDTA as an anti-coagulant and stored at 4°C until use. Genomic DNA was then extracted from the blood of patients with PCOS and the controls. All PCOS patients and controls in this study were Korean women. This SNP study with human blood samples was approved by an Institutional Review Board (IRB).

Biochemical determinations. Blood samples were collected from both PCOS and control patients. Hormone and glucose levels, including plasma FSH, LH, E₂, PRL, TSH, DHEAS, T and fasting glucose, were measured as indicators of discrimination. Insulin was measured as previously described (27,28).

Table I. Clinical and biochemical characteristics of patients for normal controls (n=105) and PCOS patients (n=233).

Characteristics	Control (n=105)	PCOS patients (n=233)
No.	105	233
BMI (kg/m ²)	20.95±2.49 (16.40-28.65)	22.96±3.86 (16.35-37.32)
Waist/hip ratio (WHR)	0.80±0.05 (0.70-0.91)	0.82±0.06 (0.69-0.95)
Hyperandrogenism and oligo- or amenorrhea	n=0 (0.00%)	n=31 (13.30%)
Hyperandrogenism and polycystic ovaries	n=0 (0.00%)	n=22 (9.44%)
Oligo- or amenorrhea and polycystic ovaries	n=0 (0.00%)	n=158 (67.82%)
Hyperandrogenism, oligo- or amenorrhea and polycystic ovaries	n=0 (0.00%)	n=22 (9.44%)
FSH levels (mIU/ml)	6.42±1.82 (3.00-11.50)	5.34±1.88 (1.00-19.90)
LH levels (mIU/ml)	3.30±1.62 (1.00-7.10)	6.73±4.67 (1.00-25.00)
E ₂ levels (pg/ml)	31.47±15.13 (5.00-64.10)	36.93±34.58 (5.00-88.50)
Prolactin levels (ng/ml)	13.24±7.48 (4.10-46.40)	13.42±9.64 (2.30-72.20)
TSH levels (μ IU/ml)	1.88±0.94 (0.03-4.06)	2.25±1.43 (0.46-10.45)
DHEA-S levels (μ g/dl)	153.03±56.46 (67.20-257.40)	194.07±75.54 (45.30-377.20)
Testosterone (ng/ml)	0.21±0.14 (0.01-0.54)	0.38±0.21 (0.06-0.86)

Genetic analysis. Exon 7 of the *SORBS1* gene was amplified by polymerase chain reaction (PCR) using 5'-TACCTCAC TGCATGCCCCACTCTC-3' as the forward and 5'-GACTGC TGGGAGGAGACATTAGAA-3' as the reverse primer in a total volume of 25 μ l. Cycling parameters were denaturation at 94°C for 5 min, 30 cycles at 94°C for 40 sec for denaturation, 68°C for 40 sec for annealing, 72°C for 40 sec for extension and 72°C for 5 min. Following PCR amplification, the PCR products were purified using a PCR purification kit (AccuPrep, Bioneer, Daejeon, Korea) and digested with *Kas* I (New England Biolabs, Beverly, MA, USA) for 3 h at 37°C. DNA fragments were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized with an ultraviolet transilluminator. The *Kas* I *GGCGCC* revealed that the restriction site contained A allele (Fig. 1A), therefore a single

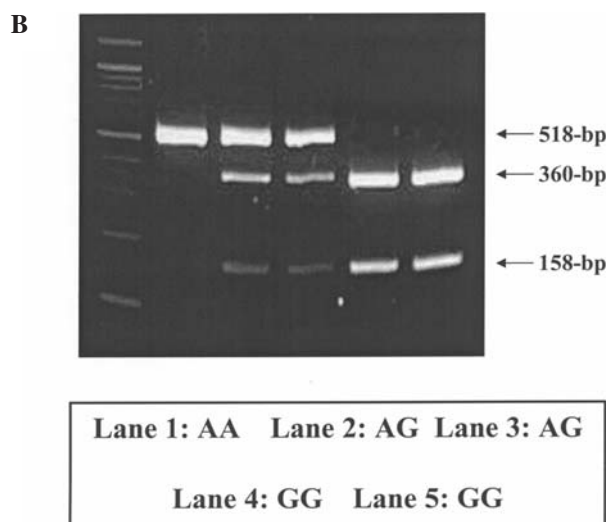
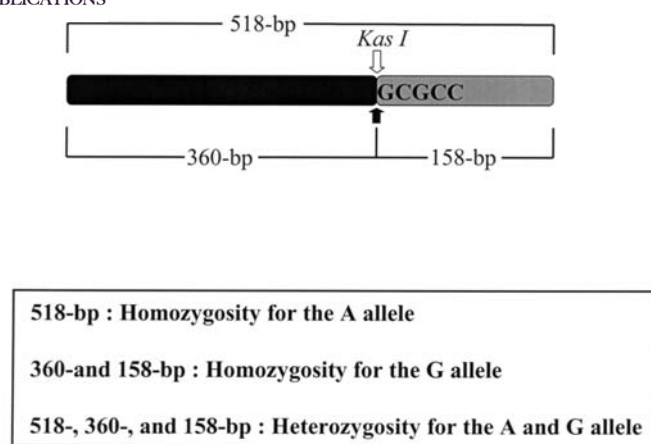


Figure 1. (A) Structure of the *SORBS1* gene. Arrow indicates the restriction of the *SORBS1* gene. The site of the G allele is marked by an arrow. When the sequence has the G allele, *Kas I* restricts the site. The products are two fragments, 360 and 158 bp. The A allele makes only one fragment, 518 bp. (B) A/G polymorphism in the *SORBS1* gene. The G allele has been restricted by *Kas I*. Shown is 2% agarose gel electrophoresis with ethidium bromide staining following *Kas I* digestion of the PCR product. The single band with 518 bp indicates an A/A genotype. Homozygosity of the G allele has two bands of 360 and 158 bp. The A/G type is indicated by three bands of 518, 360 and 158 bp.

518-bp (base pair) band indicates homozygosity for the A allele. The presence of two fragments, 360 and 158 bp, indicates homozygosity for the G allele. Three bands, corresponding to 518, 360, and 158 bp, indicate heterozygosity for the A or G allele (Fig. 1B).

Statistical analysis. Statistical analysis was performed using Hap Analysis (31), Haploview Ver. 3.32 (2006) and SAS Ver. 9.1 (2006). Logistic regression and the χ^2 test were used to analyze the association between the two groups. A p-value of <0.05 was considered statistically significant.

Results

We followed the diagnostic criteria of the ASRM/ESHRE Rotterdam consensus of 2003 regarding PCOS (27,28). According to their criteria, PCOS is diagnosed when the phenotypes of patients are found within two of three criteria, oligo- or menorrhea, clinical or biochemical hyperandrogenism and ultrasonographic polycystic ovarian morphology (27,28). We therefore looked for symptoms, body mass index (BMI), waist/hip ratio and hormone levels, including FSH, LH, E₂, prolactin, TSH, DHEA-S and testosterone levels (Table I). There were no significant differences between the clinical and biochemical features of our control and PCOS study groups. However, levels of TSH, testosterone and DHEA-S were slightly higher in the PCOS patient group than in the control group (Table I), and levels of LH were increased twice as much in PCOS patients than in the controls (Table I). Of the PCOS patients, 31 (13.3%) had hyperandrogenism and oligo- or amenorrhea, 22 (9.44%) hyperandrogenism and polycystic ovaries (PCOS), 158 (67.82%) oligo- or amenorrhea and PCOS and 22 (9.44%) hyperandrogenism, oligo- or amenorrhea and PCOS (Table I).

In order to analyze the frequency of genotypes for SNP in exon 7 of *SORBS1* gene, we used 233 samples from patients with PCOS and 105 samples from controls. Our hypothesis

was that PCOS might be caused by the aberrant expression of genetic factors, such as glucose-stimulation and secretion and/or stimulation of insulin resistance (10-12). A number of SNPs in the genes encoding the proteins involved in glucose uptake and/or insulin resistance were investigated. One of the SNPs in the *SORBS1* gene was expected to lead to a missense variant, T288A (A→G variant). This missense variant is recognized by the restriction enzyme, *Kas I*. We therefore investigated the frequency of three bands, 518, 360 and 158 bp, by restriction fragment length polymorphism (RFLP) analysis. Three genotypes were present in exon 7 of the *SORBS1* gene (Table II). The variant of *SORBS1*, A→G, was confirmed by digestion with *Kas I*. Homozygotes of the G allele did not differ significantly between the PCOS patient (0.9%) and control (0.9%) groups. In contrast, the rate of A/G genotype was twice as high in PCOS patients (23.2%) than in the control group patients (12.4%), and the frequency of the A/A genotype was similar in the PCOS (75.9%) and control (86.7%) groups, as shown in Table II.

PCOS patients were associated with a single variant of the G allele in *SORBS1*, therefore exon 7 in *SORBS1* in effective glucose uptake is significantly associated with PCOS patients. We found an association between the frequency of co-dominant alleles (p=0.040, OR=1.88) and recessive alleles (p=0.027, OR=2.06) in the PCOS patient and control groups. In addition, using the Haploview analysis, we found that the frequency of individual A and G alleles is significantly associated with a variant, A→G (p=0.039). As a result, we concluded that the A→G variant, a T228A missense, in *SORBS1* is associated with PCOS patients in a Korean population.

Discussion

It has been reported that patients with PCOS frequently suffer from obesity, type-2 diabetes, glucose tolerance and insulin resistance (1-4). These diseases are caused by the aberrant expression of target genes in PCOS patients. Of these, it has

Table II. Allele frequencies of A/G polymorphism in exon 7 of the *SORBS1* gene in PCOS patient (n=233) and control (n=105) groups.

Genotype	Case (PCOS %)	Control (%)	Co-dominant (AA or AG or GG)		Dominant (AA + AG or GG)		Recessive (AA or AG + GG)	
			OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
AA	177 (75.9)	91 (86.7)						
AG	54 (23.2)	13 (12.4)	1.88 (1.03-3.44)	0.04	0.90 (0.08-10.04)	0.93	2.06 (1.09-3.89)	0.027
GG	2 (0.9)	1 (0.9)						
Total	233	105						

been suggested that the abnormal expression of *SORBS1* is a cause of pathogenesis in PCOS (20). This results in abnormal glucose uptake and effective insulin resistance. *SORBS1* is one of the target genes for PCOS patents, indicating that it is an important adaptive protein in the insulin-signaling pathway (20-26). *SORBS1* plays a role in glucose uptake or insulin resistance in PCOS patients. An association study of the Chinese population reported that a missense mutation in *SORBS1*, T228A, is protective against obesity and insulin resistance (21). *SORBS1* contains a significant sequence homology with the Sorbin and SH3-domain in the C-terminus (21). In this study, we investigated the association between the T228A polymorphism in exon 7 of the *SORBS1* gene and PCOS in a Korean population group. A previous report has demonstrated that the T228A variant of the *SORBS1* gene has a major effect on obesity and insulin resistance in Western Pennsylvania in the United States (20).

In our study, we examined whether there is relationship between the *SORBS1* genotype and PCOS patients in a Korean population group. The frequency of recessive, AA or AG/GG genotypes is significantly associated with PCOS patient and control groups (Table II). We also performed statistical analysis, using logistic regression with a Hap analyzer as described previously, to identify whether there is relationship between the *SORBS1* genotype and PCOS patients (31). The statistical analysis revealed that there is an association between the frequency of co-dominant and recessive alleles in *SORBS1* and PCOS in a Korean population. For the association test of A and G alleles, we performed a Haploview analysis. The results revealed that the frequency of minor allele G is associated with PCOS ($p=0.039$). This suggests that a variant of minor allele G may cause pathogenesis in PCOS. These results suggest that there is a significant association between a variant of the *SORBS1* genotype, A→G, and PCOS patients in a Korean population (Table II), and that it affects insulin-stimulated glucose uptake, playing a key role in PCOS.

A previous report revealed that the T228A variant of exon 7 in the *SORBS1* gene is associated with obese populations (26). The frequency of SNPs in the *SORBS1* gene may differ

according to various standards of sampling. Since PCOS may result from various factors, analysis of the association with various angles of sampling is required. Further association analysis is therefore required for obese populations according to their levels of insulin and the presence and/or absence of hyperandrogenism in obese PCOS subjects. Since an association has been found between a number of PCOS patients and insulin-stimulated glucose uptake, candidate genes for PCOS involved in the insulin signaling pathway should be investigated in various ethnic backgrounds. Data from such genetic association studies will provide insight into the role of these genes in the pathogenesis of PCOS. In order to develop therapeutic approaches, these association studies need to be defined through the investigation of large populations of PCOS patients of different ethnicities.

Acknowledgments

We would like to thank the members of the Fertility Center and Cell and Gene Therapy Research Institute at Pochon CHA University and CHA General Hospital. We also thank Dr Sophia Chen at NIH in MD, USA for her critical comments on the manuscript. This study was supported by a grant from Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (01-PJ10-PG6-01GN13-0002).

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