

Study of the association of the T869C polymorphism of the transforming growth factor- β 1 gene with polycystic ovary syndrome

JUNG-HYUN PARK, LAN LI and KWANG-HYUN BAEK

Department of Biomedical Science, CHA University, Bundang CHA Hospital,
Seongnam-Si, Gyeonggi-Do 463-840, Republic of Korea

Received August 8, 2014; Accepted April 30, 2015

DOI: 10.3892/mmr.2015.3896

Abstract. Polycystic ovary syndrome (PCOS) is a common multifactorial disorder characterized by hyperandrogenism, insulin resistance and chronic oligoanovulation. In addition, a number of females with PCOS have ovaries with multiple cysts, an irregular or no menstrual cycle and an imbalance of female hormones compared with normal controls. The transforming growth factor β 1 (*TGF- β 1*) gene is one of the genes associated with obesity and type 2 diabetes, which are characteristic symptoms of PCOS. The present study, therefore, investigated the association between the T869C polymorphism of the *TGF- β 1* gene, a single nucleotide polymorphism (SNP) of *TGF- β 1* and PCOS. The genomic DNA from 285 patients with PCOS and 129 healthy control individuals was used in the present study. $P < 0.05$ was considered to indicate a statistically significant difference between the groups. The present study findings suggested that the frequency of genotypes provided no significant association between the T869C polymorphism in the *TGF- β 1* gene and patients with PCOS. Although the present study concluded that the T869C polymorphism in the *TGF- β 1* gene is not associated with the pathogenesis of PCOS, further studies regarding the correlation between other SNPs of the *TGF- β 1* gene and PCOS are required.

Introduction

Polycystic ovary syndrome (PCOS) is a complex syndrome and is the most common endocrine disorder in females of reproductive age globally (1). In a previous study, 4–4.8% of white females and 3.5% of African females exhibited PCOS (2). Similarly, a previous study demonstrated that

the prevalence of PCOS was 4.9% among female college students in Korea (3). According to the American Society for Reproductive Medicine (ASRM) and the European Society of Human Reproduction and Embryology (ESHRE), PCOS is diagnosed when the phenotype of patients satisfy two of the following three criteria: Oligomenorrhea or amenorrhea, biochemical hyperandrogenism or polycystic ovaries (4,5). Generally, females with PCOS also exhibit symptoms of an increased risk of infertility, obesity, hyperlipidemia, insulin resistance, type 2 diabetes (T2D), diabetic nephropathy (DN) and possibly cardiovascular disease (6–8).

The pathogenesis of PCOS is initiated by various signaling pathways: Metabolism, insulin-signaling, inflammation and angiogenesis (9). Among these pathways, insulin resistance or hyperinsulinemia triggers changes in the hypothalamo-pituitary-ovarian axis, causing excessive production of androgen, leading to aberrant follicular development. Hyperinsulinemia in PCOS inhibits the hepatic sex hormone binding globulin from increasing the levels of free testosterone (T) in the body. This promotes the secretion of luteinizing hormone (LH) with relatively low follicle-stimulating hormone (FSH) (10). For this reason, it is recognized that females with PCOS are susceptible to insulin resistance and T2D (11). A case-control genetic association investigation regarding the pathogenesis of PCOS focused on the single nucleotide polymorphisms (SNPs) affecting the inflammatory processes and the activity of transforming growth factor- β 1 (*TGF- β 1*) (12). In previous years, genetic studies have linked PCOS to a dinucleotide repeat marker, D19S884, in the *fibrillin 3* gene. Fibrillins are important molecules, which assemble into microfibrils in the extracellular matrix (ECM) to modulate the *TGF- β 1* signaling pathway (13,14). Therefore, variations in fibrillin 3 and the subsequent dysregulation of TGF- β may contribute to the pathogenesis of PCOS (15).

TGF- β is a multifunctional cytokine synthesized in a wide variety of tissue types and it is secreted from various cell types (16). The TGF- β superfamily consists of three isoforms: *TGF- β 1*, *TGF- β 2* and *TGF- β 3* (17). The members of the human TGF- β superfamily are critical modulators in apoptosis and cell survival (18). The TGF- β signaling pathway is initiated when a TGF- β superfamily ligand binds to a high-affinity transmembrane receptor complex,

Correspondence to: Professor Kwang-Hyun Baek, Department of Biomedical Science, CHA University, Bundang CHA Hospital, 335 Pangyo-Ro, Seongnam-Si, Gyeonggi-Do 463-840, Republic of Korea
E-mail: baek@cha.ac.kr

Key words: polycystic ovary syndrome, single nucleotide polymorphism, transforming growth factor β 1

composed of the activin-like kinase 5/TGF- β type 1 receptor and the TGF- β type 2 receptor. Each class of ligand binds to a specific type 2 receptor, which has a serine/threonine kinase domain (19). It subsequently recruits and phosphorylates a specific type 1 receptor. The type 1 receptor phosphorylates receptor-regulated Smads, which subsequently bind to the coSmad, Smad4. R-Smad/coSmad complexes activated by phosphorylation translocate into the nucleus to bind to gene promoters and activate the expression of the target genes involved in cell proliferation and differentiation (20,21). This TGF- β superfamily signaling demonstrates its potential role in embryonic development, cellular differentiation, hormone secretion and immune system functions (22,23). In addition to this Smad-mediated gene transcription, TGF- β signaling is involved in activating Smad-independent pathways, including the nuclear factor- κ B pathway (24), the mitogen-activated protein kinase/ERK pathway (25) and the phosphatidylinositol-3-kinase/Akt signaling pathway (26). Therefore, Smad-independent pathways in the TGF- β family signaling pathways have significant effects on the different biological functions of TGF- β , including cell cycle inhibition, immune suppression and neuroprotective effects (27,28).

The TGF- β signaling pathway has a vital role in the development of multiple tissues or cells, including folliculogenesis, which is the process of developing ovarian follicles (29). Members of the TGF- β superfamily are expressed in mammalian oocytes and thereby, the subsequent dysregulation of TGF- β has been implicated in the pathogenesis of abnormal follicle development and hyperandrogenism in patients with PCOS (30,31). A previous study indicated that the ovaries of females with PCOS exhibited all the markers of increased TGF- β activity (32).

The *TGF- β 1* gene has been suggested as a genetic factor due to the clinical symptoms of PCOS, including an increased risk of T2D. *TGF- β 1* promotes the production of the ECM in response to high levels of glucose. Therefore, *TGF- β 1* is considered to be central in the pathogenesis of DN (33).

The human *TGF- β 1* gene is located on chromosome 19q13.1-13.3 and has six known SNPs: C-988A (rs1800820), G-800A (rs1800468), C509T (rs1800469), T869C (rs1800470; Leu10/P.ro10; T29->C), G915C (rs1800471) and C11929T (Thr263Ile; rs1800472) (34,35). In a previous study, a statistically significant difference was detected between the control group and Egyptian patients with T2D in the frequencies of the *TGF- β 1* codon 10 (T869C) (36). The present study, therefore, focused on the T869C polymorphism located on exon 1, which may be one of the candidate genes associated with an increased risk of DN. The present study aimed to determine whether the T869C polymorphism of *TGF- β 1* was associated with PCOS.

Patients and methods

Study subjects. All individuals were Korean females (n=414) of which 129 were healthy controls and 285 were patients with PCOS, recruited from the Fertility Center at CHA General Hospital (Seoul, Korea) between 2008 and 2011. The diagnosis of PCOS was based on the criteria proposed by the 2003 ASRM/ESHRE Rotterdam consensus (4,5). The present study was approved by the Gangnam CHA Fertility

Center (Gyeonggi-Do, Korea). Written informed consent was provided by the patient

Phenotypic characterization of all subjects. Basal blood samples were obtained from patients with PCOS and the controls to measure the levels of the following: Plasma FSH, LH, estrogen (E2), prolactin (PRL), thyroid stimulating hormone (TSH), dehydroepiandrosteronesulphate (DHEAS), T, fasting glucose and insulin.

DNA extraction and genetic analysis. Blood samples were collected in tubes containing EDTA as an anti-clotting factor and stored at 4°C. The genomic DNA was extracted from the blood of patients with PCOS and the controls. Restriction fragment length polymorphism (RFLP) analysis was performed to determine the genotypes for the T869C polymorphism in exon 1 of the *TGF- β 1* gene. The T869C polymorphism was amplified by polymerase chain reaction (PCR) using the following primers: Forward, 5'-GTACCA GATCGCGCCCATCT-3' and reverse, 5'-TAGCCACAGCAT CGGTAGCAG-3', in a total volume of 30 μ l. In the reaction mixture (Solgent, Seoul, Korea), 100 ng genomic DNA was used as a template. The cycling parameters were as follows: Denaturation at 95°C for 5 min, 30 cycles at 95°C for 40 sec, 65°C for 40 sec and 72°C for 40 sec, followed by 72°C for 7 min. Following PCR (C1000 Thermal cycler; Bio-Rad Laboratories, Inc., Hercules, CA, USA), the PCR products of 277 bp were digested with *MspA1* I (Enzymomics, Daejeon, Korea) for 2 h at 37°C (Fig. 1A). The restricted DNA fragments were electrophoresed on a 2% agarose gel (Invitrogen Life Technologies, Carlsbad, CA, USA), containing ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) and visualized on a DNA Image Visualizer (Seoulin Bioscience Co., Ltd, Seoul, Korea).

A total of three genotypes were observed in the restricted DNA fragments: A single 277 bp band, indicating homozygosity for the T allele; the presence of two fragments, 253 bp and 24 bp, indicating homozygosity for the C allele; the presence of three fragments, 277 bp, 253 bp and 24-bp bands, indicating heterozygosity for the T allele and the C allele, respectively (Fig. 1B).

Statistics analysis. Statistical analysis for comparing the genotype frequencies of the control group and the patient group was performed using Hap analysis (HapAnalyzer Ver.1 0.1, NGRI, Seoul, Korea) and the χ^2 test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. The 2003 ASRM/ESHRE Rotterdam Consensus was followed to obtain the diagnostic criteria for PCOS. In accordance with these criteria, 285 patients with PCOS were diagnosed when they exhibited at least two of the following three symptoms: Oligomenorrhea or amenorrhea, clinical or biochemical hyperandrogenism and ultrasonographic polycystic ovarian morphology. In the present study, the control group had regular menstrual cycles and no characteristics based on the criteria proposed by the 2003 ASRM/ESHRE Rotterdam consensus. Conversely, the PCOS

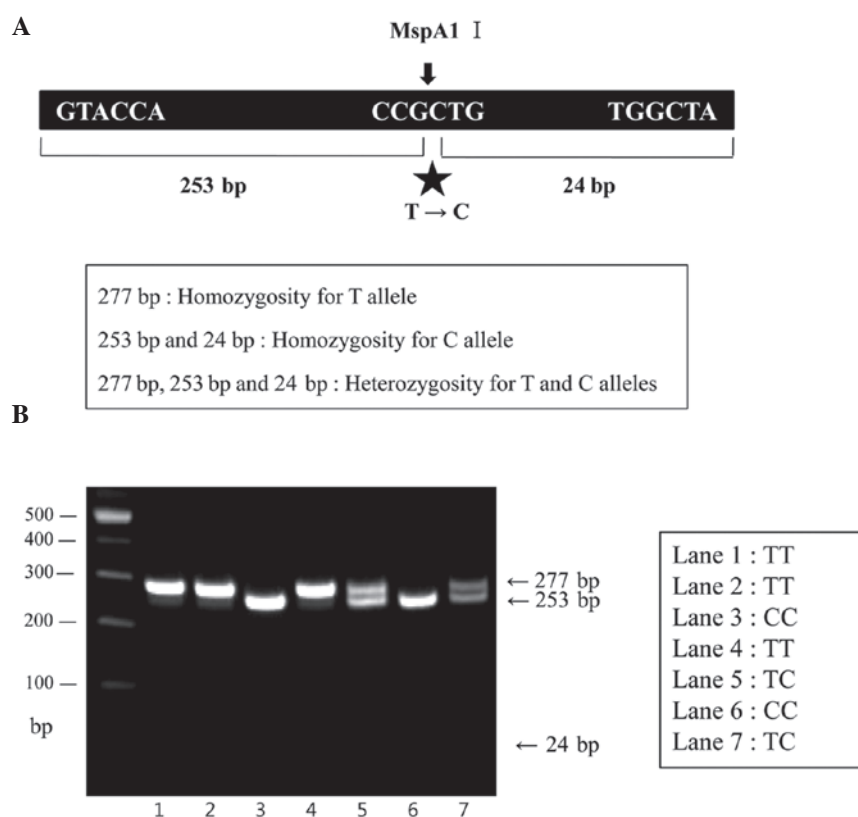


Figure 1. (A) Structure of the amplified T869C polymorphism of the *TGF-β1* gene. An arrow indicates the restriction site of *MspA1I*. (B) The T/C polymorphism in the *TGF-β1* gene. The T/T genotype is indicated by a single band at 277 bp, the C/C genotype is indicated by two bands at 253 and 24 bp, and the T/C genotype has three bands at 277, 253 and 24 bp.

Table I. Comparison of disorders and symptoms between the normal controls and patients with PCOS.

Characteristic	Controls, n=129	PCOS patients, n=285 (%)
Hyperandrogenism and oligo- or amenorrhea	0	50 (17.54)
Hyperandrogenism and polycystic ovaries	0	48 (16.84)
Oligo- or amenorrhea and polycystic ovaries	0	143 (50.18)
Hyperandrogenism, oligo- or amenorrhea and polycystic ovaries	0	44 (15.44)

PCOS, polycystic ovary syndrome.

Table II. Clinical and biochemical characteristics of normal controls and patients with PCOS.

Characteristic	Controls (n=129)	PCOS patients (n=285)	P-value
BMI (kg/m ²)	20.74±2.42 (16.39-32.56)	22.53±3.45 (16.67-28.02)	NS
Waist/hip ratio	0.78±0.05 (0.70-0.98)	0.80±0.06 (0.68-1.09)	NS
FSH levels (mIU/ml)	7.35±2.02 (3.05-20.67)	6.42±1.89 (2.64-18.86)	NS
LH levels (mIU/ml)	3.30±1.64 (0.82-7.03)	6.99±5.44 (1.20-20.08)	<0.001
E2 levels (pg/ml)	32.38±15.02 (5.06-63.38)	41.37±17.89 (8.01-86.36)	NS
Prolactin levels (ng/ml)	12.24±6.47 (4.04-46.29)	13.15±9.36 (2.30-71.54)	NS
TSH levels (μIU/ml)	1.82±0.83 (0.04-4.05)	2.30±1.25 (0.42-11.20)	NS
DHEAS levels (μg/dl)	148.98±54.85 (65.84-252.45)	178.92±67.45 (48.33-380.1)	0.01
Testosterone (ng/ml)	0.22±0.14 (0.02-0.53)	0.43±0.24 (0.07-0.85)	<0.001

BMI, body mass index; FSH, plasma follicular stimulating hormone; LH, lutenizing hormone; E2, estrogen; TSH, thyroid stimulating hormone; DHEAS, dehydroepiandrosteronesulphate; NS, not significant.

Table III. Genotypes of the T869C polymorphism of transforming growth factor- β 1 gene in control group and patients with PCOS.

Genotype	Control group n (%)	PCOS patients group n (%)	P-value
TT	41 (31.78)	78 (27.36)	0.789
TC	60 (46.51)	148 (51.92)	
CC	28 (21.71)	59 (20.7)	
Total	129	285	

PCOS, polycystic ovary syndrome.

patient group revealed that 50 patients (17.54%) had hyperandrogenism and oligomenorrhea or amenorrhea, 48 patients (16.84%) had hyperandrogenism and polycystic ovaries, 143 patients (50.18%) had oligomenorrhea or amenorrhea and polycystic ovaries and 44 patients (15.44%) had hyperandrogenism, oligomenorrhea or amenorrhea and polycystic ovaries (Table I).

Clinical and biochemical features. The clinical and biochemical features of the patients with PCOS and the control group are described in Table II, which demonstrated the body mass index (BMI), waist/hip ratio, obesity and hormone levels, including FSH, LH, E2, PRL, TSH, DHEAS and T. No particular differences were observed between the clinical and biochemical characteristics of the normal controls and the patients with PCOS. However, high levels of LH and T were observed in the PCOS patients, and the DHEA-S level was also slightly higher compared with the control group (Table II).

T869C polymorphism and PCOS. The homozygosity of the T allele of the T869C polymorphism in the *TGF- β 1* gene was confirmed by cutting with the *MspA1* I restriction enzyme (CCGTTG), resulting in the presence of a single fragment of 277-bp. The result of the RFLP analysis demonstrated that the frequencies of three genotypes were present in the T869C polymorphism of the *TGF- β 1* gene. As shown in Table III, the frequency of the T/T, T/C and C/C genotypes in the control and PCOS patient groups demonstrated similar proportions: The rate of the T/T genotype was observed in 41 controls (31.78%) and 78 patients with PCOS (27.36%), the rate of the T/C genotype was observed in 60 controls (46.51%) and 148 patients with PCOS (51.92%), and the rate of the C/C genotype was observed in 28 controls (21.71%) and 59 patients with PCOS (20.7%; Table III). These results revealed no significant association between the T869C polymorphism in the *TGF- β 1* gene and the patients with PCOS.

Discussion

TGF- β is a multifunctional cytokine, which exerts its biological function by regulating several cellular processes, including proliferation, differentiation, embryonic development, ECM formation, angiogenesis and immunity (37). Altered expression of *TGF- β 1* due to polymorphisms exerts an affect on numerous normal cellular and disease processes, including T-cell activation and proliferation, tumor development, and

asthma (38). Among the *TGF- β 1* polymorphisms, the polymorphism at codon 10 (T869C) may be associated with higher or lower *TGF- β 1* synthesis *in vitro* and may affect a variety of autoimmune-associated diseases, including rheumatoid arthritis, asthma, systemic lupus erythematosus and infectious diseases (39). In addition, TGF- β is known as an important mediator in ECM molecule production, including fibronectins, collagens and proteoglycans (40). Its overexpression is one of the most continuous molecular characteristics of pathological tissue fibrosis, which leads to multiple organ failure, including the skin, liver, lung and kidney (41). The SNP in codon 10 of *TGF- β 1* changes the amino acid sequence and affects the levels of *TGF- β 1*. For this reason, the increased and thickened ovarian stroma of patients with PCOS, which is caused by increasing fibrous tissue and collagen deposition, are signs of the dysregulation of the local TGF- β superfamily members and its signaling pathway (42).

Previous studies have suggested the direct effects of TGF- β dysregulation on females with PCOS (43,44). Ovarian folliculogenesis is regulated by a balance between extra- and intra-ovarian factors. An imbalance between extra- and intra-ovarian factors results in aberrant folliculogenesis and oogenesis disorder. Intra-ovarian factors include epidermal growth factor, fibroblast growth factors, the insulin-like growth factor family, the neurotrophin growth factor family, the TGF- β family, the vascular endothelial growth factor family, the cytokine family and other microenvironmental factors (6). The TGF- β superfamily members expressed in the ovary lead to the pathogenesis of anovulation, hyperandrogenism and abnormal follicle development in females with PCOS (30). Furthermore, folliculogenesis and follicle maturation are a series of complicated processes in which mature follicles are differentiated from primordial follicles. This developmental process can be interfered with by aberrant extra-ovarian factors, resulting in ovarian malfunction. These abnormal extra-ovarian endocrine disorders, including FSH deficiency, LH hypersecretion, hyperandrogenism and hyperinsulinemia with insulin resistance, are involved in the pathogenesis of PCOS (45). It has been reported that the majority of patients with PCOS have susceptibility to obesity and T2D. These diseases are caused by the abnormal expression of target genes in patients with PCOS. Of the target genes, the aberrant expression of TGF- β 1, a significant protein in the insulin signaling pathway, results in T2D, which has symptoms, including glucose tolerance and insulin resistance. In addition, increased levels of *TGF- β 1* in the serum are

associated with increased IL-1Ra, an anti-inflammatory cytokine. Additionally, increased concentrations of IL-1Ra develop the metabolic regulation of patients with T2D.

It has been reported that an increase in the TC and CC genotype frequency in *TGF-β1* codon 10-gene polymorphisms was statistically significant in patients with T2D, and an increased frequency of the TT genotype was significant in the controls. However, several studies investigated the association between the *TGF-β1* codon 10 gene polymorphism and T2D in the Polish and Chinese populations (46,47). The results of these previous studies revealed different frequencies of genotypes and alleles compared with those of Egyptians (36). This difference between the two previous studies of different ethnic groups may be derived from variations in the allele frequency of the different populations.

The development of low-grade chronic inflammation and the innate immune system, which regulates the effects of genes, fetal programming and metabolic syndrome are significantly involved in the pathogenesis of T2D. Since *TGF-β1* is a central mediator of the immune system through its primary immunosuppressive effect, it is a critical anti-inflammatory immune regulator (48). *TGF-β1* also affects T cells by inhibiting the activation of macrophages. Although the role of the TGF-β family in the pathogenesis of PCOS remains to be fully elucidated, reproductive abnormalities appear in knockout mice, which lose function at all levels of the TGF-β signaling pathway.

This is the first study, to the best of our knowledge, on the association between the SNP of the *TGF-β1* gene and patients with PCOS. A previous study reported that the T869C polymorphism of *TGF-β1* is associated with T2D and obesity (35), however the authors did not analyze its association with PCOS. In the present study, the results revealed no significant correlation between the T869C polymorphism in the *TGF-β1* gene and females with PCOS. Therefore, this present genetic association study indicated no evidence of the involvement of the T869C polymorphism in the *TGF-β1* gene in PCOS. However, further genotypic association investigations into other SNPs of the *TGF-β1* gene and PCOS are required. In addition, investigations regarding the *TGF-β1* gene and patients of different ethnic groups with PCOS are required.

Acknowledgements

The authors would like to thank the members of the Fertility Center and Stem Cell Institute at CHA University and CHA General Hospital. This study was supported by a grant from the Brain Korea 21 (BK21) PLUS project in Korea.

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