

Research Paper

The Association of -429T>C and -374T>A Polymorphisms in the *RAGE* Gene with Polycystic Ovary Syndrome

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Abstract

Polycystic ovary syndrome (PCOS) is a complex disorder characterized by hyperandrogenism and insulin resistance. 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 to those of normal controls. A variety of genetic factors have been involved in the pathogenesis of PCOS. Among these genetic factors, the receptor for advanced glycation end products (RAGE) that is associated with diabetes and involved in the complications of PCOS, was selected. We aimed to assess the relationship between -429T>C and -374T>A single nucleotide polymorphisms (SNPs) of *RAGE* gene with the susceptibility to PCOS. 128 controls and 265 PCOS patients were used for -374T>A polymorphism and 141 controls and 290 PCOS patients were used for -429T>C polymorphism, respectively. Genotyping of two polymorphisms were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay and statistical analysis was performed. *P* values for both alleles were higher than 0.05. Frequencies of genotype and allele of two polymorphisms in *RAGE* gene showed no significant differences between controls and PCOS patients. The initial study on the correlation between *RAGE* gene and PCOS indicates that the two polymorphisms of *RAGE* are not associated with the pathogenesis of PCOS. However, further studies regarding the association between *RAGE* gene and PCOS patients in different ethnic groups are required.

Key words: Receptor for advanced glycation end products, Single nucleotide polymorphism, Polycystic ovary syndrome.

Introduction

Polycystic ovary syndrome (PCOS) is a common and heterogeneous reproductive endocrine disorder that affects 6-8% of premenopausal women [1,2]. Its etiology is unknown and it is diagnosed by the American Society for Reproductive Medicine (ASRM) and the European Society of Human Reproduction and Embryology (ESHRE). According to ASRM/ESHRE, PCOS is defined when PCOS patients belong to two of the three criteria: oligo- or anovulation, hyperandrogenism, and polycystic ovaries [3,4]. Generally, women with PCOS have

several metabolic aberrations such as a high risk of impaired glucose tolerance [5], insulin resistance, hyperlipidemia [6,7], diabetes mellitus, and cardiovascular disease [8].

Advanced glycation end-products (AGEs) are protein or lipid products of nonenzymatic glycation that is one of the most important post-translational modifications in cells [9,10]. These compounds can be increased under conditions such as oxidative damage by hyperglycemia in patients with hyperlipidemia and diabetes mellitus [11,12]. AGE affects a variety of

cells and tissues in the body through the formation of interaction between molecules in the extracellular matrix by their receptor (RAGE) [13]. AGEs bind several receptors such as oligosaccharyltransferase-4 (AGE-R1/OST-48), AGE-R2/80K-H phosphoprotein, AGE-R3/Galectin-3, CD36, Macrophage scavenger receptor class A (SRA), and CD36 [14]. Most of AGE interacts with a cellular receptor RAGE [15]. These receptors are differently expressed based on cell and tissue types and play a role in degradation and removal of AGE [16].

RAGE is one of transmembrane receptors of the immunoglobulin superfamily. *RAGE* gene is located in the major histocompatibility complex locus (MHC) class III locus on chromosome 6p21.3 and comprises a 1.7kb 5' flanking region and 11 exons interlaced by 10 introns [17]. RAGE is expressed in a variety of cell types such as endothelial cells, dendritic cells, T-lymphocytes, monocytes, macrophages, and smooth muscles [15,18]. RAGE binds multiple ligands, including HMGB1, amyloid- β -protein, Mac-1, LPS, and AGEs [19]. RAGE has been linked to many different diseases, such as cancer, atherosclerosis, vascular disease, Alzheimer's disease, diabetic retinopathy, and diabetic nephropathy [21, 22]. In particular, patients with diabetes show increased levels of expression and accumulation of RAGE in retina and mesangial cell [22].

In normal homeostasis, RAGE binds and degrades AGE for maintaining decreased levels of AGE. However, in diabetes in particular, levels of AGE are increased. Following high levels of AGE, the high interactions between RAGE and AGE cause the secretions and activations of a variety of cytokines [24]. In PCOS, the expression levels of AGE and RAGE in serum are elevated in women with PCOS compared to those of controls [18].

To date, a few studies about the association between single nucleotide polymorphisms (SNPs) of *RAGE* gene and PCOS exist. The interaction of AGE-RAGE might lead to the pathogenesis of PCOS. Therefore, the aim of the present study was to investigate the association between polymorphisms of the *RAGE* gene and PCOS. Among 30 polymorphisms of *RAGE*, we selected -429T>C and -374T>A polymorphisms of the *RAGE* because these two polymorphisms are implicated in diabetes associated with PCOS.

Materials and methods

Study subjects

A total of 431 women which were unrelated controls and PCOS patients were recruited from the Fertility Center at CHA General Hospital (Seoul,

Korea) between 2008 and 2011. PCOS patients were diagnosed by the criteria defined by the 2003 ASRM/ESHRE Rotterdam consensus. This study was approved by the Institutional Review Boards of Fertility Center of the CHA General Hospital. Written informed consent was obtained from all participants.

Biochemical measurements

Plasma follicular stimulating hormone (FSH), luteinizing hormone (LH), estrogen (E2), prolactin (PRL), thyroid stimulating hormone (TSH), dehydroepiandrosteronesulphate (DHEAS), and testosterone (T) were measured by a chemiluminescent analyzer (Beckman Coulter Inc., Fullerton, CA, USA).

DNA extraction

Blood samples of PCOS patients and controls were collected in tubes containing EDTA as an anti-clotting factor and stored at 4°C. Genomic DNA was extracted from the blood in PCOS patients and controls.

Genetic analysis

To determine genotypes of the -429T>C and -374T>A polymorphisms in exon 1 of *RAGE* gene in PCOS cases and healthy controls, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis were carried out. DNA fragments for the -429T>C and -374T>A polymorphisms were amplified by polymerase chain reaction (PCR), using 5'-AAA ACA TGA GAA ACC CCA GA-3' as the forward primer and 5'-CCC CGA TCC TAT TTA TTC CA-3' as the reverse primer in a total volume of 30 μ l. In the reaction mixture, 100ng genomic DNA was used as a template. Cycling parameters were denaturation at 95°C for 5 minutes, 30 cycles with 95°C for 30 seconds, 58°C for 40 seconds, 72°C for 40 seconds, and 72°C for 10 minutes. After PCR, the PCR products of 222 bp were digested with *AluI* and *MluI* (New England BioLabs, MA, USA) for 2 hours at 37°C. The restricted DNA fragments were electrophoresed on a 2% agarose gel containing ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) and visualized by DNA Image Visualizer (SeoulLin Bioscience Co., Ltd, Seoul, Korea). In *RAGE* -429T>C polymorphism, three genotypes exist in restricted DNA fragments: a single 222 bp band indicates homozygosity for the T allele. The presence of two fragments, 176 bp and 46 bp, indicates homozygosity for the C allele. The presence of three fragments, 222 bp, 176 bp, and 46 bp bands, indicates heterozygosity for the T allele and the C allele, respectively. In *RAGE* -374T>A polymorphism, three genotypes exist in restricted DNA fragments: a

single 222 bp band indicates homozygosity for the T allele. The presence of two fragments, 121 bp and 101 bp, indicates homozygosity for the A allele. The presence of three fragments, 222 bp, 121 bp, and 101 bp bands, indicates heterozygosity for the T allele and the A allele, respectively.

Statistics analysis

Statistical analysis was performed by Hap Analysis (NGRI, Seoul, Korea; www.hap.ngri.re.kr) and the χ^2 test to investigate the genotypes frequencies in controls and PCOS cases. A *P* value less than 0.05 was considered to be statistically significant.

Results

We investigated the two polymorphisms -429T>C and -374T>A in the promoter region of *RAGE* gene in control and PCOS patient groups. PCOS patients were diagnosed by the 2003 ASRM/ESHRE Rotterdam Consensus. These criteria include two out of the following three criteria: oligomenorrhea and/or anovulation, clinical and/or biochemical hyperandrogenism, and polycystic ovaries (Table 1).

The clinical and biochemical characteristics of subjects were listed in Table 2. Normal and PCOS patient groups were described the body mass index (BMI), waist/hip ratio, obesity, levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen (E2), prolactin (PRL), thyroid stimulating hormone (TSH), dehydroepiandrosteronesulphate (DHEAS), and testosterone (T). No significant differences for the levels of these proteins except LH and T between

controls and PCOS patients were observed. Levels of LH and T were higher in PCOS patients compared to those of controls (Table 2).

The allelic and genotypic distribution of -374T>A and -429T>C polymorphisms were shown in Figure 1. The alleles of -374T>A and -429T>C polymorphisms were confirmed by RFLP analysis by using *Mluc I* and *Alu I* restriction enzymes, respectively. Regarding -374T>A polymorphism, no significant difference between controls and PCOS patients was identified. The TT genotype was shown in 6 controls (4.7%) and 12 PCOS patients (4.5%), TA genotype in 40 controls (31.3%) and 98 PCOS patients (37%), and AA genotype in 82 controls (64%) and 155 PCOS patients (58.5%). The frequencies of T alleles were 52 controls (20.3%) and 122 PCOS patients (23%) and A alleles were 204 controls (79.7%) and 408 PCOS patients (77%) (Table 3).

Table 1. Comparison of disorders/symptoms between the normal controls and PCOS patients.

Characteristics	-374T>A		-429T>C	
	Controls (n=128)	PCOS patients (n=265) (%)	Controls (n=141)	PCOS patients (n=290) (%)
Hyperandrogenism and oligo- or amenorrhea	n=0	n= 61 (23.02)	n=0	n=52 (17.93)
Hyperandrogenism and polycystic ovaries	n=0	n=50 (18.87)	n=0	n=51 (17.59)
Oligo- or amenorrhea and polycystic ovaries	n=0	n=114 (43.02)	n=0	n=140 (48.28)
Hyperandrogenism, oligo- or amenorrhea and polycystic ovaries	n=0	n=40 (15.09)	n=0	n=47 (16.20)

Table 2. Clinical and biochemical characteristics of normal controls and PCOS patients.

Characteristics	-374T>A		<i>P</i> value	-429T>C		<i>P</i> value
	Controls (n=128)	PCOS patients (n=265)		Controls (n=141)	PCOS patients (n=290)	
Body Mass Index (kg/m ²)	21.53±2.14 (17.31-31.64)	22.44±3.58 (17.52-27.81)	NS	20.68±2.19 (16.1-31.52)	22.15±3.02 (16.54-29.3)	NS
Waist/hip ratio	0.8±0.06 (0.65-0.94)	0.79±0.05 (0.62-1.02)	NS	0.75±0.07 (0.68-0.97)	0.82±0.03 (0.59-1.15)	NS
FSH levels (mIU/ml)	7.43±2.01 (4.08-19.51)	6.39±1.76 (2.54-18.75)	NS	7.35±1.98 (3.92-19.43)	6.35±1.71 (2.67-18.94)	NS
LH levels (mIU/ml)	3.2±1.58 (0.81-7.56)	6.71±5.29 (1.15-18.8)	< 0.001	3.13±1.46 (0.89-7.44)	6.58±5.32 (1.2-18.86)	< 0.001
E2 levels (pg/ml)	30.84±14.25 (5.63-62.79)	40.64±13.11 (7.98-75.23)	NS	29.51±13.28 (5.57-62.9)	38.75±12.18 (7.84-74.3)	NS
Prolactin levels (ng/ml)	11.7±6.36 (3.82-50.92)	13.17±8.09 (2.4-72.53)	NS	11.83±5.62 (3.75-55.4)	12.23±7.49 (2.37-75.93)	NS
TSH levels (μIU/ml)	1.74±0.71 (0.06-4.12)	2.27±1.32 (0.39-5.3)	NS	1.84±0.74 (0.05-4.08)	2.31±1.27 (0.4-5.26)	NS
DHEA-S levels (μg/dl)	150.8±52.13 (64.9-260.96)	192.46±70.37 (49.42-378.5)	0.01	147.7±53.62 (62.1-264.73)	188.5±70.29 (49.7-380.2)	0.01
Testosterone (ng/ml)	0.3±0.16 (0.03-0.47)	0.48±0.29 (0.07-0.92)	< 0.001	0.25±0.18 (0.02-0.52)	0.4±0.33 (0.06-0.87)	< 0.001

NS: not significant.

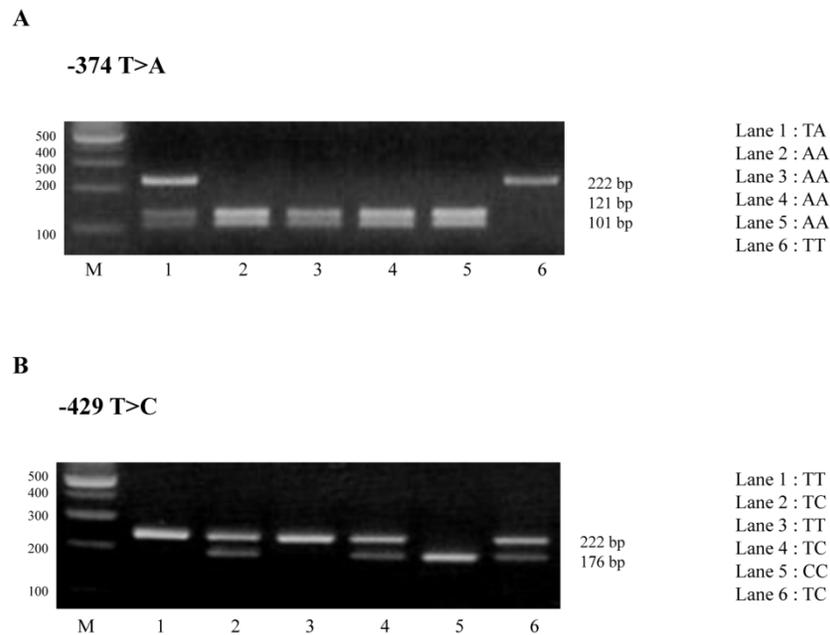


Figure 1. Results of PCR-RFLP assay in -374T>A and -429T>C polymorphisms of *RAGE* gene. (A) The amplified -374T>A polymorphism of *RAGE* gene was shown three different genotypes. The TT genotype is shown by a single band in 222bp, the TA genotype is shown by three bands in 222, 121, and 101 bp, and the AA genotype is indicated by two bands in 121 and 101 bp. (B) The amplified -429T>C polymorphisms of *RAGE* gene was shown three different genotypes. The TT genotype has one band in 222 bp, the TC genotype has three bands in 222 bp, 176 bp, and 46 bp, and CC genotype has two bands in 176 bp and 46 bp.

Also, no significant difference between controls and PCOS patients was identified in -429T>C polymorphism (Table 3). The TT genotype was shown in 103 controls (73%) and 214 PCOS patients (73.8%), TC genotype in 37 controls (26.3%) and 73 PCOS patients (25.2%), and CC genotype in 1 control (0.7%) and 3 PCOS patients (1%). The frequencies of T alleles were 243 controls (86.2%) and 501 PCOS patients (86.4%) and C alleles were 39 controls (13.8%) and 79 PCOS patients (13.6%). These results indicate that -374T>A and -429T>C polymorphisms in the promoter region of *RAGE* gene are not associated with the pathogenesis of PCOS.

Table 3. Genotypes and allele frequencies of *RAGE* in controls and PCOS patients.

-374T>A	Control (n=128) (%)	PCOS (n=265) (%)
TT	6 (4.7%)	12 (4.5%)
TA	40 (31.3%)	98 (37%)
AA	82 (64%)	155 (58.5%)
Alleles		
T	52 (20.3%)	122 (23%)
A	204 (79.7%)	408 (77%)
Allele frequency	OR(95% CI) = 1.173 (0.8139-1.691)	P value = 0.3917
-429T>C	Control (n=140) (%)	PCOS (n=290) (%)
TT	103 (73%)	214 (73.8%)
TC	37 (26.3%)	73 (25.2%)
CC	1 (0.7%)	3 (1%)
Alleles		
T	243 (86.2%)	501 (86.4%)
C	39 (13.8%)	79 (13.6%)
Allele frequency	OR(95% CI) = 0.9825 (0.6501-1.485)	P value = 0.9332

Discussion

AGEs are the products of non-enzymatic glycation and oxidation of target proteins and lipids [19]. AGEs are formed by exogenous and endogenous factors. The exogenous AGEs are significantly produced by environmental factors such as diet and smoking. The endogenous AGEs are relatively slowly accumulated under increased glucose level in blood [20]. AGEs accumulate in various environments such as aging, inflammation, and renal diseases [21]. AGEs are main factors of a variety of disease such as obesity, type 2 diabetes, cardiovascular disease, and inflammation [22]. Levels of AGEs in plasma are associated with adipogenesis that is one of the characteristics for PCOS [23].

AGE binds to RAGE that is expressed in a variety of cells such as monocytes, lymphocytes, and endothelial cells [24]. This AGE-RAGE interaction has a role in the activation of cytokine, expression of adhesion molecules, and proliferation of fibroblast by upregulating diverse signaling pathways [25]. As increased expression levels of AGEs induce the production and accumulation of AGEs, the upregulated AGE-RAGE interaction has been implicated in the pathogenesis of diabetes [26]. High levels of RAGE are shown in diabetes patients compared to those of healthy controls [27].

AGE also elevates the expression of RAGE in PCOS patients with insulin resistance [18]. According

to the increased levels of circulating AGEs in serum, AGE-RAGE interaction was elevated in PCOS patients [18]. Communications between AGE and the insulin receptor signaling (IRS) pathway have an effect on the elimination of AGEs [18]. AGEs are eliminated by IRS/PI3K pathway that is up-regulated by insulin [18]. In IRS/PI3K pathway, IRS induces autophosphorylation of tyrosine residues of insulin receptor and PI3K stimulates receptor-mediated endocytosis of AGEs [18]. On the other hand, PCOS patients that have defective IRS/PI3K pathway fail to eliminate AGEs normally [18]. Therefore, the down-regulated endocytosis and high levels of accumulation of AGEs are shown in PCOS women [18].

Interaction between AGE and RAGE also stimulates and activates nuclear NF- κ B that is one of the redox sensitive transcription factors [28]. AGE-RAGE interaction with activated NF- κ B pathway controls the expression of various inflammatory cytokines such as TNF- α , IL-1, and PAI-1 [28, 29]. Expression levels of these cytokines are increased in PCOS women compared to those of healthy controls. They have an effect on endothelial dysfunction and vascular injury that can be implicated in the pathogenesis of PCOS and infertility [30]. AGE localizes in follicular cell layers except for endothelial cells in ovaries of normal controls. However, in PCOS ovaries, AGE expression is observed in follicular cell layers and endothelial cells. Similar expression of RAGE is detected in follicular, endothelial, and stromal cells in normal ovaries and PCOS ovaries [31].

The expression levels of *RAGE* gene is regulated by -374T>A and -429T>C polymorphisms in the promoter region of *RAGE*. The -374T>A and -429T>C polymorphisms affect transcriptional effects of *RAGE*. The -374T>A polymorphism that substitutes from T to A suppresses the expression of *RAGE* [32]. On the other hand, the -429T>C polymorphism that substitutes from T to C increases the expression of *RAGE* [33].

In conclusion, the present study found that there is no significant correlation between the polymorphisms of *RAGE* gene and PCOS women. However, this association study is the first report regarding *RAGE* polymorphisms and PCOS patients in Korea. In fact, this study has two limitations. First, the number of PCOS cases and unrelated controls was not big enough. Second, only two SNPs in *RAGE* gene were used for this study. Therefore, further investigations between other SNPs of the *RAGE* gene and PCOS in different ethnic populations are required. In conclusion, this study indicated that two polymorphisms of *RAGE* gene was not associated with PCOS patients in Korean women. However,

further studies regarding the correlation between *RAGE* gene and PCOS patients in different ethnic groups are required.

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Abbreviations

AGEs: Advanced glycation end-products; ASRM/ESHRE: American Society for Reproductive Medicine and the European Society of Human Reproduction and Embryology; BMI: Body mass index; DHEAS: Dehydroepiandrosteronesulphate; E2: Estrogen; FSH: Follicle stimulating hormone; INS: Insulin receptor signaling; LH: Luteinizing hormone; NF κ B: Nuclear factor-kappa B; PCOS: Polycystic ovary syndrome; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; PRL: Prolactin; RAGE: Receptor for advanced glycation end-products; SNP: Single nucleotide polymorphism; T: Testosterone; TSH: Thyroid stimulating hormone.

Competing Interests

The authors have declared that no competing interest exists.

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