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Analysis of Connexin37 gene *C1019T* polymorphism and PCOS susceptibility in South Indian population: case–control study

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ABSTRACT

Objective: Polycystic ovarian syndrome (PCOS) is a complex and multifactorial disorder believed to be the consequence of a complex interaction between genetic, immunological, and environmental factors. The main aim of this study was to investigate the association of *Connexin37* (*Cx37*)/Gap junction alpha 4 (GJA4) gene *C1019T* single nucleotide polymorphism (SNP) with the susceptibility to polycystic ovarian syndrome (PCOS) in South Indian women.
Study design: This study comprises 98 PCOS patients and 100 healthy women without PCOS of South Indian origin. We genotyped total of seventeen selected *Cx37* SNPs including *C1019T* (rs1764391) by polymerase chain reaction and sequencing analysis. The genotype frequency and allele distributions of cases and controls were analyzed using Fisher's exact test.
Results: The genotype and allele frequencies of the *C1019T* polymorphism significantly differ between cases and controls. The frequencies of *C/C* genotype ($P = 0.009$) and '*C*' allele ($P = 0.002$) of the *C1019T* polymorphism showed a significant prevalence in cases compared to controls.
Conclusion: Our findings suggest that the *Cx37 C1019T* variation may contribute to the risk of PCOS in the South Indian women.

Introduction

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder in women of reproductive age with a prevalence of 6–10% [1]. It is characterized by the presence of polycystic ovaries, menstrual dysfunction, infertility and biochemical (elevated androgens) as well as clinical hyperandrogenism (hirsutism and/or acne) [2]. In addition it is also associated with an increased incidence of cardiovascular disease (CVD), subclinical atherosclerosis, type 2 diabetes, dyslipidaemia, impaired glucose tolerance, Obesity and insulin resistance [3,4]. The observation of familial aggregation indicates heritable tendency of the PCOS, but the etiology and pathogenesis remains uncertain.

Gap junctions are clusters of intercellular channels that aggregate at sites of close cell–cell apposition, connecting the cytoplasm of adjacent cells and allow the passage of second messengers, inorganic ions and small metabolites (less than ~1000 Da) between cells. Earlier it was shown that Intercellular communication via gap junctions between the oocyte and the surrounding granulosa cells is essential for correct functioning and development of the follicle [5]. Gap junctions mediate metabolic cooperation between granulosa cells and the oocyte by transmitting endocrine, paracrine, and growth factor signals [6,7]. Consequently, they play a role in the coordination of follicular growth, steroid hormone production [8] and the maturation of the oocyte [9].

The mammalian cumulus–oocyte complex (COC) is a multicellular unit that express multiple connexins and proper development of COC require gap junction intercellular signaling [10]. The cumulus–oocyte complex (COC) consist of the female gamete (oocyte) surrounded by specialized granulosa cells, called cumulus cells which are in direct contact with the oocyte through transzonal projections of Connexin37 (Cx37) (Gap junction alpha 4 (GJA4)) [11]. Cx37 forms gap junction channels that have conductance larger than any other known connexion [12]. Simon

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et al. showed that without Cx37, follicular development is arrested, and subsequently ovulation does not occur [13].

Single Nucleotide Polymorphisms (SNPs) in Connexin genes can have major effects on the rate of gap junction intracellular communication (GJIC) [14]. Polymorphisms in *Cx37* gene have been found in different human populations, but most of these lie outside the open reading frame [15]. Recently, Richard and co-workers identified a variant form of *Cx37* with a *C* to *T* shift at 1019, which causes a shift from proline to serine at amino acid 303 in C-terminal domain of protein sequence [16]. This C terminal domain is essential for docking between gap junctions and can affect the voltage sensitivity of the junctional conductance [17].

Several case–control studies have revealed an association between *C1019T* SNP in the human *Cx37* gene and various diseases with inconsistent results. The 1019*C* allele was significantly associated with coronary artery disease in Taiwan population [18], in contrast, 1019*T* allele was associated with coronary artery disease in Japanese population [19]. Furthermore, 1019*C* allele was significantly associated with myocardial infarction in Caucasian population from Switzerland [20], in contrast 1019*T* allele was suggested as a risk factor for myocardial infarction in Sicilian population [21]. A recent case–control study in Taiwanese population by Leu et al. [22] showed that 1019*T* allele of *Cx37* gene was associated with carotid intima-medial thickness and subsequent ischemic stroke. However the role of *Cx37* gene *C1019T* polymorphism and its key role in pathophysiology of PCOS has not been established. In the current study, for the first time we have investigated whether variants of *Cx37* gene were associated with susceptibility to PCOS in South Indian women.

Materials and methods

Study design

Ninety eight women of Indian origin with PCOS were recruited at the Infertility Institute and Research Centre (IIRC), Secunderabad, India. PCOS was confirmed by trans-vaginal ultrasound scan (TVS) at screening followed by laparoscopy. The diagnosis of PCOS was based on the Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group [23]. They had no smoking and no caffeine habits and BMI was calculated as body weight (kg) divided by body height squared (m^2). The clinical characteristics of PCOS women and controls were summarized in Table 1. Polycystic appearing ovaries were defined sonographically as the presence of multiple (>10), small (2–9 mm in diameter) follicles in the periphery (in one plane) and increased stromal echogenicity. The presence of polycystic ovaries was confirmed by ultrasound scan, followed by laparoscopy to rule out any other reproductive disorders. Women with other infertility related disorders such as endometrial cysts on ovaries, adenomycosis, ovarian adhesions and presence of cysts on pelvic organs other than ovaries, even those showed symptoms of PCOS (like hyper androgenaemia and elevated hormone concentrations) but who had normal ovaries as revealed by trans-vaginal ultrasound scan (TVS) and laparoscopic examination were excluded from the present study. In addition, the study group women showed one or more of following clinical or biochemical disturbances: infertility, hirsutism, acne, irregular menstruation, laboratory tests revealing androgen excess (serum testosterone concentration >2.5 nmol/l or plasma testosterone >40 pmol/l), and an elevated LH/FSH ratio.

Table 1
Clinical characteristics of PCOS and control group.

Variable	Total PCOS (n = 98)	Total controls (n = 100)
Age (years)	26.27 ± 3.82	26 ± 4.50
Weight (kg)	58.45 ± 12.84	56.52 ± 9.52
BMI (kg/m^2)	24.28 ± 4.25	23.73 ± 3.00
FSH (mIU/ml)	5.95 ± 1.94	6.05 ± 1.70
LH (mIU/ml)	7.68 ± 2.36	5.3 ± 1.64
LH:FSH	1.44 ± 0.88	0.90 ± 0.22
Presence of overweight and obesity	45 (45.91)	14 (14)

Data are given as mean ± S.D. or n (%).

Controls

To compare the results obtained from the patient group, a total of 100 healthy women, with regular menstrual cycles aged from 18 to 40 years (mean age: 26 years) of age were recruited as controls. They had no hirsutism, acne, alopecia, or endocrine dysfunction and had a successful pregnancy record. The absence of polycystic ovaries in the control group was confirmed by trans-vaginal ultrasound scan (TVS) method.

5 ml of venous blood sample were collected from all subjects (cases and controls) into sterile EDTA (Ethylene diamine tetra acetic acid) vacutainers for DNA isolation and stored at −80 °C until further use. All participants gave written informed consent. The study was approved by the institutional review board of the Centre for Cellular and Molecular Biology (CCMB), Hyderabad.

DNA extraction

Genomic DNA was extracted from 1 ml of EDTA anti-coagulated whole blood by using proteinase K digestion followed by a salting out procedure according to the previously described method [24].

Determination of Connexin37 genotype

The genotyping of *Cx37 C1019T* SNP (NCBI SNP CLUSTER ID: rs1764391) were analyzed by polymerase chain reaction (PCR) and sequencing analysis as per the protocols described earlier [24]. Both cases and controls were genotyped in a randomized, blinded fashion. PCRs were carried out in a total volume of 25 μl, containing 50 ng genomic DNA, 2–6 pmol of each primer, 1× Taq polymerase buffer (1.5 mM $MgCl_2$) and 0.25 U of Amplitaq DNA polymerase (Perkin Elmer, Foster City, USA). The primers for *C1019T* were 5′-ACAATGGGCTCTCATCCAGT-3′ (forward), and 5′-GCCACATTCTGGA-GAGGAAG-3′ (reverse). The primers were designed by using primer 3plus software. PCR amplification was performed in a programmable thermal cycler gradient PCR system (Eppendorf AG, Hamburg, Germany). The PCR amplification was carried out for 35 cycles (denaturation at 94 °C for 1 min, annealing for 1 min at 58 °C, extension at 72 °C for 1 min and final extension for 10 min at 72 °C). PCR products of 372 bp (+1019) was analyzed by 1.5% agarose gel stained with ethidium bromide and then sequenced with a Taq-Dye deoxy-terminator cycle sequencing kit (Applied BioSystems, USA) using an automated ABI 3770 DNA sequencer (Applied BioSystems, USA). Genotype calling was performed by using Chromas V.2 software (Technelysium Ltd., Australia).

Statistical analysis

Statistical analysis was performed using SPSS statistical package (V 11.0). SNP genotypes were tested for departures from Hardy–Weinberg equilibrium (HWE) for controls using Fisher's exact test and all SNPs were in HWE. The allele ratios and genotype distributions of cases and controls were analyzed using Fisher's exact test. '*P*' values <0.05 were considered as statistically significant. The odds ratio and 95% confidence interval (CI) values were calculated using the online Vassar Stats Calculator (http://www.faculty.vassar.edu/lowry/VassarStats.html). The sample power was analyzed by OpenEpi software.