

Association of insertion/deletion polymorphism of angiotensin converting enzyme gene with metabolic components of polycystic ovary syndrome



Susmita Banerjee¹, Anindya Dasgupta², Suparna Roy³

¹Assistant Professor, Department of Biochemistry, Diamond Harbour Government Medical College and Hospital, Diamond Harbour, West Bengal, India, ²Professor and Head, ³Associate Professor, Department of Biochemistry, Calcutta National Medical College and Hospital, Kolkata, West Bengal, India

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ABSTRACT

Background: Polycystic ovary syndrome (PCOS) has taken the prime position as an endocrine disease in women of reproductive age and is estimated to affect 2.2–22.5% worldwide and in Indian women it ranges from 9% to 12%. The primary defects that cause PCOS still remains unknown. A lot of genetic factors not excluding polymorphisms and mutations of multiple genes have been linked with PCOS, exact mechanisms which might be the cause of PCOS are yet unknown. Angiotensin converting enzyme (ACE) and its yields are related with angiogenesis of ovarian epithelium, restoration of meiosis, steroidogenesis, and follicular growth. **Aims and Objectives:** The present study was aimed to establish the association of ACE insertion/deletion (I/D) polymorphism in PCOS patients and to assess the influence of this polymorphism on the metabolic components of PCOS. **Materials and Methods:** Genomic DNA was extracted from blood obtained from 100 patients with PCOS and 100 healthy controls following ethical guidelines. DNA was amplified by polymerase chain reaction (PCR) using I/D distinct allele primers. PCR products were assessed after being exposed to gel electrophoresis. Results were analyzed in respect to biochemical parameters. **Results:** The allelic frequency and genotypic distribution of the ACE gene polymorphism is associated with PCOS in women. The concentrations of testosterone as well as luteinizing hormone/ follicle stimulating hormone ratio among the distinct genotypes were significantly different. The presence of “D” allele in a population more likely to be associated with formation of polycystic ovary and hyperandrogenism was observed. **Conclusion:** The data suggest that ACE I/D polymorphism and PCOS pathology are unassociated. However, aggravation of clinical symptoms of PCOS can be linked to the steroidogenesis which, in turn, is associated with the polymorphism.

Key words: Angiotensin-converting enzyme; Hyper-androgenism; Insertion/deletion polymorphism; Polycystic ovary syndrome; Renin angiotensin system

INTRODUCTION

Polycystic ovary syndrome (PCOS) has taken the prime position as an endocrine disease in women of reproductive age. Worldwide it has been seen to affect 2.2–22.5% of women in the reproductive age group and is one of the leading causes of infertility.¹⁻³ The syndrome consists of androgen excess, menstrual dysfunction, hyperinsulinism, and

associated systemic complications. Be the environmental or genetic factors, both are contributors to the etiopathogenesis of PCOS, resulting in a wide range of metabolic defects.^{4,8}

Although several genetic factors including polymorphism and mutations of specific genes have been linked with PCOS possibility and inheritance, the molecular mechanisms behind PCOS risk are not clearly evident.⁹

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Address for Correspondence:

Dr. Susmita Banerjee, Assistant Professor, Department of Biochemistry, Diamond Harbour Government Medical College and Hospital, Diamond Harbour, West Bengal, India. **Mobile:** +91-9903208044. **E-mail:** susmitasahoo80@gmail.com

PCOS patients other than having the clinical hallmarks of oligomenorrhea, hirsutism, infertility, and excess insulin levels exhibit altered lipid profile, obesity, high blood pressure, and an increased pro-thrombotic state.^{10,11} They have been reported to having increased predicament to the risk of type 2 Diabetes Mellitus,¹² impaired glucose tolerance and cardiovascular diseases. Hyperplasia and hypervascularity of the ovarian theca interna and stroma have been marked to be the important features of PCOS, and one of the primary causes of infertility.¹³

Genetic causes play an important role in presence of PCOS and are probably inherited as autosomal dominant trait but recent studies show that more than one gene is involved in the etiology of PCOS.¹⁴

Evidence suggests renin angiotensin system may influence ovulation, oocyte development, steroidogenesis as well as formation of corpus luteum through connections with other system.¹⁵ Angiotensin converting enzyme (ACE), encoded by the ACE gene, is a part of renin angiotensin system (RAS) and is expressed in a number of tissues such as renal tubules, breast, lung, small intestine, and ovaries. The ACE and its products are related to angiogenesis of ovarian endothelium, resumption of meiosis, steroid genesis, and follicular growth in cattle.¹⁶ Polymorphism of ACE gene is based on the insertion (I) or deletion (D) within intron 16, of a 287 base pair ALU repeat sequence resulting in genotypes: DD, II and ID. DD and II being homozygous while ID being heterozygous. Rigat et al.¹⁷ showed that the inter-personal variation of ACE concentration is related to an I/D polymorphism involving a 287-bp DNA sequence located in intron 16 which is a part of ACE gene and is also known as ACE I/D polymorphism. Yoshimura stated the connection of RAS in the progress of PCOS. However, very less reports on the role of ACE in human ovaries have been established.¹⁸

Aims and objectives

Based on these findings, the present study was aimed to estimate frequency of ACE I/D polymorphism in PCOS patients and its influence on the metabolic components of PCOS.

MATERIALS AND METHODS

Study population

This study comprised 100 PCOS patients and 100 controls from Obstetrics and Gynaecology Department of Calcutta National Medical College. The whole study was conducted according to the ethical rules and regulations of Helsinki Declaration for human studies 1975, revised in 2000.

Informed consent approved by the Ethical committee was obtained from each patient. Information of clinical and anthropometric measures were collected through pro forma. The study population was genetically homogeneous urban East Indian population.

Diagnostic criteria of PCOS

Patients with PCOS were diagnosed as per the Rotterdam PCOS consensus criteria (The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2003).¹⁹ As per the Rotterdam Criteria at least two of the below mentioned are indicators in diagnosis of PCOS: Clinical or biochemical signs of hyperandrogenism:

1. Oligo or anovulation
2. Biochemical hyperandrogenemia or clinical manifestation of hyperandrogenemia and
3. Polycystic ovaries on ultrasound (Ovulatory women with hyperandrogenemia and polycystic ovaries are considered to suffer from PCOS).

Other causes of hyperandrogenism such as hyperprolactinemia, androgen secreting tumors, cushing's syndrome, and non-classic congenital adrenal hyperplasia and patients with any chronic illness such as diabetes, hypertension, carcinoma as well as patients on long-term drug therapy were excluded from the study.

Controls

The controls were selected from healthy females by excluding the diagnosed PCOS as per Rotterdam criteria and having normal menstrual cycles (<32 days), without hirsutism or any other gynecological pathologies.

Polymorphism and genotype determination

4 ml of peripheral blood was drawn from each patient in EDTA vacutainers and genomic DNA was extracted by phenol chloroform extraction method. Stored at -20°C till polymerase chain reaction (PCR) is done. The DNA quantification was done using spectrophotometric analysis. The oligonucleotide sequence for the insertion (I) and deletion (D) polymorphism, that is both forward and reverse primers of the ACE gene was chosen from previous studies.²⁰ The genotyping for ACE gene I/D polymorphism was done by PCR followed by electrophoresis using 2% agarose gel. The 190bp fragment indicates the 'D' allele and 490bp fragment indicates 'I' allele (Figure 1). Heterozygotes show both 490 and 190bp fragments. The PCR was performed in a thermocycler in following conditions: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 45 s, and final extension at 95°C for 5 min. 'I' allele specific primer having sequence 5'-TGG GAC CAC AGC GCC CGC CAC TAC-3' and 5'-TCG CCA GCC CTC CCA TGC

CCA TAA-3' was used to confirm the accurateness of I allele. PCR conditions were 30 cycles with denaturation at 92°C for 40 s, annealing at 63°C for 40 s, and extensions at 72°C for 40 s for this amplification. The method gave a product of 335bp confirming 'I' allele. The genotyping for patient and control groups was performed following similar method. Electrophoresis was done with PCR products and analyzed in gel documentation system.

Analysis of chemical features

ACE I/D genotypic distribution in relation to body mass index (BMI), W/H ratio, and age of onset of the disease was done.

Biochemical parameters

Serum Testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin measured using ELISA technique.

Interpretation

A PCR product of 490 bp denotes a genotype homozygous for insertion (II), 190bp homozygous for deletion (DD), and the presence of 490bp and 190bp products indicate heterozygous genotype, respectively.

Statistical analysis

Data analysis was done using Statistical Package for the Social Sciences (IBM and SPSS version 21). Comparison of allelic and genotypic frequencies of patients and control subjects were done by Pearson Chi-square test. Hardy Weinberg equilibrium was assessed by Chi-square analysis. P<0.05 is considered statistically significant. Anthropometric characters and hormone levels in women with three specific genotypes were done by Mann–Whitney U test.

RESULTS

ACE I/D genotypic distribution were statistically insignificant between patients and control group with

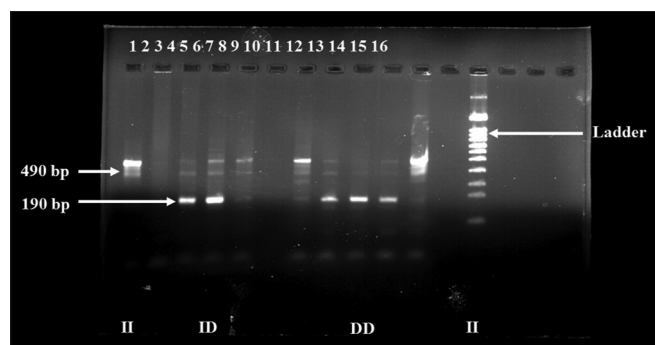


Figure 1: Gel picture showing different genotypes of ACE gene, II (insertion,homozygous), ID (insertion-deletion heterozygous), DD (deletion homozygous)

respect to BMI, W/H ratio, age of onset, and serum prolactin levels considering P<0.05 to be statistically significant.

Table 1 presents the distribution of ACE gene I/D genotypes in PCOS patients and control. The distribution between the groups with respect to genotypic distribution (P<0.05) was not statistically significant. The D and I allele frequency is 53% and 47%, respectively, in patients whereas it is 49% and 51%, respectively, in control group. Table 2 shows the comparison between three individual genotypes in patients as well as controls with respect to certain hormonal characteristics.

DISCUSSION

The distribution, as per the study, of ACE gene I/D polymorphism was analyzed in PCOS subjects to ascertain its role in the PCOS etiology. The frequencies of DD, ID, and II ACE gene polymorphism in patients with PCOS and controls are (31%, 43%, and 26%) and (34%, 29%,

Table 1: Distribution of Genotypes and allelic frequencies between PCOS and control (P<0.05 is considered significant)

Groups	Distribution of Genotypes			Allele Frequencies	
	DD	ID	II	D Allele	I Allele
Patient	31	43	26	53	47
Control	34	29	37	49	51
P-value	P=0.09 (not significant)			P=0.56 (not significant)	

Table 2: Mann–Whitney U test to compare the hormonal parameters in different genotypes among patients and controls (P<0.05 is considered significant)

Genotypes	Cases (mean rank)	Controls (mean rank)	P-value
DD (Deletion)			
Testosterone	22.9	19.06	0.314
LH	25.27	17.64	0.046*
FSH	19.4	21.16	0.645
LH/FSH ratio	26.87	16.68	0.008*
ID (Insertion/deletion)			
Testosterone	20.07	15.54	0.205
LH	23.83	9.08	0.0001*
FSH	21.33	13.5	0.032*
LH/FSH	21.65	12.92	0.017*
II (Insertion)			
Testosterone	11.38	12.68	0.644
LH	16	7.64	0.003*
FSH	12.33	11.64	0.806
LH/FSH	15.33	8.36	0.014*

LH: Luteinizing hormone, FSH: Follicle-stimulating hormone

and 37%), respectively, with $P=0.09$. In our study, both genotype and allelic distributions were insignificant when compared between cases and controls. The ACE genotypes distribution was assessed to be as per Hardy Weinberg equilibrium in the control group. However, it was seen to be deviated in the patient group. To keep Hardy-Weinberg equilibrium intact in a population, population must be very large and random. The results obtained in this study representing correlation between ID polymorphism of ACE gene and PCOS women is consistent with the result of Sun et al.,²¹ on Chinese population which mention D allele of the ACE gene has no influence on the occurrence of PCOS regardless of the criteria used for assessing patients of PCOS. Our present finding is in contrast to the study of Sun et al.,²¹ which suggested that D allele is linked with RAS and the development of polycystic ovary and hyperandrogenism. The outcome may have been influenced due to certain factors. First, in Caos study the sample size being small may limit its depiction; second, a misclassification between ID heterozygotes to the DD homozygotes might have been caused due to the method chosen.²² The error was corrected by using an additional PCR analysis to confirm DD genotypes obtained in the first PCR. A new sense primer which is insertion specific was included.

Patients and control arm in our study did not bring forth any significant differences in terms of genotype distributions. However, we observed significant differences in the concentration of testosterone, LH and LH/FSH ratio. The concentration of testosterone was higher in patients of PCOS than in controls. In the case as well as control groups, testosterone levels were highest in the DD genotype followed by ID and least in II genotypes. Higher levels of ACE were found in DD genotype compared to either II or ID genotypes,¹⁷ which implicates the fact that increased level of ACE maybe associated with increased levels of testosterone. It also indicates that I/D polymorphism of ACE gene in some way or the other may add to the expression of testosterone in ovaries. There could be several explanation of this fact. First, recent studies showed the presence of Angiotensin (1-7), its receptor Mas and, the ACE type II are expressed in human ovaries. ACE II is able to generate Angiotensin (1-7) directly from Angiotensin II or indirectly from Angiotensin I (endopeptidase). Physiologic role of this system is still under investigation but evidence from animal model shows its participation in modulation of local steroidogenesis and ovulation.²³⁻²⁵ Second, ACE is related to ovarian endothelium angiogenesis *in vitro*. ACE promoting angiogenesis of ovarian endothelium *in vivo* is still under investigation. The fact that ovary is more vascular than other organs and most of the steroidogenic cells are in contact with one capillary at least,²⁶ increased levels of

ACE can enhance angiogenesis of ovarian endothelium. Furthermore, increased blood supply may increase the supply of cholesterol which is the precursor of steroid hormone. Third, the ACE – angiotensin II product inhibits progesterone secretion stimulated by LH. Stimulation of secretion of LH through negative feedback due to reduced secretion of progesterone explains the degree of difference of LH/FSH ratio in specific genotypes of patients.²⁷ Moreover, last but not the least, increased testosterone levels can also lead to more transformation of estrogen, which through positive or negative feedback endorse modulation of the release of LH and FSH.²¹

Limitations of the study

It was a single centered study with limited resources and small study population. A multicentric study with a larger uniform study population would help in validation of our findings.

CONCLUSION

Conclusive facts pointing to the role of ACE I/D polymorphism and thus the reason of PCOS is not provided in recent genetic studies. Considerations toward it as a molecular marker along with increasing susceptibility to PCOS and commencement of the clinical symptoms would require a larger population. Although our study showed that the I/D polymorphisms in the ACE gene were not a causative factor toward the pathogenesis of PCOS there can be major variation in different genotypes. Thus, ACE I/D polymorphism is may be one of the causes of aggravated symptoms.

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Authors Contribution:

SB- Concept and design of the study, manuscript writing, statistical analysis, and interpretation of results; **AD-** Study designing, reviewing literature, statistical analysis, and revision of manuscript; **SR-** Concept, coordination, review of literature, and revision of manuscript.

Work attributed to:

Calcutta National Medical College and Hospital, 32 Gorachand Road, Kolkata - 700 014, West Bengal, India.

Orcid ID:

Dr. Susmita Banerjee - <https://orcid.org/0000-0002-8972-6128>

Dr. Anindya Dasgupta - <https://orcid.org/0000-0001-9713-1071>

Dr. Suparna Roy - <https://orcid.org/0000-0002-1879-9223>

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