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Research Article

Association of insulin-like growth factor 2 Apa1 A820G gene (rs680) polymorphism with polycystic ovarian syndrome

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ABSTRACT

Background: Hyperandrogenism is the cornerstone of polycystic ovarian syndrome (PCOS) as per androgen excess society - 2006 criteria. Insulin-like growth factor 2 (IGF 2) gene stimulates ovarian androgen secretion and is involved in the pathogenesis of PCOS. The objective of this study was to study the association of insulin-like growth factor 2 (IGF2) gene Apa1 A820G (rs680) polymorphism with PCOS.
Methods: Prospective genetic case-control study, involving 204 women with PCOS and 204 healthy, sex and age matched controls. Anthropometric and biochemical profile were taken in a well-designed proforma. Isolation of deoxyribonucleic acid (DNA) by salting out method and genotype analysis was done for all the study population using PCR-RFLP.
Results: We have demonstrated an association between IGF2 Apa1 A820G gene (rs680) polymorphism and PCOS. Frequency of G allele was 0.40 in PCOS and 0.08 in controls (OR 7.639, CI 5.08 to 11.47, and P value <0.0001) indicates that the G allele is associated with PCOS in our population. The GG genotype conferred a significant risk of developing PCOS (OR 19.645, CI 2.569 to 148.61 and P value 0.0039). We found the significant association of GG genotype with body mass index and insulin resistance in PCOS when compared with controls.
Conclusions: This study suggests that IGF 2 gene Apa1 A820G polymorphism is associated with PCOS and could be used as a relevant molecular marker to identify women with risk of developing PCOS in our population and may provide an understanding about the etiology of PCOS.

Keywords: PCOS, Gene polymorphism, IGF, Apa1 variant

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is the most common reproductive endocrine disorders of women, with a prevalence of approximately 5-10% worldwide.[1] As per the newer diagnostic criteria of PCOS, hyperandrogenism is the cornerstone of PCOS, which manifests either clinically or biochemically.[1,2]

Insulin-like growth factor 2 gene located on 11p15.5, stimulates insulin action as well as adrenal and ovarian androgen secretion, together with insulin growth factor 1 (IGF1) and IGF binding proteins (IGFBP), may play a role in the pathogenesis of PCOS.[3] Millan S et al found a significant association between homozygosity for G alleles of the Apa 1 variant in IGF2 and PCOS.[4] The G alleles increase IGF2 mRNA levels in leukocytes and possibly results in increased IGF2 expression and

secretion in liver. Insulin-like growth factor 2 is a 67-aminoacid mitogenic peptide, which may act as an autocrine or paracrine growth factor for tumor growth. Several tumors like uterine leiomyosarcoma, ovarian cancer, endometrial cancer, cervical cancer, breast carcinoma, and testicular tumors have been shown to over secrete IGF 2.[5]

Therefore the aim of the present study was to assess the association of IGF2 gene Apa 1 variant (rs680) polymorphism with PCOS, which is a common reproductive endocrine abnormality in our population.

METHODS

This study was approved by the institutional ethical committee and informed written consent was obtained from all subjects. In this prospective case-control study we included 204 PCOS patients from Anu"s fertility center, Somajiguda, Hyderabad, India from July, 2011 to January 2013.

Inclusion criteria

Subjects were ranged in age from 17 to 35 years and were diagnosed using the 2006-androgen excess society (AES) criteria:

- Hyperandrogenism, clinical or biochemical and either
- Oligo-anovulation or
- Polycystic ovarian morphology.[1,2]

Exclusion criteria

Women excluded from the study were those with inherited disorders like congenital adrenal hyperplasia, androgen secreting neoplasms, androgenic/anabolic drug use or abuse, Cushing's syndrome, syndromes of severe insulin resistance, thyroid dysfunction and hyperprolactinemia.

We have recruited 204 controls from a tertiary care hospital, Kamineni academy of medical sciences and research center, LB Nagar, Hyderabad to this study over the same period. Subjects ranged from 17-35 years and did not show hirsutism, acne or male-type alopecia. All of them had regular menstrual cycles and none of them satisfied any of the AES-2006 criteria. All the control subjects also underwent an ultrasonographic examination, and women who had any pathologic findings like polycystic ovaries were excluded from the study.

Definitions

Clinical hyperandrogenism; modified Ferriman-Gallwey (mFG) score > 6 with or without acne and/or androgenic alopecia.[6] Hirsutism was scored by studying terminal hair in nine body areas (upper lip, chin, chest, upper and lower abdomen, upper arms, thighs, and upper and lower back). The occurrence of acne was recorded by areas of distribution and degree of affection with lesions (papules, cysts, scars, or abscesses) categorized simply as mild, moderate and severe. Acanthosis nigricans (AN); dark, velvety, skin thickening on the neck, axilla, and other sites such as face, chest and knuckles were recorded. Oligomenorrhoea; absence of menstruation for more than 35 days, amenorrhea; no menstruation for more than 6 months.

The definition of polycystic ovarian morphology by ultrasound examination is the presence of >12 follicles with 2 to 9 mm diameter in the ovary. An ovarian volume of >10 ml is also suggestive. Only one ovary consistent with PCO morphology is sufficient for diagnosis.[7] All subjects underwent a trans-vaginal ultrasound or trans-abdominal ultrasound in the follicular phase to evaluate ovarian morphology and any lesions in the pelvic area.

Clinical findings

Clinical history included a questionnaire-based interview regarding socio-demographic factors, detailed menstrual and obstetric history, onset and degree of clinical symptoms of PCOS, dietary habits, drug history, and family history of PCOD, diabetes, hypertension, and cardiovascular risk factors. Physical examination for body mass index (BMI), waist to hip ratio (WHR), blood pressure, acne, hirsutism, alopecia, male pattern of hair loss, acanthosis nigricans were done.

Sampling

Two milliliters of peripheral blood was collected in EDTA for DNA isolation and 5 ml of blood in plain vial for serum preparation from all the patients and controls along with clinical data, personal history and family history.

Isolation of DNA and genotype analysis

Genomic DNA was isolated from the peripheral blood of subjects using salting out method in our laboratory.[8,9] The DNA was stored at -200 C until processing. Genotyping for the IGF2 Apa1 gene polymorphism (rs680) was performed by polymerase chain reaction (PCR), with the use of specific published primers.[10] Forward primer: 5'-CTTGGACTTTGAAGTCAAATTGG-3'; Reverse primer: 5'GGTCGTGCCAATT ACATTTCA-3' synthesized from sigma - Aldrich Chemical Pvt Limited (Bangalore, India), followed by restriction fragment length polymorphism (RFLP) analysis. A three-step PCR was performed using XP thermal cycler as described by us earlier.[11] Briefly the PCR conditions included an initial denaturation at 940C for 5 minutes, followed by 30 cycles of denaturation at 940C for 30 seconds, annealing at 550C for 30 seconds and extension at 680C for 45 seconds, final extension at 680C for 5 minutes. The 292 bp amplified PCR product was digested with Apa1 enzyme at 370C for 2 hours and electrophoresed on 2%

agarose gel with ethidium bromide. Bands of 229 bp were observed in case of GG genotype, 292 bp and 229 bp in AG genotype and an undigested 292 bp band in AA genotype. Restriction enzyme digested PCR products were imaged and analyzed by documentation in UVI Tech gel documentation system (UVI Tech Ltd., Cambridge, United Kingdom).

Data and statistics

Body mass index = weight/height^2 (kg/m^2) and Insulin resistance (Homeostatic Model Assessment score) was calculated by using the formula: fasting serum insulin (uU/ml) x fasting plasma glucose (mg/dl)/405.[12]

Statistical analysis was performed using "Medcalc" statistical software (MSS), USA. Chi squre test (X2), odds ratio (OR), and 95% confidence interval (CI) were done to assess the association between the groups. One-way ANOVA with Bonferroni post hoc test was performed using "Graphpad Insta3" software. A p-value of <0.05 was considered statistically significant.

RESULTS

Table 1: Comparison of mean values of anthropometric and biochemical characteristics in PCOS patients and controls with their mean and standard deviations.

Parameter	PCOS (n=204)	Controls (n=204)	P value
Age (years)	28±3.6	28±5.1	1.0000
BMI (kg/m^2)*	27.12±4.93	23.4±3.2	<0.01
WC (inches)*	37±4.3	30.36±3.3	<0.01
HC (inches)	39.4±4.1	38.11±3.7	0.0008
WHR*	0.93±0.04	0.79±0.5	<0.01
Fasting glucose mg/dl	88±8.6	86.85±7.1	0.0678
Fasting insulin µIU/ml*	16.94±7.26	6.66±3.19	<0.01
HOMA score*	3.73±3.8	1.44±0.75	<0.01

*Significant values (p <0.05); BMI, Body mass index; WC, waist circumference; HC, Hip circumference; HOMA; Homeostatic modified assessment score.

The age range was 17 to 35 years for both patients and controls. The clinical features of hyperandrogenism include 92% of hirsutism, 88% of acne and 66% of androgenic alopecia as per the Androgen Excess Society (AES) 2006 - criteria of polycystic ovary syndrome (PCOS).[1,2] Comparison of mean values of anthropometric and biochemical features of PCOS cases and controls were shown in Table 1. All the subjects of PCOS (100%), showed polycystic ovary in our study, due to strict adherence to the AES-2006 criteria.

Table 2: Genotypes and alleles of IGF2 polymorphism identified in the study.

IGF 2 / Apa 1 (A to G change)	AA	GA	GG	A allele	G allele
PCOS	58 (28.4%)	128 (62.8%)	18 (8.8%)	0.60 (244)	0.40 (164)
Controls	172 (84.5%)	31 (15%)	01 (0.5%)	0.92 (375)	0.08 (33)

(Allele frequency odds ratio – 7.639, 95% CI-5.083-11.47, p < 0.05).

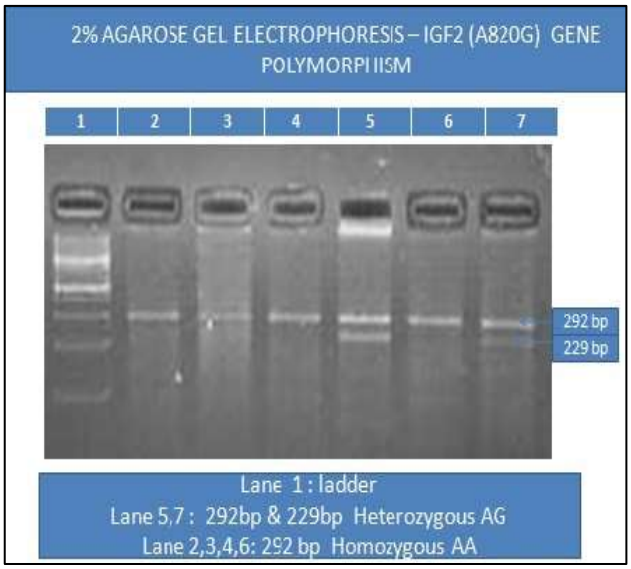

Figure 1: IGF 2 Apa 1 A820G gene polymorphism - 2% agarose gel electrophoresis.

Table 3: Statistical analysis of genotypes of IGF2 polymorphism identified in the study.

Genotype	PCOS	Controls	OR,	(95% CI),	P value
GG versus GA+AA	18/186	01/203	19.64,	2.5-148.6,	0.0039
GG+GA versus AA	146/58	32/172	13.53	8.33 - 21.96	< 0.01
GA versus GG+AA	128/76	31/173	9.3,	5.8 - 15.3,	< 0.01
AA versus GG+GA	58/146	172/32	0.07,	0.04 - 0.12,	< 0.01

PCOS - Polycystic ovarian syndrome, OR - Odds ratio; CI - Confidence interval, p - significance <0.05.

A PCR product of 292 bp was obtained, which on digestion with restriction enzyme gave fragments of 229 bp indicating GG genotype, 292/229 bp indicating AG genotype and 292 bp indicating AA genotype (Figure 1). The homozygous GG genotype was seen in 8.8% of patients with PCOS when compared with 0.5% of healthy controls (Table 2). Data showed that Recessive (GG versus AG+AA), co-dominant (AG versus GG+AA) genotype pattern of inheritance exhibited a significant association with PCOS (Table 3). The GG genotype was associated with PCOS (OR, 19.64; 95% CI 2.58 to 148.6, P 0.0039). Frequency of G allele was 0.40 in PCOS and 0.08 in controls, (p <0.01); (OR 7.639, 95% CI 5.08 to 11.47) (Table 2). Height, weight, body mass index, waist/hip ratio, fasting insulin and HOMA score showed a significant elevation with GG genotypes of PCOS (p <0.05) rather than AG and AA genotypes when compared with age-matched healthy controls (Table 4). The genotype and allele frequencies for the 204 PCOS patients (18 G/G, 128 G/A and 58 A/A) were in Hardy-Weinberg equilibrium.

Table 4: Distribution of Apa1 IGF2 genotypes, height, weight, BMI, W/H, FG, FI and HOMA score in patients and controls and their comparison with mean and standard deviation.

Parameters	A/A genotype		A/G genotype		G/G genotype		P value
	PCOS	Controls	PCOS	Controls	PCOS	Controls	
Age in years	27.96±3.79	28±5.08	28.4±3.6	29±5.3	27.8±4.05	23	> 0.05
Ht (cm)	157.49±6.19	157.7±5.04	156.6±6.17	156.56±5.4	153.93±5.16	152	< 0.01
Wt (kgs)	67.34±14.02	58.6±6.4	67.79±10.93	60.4±12.2	71.83±11.88	54	< 0.01
BMI (Kg/m^2)	26.76±5.6	23.25±3	27±4.6	24.4±4	28.8±4.9	23	< 0.01
W/H	0.92±0.05	0.78±0.05	0.94±0.035	0.8±0.05	0.94±0.049	0.9	< 0.01
F glu mg/dl	87.98±13.5	88.76±12.28	88.47±10	89.03±8.8	87.94±10.7	98	> 0.05
F ins uIU/ml	12.94±10	6.8±3.4	10.6±6.7	6.4±2.6	19.9±9.8	4.2	< 0.01
HOMA score	3±2.5	1.4±0.8	3.4±2.5	1.4±0.6	6.9±4.5	1.02	< 0.01

Data are shown as mean ±SD; P values were evaluated by one-way ANOVA with Post Boneferroni hoc test; Significant values (p is <0.05)Abbreviations: Ht, height; Wt, weight; BMI, body mass index; W/H, waist to hip ratio; F glu, fasting glucose; F ins, fating insulin; HOMA, homeostatic model assessment score.

DISCUSSION

The age range was 17 to 35 years for both patients and controls. The present study showed 92% of hirsutism, 88% of acne and 66% of androgenic alopecia as clinical manifestations of hyperandrogenism in our PCOS patients which was more than a meta-analysis, which showed 65-75% hirsutism, 15-25% acne, and 10-40% of androgenic alopecia.[1,2] Comparison of mean values of anthropometric and biochemical features of P COS cases and controls were shown in Table 1.

Hyperandrogenism is the cornerstone of polycystic ovarian syndrome (PCOS) as per androgen excess society criteria-2006.[1,2] There are several genetic studies of PCOS which have focused on finding an association with selected candidate genes.[13] Insulin-like growth factor 2 (11p15.5) gene stimulates insulin action as well as adrenal and ovarian androgen secretion, together with IGF1 and IGF binding proteins, may play a role in the pathogenesis of PCOS.[3]

Vafiadis et al, demonstrated G alleles of the Apa1 IGF2 polymorphism was associated with increased mRNA levels in leucocytes compared with the A alleles.[14] It was hypothesized that this functional polymorphism could result in increased liver IGF2 expression and secretion.[15] IGF2 stimulates adrenal and ovarian androgen secretion, and together with IGF1 and IGF binding proteins, suggested to play a role in the pathogenesis of PCOS.[16]

The present study analyzed for the first time IGF 2 Apa 1 A820G polymorphism in South Indian women with PCOS. Our data showed a significantly increased frequency of IGF 2 G allele with PCOS and a sevenfold increase in risk in women carrying GG genotype. Similar to our study, Millan S et al, found a significant association between homozygosity for G alleles of the Apa 1 variant in IGF 2 and PCOS.[4]

There was significant increase in mean values of weight, body mass index, fasting insulin, and Homeostatic model assessment score - insulin resistance (HOMA-IR) in case of GG genotypes when compared to AG and AA genotypes, which indirectly gave good suggestion that the GG genotype or PCOS with homozygous G allele are associated with obesity and insulin resistance which were implicated in the pathogenesis of PCOS. Similarly O'Dell and colleagues, have reported significantly higher body mass index in GG genotype compared to AA genotype in middile-aged Caucasian men.[16] Obesity and insulin resistance are frequent findings in hyperandrogenic women.[17] Obesity affects approximately 50% PCOS patients, similarly in our study population, 70% of PCOS

patients were obese (BMI >25 kg/m2), as per Asia-Pacific definition of obesity.[18,19] This high prevalence can be attributed to food habits and life styles of Indian women.

Homeostatic model assessment score (HOMA), is a good indicator of insulin resistance (IR). In our study, HOMA score was significantly higher in PCOS compared to controls similar to the findings of Chae SJ et al.[20] The prevalence of insulin resistance is greater in obese than non-obese patients, as per the meta-analysis, between 50% and 70% of women with PCOS have demonstrable IR and hyperinsulinism.[1,2] Similarly we have observed 59% of PCOS women with IR and hyperinsulinism in our study.

To the best of our knowledge, this is the first study in the South Indian population, which examined the association of IGF 2 Apa 1 A820G polymorphism (rs680) with PCOS.

CONCLUSION

To conclude, our study suggests that Apa1 A820G IGF 2 (rs680) gene polymorphism could be used as a relevant molecular marker to identify women at risk of developing PCOS in our population.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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