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Original article:

Study of Incidental finding of Primer induced Mutagenesis Seen in Subjects with respect to PPARγPro12Ala Polymorphism in Polycystic Ovary Syndrome (PCOS)

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ABSTRACT

Introduction: PCOS is the commonest cause of endocrinal disorder in women of reproductive age. Insulin Resistance is most frequently associated with PCOS. PPARγPro12Ala Polymorphism; by reducing insulin resistance in PCOS subjects has emerged as one of the promising modality of treatment of this syndrome.

Aims & Objectives: To explain the phenomenon of Primer induced Mutagenesis with the help of PPARγPro12Ala Polymorphism in subjects with PCOS.

Materials and Methods: A hospital based case control study was carried out in 50 diagnosed cases of PCOS (15-45 years of age); according to revised Rotterdam Criteria along with 50 Age and BMI matched apparently healthy controls. PPARγPro12Ala Polymorphism was detected through DNA extraction from whole blood followed by PCR and RFLP using $BstU_1$ Fast Digest. When the C G substitution at nucleotide 34 is present (missense mutation CCA to GCA), the mutagenic downstream primer introduces a $BstU_1$ restriction site (CG || CG). The expected products after digestion with $BstU_1$ are 270 bp for normal homozygotes, 227 bp and 43 bp for Pro12Ala homozygotes and 270 bp, 227 bp and 43 bp for heterozygotes. Statistical data analysis was done through spss version 16 using independent sample t-test, Pearson's correlation coefficient and chi- square test.

Results and Conclusion: There was no significant difference in genotypic distribution of C/G genotypes between cases and controls. Cases with CG genotype were associated with higher insulin sensitivity as compared to CC genotype though it was not statistically significant.

Keywords: - Primer induced mutagenesis, PPARγPro12Ala Polymorphism in PCOS.

Introduction:

PCOS or Polycystic Ovary Syndrome is the most common cause of anovulatory infertility[1]. According to revised Rotterdam criteria 2003; PCOS is diagnosed as having any two of the following 3 criteria (1) Oligo-ovulation or anovulation as menstrual irregularities; (2) signs of androgen excess like hirsutism, acne, alopecia; and (3) polycystic ovaries (≥12 cysts) as on ultrasonography[2]. Its documented prevalence in most of the literatures is found to be 5 - 10% in women of childbearing age[1]. Although multifactorial; the major contributor for its causation has found to be Insulin Resistance. Insulin Resistance is seen in 30 - 40% women with PCOS as against hyperinsulinemia in about 50 – 70% of women with PCOS[3,4]. Insulin Resistance means, insulin is normal or high in blood, but it cannot exert its action completely by binding to its receptors on cells. Thus, by decreasing Insulin Resistance; the outcome of PCOS can be improved.

Peroxisome Proliferator Activated Receptor gamma (PPAR-γ); also known by the names of "Glitazone Receptor" or "Thiazolidinedione Receptor" act as receptors for Thiazolidinedione (TZD) group of drugs[5].

These drugs have hypoglycaemic effect in Type-II DM, by reducingInsulin Resistance[6,7]. This fact can be utilised for the treatment of PCOS also. PPARγ is a type II nuclear receptor encoded by PPARγ gene (at 3p25)[8]. PPARγ forms heterodimer with Retinoid X Receptor (RXR)[9], which then binds to PPRE (Peroxisome Proliferator Hormone Response Elements) & regulate transcription of various genes[9]. This in turn leads to either increased or decreased transcription of these genes thereby causing change in Insulin Resistance[10].

PPAR-γ Pro12Ala polymorphism involves a missense mutation of **CCA** to **GCA** in codon 12 of exon B of PPAR-γ gene. This exon encodes for $-NH_2$ terminal residue of PPAR-γ_2. This mutation leads to Proline to Alanine substitution. As a result PPAR-γ_2 is expressed excessively. This in turn causes stimulation of insulin sensitivity by promoting insulin action in adipose tissues.[11-14]

Aims & Objectives: To explain the phenomenon of Primer induced Mutagenesis with the help of PPARγPro12Ala Polymorphism in subjects with PCOS.

Materials and Methods: A hospital-based observational case-control study was conducted in the Department of Biochemistry in collaboration with the Department of Obstetrics and Gynaecology, Lady Hardinge Medical College and Smt. Sucheta Kriplani Hospital, New Delhi, after being approved by the institutional ethical committee from November 2015 to March 2017. The study population consisted of convenient sample size of 100 subjects; due to time constraint with 50 diagnosed cases of PCOS in the age group of 15 to 45 years, as per revised Rotterdam criteria along with 50 Age and BMI matched healthy women volunteers. Women with related hormonal disorders like congenital adrenal hyperplasia (CAH), Cushing's syndrome and androgen-secreting tumours and patients on treatment with oral contraceptive pills (OCP) and high dose androgens were excluded from the study. Bilingual written informed consent was obtained from the study subjects. Detailed history and clinical examination of study subjects was carried out and Demographic parameters were recorded from the study subjects. Subsequently, fasting venous blood samples (2 – 3 ml) were collected from the subjects on day 2 to 5 of the menstrual cycle and were used to extract deoxyribonucleic acid (DNA) by commercially available kits from Qiagen (Netherlands) followed by PCR targeting PPAR-γ gene using following primers.

Forward Primer

5' GCATGGATCCCAATGC 3' (18 bp)

Reverse Primer

5'GATATGTTTGCAGACAGTGTATCAGTGAAGGA ATCGCTTTCCG 3' (43 bp).

Each 25µL PCR reaction mixture consisted of 100ng of genomic DNA template; along with 5pmol of each primer; 2.5 µl of 10X PCR buffer thereby forming final concentration as 1X; 200 µM of dNTP mix containing dATP,dCTP,dTTP and dGTP and 0.5U of Taq DNA polymerase.

PCR reactions were carried out in a thermal cycler (Palm-Cycler from Genetix Brand); under following conditions:-

A. Initial Denaturation at 94^{o} C X 5 minutes.

B. 35 cycles of

Denaturation at 94^{o}C for 30 seconds.

Annealing at 52^{o}C for 30 seconds.

Extension at 72^{o}C for 30 seconds.

C. Final Extension at 72°C for 5 minutes.

Amplicon of 270 bp so obtained was resolved on 2.5% agarose gel.

Restriction Fragment Length Polymorphism (RFLP)

The amplicons were digested with restriction enzyme $BstU_1$ Fast Digest from Thermo Scientific. A total of 30 µl reaction mixture was prepared in a PCR tube using 10µl of above PCR product; 10U $BstU_1$ Fast Digest and 2 µl of 10X Fast Digest Green Buffer. This was then incubated at 37°C for 10 minutes. 10 µl of this mixture was then loaded on 2.5 % agarose gel for visualisation of digested bands.

When the C→G substitution at nucleotide 34 is present (missense mutation CCA to GCA), the mutagenic downstream primer introduces a $BstU_1$ restriction site (CG || CG). The expected products after digestion with $BstU_1$ are 270 bp for normal homozygotes, 227 bp and 43 bp for Pro12Ala homozygotes and 270 bp, 227 bp and 43 bp for heterozygotes.[14]

The data were analyzed statistically by using SPSS ver. 16. Intergroup comparison of biochemical parameters between cases and controls was done using Independent Sample 't' test (for parametric data) and the Mann Whitney U test (for non-parametric data). The p value < 0.05 was considered as statistically significant. Categorical data analysis for the study of PPAR-γ polymorphism was done using Chi-Square test with odd's ratio as the risk estimate.

The Data and reagent availability; used in the present study is assured for further research.

Observations & Results:-

The Demographic and Anthropometric parameters are depicted in (Table. 1). It was observed that the mean weight, waist circumference and waist-to-hip ratio were significantly higher in cases as compared to controls ($p < 0.05$).

Variable	Cases (n=50) (Mean ± S.D.)	Controls (n=50) (Mean ± S.D.)	*p* value
Age (years)	25.7 ± 4.7	26.9 ± 4.7	0.21
Height (cm)	153.5 ± 5.3	153.3 ± 4.5	0.86
Weight (Kg)	64.6 ± 12.2	54.7 ± 9.8	**<0.001****
WC (cm)	88.66 ± 1.21	73.04 ± 9.88	**<0.001****
WHR	1.01 ± 0.28	0.82 ± 0.07	**<0.001****

***p* - value < 0.05 : Significant**
** ***p* - value < 0.001 : Highly Significant**

Table. 1 Demographic profile & Anthropometric parameters.

The data of PPAR-γ Pro12Ala polymorphism revealed the presence of CC (Pro/Pro homozygotes) in 78% of cases and 66% of controls. Also, CG (Pro/Ala heterozygotes) was observed only in 22% of the cases and 34% of controls. However, this difference in the genotypic distribution of C/G genotypes was not found to be statistically significant. No GG (Ala/Ala) genotype was seen in this study population. (Table. 2)

Genotype	Cases (n=50)		Controls (n=50)		*p*-value
	n	%	n	%	
CC	39	78	33	66	0.181
CG	11	22	17	34	

Table. 2 Genotypic distribution of PPARγ Pro12Ala Polymorphism in the study Population

Discussion

Polycystic ovary syndrome (PCOS) is a common endocrinal and metabolic disorder, characterized by menstrual irregularities most common being oligomenorrhoea, chronic anovulation and hyperandrogenism. It results from the interaction of genetic predisposition and environmental risk factors. Since it has multifactorial etiology, its presentation is quite pleomorphic and variable in different patients. One major cause for variability in its presentation can be because of using different criteria for its diagnosis.

This study was conducted as an effort to visualise polymorphism of the PPAR-γ gene and its possible role in the etiopathogenesis of PCOS in a better way. Also, there are very few Indian studies regarding polymorphism in PCOS[3]. Hence, the present study also paves way for expanding and further exploring our latest knowledge about PPARγ Pro12Ala polymorphism.

PPAR-γ Pro12Ala polymorphism in PCOS has shown variable results in different populations till now. This variation can be because of differences in geographical distribution, ethnicity, lifestyle and sample size of the various studies conducted so far[3]. In the present study; genotypic analysis revealed a relatively lower frequency of Pro/Ala heterozygotes (22%) as compared to Pro/Pro homozygotes (78%) in cases as compared to 34% and 66% respectively in controls. Similar genotypic frequency distribution was also reported by authors of several other studies in PCOS[15-18]. Ala/Ala homozygotes (GG) were not observed in this study. This could be due to the relatively smaller sample size of the study.

After extracting DNA from the whole blood samples; Polymerase Chain Reaction was carried out. Subsequently; during performing RFLP of PPAR-γ gene, (using appropriate primers); it was observed that, only when the C⟶ G substitution at nucleotide 34 was present (missense mutation CCA to GCA), the mutagenic reverse primer introduced a second mutation and created $BstU_1$ restriction site (CG || CG). It was further seen that; the process of RFLP got completed and appropriate restriction digestion occurred only if both the

mutations were present[16]. Thus, besides the study of PPAR-γ Pro12Ala polymorphism; the interesting phenomenon of Primer Induced Mutagenesis was also observed.

Conclusion

The present study showed that, Pro/Ala genotype (CG) may be associated with high insulin sensitivity; depicting its protective role in PCOS. There was no significant difference in genotypic distribution of C/G genotypes between cases and controls. Also, cases with CG genotype showed higher insulin sensitivity as compared to CC genotype (not statistically significant). In the present study, no GG genotype was found. Primer induced mutagenesis was seen as a peculiar feature in the present study.

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