INSR SILENT POLYMORPHISM INFLUENCES INSULIN RESISTANCE IN INDIAN WOMEN WITH PCOS
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ABSTRACT

Objectives of study
Polycystic Ovarian Syndrome (PCOS), a major cause of infertility is also strongly associated with insulin resistance. Defects in insulin receptor signaling are considered as one of the major molecular pathogenesis for insulin resistance. To investigate the possible mechanism of this signaling defect at genetic level, single nucleotide polymorphism (SNP) [His 1085 C/T; rs 1799817] at the exon 17 of insulin receptor gene (INSR) was studied.

Brief Methodology
It is a case control study with cases of PCOS selected from the outpatient department of Safdarjung Hospital, New Delhi, after approval from institutional ethical committee. SNP analysis was performed with PCR-RFLP technique using designed primers and Pml I (Eco721) restriction enzyme. Serum fasting insulin was measured by ELISA technique and HOMA-IR was calculated mathematically.

Summary of Results
A higher frequency of the wild type CC genotype was observed in PCOS women than in controls. Also, HOMA-IR, a tool for estimating insulin resistance, was significantly high in PCOS women with the C allele. Non-PCOS women having C allele were also insulin resistant.

Conclusions
His1085His silent polymorphism at exon 17, rs 1799817 of INSR is associated with insulin resistance in Indian women with PCOS. Presence of C allele (rs1799817) could be developed as a marker for insulin resistance and metabolic complications in women with PCOS.

DETAILED SUMMARY
Polycystic ovarian syndrome (PCOS), the most frequent endocrinopathy in women is a syndrome of chronic anovulatory infertility characterized by androgen excess and polycystic ovaries. Although the cause of PCOS is not well understood, insulin resistance may be a key factor. Women with PCOS have increased risk of type 2 diabetes, which is mainly a insulin resistant state. Genes involved in insulin action have been considered good candidates for causation of PCOS. The INSR gene is located on short arm of chromosome 19 (19p13.2). It is 120,000 bp long, composed of 22 exons and 21 introns. Human insulin-receptor (INSR) is a transmembrane glycoprotein receptor on the surface of all cells. It mediates the action of insulin upon target cells. Mature human INSR is a hetero-tetramer of two α-subunits and two β-subunits. Exons 1-11 encode the α-subunit of the receptor which is the insulin binding region. In contrast, exons 12-22 encode the β-subunit, which is the catalytic subunit of which exons 17-22 code for protein tyrosine kinase domains and their activity is required for transmembrane signaling to mediate the biological effect of insulin. Hence, any functional and structural defects in
insulin-receptor might impair the biological response to insulin, thus leading to insulin resistance. Any defects of INSR in number or functions might decrease the action of insulin and cause insulin-resistance. Previous studies demonstrated that mutations in INSR gene were detected in many patients with severe or moderate insulin resistance. The tyrosine kinase domain (exon 17-21) mutations of the insulin receptor have been shown to cause severe hyperinsulinemia and insulin resistance. Several polymorphisms in the exon 17 region of INSR gene have been studied by various workers in diseases especially type 2 diabetes, insulin resistance and polycystic ovarian syndrome. The polymorphism rs1799817 (C/T) on exon 17 of INSR gene (His1085His) has been proved to be associated with PCOS. This SNP has been studied in cancers of breast and colorectum in USA and Iran respectively as polymorphisms in pathways controlling energy homeostasis have been proposed to influence the risk of chronic conditions including cancer. Hence, this particular polymorphism site (db SNP rs 1799817) seems to influence insulin resistance and disorders associated with it. It could unravel a pathway in causation of insulin resistance and pathophysiology of PCOS. The aim of this case-control design study was to analyze the exon 17 C/T (H1085H) SNP rs 1799817 as a molecular marker for PCOS in our patients.

MATERIALS AND METHODS

Subjects
Women diagnosed with PCOS were selected from the Gynecology OPD of Safdarjung Hospital, New Delhi. PCOS was diagnosed according to Rotterdam Criteria, 2003. A total of 50 PCOS cases and an equal number of age matched healthy control women were recruited for the study.

Clinical and Biochemical Parameters
The anthropometric data - height, weight, body mass index was obtained from all subjects after taking informed written consent. 10 ml fasting venous blood was drawn. Sugar was measured using glucose oxidase-peroxidase method. Insulin levels in serum were estimated using ELISA based kit procured from Diasorin Ltd, Germany.

HOMA-IR (Homeostatic Model Assessment-Insulin Resistance), a parameter for estimating insulin resistance was calculated mathematically by the formula:

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\text{HOMA-IR (mass units)} = \frac{[\text{glucose (mg/dl)} \times \text{insulin (mIU/L)}]}{405}.
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Body mass index (BMI) was calculated as [weight in kg/(height in meter)].

SNP analysis
Genomic DNA was extracted manually by the chloroform-phenol method from the blood collected in EDTA vial. The extracted DNA was dissolved and stored in TE buffer. Polymerase chain reaction was performed in peltier thermocycler PTC-200 from MJ Research to produce multiple copies of the region of our interest in exon 17 of INSR gene.

Primers used for this reason were designed and checked for compatibility using the IDT software available at http://eu.idtdna.com/scitools/applications/fusionprimers/default.aspx.

Primers used were synthesized by Eurofins Genomics India Pvt Ltd, Bangalore, India:

F: 5' AGTAGTCTCAGGTGGGAAGTAG 3'
R: 5' GGAGAGATGGGGATTGAGAAG 3'

Restriction enzyme was selected and checked using sequence manipulation suite available at URL: http://www.bioinformatics.org/sms2/rest_digest.html.

Eco721 (Pml I) enzyme was obtained from Fermentas catalogue #ER0361.

PCR mix was incubated overnight with the restriction enzyme at 37 °C.

Restriction digest and PCR product were electrophoresed in 2% agarose gel and visualized in Syngene ChemiG:Box XR gel documentation system.

Our PCR product was of 711 bp length, which after complete digestion by Eco 721 gives rise to two fragments of 547 bp and 164 bp in case of heterozygous genotype.

Statistical Analysis
Univariate analysis of all continuous variables between PCOS and control groups were done by unpaired t tests. Results are expressed as mean±S.D. The association between genotype and PCOS was
analyzed using chi-square test. P<0.05 was considered to be statistically significant. All analyses were performed using MS excel and GraphPad Prism Quick Calculator. Synergy factor was calculated using excel program provided by Cortina-Borja et al, 2009.

Results
The allelic and genotypic distributions of INSR exon 17 rs1799817 (H1085H) polymorphism for both cases and control group were consistent with the Hardy-Weinberg equilibrium [cases: chi sq=0.58, df=1, p=0.95; controls: chi sq=0.0032, df=1, p=0.99]. In our study group, 34% (17/50) PCOS cases and 8% (4/50) of controls were of CC genotype; 44% (22/50) of cases and 40% (20/50) of controls were CT and 22% (11/50) cases and 52% (26/50) of controls were of TT genotype. The frequencies of CC and CT genotypes were significantly high in PCOS women when compared with controls (chi-square=14.2, df=2, P=0.0008). Frequency distribution of C allele and T allele of the INSR His1085 C/T polymorphism was significantly different between the PCOS cases as compared to healthy controls (chi-square=6.94, df=1, P=0.008).

To understand the influence of this polymorphism on insulin resistance and BMI in PCOS cases, serum fasting insulin, BMI and HOMA-IR values were compared in CC, CT and TT genotypes. BMI was not significantly different between the genotypes. However, serum fasting insulin and HOMA-IR were found to be significantly high in CC genotype cases when compared to cases of CT and TT genotypes (p<0.001). Interestingly however, serum fasting insulin and HOMA-IR was significantly high in CC genotypes of control group as well as compared to CT/TT genotypes. S. fasting insulin, BMI and HOMA-IR values in CT vs TT genotype was non-significant in both cases and controls.

To measure the combined effect of insulin resistance (HOMA-IR > 2.5) and INSR rs 1799817 polymorphism, we calculated their synergy factor (SF) to allow assessment of binary interactions viz presence of C allele and insulin resistance in causation of PCOS. In present study, insulin resistance and INSR (rs1799817) polymorphism act non-synergistically (SF=2.73, p=0.3) in causation of PCOS.

Discussion and Conclusions
In the present study, one SNP at exon 17 of INSR gene, which encodes the protein tyrosine kinase domain, was studied in PCOS cases and healthy age matched controls. Studies based in China, Korea, North-America, Iran and Mumbai, India show that exon 17 of INSR participates in the development of PCOS.

The His1085His [rs1799817, (CAC -CAT)] polymorphism in INSR gene is a synonymous/silent polymorphism where the protein synthesized remains same, i.e. Histidine.

The His1085His polymorphism might play an important role because the ATP binding site of exon 17 is located in this region, which is a key factor to fix phosphorus during autophosphorylation responses. Although it is unclear how a silent polymorphism can alter the risk of insulin resistance and associated diseases, there is emerging evidence for a possible role of silent polymorphisms in altering protein function. Because this synonymous SNP has no known influence on biological functions, the association between INSR H1085H and diseases could be due to linkage disequilibrium with nearby functional variants.

A significant association of this silent polymorphism His1085His (rs1799817) in exon 17 has been reported in diseases like type 2 diabetes, polycystic ovarian syndrome and cancers16, 20-24. Specific studies on this particular polymorphism of INSR in PCOS patients of Iran found that mutant T allele is associated with the disease31. However, a parallel study in Iraq found no association. Previous studies based in Turkey and Croatia also showed no association.

In the present study, there was a higher prevalence of CC and CT genotype in PCOS cases and this genotype is associated with increased insulin resistance. These are in contrast to previous studies in which T allele has been implicated in PCOS. However, this is consistent to findings in insulin resistant cases and type 2 diabetes, where the T allele was found to be protective.

In a Chinese study based on the relationship between insulin resistance and His1085His polymorphism, the frequency of the "T" allele was higher in the controls when compared with the insulin-resistant
subjects. In a South Indian study on type 2 diabetics, T allele was more in subjects with normal glucose
tolerance as compared to cases, emphasizing the protective status of T allele towards insulin resistance.
So, in insulin resistant cases of PCOS, the C allele is more frequently observed.
In conclusion, CC and CT genotype of INSR H1085H (rs1799817) SNP is significantly associated with
PCOS cases, especially with increased insulin resistance. Even, controls with C allele demonstrated
high insulin resistance. However, more number of cases can be tested to confirm the findings.