Genetic Variations in Women with Insulin Resistance

Mohammad Ateab, Mohammad Nader, and Ismail Aziz

***Abstract*—**This study explores on the relationship between the polycystic ovary syndrome (PCOS) and insulin resistance which caused by variation in the 5-prime flanking region of the insulin gene, a variable number of tandem repeat (VNTR) regulates transcription of the gene.

The study includes 50 Iraqi women with polycystic ovary syndrome (PCOS) and 25 healthy women, Blood samples were collected from the the Medical City hospital, Kamal al-Samarrai Hospital and private medical laboratories (in Baghdad), during the period from November, 2011 to May, 2012. The age of infertile and fertile women was ranged from 16 to 45 years.

The molecular study was focused on the 42% of PCOS women with insulin resistance. By sequencing for 50 samples, one separate segment (INS-VNTR) containing nucleotide 360 of insulin gene was amplified by using specific primer..The result of sequencing refers to a number of mutations found in women with PCOS (substitution = 69.86% , deletion =10.95% and insertion =19.17%) . Some of mutations were repeated in a number of patients with the same type and locations. The results showed 9.52% of patients having deletion in adenine in 388 position (TAC/ T_C) , 9.52% of patients having deletion in adenine in 681 position (CCA/ CC_) , 9.52% of patients having insertion adenine in 638 position (GCT/ GACT) , 9.52% of patients having Missense mutation in 447 position (GAG/ AAG) , 9.52% of patients having Missense mutation in 84 position (CAG/ AAG) and 9.52% of patients having silent mutation in 88 position (GGG/ GGT).

The results showed 14.28% of patients having Missense mutation in 455 position (GGG/ AGG) , 14.28% of patients having Missense mutation in 434 position (CCA/ TCA) and 14.28% of patients having silent mutation in 639 position (CGG / CGT).

The results showed 19.04% of patients having insertion in thymine in 94 position (CTG/ CTTG). Also compound mutations have been detected among most of PCOS with insulin resistance

***Keywords*——** polycystic ovary syndrome, variable number of tandem repeat , Mutations , Sequencing

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I. Introduction

POLYCYSTIC ovary syndrome (PCOS) is one of the most common female endocrine disorders, but there is strong evidence that it can to a large degree be classified as a genetic disease [1]. PCOS produces symptoms in approximately 5% to 10% of women of reproductive age. It is thought to be one of the leading causes of female subfertility and the most frequent endocrine problem in women of reproductive age [2].

The principal features are an ovulation, resulting in irregular menstruation, amenorrhea, ovulation-related infertility, and polycystic ovaries; excessive amounts or effects of androgenic hormones, hirsutism , and insulin resistance, often associated with obesity, Type 2 diabetes, and high cholesterol levels . Not all women with PCOS have polycystic ovaries (PCO), nor do all women with ovarian cysts have PCOS; although a pelvic ultrasound is a major diagnostic tool, it is not the only one. The diagnosis is straightforward using the Rotterdam criteria [3].Women with PCOS is more insulin resistant, and up to 40% of these patients have impaired glucose tolerance. They are at an increased (3–7 times) risk of developing early-onset type 2 diabetes, the risk being greater in obese PCOS patients and those with a family history of type 2 diabetes . Insulin plays both direct and indirect roles in the pathogenesis of androgen excess in PCOS. Although women with PCOS have peripheral insulin resistance, ovarian steroidogenesis appears to be hypersensitive to insulin [4].

In addition, insulin can stimulate human theca cell proliferation, and can also enhance ovarian growth and follicular cyst formation in rats [5]. A highly polymorphic stretch of DNA lying 360 bp upstream of the initiation of transcription of the INS gene called Variable Number of Tandem Repeats (VNTR). There are three classes of (VNTR) in the insulin gene (class I, class II and class III) .Any mutations in this region may be effect on insulin gene (INS) expressions. Genotype of the VNTR-INS gene was different among various population groups. Several studies and research focused on the genetic variations of genes that have an impact on causing Polycystic Ovary Syndrome (PCOS) such as INS gene (VNTR) and other region [6].

The aim of this study is to detect the relationship between PCOS and insulin resistance caused by mutations in insulin gene (INS-VNTR) and hormonal disturbance with other parameters, to achieve this aim the following steps were done:

1. Identification of some genetic mutations in insulin gene (INS-VNTR).

2. Determination the presence of relationship between PCOS and insulin resistance.

II. Materials And Methods

A. Subjects

This study was carried out through period between"1August 2011 to 30 of March 2012" in Institute of Genetic Engineering & Biotechnology for post Graduate Studies at University of Baghdad. Two study groups have been investigated:

1. Patients group (PCOS group)

This study has included fifty infertile Iraqi women with PCOS. Patients were selected from the Medical City Hospital and Kamal Al-Samarrai Hospital in Baghdad.

2. Healthy control group (fertile)

Healthy control group consists of twenty five healthy fertile women of different ages (16-45 years). venous blood samples (5 ml) Have been collected from each women of both PCOS and healthy control.

B. Genomic DNA isolation

Total genomic DNA isolated from the whole and frozen blood collected in EDTA anticoagulant tubes for molecular studies was applied using genomic DNA purification kits (Bioneer) /Korea. The isolation of DNA was based on five steps process using salting out methods [7].

C. Agarose Gel Electrophoresis

After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA [7].

The reagents of Gel Electrophoresis:

- Agarose .
- 1 X TBE Buffer.
- Bromophenol Blue in 1 % glycerol(loading buffer).
- Ethidium Bromide.
- DNA Ladder Marker (100 bp)

D. Insulin gene (INS) mutations detection

The insulin (INS) gene locates at chromosome 11p15.5and is one of established susceptibility locus to type II diabetes. In the 5-prime flanking region of the INS gene, a variable number of tandem repeat (VNTR) regulates transcription of the gene. Therefore specific primers were designed to amplification (INS-VNTR) region in this study (PCR). The selection of these regions is based on their among most common insulin resistance that leads to PCOS [8].

PCR process was performed using specific primer was designed for (INS-VNTR) region by using (another studies, NCBI and Primer 3 programe). The sequences of these primers were listed in (table: I)

TABLE I
SEQUENCES OF PRIMER

Name of primers	Sequence of primers	PCR Product size
F primer (*INS-VNTR*)	5'-AGC AGG TCT GTT CCA AGG-3'	360 bp
R primer (*INS-VNTR*)	5'-CTT GGG TGT GTA GAA GAA GC-3'	

Optimization of PCR reaction was accomplished after several trials for annealing phase (58 , 60 , 62 oC) , It was then selected temperature of 62 oC that give the best results for polymerase chain reactions. The PCR reaction was carried out as shown in table (II).

TABLE II
PCR PROGRAMS FOR (INS-VNTR) REGION

Steps	Temperature (ºC)	Time (minutes)	No. of Cycles
Initial denaturation	96	2	1
First loop:			
Denaturation	96	1	30
Annealing	62	1	
Extension	72	1	
Final extension	72	10	1

E. PCR products sequencing

The PCR products (75 samples) of the analyzed (INS-VNTR) gene and primers were sending to Macrogen Company (U.S.A) for sequencing. More information available on web site: (http://www.macrogen.com). Steps to send samples (PCR products and primers) for (INS-VNTR) region as shown below:

1. By using cool box PCR products (50 µl) and primers (F&R) 50µl for each Samples were sent to Alexander company for medical surface (Sadoun Street - Baghdad – Iraq) with writing important notes on each sample.
2. Alexander company sent the samples to the United States of America (Macrogen company) in order to detect sequences for samples (in our study) to detection any mutation and alterations in (INS - VNTR) gene

F. Blood Glucose assay

Glucose blood levels performed by using The ACCU-CHEK Active System, GC model, Germany. Accu-Chek Advantage meets these needs by providing accurate and dependable glucose measurement using a tiny drop of blood, for all blood sample types (capillary, arterial, venous and neonatal) over a wide range of hematocrit. It delivers this performance at temperatures from 57 to 104 °F, and at altitudes to above 10,000 feet. The principle of the ACCU-CHEK Active System depended on some parameters (Electron Transfer Reactions, Enzyme Reactions, Mediators and Amperometry) and after that read the result [9].

III. Results And Discussion

A. Age distribution of the samples

The age of all PCOS women was ranged less than 25 to more than 35 years. Table (3-1) revealed that 42% of the study PCOS women were between (25 -35) years, followed by 30% of patients whose age ranged between (>25) years and 28% of patients whose age (<35) years of total PCOS patients. This may leads to conclude that group 2 and group 3 constituted the greatest number groups in the present study. The results agreed with other studies that reported affecting 4% to 12% of women of reproductive age. [10].

Figure axis labels are often a source of confusion. Use It is believed that the reason for this is due to genetic differences and geographic locations in addition to the environmental conditions and surrounded by physical and chemical effects.

TABLE II
DISTRIBUTION OF PCOS WOMEN AND CONTROL GROUP MEAN ACCORDING TO THEIR AGES

Age group (years)	PCOS group		Control group	
	No.	%	No.	%
Less than 25	15	30	7	28
25-35	21	42	14	56
More than 35	14	28	4	16
Total	50	100%	25	100%

B. DNA Isolation

The genomic DNA which extracted from blood of PCOS showed good concentration (3- 6µg/ml), (Figure I).

Genomic DNA from the samples was isolated following the standard protocol which Used in many research and genetic studies [7].

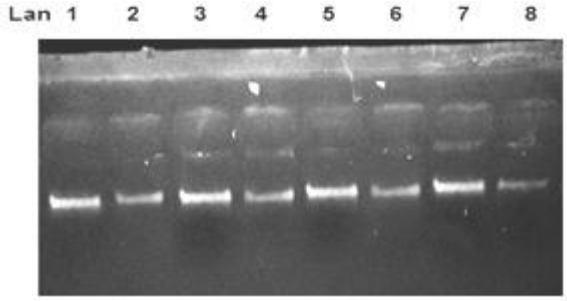

Fig 1: Chromosomal DNA bands on 1% agarose gel at 100 volt for 20min. DNA sample were extracted from some PCOS women

C. PCR analysis

The present study used PCR technique to detect region of the insulin gene (INS-VNTR).The PCR results revealed that identical bands related to the (INS-VNTR) region was present. (INS-VNTR) PCR amplified regions showed a molecular weight of 360 bp (Figure II).

Yuping et al.,(2009) showed that the PCR (INS-VNTR) products were 360 bp and this corresponds with our study [12].

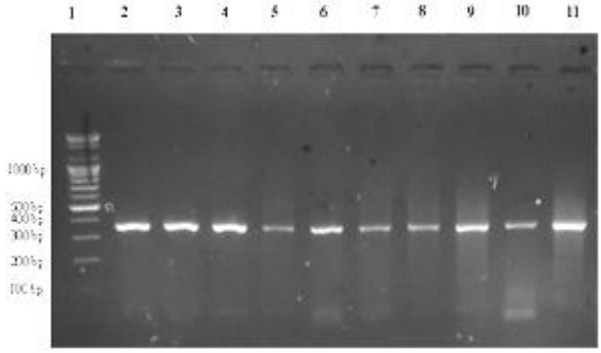

Fig 2: PCR products of (INS-VNTR) region on 2% agarose gel at 100 voltages for (35-50) min.
Lane 1: DNA ladder.
Lane (2-11): PCR products of the (INS-VNTR) region from PCOS women

D. Insulin gene VNTR alterations

The insulin gene VNTR was screened by sequencing from 50 PCOS, all results were directly compared with human reference insulin gene VNTR sequence (http:NCBI Reference Sequence) by software program (BioEdit Pro.version:7.0.0) that available in web site (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Used this method of Mahdi,(2012) in the study of genetic mutations and variations of TPo gene (exon 8 and 9) in Iraqi women with PCOS [13].

E. Percentage of mutations

There was many alternation in insulin gene (INS-VNTR). The study have detected by sequencing (73) mutations in (21) women of the total (50) women with PCOS ranged about (42%).While the percentage of genetic mutations in women who suffer from insulin resistance (PCOS group) about 64% (16 patients of the total 25).The percentage of mutations in the healthy group generally ranges (40%).

Through a review of previous studies from the last several years, the genetic etiology of PNDM (permanent neonatal diabetes mellitus) TNDM (transient neonatal diabetes mellitus) was became clearer. Where the proportion of genetic mutations percentage about40% of patients [14] these results are similar to the results of other studies [15].

The rates of mutations in diabetes (INS-VNTR) may be different because of damage in other genes affect the work of the insulin gene. Allelic variation at VNTRs may play an important general role in human disease [16].

F. Effect of mutations

The effect of mutation on insulin gene (INS-VNTR) was represented by effecting on regulation of insulin secretion and action. Table (4-4) showed that there was (50.68%) missense leading to the impact on phenotype that lead to Replacement in amino acids (Transition = 31.91% and Transversion = 68.08%) in PCOS group. The deletion and insertion mutations leads to frameshift there was about (30.13%) in this study . These mutations resulted in a completely different translation (defect protein) [17]. A silent mutation which code for the same amino acid and alteration in the DNA sequence [18] Where results of

this study showed that the percentage of Silent mutations (19.17%)

Hypothetically, all the genes coding for the proteins involved in the regulation of insulin secretion and action, when mutated, may contribute to an increase in insulin resistance and to the development of metabolic syndrome (such as PCOS). The gene variants may be localized in the coding region or in the regulatory regions, such as the promoter. Because of its role in the insulin secretion, a promising gene that may be involved in the development of metabolic syndrome is the gene codifying for insulin itself [19]

TABLE IV
PERCENTAGE OF MUTATIONS EFFECT IN PCOS WITH INSULIN RESISTANCE, PCOS GROUP AND CONTROL GROUP

Effect of mutations	Percentage		
	PCOS with insulin resistance n= (16 / 25)	PCOS group n=(21 / 50)	Control group n= (10 / 25)
Missense	71.21 %	50.68 %	38.09 %
silent	10.60 %	19 .17 %	47.61 %
Frameshift	18.18 %	30.13	14.28 %

G.Average level of insulin and glucose in mutant patients

In the group of women who suffered from PCOS found that the mutation rate was 42% (21) of the total (50) patients, and founded that the rate of fasting insulin levels for this group of mutations (11.94IU/ml) and the standard rate of fasting glucose (113mg/dl),table (3-3) shows these values.

The effect of INS-VNTR on insulin levels has been largely investigated, with increased transcriptional activity of the insulin gene in pancreas [20]

In humans, expression levels of insulin are regulated in part by a direct transcriptional influence of a polymorphic insulin gene (INS-VNTR) genetic element associated with the proinsulin gene promoter region. Higher expression levels of proinsulin, due to the presence of the VNTR III in the thymus [21]

Vafiadis et al.,(1997) explain that the INS-VNTR situated 5′-upstream of the INS promoter, is regarded as a causal variant at the locus after a close correlation was found between VNTR haplotypes and insulin thymic expression [22]. However, comprehensive resequencing of the locus revealed two polymorphisms that are potentially functional and genetically indistinguishable from the VNTR. Therefore, direct evidence is required to support that the VNTR allele controls insulin transcript expression [23]

TABLE V
AVERAGE LEVELS OF INSULIN AND GLUCOSE IN MUTANT PATIENTS

Mutations Percentage in PCOS group n=(21/50)	Average level of fasting insulin (IU/ml)	Average level of fasting glucose (mg/dl)
42 %	11.94	113

IV. CONCLUSION

1. Polycystic ovaries syndrome can be considered as a complex metabolic syndrome triggered by the interact effect of genetic and environmental factors.
2. There was an association between Polycystic ovaries syndrome (PCOS) and insulin resistance because the insulin plays an important role (direct and indirect) in reproductive function.
3. By carrying out the DNA sequencing, many mutations (substitution, deletion and insertion) have been reported in insulin gene (INS-VNTR) which play an essential role in insulin expression

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