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KAHRAMANMARAŞ SÜTÇÜ İMAM UNIVERSITY
RADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

INVESTIGATION OF SNP (rs10889677) LOCATED AT THE miRNA BINDING SITE OF THE IL23R GENE IN PATIENTS WITH POLYCYSTIC OVARY SYNDROME

HAMA HUSSEIN HAMA HAMA

MASTER THESIS

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Submitted in candidature for the degree of Master in

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M.Sc. thesis entitled “Investigation of SNP (rs10889677) located miRNA binding sites of the IL23R gene in patient with polycystic ovary syndrome” prepared by Hama Hussein Hama HAMA who is a student at Bioengineering and Sciences Department, Graduate School of Natural and Applied Sciences, KahramanmaraşSütçü Imam University, was certified on date(18/8/2016)by all of jury members, whose signatures are given below**.**

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POLİKİSTİK YUMURTALIK SENDROMLU HASTALARDA IL23R GENİNİN miRNA BAĞLANMA YERİ SNP'SİNİN (rs10889677) ARAŞTIRILMASI

(Yüksek Lisans Tezi)

ÖZET

IL-23reseptörü (IL-23R), T hücrelerinin T-yardımcı 17 fenotip hücrelere dönüşmesi için kılavuzluk rölü üstlenmektedir. IL-23R tek nükleotid polimorfizmi (SNP), Crohn hastalığı ve romatizmal artrit dahil olmak üzere birçok otoimün hastalıkları ile ilişkili bulunmuştur. miRNA'lar birçok fizyolojik ve patolojik süreçte mRNA'nın 3' translasyona uğramayan bölgesine (UTR) bağlanarak gen ekspresyonu seviyesini düzenlemekte rol oynar. Mevcut çalışmada, IL23R geninin 3'UTR bölgesindeki miRNA bağlanma bölgesinde gözlenen tek nükleotid polimorfizmi (SNP) (rs10889677) ile bir otoimmün hastalığı olan Polikistik Yumurtalık Sendromu (PCOS) arasında bir ilişki olup olmadığını araştırılmıştır. Bunun için 42 PCOS hastası ve 37 PCOS hastası olmayan (kontrol) sağlıklı kadın birey kullanıldı. Hastaların ortalama yaş oranı 24.22 ± 5.22 iken kontrollerin ortalama yaş oranı 24.73 ± 6.16 idi. Androjen hormonlar da dahil olmak üzere bir grup hormonun (Luteinize Edici Hormon, Estradiyol, Testosteron, Dehidroepyiandrosteron, Tiroid Uyarıcı Hormon, Yumurtalık Uyarıcı Hormon) kan seviyeleri ölçüldü. Aynı bireylerin tüm kanlarından DNA izolasyonu yapıldı ve bu genomik DNAlar kullanılarak rs10889677 polimorfizmi üzerinden genotipleme gerçekleştirildi. Sonuç olarak,, AA, CA ve CC, genotipleri için görece olasılıklar oranı (OR) sırasıyla 1.06, 0.72, and 1.65olarak bulunmuştur. Her ne kadar genotipler ve gruplar arasında yapılan ki-kare testi istatiksel olarak anlamlı bir ilişki vermediyse de (p=0.07), OR değerleri CC genotipinin PCOS hastalığı için bir meyil oluşturabildiğini düşündürmektedir.

Anahtar Kelimeler: PCOS , IL23R , miRNA bağlanma yeri , SNP

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INVESTIGATION OF SNP (rs10889677) LOCATED AT THE miRNA BINDING SITE OF THE IL23R GENE IN PATIENTS WITH POLYCYSTIC OVARY SYNDROME

(M.Sc. THESIS)

ABSTRACT

IL-23 receptor (IL-23R) guides T cells toward the T-helper 17 phenotype. IL-23R single nucleotide polymorphisms (SNPs) have been associated with several autoimmune diseases, including Crohn's disease and rheumatoid arthritis. miRNAs play a role in a variety of physiological and pathological processes to regulate the levels of gene expression through their binding to 3' Untranslated Region (UTR) of mRNAs. In the present study, it has been investigated whether there is a relationship between the SNP (rs10889677) observed in the gene of miRNA binding site of IL23R mRNA and Polycystic Ovary Syndrome (PCOS), which is an autoimmune disease. Study included 42 PCOS patients and 37 female individuals with no diagnosis of PCOS. Mean age of patients was 24.73±6.16 and it was 24.22±5.22 for control. Different hormones including certain androgen hormones (Luteinizing hormone, Estradiol, Testosterone, Dehydroepiandrosterone, Thyroid-Stimulating Hormone, Follicle-stimulating hormone) were evaluated. DNAs were isolated from the whole bloods of the same individuals, and by using these genomic DNAs, genotyping was performed through the rs10889677 polymorphism. As a result, odds ratios (OR) for AA, CA and CC genotypes, were found to be 1.06, 0.72, and 1.65, respectively. Although no statistically significant association has been determined for the study groups and the genotypes through chi-square test ($p=0.07$), OR values suggested a tendency for CC to cause a slight susceptibility for the disease.

Key Words: PCOS, IL23R, miRNA, SNP.

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Hama Hussein Hama HAMA

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LIST OF ABBREVIATIONS

PCOS	: Poly cystic ovary syndrome
PCOM	: Poly cystic ovary morphology
GWAS	: Genome-wide association study
IL	: Interleukin
IL23R	: Lnterleukin-23 receptor
SNP	: Single nucleotide polymorphism
Th cell	: T helper cell
Th17/ThIL-17	: T helper 17 cells
UTR	: Untranslated region
3’-UTR	: 3’-Untranslated region
MiRNA	: MicroRNA
mRNA	: Messenger RNA
Pre-miRNA	: Precursor-miRNA
Pri-miRNA	: Primary-miRNA
DCs	: Dendritic cells
NKT	: Natural killer T
T2DM	: Diabetes mellitus 2
CDV	: Cardiovascular disease
CD	: Crohn’s disease
IBD	: Inflammatory bowel disease
RA	: Rheumatoid arthritis
RHF	: Rheumatoid Heart Fever
BC	: Breast cancer
SSC	: System Sclerosis
SLE	: Systemic lupus erythematosus
NSCLC	: Non small lung cell cancer
MAF	: Minor allele frequency
RAF	: Risk allele frequency
RISC	: RNA-induced silencing complex

BMI	: Body Mass Index
FSH	: Follicle-stimulating hormone
LH	: Luteinizing hormone
E2	: Estradiol 2
DHEA	: Dehydroepiandrosterone
TSH	: Thyroid-Stimulating Hormone
STAT	: Signal trancducer and activator of transcription
TNF-α	: tumor necrosis factor-α
IL-12Rβ1	: interleukin-12 receptor β1 subunit
INF-γ	: interferon gamma
PCR	: polymerase chain reaction
RFLP	: Restriction Fragment Length Polymorphism
Bp	: basepair
Ml	: Microliter
Ml	: Millilitre
Ng	: Nanogram
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide triphosphate
EDTA	: Ethylenediaminetetraacetic acid
EtBr	: Ethidiuam bromide
TAE	:Tris-acetate-EDTA
TBE	: Tris/Borate/EDTA
TAE	: Tris/acetate buffer
UV	: Ultraviolet light
ANOVA	: One-way analysis of variance
OR	: Odds ratio
95% CI	: 95% confidence interval

1. INTRODUCTION

Polycystic ovary syndrome (PCOS) was first termed in medical literature by Stein and Leventhal who, in 1935, explained some women sufferance from hirsutism, amenorrhea, and expanded ovaries with multiple cysts. Nowadays, it is introduced as a common, heterogeneous, heritable disorder influence women during their lifetime. PCOS is described by hyperandrogenism, ovulatory dysfunction, and polycystic ovaries. However, there is remarkable inter individual variations in presentation. Although not asked for identification, the being of insulin resistance and hyperinsulinemia is concerning and places the influenced patients at increase danger of diabetes mellitus and cardiovascular disease. Thus, PCOS oppositely impacts endocrine, metabolic, and cardiovascular health (Sirmans and Pate, 2013). PCOS is a highly complicated disorder impacted by both genetic factors and environmental (Kosova and Urbanek,, 2013).

Polycystic ovary syndrome (PCOS) is the widely recognized endocrinopathy in female of reproductive age. Familial clustering of status indicate that genetic factors has a significant role in its etiology. Several researches of relatives with some statues of PCOS have shown outcomes referring to an autosomal dominant phenotype (Franks et al., 1997). PCOS is clinically elucidated by the existence of three main clinical characteristics. Firstly, hyperandrogenism (HA), second, oligo-anovulation (OA) and third polycystic ovary morphology (PCOM). These clinical characteristics change according to irregular cycles, metabolic syndrome disease or endometrial cancer and infertility. It is subsequently logical to suppose that a private etiology could evolve these properties (Cui et al., 2015). Genome wide association studies GWAS) demonstrated that the heredity was involved in PCOS. Eleven candidate genes including 15 susceptibility single nucleotide polymorphisms (SNPs) were identified as being associated with an elevated danger of PCOS in Chinese population (Chen et al., 2011; Shi et al., 2012)

Single Nucleotide Polymorphism (SNP) is a genomic variation resource. An SNP is a single nucleotide alteration in DNA. SNPs are the most common and most wide spread in human genome causing genetic polymorphism in 90% of human DNA polymorphisms. Discovery of SNPs has been carried out via surveys at the genome-level and concerted efforts focusing on specific genomic regions based on limited protein and DNA sequence information (Zhao et al., 2003). Susceptibility of genetic diseases are commonly associated with polymorphisms (SNPs) in people. The mechanisms through which SNPs manifest their effects are not fully known yet. However, the impact of a group of diseases resulted by missense mutations arising from SNPs has been solved, and a group of SNPs seen in the general population has been determined (Wang and Moult, 2001).

Genotyping technology advanced and utilized the SNP assays, and this, subsequently, implemented most human genome assembly methods to connect genetic diversity with susceptibility of diseases (Hamdy et al., 2015).

Interleukin-23 receptor (IL23R) is an essential component in T helper 17 cell-mediated inflammatory process, and it has a significant part in pathogenesis of multiple cancers. Variations in the gene that encodes IL23R have been specified as the displaying reason in process of immunopathology and also, suggested that these variations may change the danger of pathogenesis (Chen et al., 2010). The IL23R gene is placed on the short arm of chromosome 1 at position 31.3 (Kim et al., 2009).

IL-23 is a new recognized member of the IL-12 family of heterodimeric cytokines which modulate T helper cells into subpopulations, and both IL-23 and IL-12 are interested goals to remedy of autoimmune disorder.

microRNAs are a new class of small non coding RNAs contain 18-25 nucleotides. They can bind to the 3’ untranslated regions (UTRs) of target mRNAs and acts as post-transcriptional regulators of gene expression, by inhibition translation or promoting mRNA degradation. Thus their function was originally reported among normal development, also today it is known that the miRNAs have a significant role as oncogenic integral components and tumor suppressor networks (Sokolova, 2011). MicroRNAs have a role in a lot of physiological processes in the cell, such as migration, differentiation, proliferation and apoptosis (Tokarz and Blasiak , 2012).

Binding site polymorphisms of microRNA that are involved in disease danger and prognosis are quickly being discovered and expected as this genetic variations may be potentially harmful, and they may deeply influence the health of people (Christensen et al., 2009).

Biological functions performed by miRNA mostly occur through their binding to the 3′ untranslated regions of the target messenger RNAs. This binding leads to the inhibition of the gene’s expression. One miRNA may target several genes. Thus, it is known as an expert controller for gene expression. According to bioinformatics, more than %30 of whole human genes are regulated by miRNA. However, only 1–3% of the human genome contain miRNA (Wu et al., 2011).

The 3'untranslated regions (3'UTRs) of mRNAs has an important effect on posttranscriptional process in gene expression, for instance by changing of mRNA stability, localization and translation (Winter et al., 2008).

The SNPs located at the 3’-UTR of the target mRNA, through which regulatory proteins and miRNAs can regulate it, may impact the translation and stability of the mRNA and moreover effect of receptor protein amount represented by a cell (Wang et al., 2012).

In the present study, our aim was to determine the genotypes of PCOS patients and their age-matched controls in terms of an SNP, namely rs10889677, found in miRNA binding site of IL23R gene, which is transcribed at the 3’UTR in its mRNA.

2. LITERATURE REVIEW

2.1. miRNA Discovery

miRNAs are single stranded RNAs that contain about 22 nucleotides in length and that are recently identified. It is transcribed by gene inside nucleus by the RNase-II type enzyme and its synthesis is finished in cytoplasm by RNase-III type enzyme and Dicer from endogenous hairpin-shaped transcripts. miRNAs adjust gene expression at the post-transcriptional level (Ambros ,2001).

Discovery of miRNA for the first time was made by Victor Ambros and his partner when they cloned *lin-4*, a gene involved in *C. elegans* expansion regulation, that does not encode for any protein but makes a ~22nt long RNA molecule from a longer hairpin-shaped RNA precursor (Lee et al .,1993; Wightman et al., 1993). Further, they and others detected that the *lin-4* RNA acted as an antisense translational repressor and could regulate expression of the *lin-14* gene binding to 3'untranslated region (UTR) in complementary sequences (Lee et al., 1993; Wightman et al., 1993). *Lin-4* was found in a large number of species. In the year of 2000, a new tiny RNA, *let-7* was notified in *C. elegans (*Reinhart *et al., 2000).*

Interestingly, *let-7* was also discovered to be much conserved in an assortment of organisms (Pasquinelli et al., 2000). Since then a large number of similar small RNA sequences were found in an expansion range of organism in viruses, plants and from nematodes to vertebrates. miRNAs are now taught as a conserved class of small non-coding RNAs which are thought to adjust more than 60% of the human protein-coding genes (Friedman et al 2009), and are implicated in many biological processes such as development, differentiation, proliferation, antiviral defense, and tumorigenesis (Ambros, 2001; Bartel, 2004; Bennasser et al., 2004; He and Hannon, 2004; Kim, 2005) . Until November 2011, nearly 1500 human mature miRNAs have been commented in the miRBase database Release (Griffiths-Jones, 2004).

2.2. Definition of miRNAs

(Micro)RNAs are a recently discovered as a short non-coding class, the molecule length of endogenous RNA contain 18-25 nucleotides only. Since identification of miRNA return to

1993, it’s very small and highly abundant molecule which have been explained that it has an important role in pathological and biological processes (Lee et al., 1993). Work in this area has demonstrated that miRNAs may regulate cellular gene expression at the transcriptional or post-transcriptional level; by suppressing translation of protein coding genes, or cleaving target mRNAs to induce their degradation, through imperfect pairing with target mRNAs of protein coding genes (Barte, 2004).

The interest in and understanding of miRNA-directed gene regulation has increased exponentially over the last five years in particular. This is reflected in the rapid expansion of literature describing novel miRNA genes, about their functions and regulatory targets. 10,581 sequences of mature miRNA have been recorded in primates. Besides, more than 940 mature miRNAs have been represented at the same time; while, prognoses of computation evaluated that this number may be over 1,000 (Berezikov et al *., 2005).* It is obvious that the miRNA story is in rapid evolution (Heneghan, 2012).

2.2.1. The biogenesis of miRNA

The synthesis process begins in the nucleus, where miRNAs are encoded by DNA genomic and transcribed by RNA polymerase II or III into pri-miRNA. The nucleic acid enzyme (Drosha) is cutting pri-miRNA to form a precursor miRNA (premiRNA). Through the shuttle protein, Pre-miRNA is exported into the cytoplasm, by exportin 5, then Pre-miRNA is cut by another nucleic acid enzyme (Dicer) into approximately 22 nucleotides of double-stranded miRNAs. When two strands in duplex miRNAs are divided into two miRNAs, one of them is called the mature miRNA, but another is degraded. Single stranded mature miRNAs are related to the RNA-encourage silencing complex (RISC), and they become the active miRNAs. The active complex reacts with target mRNA at its 3’UTR region through the complete or incomplete pairing machinery to induce the inhibition or degradation of translation of the target mRNA. Active miRNAs function through silencing the expression of genes at their post transcriptional level. (Yan et al.,2015 ; Du and Zamore, 2005)

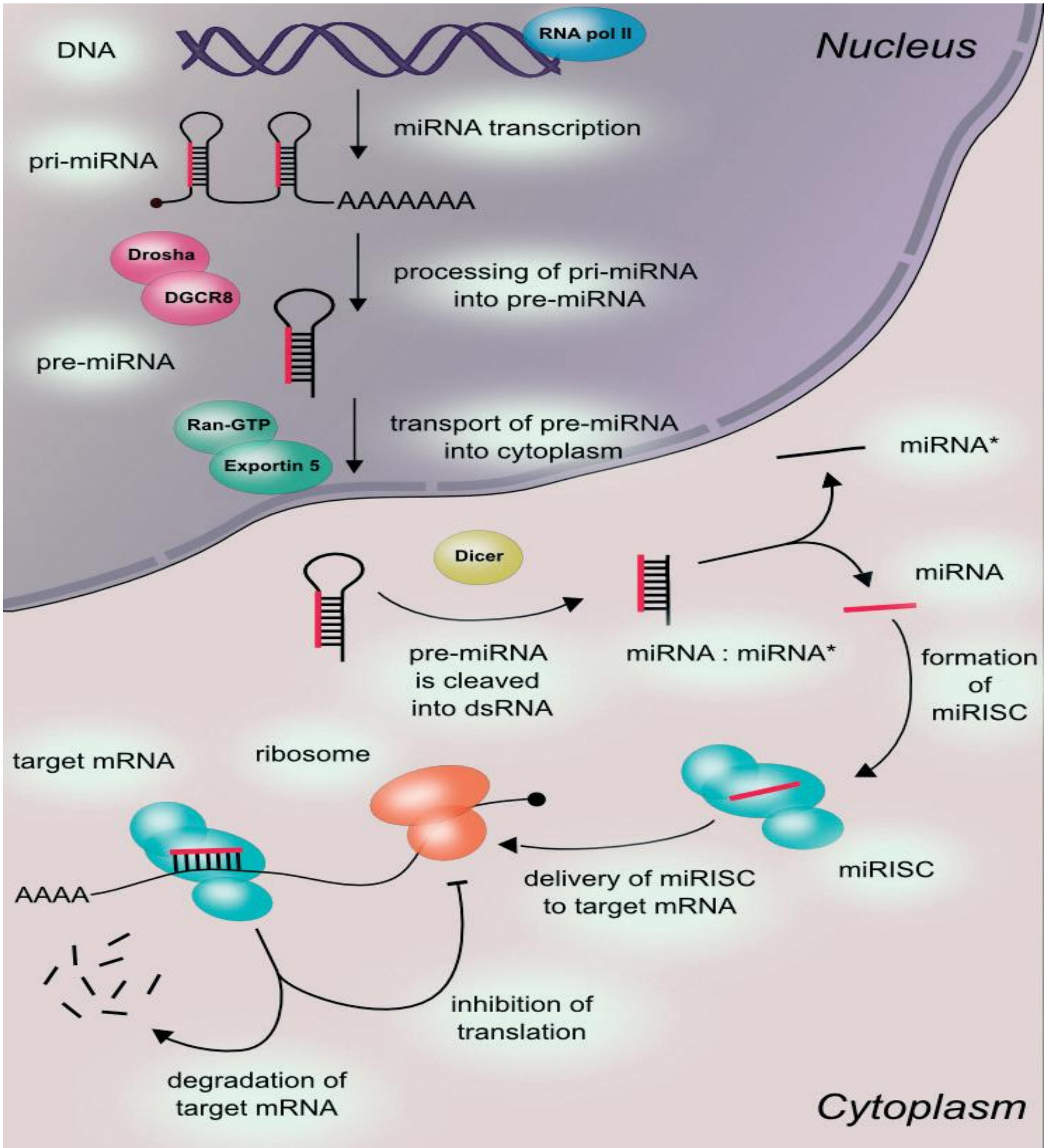


Figure 2.1 Schematic representation of microRNA (miRNA) biogenesis (Tokarz, and Blasiak, , 2012).

2.2.2. The function of miRNA

Alvarez-Garcia and Miska (2005) claimed that the greatest microRNAs in animals are assumed to work through the inhibition of effective mRNA translation of target genes during of ellipse in base combining with the 3'-untranslated region (3'UTR) of target mRNAs. The fundamental mechanism is not understood yet, nonetheless it indicates to contain the inhibition of translational commencement. They also indicate that the objects of MicroRNA are predominately obscure, however they estimated that range from one to several object genes for a certain microRNA. Moreover, it is clarified that miRNA might function as positive organizator in many cases. Hence, it is assessed that a single miRNA may organize as various as 200mRNAs with 1-4% genes in human genome. Also there are hundreds of evidence evaluating that miRNA has a significant functions in several key biological procedures like cell growth, cell proliferation, embryonic development, tissue differentiation and apoptosis. Likewise, earlier studies found that miRNA played a significant functions in cross-species gene expression variation, cellular signaling network, and coregulation with transcription factors (Lu et al., 2008).

Similarly, dysfunction of miRNA biogenesis and dysregulation of miRNAs, modification of miRNAs and their purposes can consequence in deferent diseases (Lu et al., 2008)

There are connection between modified miRNA expression patterns and development of cancer. On the grounds that the efficiency of miRNA– mRNA interaction can be affected by characteristics of thermodynamic of binding and hybridization site, polymorphisms in 3UTR of target mRNA may alter miRNA– mRNA binding and also alter target regulation of gene thereby effecting individuals' danger of cancer (Ahangari et al., 2014).

2.2.3. miRNAs and Diseases

Profiling and functional investigation of miRNAs and their targets have identified miRNAs as critical regulators of a variety of cellular processes for instance proliferation, differentiation, apoptosis, adipogenesis and metabolic integration. Aberrant miRNA expression has been documented in many pathological processes, leading to both benign and malignant diseases (Heneghan et al., 2009; Sonkoly and Pivarcsi, 2008).

Perhaps the greatest emphasis thus far has been on elucidating the precise role of miRNAs in carcinogenesis and great progress has been achieved in that field. Efforts to explore the role of miRNAs in benign diseases [e.g. diabetes mellitus, obesity, heart failure, infectious, inflammatory and auto-immune conditions including viral hepatitis, inflammatory bowel disease, and rheumatoid arthritis] have identified significant involvement of miRNAs in the pathogenesis of these conditions also (Sonkoly et al., 2014; Bala et al.,2008)

2.3. Definition of Poly-cystic Ovary Syndrome (PCOS)

Polycystic ovary syndrome (PCOS) is an endocrine disease that effects on women at reproductive age. The disease is described by presence of some clinical characteristics such as irregular of menstruation, hyperandrogenism, insulin resistance (hyperinsulinaemia) and often central obesity. PCOS is a significant cause for infertility, cardiovascular disease (heart diseases), type 2 of diabetes mellitus, dyslipidemia, and endometrial cancer, therefore it should be diagnosed in the early stage (Xita et al ., 2002) .

The medical literature referred to it first in 1935(Stein and Leventhal). It was described much earlier in 1328 by the French Rabbi Levi Ben Gresham (Salehi et al., 2004). Different family studies have been proposed that polycystic ovary syndrome (PCOS) has a genetic basis. These studies have suggested that because of familial collecting of hyperandrogenemia essentially relatives of PCOS patients, it is a genetic trait. PCOS impacts 4% to 12% of women of reproductive age. Despite its frequency, the PCOS diagnosis is difficult (Sheikhha et al., 2007).

2.3.1. The etiology of PCOS

The last studies suggested that genetic factors have a major role in the PCOS etiology. While the mode of inheritance of PCOS stays unknown, and recent researches indicate that this disorder could be a complex trait. This means that various genes are interacting with environmental factors to excide the phenotype (Sheikhha et al., 2007).

PCOS is a variable combination syndrome of menstrual irregularity, acne or hirsutism, and obesity. PCOS can be identified in adolescence and it has early childhood preceding. PCOS is the highly concerted endocrine reason of an ovulatory infertility and a leading risk factor for the metabolic syndrome and in turn, T2DM development in women.

PCOS is considered to increase a woman's risk in evolving cardiovascular diseases. Therefore, identification of the PCOS risk in women and implementing remediation at once is probably an efficient approach in preventing several of the long-term complications associated for this syndrome (Sørensen et al ., 2014). PCOS usually develops in adolescents and arises as a result of a genetically resolved disorder.

PCOS is identified as an important metabolic and reproductive disorder. It is associated with troubled insulin activity and dysfunction of pancreatic B-cell. These defects allow an increased hazard for glucose intolerance that enhances the danger for type-2 diabetes mellitus. Insulin resistance (hyperinsulinemia) is connected with weight gain, which makes it conceivable to put on weight effectively regardless of good sustenance and activity. It additionally prompts high androgens which might prompt skin break out, facial hair development, and male pattern baldness on head (Diamanti-Kandarakis, and Papavassiliou, 2006).

The mechanisms of genetic uniform functional the polycystic ovary syndrome (PCOS) and hyperandrogenism remain largely unbeknown. Given the big number of genetic variants discovered as relatives to these disorders, the developing picture is that of a complex multigenic attribute in which natural impacts assume a critical part in the statement of the phenotype of hyperandrogenic. PCOS is described by adrenal androgen secretion and increased ovarian (Escobar-Morreale et al., 2005).

The PCOS prevalence development in people emphasizes where the gene pool has a relation but environmental agent are assuming an ever more significant role. An increasing weight in sensitive people is related to PCOS development. The development of obesity is linked to the development of PCOS in susceptible individuals. The modern living environment in developed countries is characterized by low daily energy expenditure and an abundant and inexpensive food supply, making positive energy balance common. However, even this view is too simplistic and it is likely that other factors in the environment, perhaps unrecognized or poorly understood (e.g. exposure to environmental toxins), may also play a role (Diamanti-Kandarakis et al., 2006).

Both the polycystic ovary syndrome (PCOS) and obesity commonly occur in women of reproductive age. Fifty percent %50 of all patients with PCOS are obese, and the presence of obesity influence the manifestations of clinical of PCOS (Salehi et al., 2004). Also Women with polycystic ovary syndrome (PCOS) are mostly assumed to be at increased danger for cardiovascular disease (CVD), given the high predominance of the metabolic syndrome X among them. Long-term studies of well recognized women with PCOS are lacking any relation to primary cardiovascular case such as stroke or myocardial infarction, therefore this correlation stays more speculative than substantive. Epidemiological studies that have focused on separate signs and stigmata of PCOS, such as polycystic ovaries, chronic anovulation or hyperandrogenism, discover mixed results of them (Legro, 2003).

Dysfunction of thyroid may also be associated with problems of fertility. Women without diagnosis of PCOS may often be asked to receive thyroid gland testing. Diseases causing overt hypothyroidism or hyperthyroidism, can also induce (monthly) menstrual disorders and consequences of fertility. Polycystic ovary syndrome (PCOS) are nearly associated. Based on several of studies showing an importance role of prevalence of Hashimoto's thyroiditis in women with PCOS than in controls. However, the mechanisms are not as clear for this association. Surely, genetic susceptibility give a share in a significant part to the development of PCOS and HT. However, an exact genetic background has not yet been determined. Polymorphisms of the PCOS refer to a gene that could be involved in the pathogenesis of HT and PCOS (Gaberšček et al., 2015).

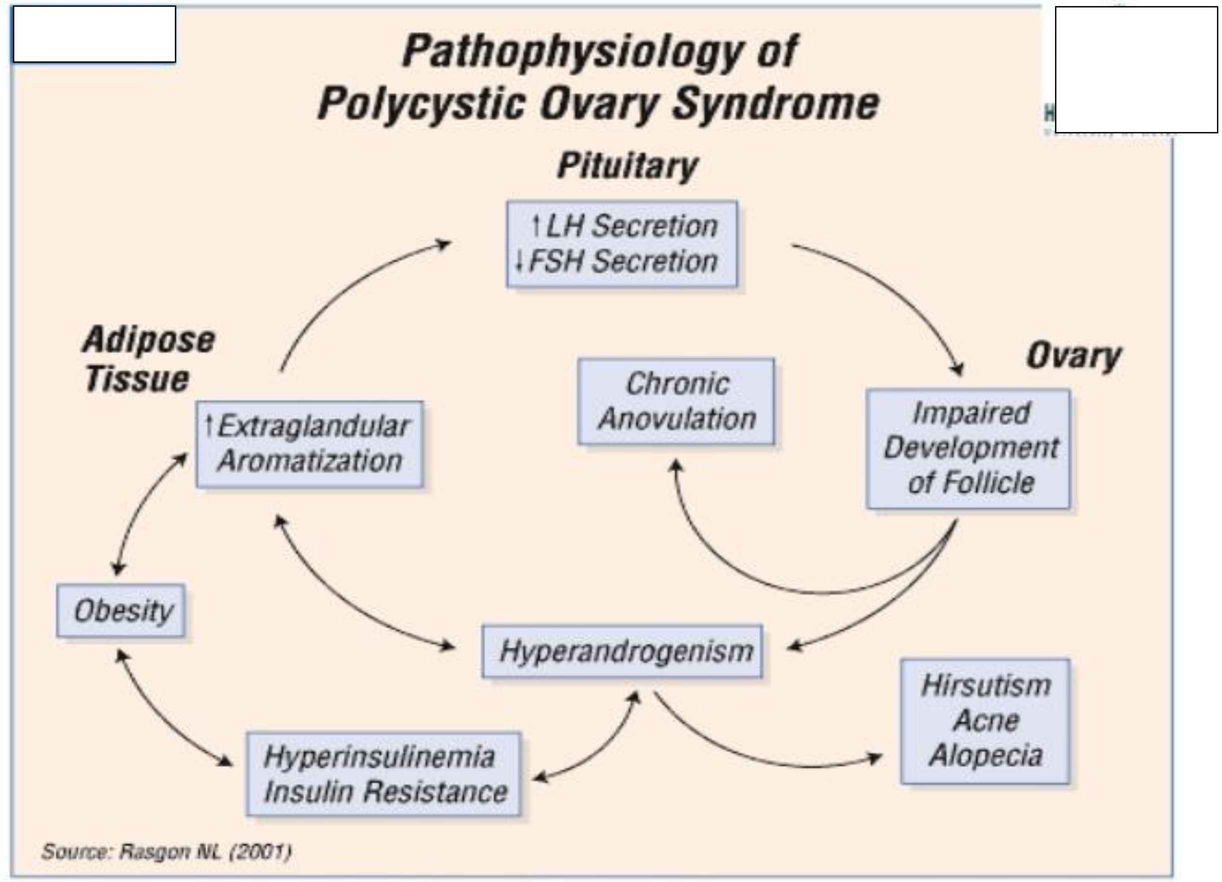


Figure 2.2 Pathophysiology of poly cystic ovary syndrome

2.4. IL23 Gene & IL23 Receptor

Interleukin-23 receptor (IL23R) can react with IL-23 and, thus, is involved in the T-helper 17 (Th17) cell-mediated inflammatory process as well as tumor genesis. Th17 cells belong to pro-inflammatory CD4+ effecter T-cells and can mediate tissue inflammation by secreting high levels of the proinflammatory cytokine IL-17 in response to stimulation. IL-23R was involved in multiple significant biological processes (Zhou et al ., 2013).

Interleukin-23 (IL-23) is a heterodimeric cytokine formed by the IL-23-specific p19 subunit and the IL-12p40 subunit (Chen et al., 2015). IL-12 is important for the development of T helper (Th) 1 cells that are fundamental for host immune and tumor suppression (Languish et al., 2005)**.** Interleukin‑23 receptor (*IL‑23R*) gene has been found to be related to several different autoimmune diseases and regulate the genes expression affected in inflammation.

IL‑23 is generally represented on the cell surface of T lymphocytes, natural killer cells, and dendritic cells (Parhamet al., 2002; Trinchieri et al., 2003). IL‑23R, a member of hemopoietin receptor superfamily, is the private component of the heterodimeric receptor complex for IL‑23 signaling cascade. Therefore, IL‑23 and IL‑23R signaling pathway plays an important role in immune regulation (Li et al., 2015).

Interleukin-23 (IL-23), a key regulator of T helper type 17 (Th17) cells, is released by dendritic cells (DC) in response to immune danger. However, apparently little is discovered regarding agents that regulate its secretion (Wang et al., 2011).

The novel inflammation pathway IL-23/IL-17 axis has given evidence to serve as a helpful biomarker for renal disease activity and for predicting the response to immunosuppressive therapy. IL-23R affects the IL23/IL17 axis by developing the expression and production of IL-17A and IL-17F in Th17 cells (Hamdy et al., 2015). Today, several researches have detected that some single nucleotide polymorphisms (SNPs) in the *IL23R* gene are connected to immune-related diseases, including inflammatory bowel disease (Jiang et al., 2010), chronic inflammatory disease (Song et al ., 2013 ; Einarsdottir et al .,2009), Crohn’s disease , rheumatoid arthritis (RA) and systemic sclerosis (SSc) (Faragó et al ., 2008). AML disease

(Qian et al., 2013), psoriasis (Nair et al., 2008), systemic lupus erythematosus (SLE) and (Safrany et al., 2010) and some genetic variant of IL23 may contribute to the pathological development from hepatitis to hepatocellular carcinoma (Xu et al., 2013).

Moreover, previous researches have mentioned that IL-23R exerts an immune surveillance function via CD8þ T-cells and accelerates tumor growth. These findings propose that IL-23R may play a significant role in cancer progression and development. Also, some case-control surveys have investigated the association of IL-23R polymorphisms with the danger of cancer, containing esophageal squamous cell carcinoma, ovarian cancer, bladder cancer, acute myeloid leukemia, breast cancer, gastric cancer, lung cancer, colorectal cancer, and nasopharyngeal cancer (Hamdy et al ., 2015)

2.4.1. IL-23R genetic variants

Few surveys have reported the association of particular IL-23R variants and ovarian cancer in the Chinese population (Zhang et al., 2010). There are currently no studies that have proved evidence of IL-23R variants association with Rheumatic heart fever (RHF). However, numerous surveys have been released documenting association of IL23R variants with different auto-inflammatory/auto-immune related disorders such as Crohn's disease, rheumatoid arthritis, ankylosing spondylitis, psoriasis arthritis and inflammatory bowel disease. Table (2.1) refer to summary of common IL23R variants association with auto-inflammatory/autoimmune disease (Leaupepe , 2015).

Table 2-1 A summary of Auto-inflammatory & Autoimmune disease that are associated with common IL23R SNPs.

IL23R SNP of interest highlight yellow.

IL23R SNP	**Auto-inflammatory & Autoimmune disease**	**Risk Allele**	**Chromosome position**	**Source/Previous Studies**

Rs1004819	Crohn's disease, Ankylosing Spondylitis, Ulcerative Colitis, Celiac Disease, Inflammation bowel disease, Psoriasis,	C	1 : 67204530	Duerr *et al* (2006), Burton et al (2007), Glas *et al* (2009)
Rs10489629	Crohn's disease, Ankylosing Spondylitis, Inflammation bowel disease, Psoriasis, Celiac disease	A	1 : 67222666	Burton *et al* (2007), Park *et al* (2009), Einarsdottir *et al* (2009)
Rs10889677	Ankylosing Spondylitis Crohn's disease, , Ulcerative Colitis, Psoriasis	A or C	1 : 67259437	Huber et al (2008), Duerr et al (2006), Burton et al (2007), Silverberg et al (2009), Farago et al (2008), Einarsdottir et al (2009)
Rs11209026	Inflammation bowel disease, Psoriasis, Crohn's disease, Ankylosing Spondylitis, Ulcerative Colitis, Psoriasis	A or G	1 : 67240275	Duerr et al (2006), Burton et al (2007), Weersma et al (2008), Liu et al (2008), Fischer et al (2011), Hollis-Moffatt et al (2009), Chang et al (2008)

Rs11209032	Crohn's disease Ankylosing spondylitis, Inflammatory bowel disease,	A	1 : 67274409	Duerr et al (2006), Brown (2008), Burton et al (2007) Jiang et al (2010),
Rs11465770	Crohn's disease	T	1 : 67168280	Bin et al (2009)
Rs11465788	Crohn's disease, Ulcerative colitis	C	1 : 67182611	Bin et al (2009), Juyal et al (2011)
Rs11465804	Ankylosing spondylitis, Inflammatory bowel disease, Crohn's disease, Psoriasis, Celiac disease	G	1 : 67236843	Brown (2008), Burton et al (2007), Deshmukh et al, Kanaan et al (2012)
Rs11805303	Crohn's disease, Inflammatory bowel disease, Ulcerative colitis	T	1 67209833 :	Burton et al (2007), Lv et al (2012), Fisher (2008)
Rs1343151	Ankylosing spondylitis, Rheumatoid arthritis	T	1 67253446:	Duerr et al (2006), Burton et al (2007),
Rs1495965	Ankylosing spondylitis	A	1 : 67287825	Burton et al (2007), Mizuki et al (2010),

Rs2201841	Crohn's disease, Psoriasis, Ankylosing spondylitis, Ulcerative Colitis, Celiac Disease, Inflammation bowel disease	C or T	1 : 67228519	Duerr et al (2006), Huber et al (2008), Einarsdottir et al (2009), Sáfrány et al (2009)
Rs7517847	Crohn's disease, Ankylosing Spondylitis	G or T	1 : 67215986	Tung et al (2011), Lee et al (2012)
Rs7530511	Psoriasis, Crohn's disease, Ankylosing spondylitis	T	1 : 67219704	Nair et al (2008), Huber et al (2008), Taylor et al (2008),

2.5. Introduction to Single nucleotide polymorphism

Single nucleotide polymorphisms, frequently called SNPs (pronounced “snips”), are the most common type of genetic variation among people. Each SNP represents a difference in a single DNA building block, called a nucleotide. For example, a SNP may replace the nucleotide cytosine (C) with the nucleotide thymine (T) in a certain stretch of DNA. SNPs occur at a rate of one per 300 nucleotides that accounts for about 10 million SNPs in the human genome.

Scientists can use SNPs as a biological markers to identify genes with disease. When SNPs found near a gene or a close to a gene, they might have an effect on the functions of the gene and contribute to the disease. While most SNPs are harmless and do not affect health and development as a whole, some of the SNPs have shown to be important in the diagnosis of disease in human. Studies have shown that the presence of SNPs may help the response of a patient to certain drugs, and increase susceptibility to environmental factors such as toxins and the risk of developing disease SNPs are also useful to detect disease associated with gene inheritance within families. Further researches will work to find relations between SNPs and complex disease such as heart disease, diabetes, and cancer (https://ghr.nlm.nih.gov/).

2.5.1. Definition and structure

Single-nucleotide polymorphisms (SNPs) can be used for genetic mapping, which is important for gene description of human disease and biological functioning (Griffin and Smith, 2000).

SNPs are useful in the understanding of the whole-genome structure. It is estimated that SNPs occur at every 1000 among the human population, which accounts for an overall of several millions. 90% of inter-individual mutability is due to SNPs differences, which roughly equals to 100,000 amino acids (Riva and Kohane , 2002).

Nucleotides are the basic constituents of the genome, there are four types of nucleotides: Adenine (A), guanine (G), Thymine (T), and Cytosine (C). A single nucleotide polymorphism (SNP) is a variation in a single base pair in a DNA sequence. Each DNA fragment that carries the certain nucleotide in a given position is called an allele. For example, there are two nucleotides in the following two DNA fragments in the fourth position: CCACGTT and CCATGTT. In this case, we say that the SNP has two alleles, C and T. Unlike polymorphism, SNPs consist of two alleles, whereas in polymorphism there are three or four alleles, meaning three of four different nucleotides can be found at the same position. Thus, the SNP is mostly referred to as the bi-allelic polymorphism with the minor allele frequency (MAF) being larger than 1. If the two alleles are similar, then they are called homozygous, if the two alleles are different, they are called heterozygous. The most frequent allele in a locus is called the wild type whereas the second most repeated is the mutant allele (Su et al., 2007).

2.5.2. The history and international SNP Map

1.2 million SNPs have been identified that are distributed across the human genome, with an average density of one SNP per 1.9 kilo bases. The determination of these SNPs were made by the use of two projects: The SNP association and the analysis of clone overlaps by the International Human Genome Sequencing Consortium. The map integrates all publicly obtainable SNPs with described genes and other genomic characteristics. Nearly 60,000 SNPs are contained within exon regions, 85% of exons are within distance of a 5 kb of the closest SNP. Human genome has a huge nucleotide diversity, in a way that is compatible with a standard population genetic model of human history. This high-density SNP map is essential and important source of haplotype variation knowledge in human genome, through which important genes needed for diagnosis and cure of diseases can be identified (Sachidanandam et al., 2001).

Single-nucleotide polymorphism (SNP) mapping in *Caenorhabditis elegans* is a strong and elegant tool for determining the chromosomal position of a mutant gene. SNP designed as a way to clone a gene in *C. elegans* was first used by Jakubowski and Kornfeld (Davis and Hammarlund, 2006).

Most genomic variations are attributable to single nucleotide polymorphisms (SNPs), therefore show the highest decision for tracking disease genes and population history. It has been suggested that a dense map of 30,000±500,000 SNPs can be used to scan the human genome for haplotypes associated with common diseases (Altshuler et al., 2000).

2.5.3. Importance of SNPs

The occurrence of SNPs is one per 1000-2000 bps, and the number of all SNPs in human genome is estimated to be 1,433,393 by the year 2000 (Venter et. al 2001). SNPs can be found everywhere in coding and non-coding regions of the DNA. Protein coding regions of the DNA have less than 1% of the SNPs, this suggests the very little contribution of the SNPs in phenotypic variations (Pungliya, 2001).

The SNPs supply the most perfect and densely arranged system of genome landmarks available. Above all, this enables surveyors to improve the decision of linkage maps, in particular in the exact mapping and survey of genes participating to complex disorders, where the contributory influence of individual genes is small or where genetics is compounded by multiple locus interaction and environment. As well as enabling most important mapping, SNPs provide a new and fascinating layer of detail to our understanding of human variability and what this tells us about disease susceptibility, populations, and evolution (Phillips, 2007).

2.5.4. SNPs in miRNA binding sites influence and let7 family of miRNA

The concept of single nucleotide polymorphisms within protein-coding genes can affect the functions of protein, and successively affect the individual capability to cancers, has been well documented. As delineated above, miRNAs bind to the 3' untranslated region (UTR) of their target genes – regions and have regulatory impact on gene expressions which are evolutionarily extremely preserved suggesting a main role for these regions in natural selection. Hundreds of mRNAs can be regulated by a single miRNA jointly; the potential of cellular transformation is high which derived from dysfunction of a single miRNA. Recent research has been declared that SNPs, in their complementary binding site on mRNA or in miRNA sequences can be resulted in diverse functional consequences, and can denote ideal candidate biomarkers for prognosis, disease diagnosis and outcome (Saunders et al., 2007; Chen and Rajewsky 2006).

miRNA binding site of a miRNA target contain a single nucleotide polymorphism (SNP) may disrupt miRNA–target interaction, resulting in the deregulation of expression of target gene (Kontorovich et al., 2010).

The binding of microRNA to mRNA is important for protein expression and regulating the mRNA level. Moreover, single-nucleotide polymorphisms can impact this binding and might establish in the microRNA target site, which can make illegitimate binding sites or cancel existing binding sites. Also, in microRNA, polymorphisms have a differentiation effect on protein expression and the danger of certain human diseases can be affected by another type of genetic mutability. Within microRNA-binding sites, different methods have been used to identify functional polymorphisms and make declaration. These polymorphisms in predicted microRNA-binding sites have been tested in big case–control survey (Chen et al., 2008)

Much previous research has shown the deregulation of miRNA or miRNA-associated genes in ovarian cancer. However, the effect of low penetrance, common genetic variations in the miRNA pathway genes on ovarian cancer redisposition has not been widely explored. It has been showed that SNPs in miRNA processing genes and miRNA binding sites were associated with ovarian cancer predisposition (Liang et al., 2010).

The first known human miRNA is called Let-7. In 2005, it was known that let-7 is very important for normal cell differentiation of stem cells in the sample organism, and that numerous human genes encoding let-7 family members were mapped to regions of the genome with known deletions in cancer, which also suggested that let-7 might be a tumor suppressor (Slack, 2013).

In *Caenorhabditis elegans*, the let-7 family of microRNAs was found, and is functionally saved from worms to humans. In nine different chromosomes, the human let-7 family has 13 members, and deregulated let-7 expression appear in many human cancers. A growing body of evidence recommends that restoration of let-7 expression may be useful in therapy of cancers (Boyerinas, 2010).

The worldwide genetic regulator, let-7 family, binds to the 3' untranslated regions of their target mRNAs. These mRNAs might also carry SNPs on their miRNA binding sites. The effect of such SNPs on target gene, which were present at the binding site, were assessed in the regulation of breast cancer and risk for non–small cell lung cancer (NSCLC) (Chin et al ., 2008; Slack , 2013).

2.6. The 3' untranslated regions

The 3' untranslated regions (3'UTR) are involving in the adjustment of gene expression at numerous levels. At the pre-messenger RNA (mRNA) level, 3'UTRs are involved in the mRNA 3' end structure and polyadenylation, while they select characteristics such as mRNA stability/degradation, nuclear export, subcellular localization and translation effectiveness at the mature mRNA level (Brendle et al., 2008).

The 3'UTR plays a significant role in the translation, localization and stability of the mRNA. Study on the pathophysiology of diseases and mutations influencing the functionality of the 3' untranslated is till now not dense. Nevertheless, the obtainable information about this mRNA region proposed that it is many times neglected during the genetic screening of disease-associated candidate genes, but it actually plays a significant role in many different diseases and disease improvement (Conne et al., 2000). Secondary structure of 3′-UTR of mRNA and termination codon, or polyadenylation signal may be affected by mutation that is reason to disease and translation de-regulation. The obtainable data propose a relation between mutations in 3’UTR and disease. However, this relationship is required to be proven accuracy (Chatterjee and Pal, 2009).

3’UTR regulatory components are viewed to have a role after transcription at the mRNA level, but in principle could also profit at the DNA level as distal promoters to modulate transcription (Merritt et al ., 2008).

There were a lot of significant sequences located at 3' UTR region at the end of mRNA transcripts that make an influence on the fate of mRNA. The known data suggest that sequence characteristics and 3' end processing that contain binding protein association and selected signaling pathways affect 3' UTRs to delineate their significance in the mammalian regulatory procedure.

More famous aspects, for instance, processes of cleavage and polyadenylation that makes effect on stability and location of mRNA have also been showed as other roles of 3' UTR (Matoulkova et al., 2012).

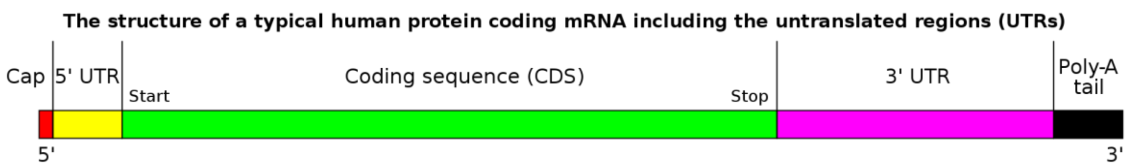


Figure 2.3 the structure of a human protein coding mRNA include untranslated region.

2.7. rs10889677

It has been investigated that the rs10889677 situated in the 3 UTR might cause overexpression of the IL23R, which, in turn, leads to heavy diversity of T-cells forward a Th17 subpopulation through an increased releasing of other proinflammatory cytokines (Hamdy et al., 2015).

The rs10889677 SNP in IL23R maybe can cause overexpression of the receptor, driving differentiation of Th1 helper T cells forward a Th17 subpopulation, resulting in an increased release of IL-17 from these cells. This will consequently reason to the release of extra cytokines (e.g. TNF), leading cancer and chronic inflammation. The polymorphism of rs10889677 was connected to breast cancer (BC) below the allelic, dominant, heterozygous, and homozygous models (Liu et al., 2015). Previously, an important association between rs10889677 and the development of rheumatoid arthritis has also been suggested (Hamdy et al., 2015). In accordance with this, Zhang et al. (2010) implied that allele's frequencies of rs10889677 varied considerably among patients and controls with ovarian cancer. The allele's C frequency of rs10889677 was expressively improved in ovarian cancer cases compared to controls.

3. MATERIALS AND METHODS

3.1. Sample collection

The samples were collected from the pathology department at KahramanmaraşSütçü İmam University hospital. A total of 79 samples were analyzed. The study included 42 patient samples and (37 healthy controls) that were storied and the clinical characteristics of both patients and control samples determined, that including (AGE, BMI, LH, E2, TESTO, DHEA and TSH). The samples stored at 4˚C until further analysis.Informed consult was taken from all participates and the study approved by local ethics committee.

3.2. Instruments, Chemical Materials and Solutions

In this study employed instruments, chemicals and solutions as shown in tables below.

Table 3-1 Utilized instruments, brands and product place

Instruments	Brands	Manufactures
Heating magnetic stirrer	Velp	Italy
Refrigerator	Bosch	Turkey
Incubator	Uniequip	Germany
Vortex	Scilogex mx-s	Germany
Centrifuge(1-14)	Sigma	UK
Micropipettes set	Pipetman	Germany
Biological safety	Bilser	Turkey
Mini centrifuge	MyFUGE	Germany
Thermal cycler (PCR)	VWR	USA
gel electrophoresis	MUPID-EXU	JAPAN
Microwave	Arcelik	Turkey
PCR tube 0.2 Ml		
Spectrophotometer(nanodrop)	VWR(mySPEC)	USA
Ultraviolet translluminator	L.PIX(Loccus)	USA
Edta tube 0.5 ml		
Precision balances	KERN PLS	Germany

Table 3-2 Utilized solutions, and their chemicals content

Solutions	Content
10X TBE	108,9 g Tris 55 g boric acid 9,37 g EDTA 1000 ml distilled water
Ethidium bromide	0,5 Ethidium bromide 1 ml distilled water
DNA loading Dye	10 mMTris-HCl (pH 7.6), 0.03% bromophenol blue 0.03% xylene cyanol FF, 60% glycerol and 60 mM EDTA
Proteinase K (10 mg/ml	Dissolve as 10 mg/ml in 10 mMTris pH 7.5, stored at - 20 C

Table 3-3 Genomic DNA extraction kit content for 250 samples (Jena Bioscience, Germany):

Agents	250 preps
Blood Lysis Buffer	2*137 ml
Lysis Buffer	80 ml
Binding Buffer	80 ml
RNase A (50mg/ml)	5*7.5 mg before use, add 150 µl double distilled water as indicated o the bottle) store at -20 º C)
Proteinase (10mg/ml)	5*5 mg before start add 500 µl dd-water to the Proteinase K tube store at -20 º C
Activation Buffer	30 ml
Washing Buffer	Add 120 µl 96-99% Ethanol to each bottle (not included in the kit)
Elution Buffer	25 ml
Spine columns	250 pieces
2 ml collection tube	250 pieces

3.3. DNA Extraction

There are many different published protocols for isolation DNA. In our study weextracted DNA from the 79 blood samples by using Jena bioscience protocol .the protocol is prepared for the genomic DNA isolationfrom whole blood .online published http:www.jenabioscience.com.

A. Sample Preparation:

Mix 200 µL (1 volume) of whole human blood with 1 ml (5 volumes) of Blood Lysis Buffer in an appropriately sized tube and Incubate for 10 minutes on ice after , Mix by vortexing briefly 2-3 times during incubation after Centrifuge at 10,000 g for 10 minutes to pellet the white blood cells then discard supernatant completely.

B. Cell Lysis:

Add 300 µLLysis Buffer and 2 µLRNasefor the cell pellet, Vortexes vigorously for 30-60 sec, Add 8 µL Proteinase K and mixing by pipetting and Incubate at 60 °C for 10 minutes and cool down for 5 minutes after , Add 300 µL Binding Buffer after vortex briefly, after that Place tube on ice to 5 minutes then Centrifuge for 5 min at 10,000 g

C. Column Activation:

put a spin column into a 2 ml collection tube after increase 100 µLinActivation Buffer into the spin column after that Centrifuge at 10,000 g for 30 sec and at once proceed to the another step then Discard flow-through

D. Column loading:

Pipet supernatant directly into the spin column, Centrifuge for 1 min at 10,000 g and Discard flow-through

E. Primary Washing:

Increase 500 µL Washing Buffer to the spin column, and Centrifuge for 30 sec at 10,000 g after Discard flow-through

F. Secondary Washing:

Add 500 µL Washing Buffer into the spin column, and Centrifuge for 30 sec at 10,000 g
After Discard flow-through after that Centrifuge again at 10,000 g for 1 min to remove residual Washing Buffer and Discard the 2 ml wash tube and put column into the elution tube

G. Elution of DNA:

Increase 40-50 µLin Elution Buffer to the center of the column and Incubate at room temperature for 1 min after that Centrifuge at 10,000 g for 2 min then Store the eluted DNA at 4 °C or -20 °C.

After finishing the process of DNA extraction we used (Micro volume spectrophotometers, my SPEC) to determination of concentration and purity of DNA.

Figure 3.1 Micro volume spectrophotometers, mySPEC to determine the concentration of DNA.

3.4. Poly Cystic Ovary Syndrome

Polymerase Chain Reaction (PCR) depends on the utilization of the capacity of DNA polymerase to create a new strand of DNA complementary to the presented template strand. Because nucleotides can be added by DNA polymerase only onto a preexisting 3'-OH group, also primer is necessary to add nucleotides by DNA polymerase. Specific region of template sequence will be delineated by this requirement. At the final of process create billions of copies in specific sequence of DNA. PCR technique was used in order to amplify a certain region of IL23R gene. All the materials used in PCR reactions were sterilized, and PCR mixtures were prepared in PCR tubes. PCR reactions were performed by using VWR thermal cyclers

3.4.1. Primer Design

In general, the optimum length for primers is 18-22 bp. This length is suitable for accuracy and short enough for primers to bind to the template at the annealing temperature. Primer is a strand of nucleic acid that serves as a starting point for DNA synthesis. Selection of primers was depended on the target region in the sequence. A pair of primer of mRNA sequences (forward ATCGTGAATGAGGAGTTGCC, reverse TGTGCCTGTATGTGTGACCA) for IL23 Gene were designed. Online primer design program www.thermoscinentfic.com/onebic was employed. The sequence of the primers, annealing temperature and the length of PCR products are exhibited in Table 3.8.

Table 3-4 Primer sequences, PCR product size of target region of IL23 gene and annealing temperature

primer name	Sequence 3, to 5,	Optimal Annealing Temperature	PCR Product Size
IL 23 FW Primer RV Primer	ATCGTGAATGAGGAGTTGCC TGTGCCTGTATGTGTGACCA	57 °C	470 bp

3.4.2. PCR Process

A DNA sample was used to carry out the PCR for each primer pairs. The components presented in Table 3.5 were mixed in a PCR tube for each sample, and the tube was vortexed gently then briefly centrifuged. The tubes were placed in a thermal cycler and the appropriate program was set.

Table 3-5 the components of PCR reaction and their quantities in 30 µL total volume

Chemical Substances	Quantity (µL)
Buffer	3µL
MgCl2	1.5µL
Dntp	3µL
Forward primer	1.5µL
Revers primer	1.5µL
Taq DNA polymerase	0.5µL
dH2O	17 µL
DNA	2µL
Mixture	30µL

The thermocycling program was set to run 36 cycles according to the following parameters as indicated in Table 3.6.

Table 3-6 Thermal cycling Conditions of PCR reaction

Step	**Temperature**	**Time**
1. Pre denaturation at	96°C	5 minutes
2. Denaturation at	94°C	30 second
3. Primer annealing at	57°C	30 second
4 .Extension at	72°C	40 second
5. Final extension	36 cycles 72°C for	7 minutes
Hold	4°C	0

The final products were analyzed by 2.0% agarose gel electrophoresis and stained with ethidium bromide. The gel was run at 100 volts for 30 minutes. The DNA fragments were illuminated by UV-light. Our product is 470 bp according to the obtained image in PCR results by gel electrophoresis.

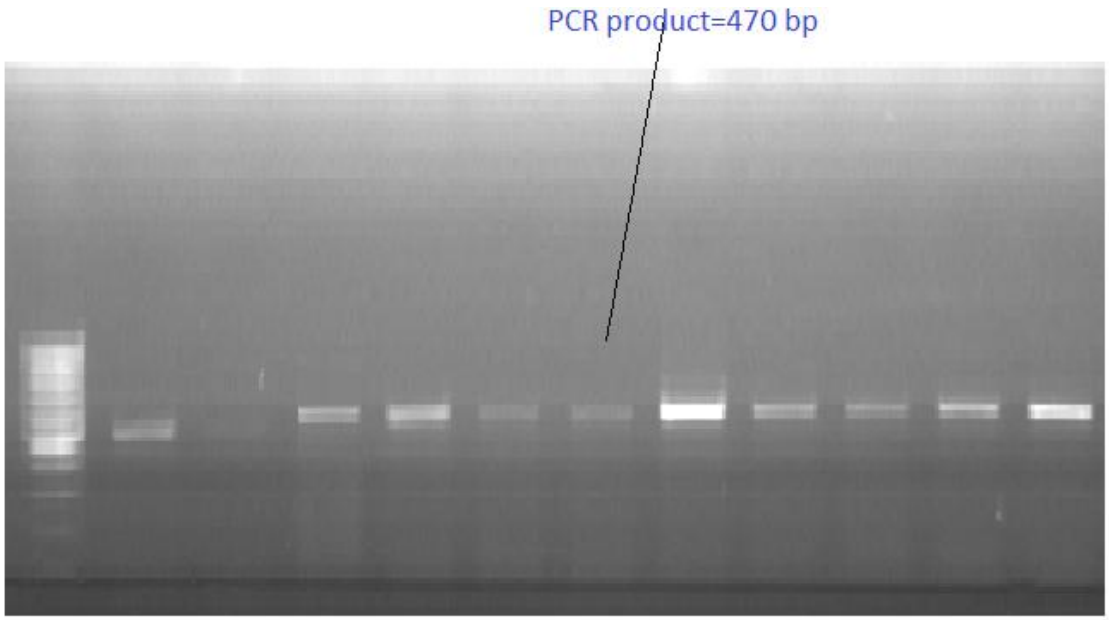


Figure 3.2 Gel images of the PCR reaction products analyzed by electrophoresis in a 2% agarose gel with ethidium bromide staining...

3.5. PCR-Restriction Fragment Length Polymorphism

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) is an old method that depends on the use of restriction enzymes to differentiate between SNPs. It is an easy method to analyze the SNPs and it is an approved way to use in the field. Restriction enzymes has the capacity to cut DNA at a very specific sequence pattern. The presence of a SNP will be detected trough the different sizes of fragments as result of the restriction.

For the restriction, The PCR products were digested with one unit ofMnlI enzyme at 37°C for fifteen minutes time of incubation. The components of the digestion mixture were shown in Table 3.7. The segments produced at the end of this digestion were used to identify the rs10889677 SNPs mutation. Restriction fragments analyzed after incubation on 2% agarose gels and visualized by using UV Tran’s illuminator.

Table 3-7 the reaction condition for PCR/RFLP and their quantities

Chemical Substances	**Quantity (µL)**
Restriction Enzyme (MnlI)	1µL
10X Buffer	5µL
dH2O	24µL
DNA	20µL
Total	50µL
Incubation Temperature	37°C
Incubation time	15 minutes

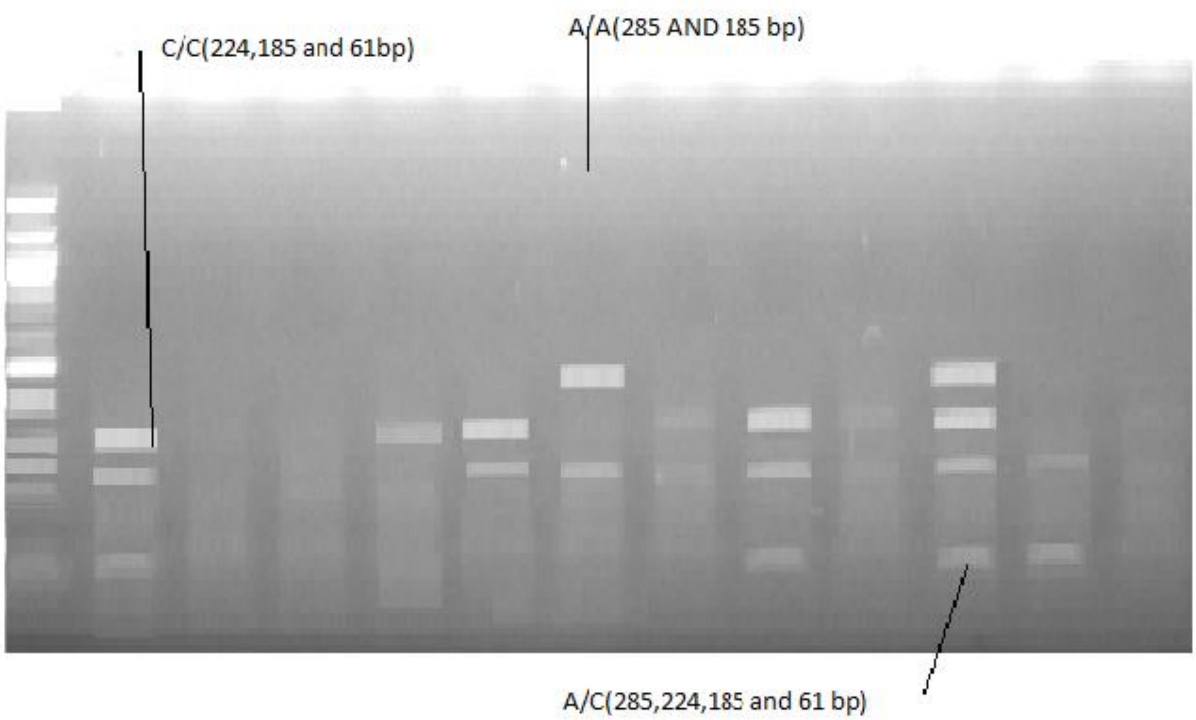
3.5.1. Estimation of the Digested Products (PCR-RFLP)

After digestion with the restriction enzyme,MnlI,fragments were loaded onto 2% agarose gel and stained with a compound that makes the DNA visible under UV light(Ethidium bromide (EtBr)) along with the DNA Ladder. DNA sampleswere electrophoresed under electric field at 100 volts up to 30 minutes. The DNA fragmentswere visualized by UV illumination and the sizes estimated by known sizecomparison.MnlI*cuts* the 470 bp PCR product at positions 185 and 409 bp. The first position, although recognized by the enzyme, does not have a SNP, meaning it will always have C nucleotide at the target position thereby producing two fragments of 185 and 285 bp. The second recognition site, however, can have a SNP, which produces different sizes of fragments depending on the nucleotide it has at the right position. In case this SNP has a nucleotide. Then the site will not be recognized by the enzyme, but when it has C nucleotide, it will be recognized. This recognition will produce two smaller fragments of 285 (which was cut because of the first recognition site) as 224 and 61. Accordingly, if one genoci DNA sample is AA homozygous, then only one (first) recognition site will exist, therefore two fragments will be seen; 185 and 285 bp. If the sample is CC homozygous, it will produce three bands because the first recognition site, which does not have SNP, initially produce two products as it does in AA genotype (185 and 285). Then, however, the second recognition site will cut the 285 bp-product once more, producing 224 and 61 products. Therefore, at the end, you will have three bands which are at 285, 185, and 61 bp length. When considered together; an allele producing two bands (285 and 185 bp) and C allele producing three bands (224, 185 and 61 bp), AC heterozygous genotype will show four bands because, in total, four different-sized products will be generated. The gel patterns obtained for the (A/A), (A/C), and (C/C) genotypes are shown in Table3.8. And Figure3.3. Below.

Table 3-8 The sizes of the fragments obtained after the digestion Reactions of rs(10889677) SNPs for the (A/A), (A/C), and (C/C) genotypes

A/A **(Normal homozygote)**	**A/ C** **(Heterozygote)**	**C / C** **(Mutant homozygote)**
285	**285**	**224**
185	**224**	**185**
	185	**61**
	61	

Figure 3.3 The gel patterns obtained after loading the digested fragments for the (A/A), (A/C), and (C/C) genotype



3.6. Statistical Analysis

Statistical Package for Social Sciences software (SPSS 19) was used for analysis. Descriptive parameters were shown as Mean± Standard Deviation or in percentages. Independent samples t-tests and Pearson’s chi-square tests were used to analyze the differences in means and proportions between groups. Odds ratios and their confidence intervals based on genotype frequency were measured by MedCalc easy to use statistical software. Comparisons between groups were tested with ANOVA. A p value below 0.05 was considered as significant.

4. RESULTS

Seventy nine cases were involved in this study, 42 female patients with polycystic ovary syndrome and 37 female individuals not diagnosed with PCOS (control). Mean age of the patients was 24.73±6.16 years while it was XX±YY years for control. The statistical analysis showed non-significant differences between age of the patients and control, ($p=0.69$). BMI was significantly increased in patients, ($p=0.001$). Different blood parameters were evaluated, no significant differences were found in FSH, E2 and TSH ($P>0.05$), while there were a significant increase found in LH, Testosterone and DHEA ($P<0.05$) data showed in Table 4.1.

Table 4-1 Comparison of clinical and demographic characteristics of the study group

PARAMETERS	CONTROL	PCOS	P VALUE
AGE (year)	**24.22±5.22**	**24.73±6.16**	**0.69**
BMI (kg/m2)	**22.63±3.5**	**29.44±6.48**	**0.001**
FSH (U/L)	**6.19±1.38**	**6.13±1.89**	**0.88**
LH(U/L)	**3.92±1.96**	**6.91±4.47**	**0.001**
E2 (pmol/L)	**45.4±12.87**	**47.41±21.36**	**0.62**
TESTO(nmol/L)	**31.86±15.25**	**58.79±35.37**	**0.001**
DHEA (ng/ml))	**191.16±61.35**	**261.76±114.50**	**0.001**
TSH(mIU/L)	**1.65±1.09**	**1.9±1.19**	**0.33**

Table 4.2. Showed the different genotypes and clinical characteristics of the study groups. Thirty seven AA, 31 of CA and 11 of CC were found. Mean of age of the genotypes was none significantly different, BMI was increased none significantly in CA and CC in comparison with the AA. FSH was also none significantly increased, ($p=0.49$). LH was decreased in CA and CC non- significantly. About the rest of the parameters, E2, Testosterone, DHEA and TSH the differences were not significant, ($p>0.05$).

Table 4-2 Comparison of clinical characteristics according to the genotypes.

PARAMETERS	AA N=37 **Mean**± S.D	CA N=31 **Mean**± S.D	CC N=11 **Mean**± S.D	PVALUE
AGE (year)	23.67±6.07	25.22±5.21	24.90±5.66	**0.66**
BMI (kg/m2)	25.05±6.41	27.13±6.05	27.25±6.25	**0.51**
FSH (U/L)	6.06±1.88	6.12±1.50	6.66±1.19	**0.49**
LH (U/L)	5.73±4.38	5.49±3.65	4.92±1.51	**0.78**
E2 (pmol/L)	48.99±16.63	46.70±17.09	42.15±17.45	**0.41**
TESTO (nmol/L)	46.26±19.75	47.22±43.05	43.46±17.55	**0.92**
DHEA (ng/ml)	238.87±112.53	209.51±69.79	233.45±125.68	**0.62**
TSH(mIU/L)	1.49±.99	1.96±1.19	2.07±1.40	**0.25**

Also Table (4.3), showed different genotypes for the patients and the control. The genotypes were not significantly associated with PCOS patients compared to control. P values were, (0.62), (0.86) and (0.37) for AA, CA and CC, respectively. The genotype frequency did not differ significantly between the patients and control. (p>0.05). Odds ratio and their interval confidence intervals were measured. OR for AA was 1.06, and CA 0.72 and for CC the OR was 1.65. THE OR and 95% CI for each genotype were given in the table.

Table 4-3Comparison of genotypes separately between PCOS patients and control

GENOTYPE	PATIENTS N= 42	CONTROL N=37	P VALUE	ODDS RATIO 95% CI
AANormal homozygote	20(47.61%)	17 (45.94%)	0.66 NS	1.06 (0.44-2.59)
CA Hetero zygote	15 (35.71%)	16 (43.24%)	0.86 NS	0.72 (0.29-1.80)
CCWild type homozygote	7 (16.66%)	4 (10.81%)	0.37 NS	1.65 (0.44-6.16)

The table (4.4.), Show 3 genotypic distribution between patients and control and the susceptibility of the patients to develop PCOS.P value 0.07Non significant difference were found within group

Table 4-4Comparison of genotypes altogether between PCOS patients and control

GENOTYPE	PATIENTS N= 42	CONTROL N=37	P VALUE	ODDS RATIO 95% CI
AA **Normal homozygote**	20(47.61%)	17 (45.94%)	0.07 NS	1.06 (0.44-2.59)
CA **Hetero zygote**	15 (35.71%)	16 (43.24%)		0.72 (0.29-1.80)
CC **Wild type homozygote**	7 (16.66%)	4 (10.81%)		1.65 (0.44-6.16)

5. DISCUSSION

Polycystic ovary syndrome (PCOS) is referred to a very complex heterogeneous endocrine disease and which gives the symptoms of amenorrhea (or oligomenorrhoea), hyperandrogenism, obesity, hirsutism, insulin resistance, and is the 7-fold approximate increased danger of diabetes mellitus (type 2). It is also the major reason behind the female infertility. The PCOS prevalence in women during the reproductive-age has been observed at a rate of 4%-12%. Familial gathering of this syndrome is well documented. In our study, we worked with the women who were at reproductive age. When we collected information about our patients, we did not ask about their family history of having PCOS. This was one of the limitations of our study, therefore, we could not check whether there was a familial relation for this disease in the population we studied.

The PCOS etiology is unclear now, but both genetic factors and environmental factors might cause the PCOS pathogenesis. This may be clarified by the presence of a permanent malicious cycle of pathological impacts in which the excessive androgen levels lead to a visceral abdominal fat arrangement and simplify the expansion of androgen secretion in adrenal glands or the ovaries (Sheehan, 2004). We measured the androgen levels (testosterone, dihyrdoepiendostosrone) for both the patient and the control group. These two hormones were significantly higher (p=0.001 for both) in our patient group. This observation is well matched with previous studies, therefore increased the credibility of our research.

PCOS is typically occurred among the adolescent, and is a heterogeneous syndrome, previously described by ovulation features (amenorrhoea, oligomenorrhoea, and irregular periods) along with the symptoms of increased androgen secretion (hirsutism, alopecia, acne (Franks et al., 1997). The properties of biochemical abnormalities include increase in serum concentrations of androgen, as the most consistent endocrine abnormality, and luteinizing hormone (LH). However, standard of follicle stimulating hormone (FSH) stays normal in these patients (Zawadzki et al., 1992). Again, expected as considering the previous results, LH in PCOS groups was elevated significantly (p=0.001) in the patients compared to the control group while FSH stayed the same (p=0.88).

Obesity has more effects on PCOS, yet the prevalence of being obese differ from one body to another, and is partially determined by the nature of the clinic where the patient is first referred (Franks, 1995). It is proved that clinical riskiness of PCOS are added by obesity and the danger of metabolic dysfunction is therefore expanded. The subjects included in our study were compared according to their Body Mass Index (BMI), which is the measurement of people's obesity status. In our groups, in accordance with the previous observations, the patients were determined to have a significantly higher ($p=0.001$) BMI compared to the controls.

Biochemical tests for our study showed results that were, in general, in an agreement with the previous studies. In addition to this, the mean ages of our groups, when compared, were found to have no statistically significant difference between them. When altogether these findings were considered, we can say that our control group was picked well, consequently, the conclusion we will reach at the end, has a good potential to reflect of high percentage of the truth.

Single-nucleotide polymorphisms (SNPs) are common genetic variations in DNA sequence which appear in which one single nucleotide in (A, T, C, or G) within the genomic DNA sequence is converted. Many SNPs, or two in every three SNPs, include the alteration of thymine (T) with cytosine (C). SNPs appear each 100–300 bases during the genome of human. From the standpoint of evolution, SNPs are usually stabilized and there is not more change from generation to generation (http://www.duerinck.com/snp.htm).

Nonattendance of four-repeat-units allele located in a polymorphic area of pent nucleotide $(TTTTA)_n$ duplicate in the CYP11A gene, encoding for cytochrome P450scc, is associated to PCOS. It was assumed that androgen production increase could be resulted from up-regulation of this enzyme. As well as, PCOS does not appear to be associated with the alleles located in CYP19 gene that encodes for cytochrome P450arom. In PCOS, researchers observed that there is association between androgens action and androgen receptor gene (AR) polymorphisms (Franks, 1995).

Two SNPs rs1884444 and rs10889677 in IL23R gene were confirmed with according to the Chinese genotype data found in Hap Map database, in which rs10889677 is localized at the 3-untranslated region (3-UTR) and arranged by microRNAs (miRNAs) and protein regulator. Also, there are a variant number of SNPs in the 3-UTR region that can influence the translation and stability of the mRNA and the quantity of receptor protein that a cell could express. Nowadays, by many studies, it was revealed that having rs10889677A allele in the 3-

UTR, persons exhibited a promoted quality of both protein and mRNA production of IL23R that subsequently facilitates the absence of binding ability for the miRNAs (Let-7e and Let-7f) (Zheng et al., 2012).

The SNPs in IL-23R (rs10889677 that is found in the 3' UTR) could result in receptor's overexpression (e.g. by increasing mRNA stabilization), leading differentiation of Th1 helper T cells towards a Th17 subpopulation, which leads to an excess liberate of IL-17 from these cells. This would then trigger the liberation of another cytokines (e.g. TNF), causing the involved organ to have a chronic inflammation, such as the PCOS (Huber et al., 2008). Some research showed that genetic variants of IL-23R gene, i.e. rs10889677, may regulate its expression (Zheng et al., 2012). Zhou et al. (2013), have found that rs10889677 SNP might be more associate as a concerted genetic vulnerable agent to multiple solid tumors.

An utilitarian rs10889677AC SNP has been related with genetic vulnerability of solid tumors, containing ovarian cancer, oral cancer, gastric cancer ,breast cancer, lung cancer and nasopharyngeal carcinoma (Chien et al ., 2012). However, no results have been made on how much the genetic variant affects the risk of cancer. A recent meta-analysis containing five properties (6731 patients and 7296 healthy controls) methodically tested relation among the rs10889677AC SNP and multiple cancer risk.

According to the new discovery by other researchers, IL-23 might play the most important role in inflammatory processes in PCOS. rs2201841CC and the rs10889677AA genotypes have displayed an accumulation in RA and Crohn's patients; logistic regression analysis detected the way of liability of these alleles in the disease development.

In our study, we performed chi-square test in order to evaluate the possible correlation between rs10889677 SNP and PCOS. At the end of the analysis, we observed no relation between the two variables. However, when we checked the Odds Ratios of the genotypes in two groups, we determined that CC homozygous genotype was nearly 60% higher in the patient group suggesting a slight tendency for this genotype to cause a genetic susceptibility for the disease. However, this susceptibility is far from being convincing especially when A/C genotype is considered. A/C genotype is almost 30% lower in the patients where AA genotype was 4% lower. This observation makes it difficult for us to oversee the role of any alleles in the initiation or the progression of the disease because of the fact that the direction of the changes in the patients seemed independent of the presence of any allele.

So as to reach a better conclusion about the correlation between SNP we studied and PCOS, further investigations need to be conducted with a larger number of subjects. In further experiments, it should be also considered to performing sequencing instead of PCR-RFLP analysis since that method will provide the researcher with clearer results. Lastly, one of the limitations of our study was to do PCR analysis only once. Since cross-contamination of samples was quite possible, any research on this topic must repeat the experiments at least three times before presenting their results

6. CONLUSIONS

1-BMI, LH, TESTO, and DHEA were significantly higher in PCOS patients.

2-E2, FSH and TSH levels were similar for the patients and the control.

3-No statistically significant relation has been found between the presence of PCOS and the genotypes in the population we studied.

4-CC genotype showed a higher OR in patients compared to the control while AC genotype was lower and AA genotype did not differ.

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