Association between Interleukin-32 and Interleukin-17A Single Nucleotide Polymorphisms and Serum Levels with Polycystic Ovary Syndrome

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ABSTRACT

Polycystic ovary syndrome (PCOS) is correlated with low-grade chronic inflammation. Interleukin-17A (IL-17A) and Interleukin-32 (IL-32) are two members of the pro-inflammatory cytokines which act as significant components of the immune system during certain inflammatory diseases. Along with immunological processes, genetic factors play major roles in predisposition to PCOS. There are myriad single nucleotide polymorphisms (SNPs) within IL-17A and IL-32 genes that may affect their production and the susceptibility of individuals to PCOS. The objective of the present research was to investigate the association between IL-17A (rs2275913) and IL-32 (rs9927163, rs4786370) SNPs, and also their serum levels with susceptibility to PCOS in a group of Iranian women.

In this case-control study, 150 PCOS patients (mean age of 29.1 years) and 150 healthy women (mean age of 26.1 years) were analyzed in terms of IL-17A and IL-32 SNPs via polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Furthermore, serum levels of IL-17A and IL-32 cytokines were measured through the use of ELISA method.

There were significant differences between PCOS and healthy women regarding IL-17A rs2275913 alleles, genotypes frequencies (*p*=0.005, and 0.01, respectively) and the allelic distribution of IL-32 rs9927163 SNP (*p*=0.03). Additionally, significant differences were indicated between two groups concerning the AG genotype against AA+GG genotypes (*p*=0.009) and the GG genotype against AA+AG genotypes (*p*=0.006) in IL-17A rs2275913 SNP. In the matter of IL-32 gene SNPs, GC haplotype frequency was significantly different between patients and controls (*p*=0.05). Furthermore, IL-32 serum level was not significantly different between the two studied groups and the serum level of IL-17A was not detectable.

In conclusion, IL-17A and IL-32 SNPs might be associated with predisposition to PCOS in Iranian women.

Keywords: Interleukin-17A; Interleukin-32; Polycystic ovary syndrome

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a prevalent and convoluted endocrine disorder affecting 6-15% of

women at gestational ages (20-35 years old). As a matter of fact, PCOS is a metabolic syndrome with a wide spectrum of complications, from high blood pressure, cardiovascular issues, dyslipidemia, and hyperinsulinemia, to insulin resistance, type II diabetes mellitus, and infertility.[1] PCOS is also considered as a reproductive disorder with no exact etiology. On the other hand, there is strong evidence corroborating the role of genetic factors in the etiology of PCOS, and the fact that it is a familial disorder. The chances of mothers and sisters of PCOS women developing PCOS are 35% and 40%, respectively.[2,3] Therefore, family history can be considered an important risk factor in the development of this syndrome. Recent studies have suggested that more than one gene is probably involved in the pathogenesis of PCOS, supporting the view that PCOS is a heterogeneous disorder.[4] Additionally, emerging documents suggest that low-grade chronic inflammation can be the main cause of ovarian dysfunction, insulin resistance and other complications of PCOS.[5] Elevated leukocyte counts and increased inflammatory factors, including IL-18, tumor necrosis factor-alpha (TNF-α), IL-6, monocyte chemo-attractant protein-1 (MCP-1), and macrophage inflammatory protein-1 (MIP-1) are reported in women suffering from PCOS.[6-8]

Interleukin-17A (IL-17A) is a member of the pro-inflammatory cytokines family, whose main source is T helper 17 cells. The production of IL-17A is dependent on IL-6 and transforming growth factor-beta (TGF-β).[9] IL-17A stimulates the production of different pro-inflammatory mediators such as IL-1, IL-6, TNF-α, Nitric Oxide Synthase-2 (NOS-2), metalloproteinases, and chemokines, a process which ensues the recruitment of neutrophil and monocyte and the induction of inflammatory responses.[10]

One of the most important SNPs of IL-17A is rs2275913 (A/G), which is located on the promoter of IL-17A gene on chromosome 6p12.2. The A allele of this SNP has more tendency to activate the nuclear factor of activated T cell (NFAT), an important transcription factor in IL-17A production, compared with the G variant form.[11] Although several studies[12-17] have reported the correlation between several autoimmune diseases, gastric disorders, and pregnancy-related diseases and IL-17A rs2275913 SNP, there is no published study concerning PCOS.

Yet another cytokine with pro-inflammatory properties is interleukin-32 which is frequently produced by natural killer cells, T cells, monocytes, and endothelial cells.[18] IL-32 acts as a significant component of innate and adaptive immunity during inflammatory diseases such as rheumatoid arthritis, atopic dermatitis, cancers and chronic obstructive pulmonary diseases.[19,20]

IL-32 gene has been observed to be located on human chromosome 16p13.3; moreover, the two important single nucleotide polymorphisms in IL-32 gene are rs4786370 (C/T) and rs9927163 (G/T). The IL-32 rs4786370 SNP is located on the promoter region, enhancing the expression of IL-32 gene,[21] while IL-32 rs9927163 SNP is located in the intron region and may regulate the IL-32 gene expression.[22] Several studies have demonstrated the association between IL-32 gene SNPs (rs9927163, rs4786370) and cardiovascular disorders,[23] rheumatoid arthritis[24] and acute lung injury,[22] whereas no study has specified the importance of these SNPs in PCOS.

Given the essential functions of IL-17A and IL-32 in chronic inflammatory diseases, and the limited data concerning their association with PCOS, the primary objective of the present study was to investigate the probable association between IL-17A (rs2275913) and IL-32 (rs9927163, rs4786370) SNPs, as genetic factors, and susceptibility to PCOS among a group of Iranian women. The second aim was to compare the sera levels of IL-17A and IL-32 between women complicated with polycystic ovary syndrome and healthy women.

MATERIALS AND METHODS

Subjects

In the present research, 150 women diagnosed with PCOS (mean age of 29.1 years) who referred to the infertility research center of Shiraz (Ghadir Mother and Child Hospital) were enrolled as the case group. The sample size was calculated using $n_1 = n_2 = 2 (Z_{\alpha/2} + Z_\beta)^2 \sigma^2 / (\mu_1 - \mu_2)^2$ formula. All patients were diagnosed by the same gynecologist on the basis of clinical symptoms and Rotterdam criteria.[25] Patients had ovulatory dysfunction and polycystic ovaries based on ultrasound evidence. Moreover, the patients had no history of immunological disorders such as autoimmunity, immune deficiencies, cancer, and active infection. Further included were 150 healthy women (with a mean age of 26.1 years) as the healthy control group. The inclusion criteria for healthy women were

the same as that of the patient group except for PCOS. Moreover, the healthy group had no history of pregnancy-related disorders and had at least one delivery without any complication. The control group included women with no family history of PCOS. Written informed consent was obtained from all participants regarding the collection of blood samples and the following tests. The study was approved by the local Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (N: IR.SUMS.REC.1396.S513). Demographic and clinical characteristics of the two studied groups are shown in Table 1.

Table 1. Demographic and clinical characteristics of patients with polycystic ovary syndrome and healthy controls

Variables		Patients n=150	Controls n= 150	p value[*]
Age (year)		29.11±6.23	26.13±4.73	<0.001[*]
Weight (kg)		68.8±10.4	62.3±5.5	<0.050[*]
Height (m)		1.62±0.05	1.59±7.11	>0.050
BMI (Kg/m^2)		26.38±4.04	23.96±5.2	<0.050[*]
Menses				
	Regular	19.7 % (n=30)	95.1 % (n=143)	<0.050[*]
	Irregular	30.7 % (n=46)	4.9 % (n=7)	
	Amenorrhea	1.6 % (n=2)		
	Hyper menorrhea	0.8 % (n=1)		
	Oligomenorrhea	47.2 % (n=71)		
FSH (mIU/ml)		5.28±2.07	10.89±3.93	<0.050[*]
LH (mIU/ml)		7.02±4.67	3.56±1.59	<0.050[*]
TSH (micIU/ml)		2.57±1.65	2.10±1.45	>0.050
Prolactin (ng/ml)		16.64±11.19	14.07±14	<0.050[*]

Data are means ± SD. [*]Significant P value calculated using Independent Samples T-test.

BMI: body mass index, FSH: follicle stimulating hormone, LH: luteinizing hormone, TSH: thyroid-stimulating hormone
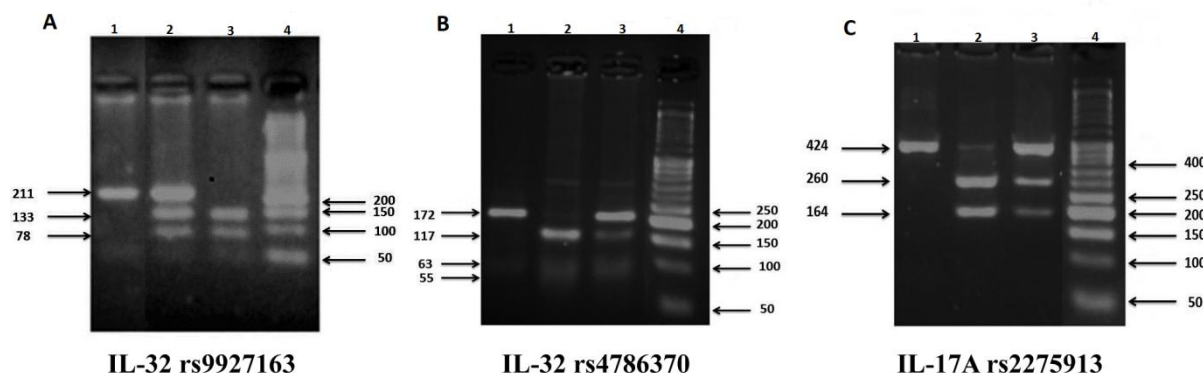

Figure 1. Typical examples of a 3% agarose gel electrophoresis for identification of IL-17A, and IL-32 polymorphisms in patients with polycystic ovary syndrome and healthy controls after enzymatic digestion.

A. IL-32 rs9927163 polymorphism. Lane 1: TT (band size: 211 bp); lane 2: TG (bands sizes: 211, 133, 78 bp); and lane 3 represents GG (bands sizes: 133 and 78 bp). Lane 4 shows DNA marker (50 bp).

B. IL-32 rs4786370 polymorphism. Lane 1: TT (bands sizes: 172 and 63 bp); lane 2: CC (bands sizes: 117, 63 and 55 bp); and lane 3 represents TC (bands sizes: 172, 117, 63 and 55 bp). Lane 4 shows DNA marker (50 bp).

C. IL-17A rs2275913 polymorphism. Lane 1: AA (band size: 424 bp); lane 2: GG (bands sizes: 260, 164 bp); and lane 3 represents AG (bands sizes: 424, 260 and 164 bp). Lane 4 shows DNA marker (50 bp). bp: Base pair.

Table 2. Primers sequences, restriction enzymes and products sizes (bp) after digestion in an study evaluating IL-32 and IL-17A in polycystic ovary syndrome

SNPs	Primers sequences	Restriction enzymes	Products sizes (bp) after digestion
IL-17A rs2275913	F: 5'- GCCAAGGAATCTGTGAGGAA-3'	XagI	AA: 424
	R: 5'-TGCCTGCTATGAGATGGACA-3'		AG: 424, 260, 164
			GG: 260, 164
IL-32 rs9927163	F: 5'-CTGGAAACGACTCGGAGAAT-3'	BcnI	TT: 211
	R: 5'-CCACATCATGGAAACCCCTA-3'		TG: 211, 133, 78
			GG: 133, 78
IL-32 rs4786370	F: 5'-TTGCATTGCCTGTAAATTGC-3'	BspLI	TT: 172, 63
	R: 5'-AACCGCCTTTCCACATACAG-3'		TC: 172, 117, 63, 55
			CC: 117, 63, 55

SNP: single nucleotide polymorphism; IL-17A: Interleukin-17A; IL-32: Interleukin-32; bp: Base pair

DNA Extraction, SNP Primers Design, and Genotyping

Genomic DNA was extracted from 1 ml anti-coagulated whole blood samples using Prime Prep Genomic DNA Extraction kit (GeNetBio, South Korea) according to the manufacturer's guidelines. Primer sequences (Eurofins Scientific, Ebersberg, Germany) were designed by the Gene runner 4.0 software and evaluated by primer BLAST on NCBI. SimpliAmp thermal cycler (applied Biosystems by Life Technologies, California, USA) instrument was used for amplification. IL-17A rs2275913 and IL-32 gene SNPs (rs9927163 and rs4786370) genotyping was performed via polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique.

PCR amplicons were digested using XagI, BspLI, and BcnI restriction enzymes (Thermo scientific, Lithuania), and RFLP products were separated by 3% agarose gel electrophoresis (Figure 1). Primer sequences, restriction enzymes and products sizes employed in genotyping are listed in Table 2.

Enzyme-Linked Immuno Sorbent Assay (ELISA)

For cytokine assay, 5 mL of peripheral venous blood samples were collected from 47 out of 150 PCOS and 41 out of 150 healthy subjects. The selection of cases and controls for cytokine assay was randomly and based on the availability of sera and limitation of the ELISA kit capacity. The samples were centrifuged at 2500 rpm for 15 min, and the separated serum samples were stored at -70 ℃ until the analysis date. Serum concentrations of IL-17A and IL-32 were measured by commercial ELISA kits (from eBioscience, San Diego,

CA, USA, and Bioassay Technology Laboratory, Shanghai, China, respectively) based on the manufacturer's instructions. The plates were read at 450 nm using multi-detection microplate reader (Bio-Tek synergy HT multi-Detection microplate reader, Bradenton, USA). The minimum detectable levels of IL-17A and IL-32 were 4 pg/mL and 0.5 pg/mL, respectively.

Statistical Analysis

All statistical analyses were carried out using SPSS Statistics for Windows, version 16.0 (SPSS Inc., Chicago, Ill., USA). p values < 0.05 were considered as statistically significant. Hardy-Weinberg (HW) equilibrium test was used to calculate the genetic variation of populations at equilibrium. Independent Samples T-test was applied to the comparison of different demographic and clinical features between PCOS and control groups. The genotype and allele frequencies between the case and control groups were determined through the use of Chi-Square or Fisher's exact test. The association between PCOS and different genotypes was evaluated by odds ratio (OR) with a 95% confidence interval. Haplotype frequencies were calculated by Arlequin software (version 3.5.2.2). The degree of linkage disequilibrium among polymorphisms was estimated by SNP Analyzer software 2.0.[26] Furthermore, the association between different genotypes and clinical characteristics of PCOS patients was determined using one-way ANOVA and Kruskal-Wallis tests. Finally, the differences in cytokine levels were examined by Independent Samples T-test.

RESULTS

Genotypes and Alleles Frequencies of IL-17A rs2275913 and IL-32 (rs4786370 and rs9927163) SNPs

In this study, 150 PCOS patients and 150 healthy women were analyzed for IL-17A and IL-32 gene single nucleotide polymorphisms using PCR-RFLP technique as was described in the Material and Methods section. Table 3 illustrates the frequency distribution of genotypes and alleles observed in both groups. It was found that both studied populations were

in Hardy-Weinberg (HW) equilibrium concerning all three SNPs. Chi-Square test showed that there were significant differences between the two groups regarding IL-17A rs2275913 allele (p=0.005, CI = (1.192–2.820), OR=1.833) and genotype frequencies (p= 0.01); in fact, both G allele and GG genotype of rs2275913 SNP were observed at a higher ratio in PCOS patients. There was no difference between patients and healthy subjects concerning the genotypes of IL-32 SNPs; however the allelic distribution of IL-32 rs9927163 SNP was significantly different between the groups (p=0.03), but OR was <1 (OR=0.696).

Table 3. Comparison of allele and genotype frequencies for IL-17A rs2275913, IL-32 rs9927163, and IL-32 rs4786370 SNPs between patients with polycystic ovary syndrome and healthy controls

alleles/ Genotypes	Patients Number (%)	Controls Number (%)	OR (95% CI)	*p* value
IL-17A rs2275913	2n =300	2n =300		
G allele	260 (86.7)	234 (78)	1.833 (1.192 – 2.820)	0.005[*]
A allele	40(13.3)	66 (22)		
AA	16 (10.7)	22 (14.7)		0.01[*]
AG	8 (5.3)	22 (14.7)		
GG	126 (84)	106 (70.6)		
AA vs. AG + GG	16/134 (10.7/89.3)	22/128 (14.7/85.3)	0.694 (0.349 – 1.382)	0.299
AG vs. AA + GG	8/142 (5.3/94.7)	22/128 (14.7/85.3)	0.327 (0.141- 0.762)	0.009[*]
GG vs. AA + AG	126/24 (84/16)	106/44 (70.6/29.4)	2.179 (1.244 – 3.816)	0.006[*]
IL-32 rs9927163	2n =300	2n =300		
T allele	167 (55.7)	193 (64.3)	0.696 (0.501 - 0.967)	0.03*
G allele	133 (44.3)	107 (35.7)		
TT	50 (33.3)	64 (42.7)		0.11
TG	67 (44.7)	65 (43.3)		
GG	33 (22)	21(14)		
TT vs. TG + GG	50/100 (33.3/66.7)	64/86 (42.7/57.3)	0.671 (0.420 – 1.073)	0.096
TG vs. TT + GG	67/83 (44.7/55.3)	65/85 (43.3/56.7)	1.055 (0.669 – 1.665)	0.816
GG vs. TT + TG	33/117 (22/78)	21/129 (14/86)	1.732 (0.949 – 3.162)	0.073
IL-32 rs4786370	2n =300	2n =300		
C allele	134 (44.7)	126 (42)	1.115 (0.807 – 1.540)	0.510
T allele	166 (55.3)	174 (58)		
TT	48 (32)	47 (31.3)		
TC	70 (46.7)	80 (53.4)		0.341
CC	32 (21.3)	23 (15.3)		
TT vs. TC + CC	48/102 (32/68)	47/103 (31.3/68.7)	1.031 (0.634 – 1.677)	0.901
TC vs. TT + CC	70/80 (46.7/53.3)	80/70 (53.4/46.6)	0.765 (0.486 – 1.205)	0.248
CC vs. TT + TC	32/118 (21.3/78.7)	23/127 (15.3/84.7)	1.497 (0.828 – 2.705)	0.181

[*]Significant *p* value calculated using Chi-Square test, SNP: single nucleotide polymorphism, OR: odds ratio, CI: confidence interval, IL-17A: Interleukin-17A, IL-32: Interleukin-32

The odds ratio was calculated so as to determine the association between PCOS and different genotypes of IL-17A and IL-32 SNPs. As shown in Table 3, comparing the AG genotype against AA+GG genotypes in IL-17A rs2275913 SNP, the odds ratio value is 0.327 (p=0.009), and the comparison of individuals concerning the GG genotype against AA +AG genotypes indicates an odds ratio value of 2.179 (p=0.006).

In total, our data indicated an association between the G allele and GG genotype in IL-17A rs2275913 SNP with a higher chance of susceptibility to PCOS in Iranian women.

Assessment of Genotypes Combination, Haplotypes Frequencies, and Linkage Disequilibrium of IL-32 Gene SNPs in PCOS and Control Groups

Genotype combination frequencies of IL-32 SNPs (rs9927163 and rs4786370) were calculated by Chi-Square test. None of the combined genotypes models were different between the two studied groups, while in the model associated with rs9927163 GG/ rs4786370 CC combined genotypes, the p value was less than 0.05 (CI=0.092 – 0.907, OR = 0.289, p=0.02).

The haplotype frequencies of both IL-32 gene SNPs were also investigated and the linkage disequilibrium degree between IL-32 rs4786370 and rs9927163 SNPs was further estimated. In terms of GC haplotype, there was a statistically significant difference between the groups (Table 4, p=0.05).

The IL-32 gene SNPs did not display a high degree of genetic linkage in PCOS and control groups.

The Association between Genotypes and Clinical Features of PCOS Patients

The association between different genotypes and the clinical features of PCOS patients was investigated using one-way ANOVA and Kruskal-Wallis tests. Results indicated that there was no significant association between the clinical characteristics and genotypes.

IL-17A and IL-32 Serum Levels

Evaluation of IL-32 serum concentration by commercial ELISA kits indicated that the level of IL-32 was not statistically different between healthy and PCOS women (p=0.9, Figure 2). In our study, IL-17A serum levels were undetectable in most cases and controls. Moreover, there was no significant association between the sera levels of IL-32 and genotypes.
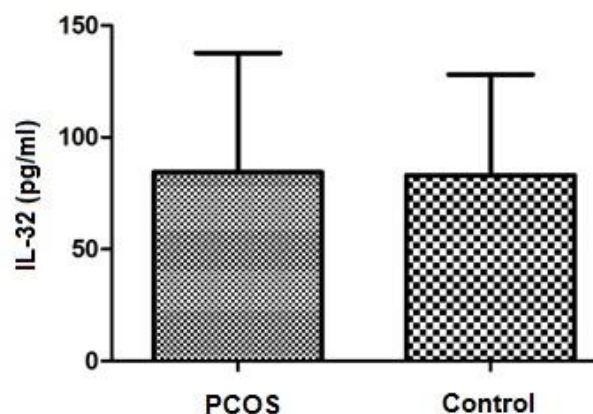

Figure 2. IL-32 serum levels in control and PCOS groups. The graph represents the Mean±SD. _P_ value was calculated by Independent Samples T-test
IL-32: interleukin-32, PCOS: polycystic ovary syndrome

Table 4. IL-32 SNPs (rs9927163 and rs4786370) haplotype frequencies in patients with polycystic ovary syndrome and healthy controls

Haplotypes (rs9927163/rs4786370)	Patients Number (%) 2n=300	Controls Number (%) 2n=300	OR	95% CI	_p_ value
TT	124 (41%)	135 (44%)	0.861	(0.623 – 1.189)	0.36
GC	89 (30%)	68 (23%)	1.439	(0.997 – 2.076)	0.05*
GT	42 (14%)	39 (13%)	1.089	(0.681 – 1.740)	0.72
TC	45 (15%)	58 (20%)	0.736	(0.480 – 1.128)	0.16

*Significant p value, calculated by Chi-Square test. OR: odds ratio, CI: confidence interval, SNP: single nucleotide polymorphism, IL-32: Interleukin-32, PCOS: Polycystic ovary syndrome

DISCUSSION

IL-17A and IL-32 are associated with the pathogenesis of numerous autoimmune and inflammatory diseases like PCOS.[19,20,27] In addition to the functions of the immune system, genetic factors play crucial roles in the development of certain diseases. Therefore, in the present study, we investigated the association between IL-17A and IL-32 genes polymorphisms and PCOS.

The IL-17A rs2275913 and IL-32 gene polymorphisms (rs9927163 and rs4786370) were reported to be associated with several immunological diseases,[12,13,22-24,] and reproductive disorders.[14-17] To best of our knowledge, no published study has investigated IL-32 and IL-17A SNPs in PCOS. The results of the present study showed that the distribution of rs2275913 SNP in IL-17A was different between Iranian women complicated with PCOS and healthy women. Both GG genotype and G allele were found in a higher proportion in the PCOS group compared with the controls. The present study is the first to report the relationship between IL-17A rs2275913 and PCOS; on the other hand, several studies have evaluated the association between this SNP and other diseases. In a study, Rolandelli et al. reported that both the A allele and AA genotype were in lower frequencies in tuberculosis population and that the AA genotype was positively associated with protection against active tuberculosis.[28] Moreover, Holster et al. showed that IL-17A rs2275913 polymorphism did not play any significant roles in bronchiolitis, while GA or AA genotypes were proved as protective factors with regards to asthma.[29] The published results of another study indicated that the AA genotype and A allele from rs2275913 SNP were associated with the susceptibility to cervical cancer in Chinese women.[30] Few studies have surveyed the association between IL-17A rs2275913 SNP and pregnancy-related diseases such as pre-eclampsia and recurrent pregnancy loss (RPL).[14,16] In our previous results, no significant association was observed between IL-17A rs2275913 SNP and susceptibility to pre-eclampsia,[14] which is in accordance with another study conducted by Wang et al.[15] Additionally, certain studies have surveyed the association of IL-17A gene polymorphisms with RPL; however, the reported data are conflicting. In some studies, no correlation was observed between IL-17A rs2275913 SNP and predisposition to RPL,[17,31] yet

others reported this SNP to be correlated with a high risk of RPL.[16,32] In general, further research is required on worldwide populations regarding the association between rs2275913 SNP and PCOS.

In the present study, no statistical differences were observed between PCOS and healthy women regarding genotype and allele distribution of IL-32 gene polymorphism at position rs4786370, yet the allelic frequencies of IL-32 rs9927163 SNP were different between the two studied groups. Additionally, the haplotype frequency distribution of both IL-32 gene SNPs was statistically different between the two studied groups. Interestingly, there is not any published study regarding the role of this haplotype in PCOS and other diseases. The cases and the controls were not correlated in terms of the degree of genetic linkage among the IL-32 SNPs. No studies were found concerning the role of IL-32 polymorphisms in PCOS and pregnancy-related disorders; regarding susceptibility to other diseases, however, a certain study reported that rs4786370 SNP has a functional effect on lipid profiles in rheumatoid arthritis patients.[24] In another study, it was suggested that TG genotype was associated with an increased risk of infection-associated acute lung injury.[22]

In quantifying the serum levels of IL-32, our results indicated that the concentration of this cytokine was not significantly different between the two groups. Some studies have measured the serum concentration of IL-32 in other diseases. You-Jung HA et al. reported that elevated serum levels of IL-32 were detected in Behcet's disease when compared with those in the healthy controls.[33] In a study on the association between colorectal cancer and genetic variation of IL-32 gene, the plasma levels of IL-32 from patients were no different from that of the healthy controls.[34]

In our study, IL-17A levels were undetectable in most cases and controls. The short serum half-life of IL-17A, like some other cytokines, might account for the unsuccessful detection of this cytokine in our samples.[35] It is recommended that more sensitive approaches be employed in the detection of IL-17A in the serum samples. Additional challenges might be caused by analytical and methodological variables. Investigating the roles of IL-17 in the interaction between PCOS and gingival inflammation, a group of researchers observed that IL-17A concentration was increased in females with PCOS (with and without gingivitis) compared with healthy women.[9] In contrast,

another study showed that the serum level of IL-17 in women with PCOS was significantly lower than the control group.[27] As ethnicity has a great impact on the cytokines gene polymorphisms and their production,[36] the discrepancy between the aforementioned studies might be related to ethnics of the studied populations.

There were certain limitations in our study which might have affected the results. The sample size and study of a limited number of SNPs are among the most important limitation points. Moreover, functional assays regarding the studied SNPs are also required if we are to find the exact roles of these SNPs in susceptibility to PCOS.

In summary, IL-17A rs2275913 and IL-32 rs9927163 SNPs might be associated with susceptibility to PCOS among Iranian women.

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