Original Article

Association of rs13429458 and rs12478601 Single Nucleotide Polymorphisms of *THADA* Gene with Polycystic Ovary Syndrome

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Abstract

Background: It is thought that genetic factors are influential in the etiology of polycystic ovarian syndrome (PCOS), the most frequent endocrinological disorder of females in their reproductive age. This study was carried out to elucidate the association of rs13429458 and rs12478601 single nucleotide polymorphisms (SNPs) of the *THADA* gene and the risk of the PCOS among a population of Iranian female patients.

Materials and Methods: This case-control study contains 66 infertile women with PCOS (patient group) and 44 healthy women without PCOS (control group) that referred to the IVF Unit of the Infertility Research Center of the Academic Center for Education, Culture and Research (ACECR). The polymerase chain reaction (PCR) was utilized to amplify genome DNA as well as direct sequencing to determine SNPs. The *THADA* rs12478601 and rs13429458 genotypes were consequently examined with amplification refractory mutation system-PCR (ARMS-PCR).

Results: In this study, we observed that rs13429458 polymorphism was not associated with PCOS risk in two groups (P=0.42). On the other hand, data analysis indicated that the rs12478601 genotype significantly increased the risk of PCOS in the case group (P=0.032) in compared with control group. We found that the "T" allele of rs12478601 in the THADA gene had a significant relation to PCOS in the case group (odds ratio [OR]: 2.574, 95% confidence interval [CI]: 1.439-4.604, P=0.001).

Conclusion: This study has presented further evidence that TT and CT genotype of *THADA* rs12478601 is associated with a high risk of PCOS.

Keywords: Polycystic Ovarian Syndrome, Single Nucleotide Polymorphisms, *THADA*

Citation: Naserpoor L, Jannatifar R, Roshanaei K, Khoshandam M, Kallhor N. Association of rs13429458 and rs12478601 single nucleotide polymorphisms of THADA gene with polycystic ovary syndrome. Int J Fertil Steril. 2021; 16(1): 36-41. doi: 10.22074/IJFS.2021.524795.1090.

Introduction

Polycystic ovarian syndrome (PCOS) is the most widespread endocrinopathy with a prevalence of 5-10% in the world (1). Also, clinical and/or biochemical hyperandrogenism and polycystic ovaries in ultrasound are considered as key characteristics of this syndrome (2). Despite of incomplete information about PCOS causes, it has indicated genetic factors are one of important factors (3). Several findings suggest that environmental factors, including enhanced caloric intake, endocrine disruptors, and lifestyle are involved in the pathogenesis of PCOS (4). Although there are studies that have emphasized the impact of hereditary factors, and relation to strong familial clustering (5, 6). Various studies have shown several genes involved in molecular pathways and mechanisms possibly linked to PCOS, including central energy metabolism, insulin secretion and action, gonadotropin action, and steroid hormone biosynthesis (7). DNA technology development brings knowledge of PCOS-related genetic factors (8). *THADA* (thyroid-associated protein) as a target gene in the thyroid tumor has been located on chromosome band 2p21 which was determined first by Rippe et al. (9). *THADA* expression has been reported in the pancreas, thyroid, testes, thymus, adrenal medulla, adrenal cortex, small intestine, and stomach (10, 11). Because *THADA* is of fairly large size (370 kb) and was controlled by many regulatory elements, variants within *THADA* have been associated with a range of diseases (12). To explore possible relationships between single nucleotide polymorphism (SNP) and phenotypic variation, different computational tools like SNP Nexus, UTRscan, Human Splicing Finder (HSF), Repeat Masker and RNAsnp Web Server PupaSuite were used for prioritization of high-risk SNP in [interionic and exonic 5′ and 3′-untranslated region (UTR) SNPs.

These polymorphisms have been identified in the intronic region of THADA. The bioinformatics web tools, test SNPs with potential pathological results, to select markers for PCOS susceptibility.

SNPs within *THADA* have also been related to type 2 diabetes. Two SNPs of *THADA*, rs13429458 and rs12478601,

Received: 14/February/2021, Accepted: 25/May/2021
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Royan Institute
International Journal of Fertility and Sterility
Vol 16, No 1, January-March 2022, Pages: 36-41

were determined to be related to PCOS (13). Based on previous studies, we tried to explore the association between these SNPs (rs13429458, rs12478601 (and PCOS risk. This is a first study that investigating that this polymorphism among some of the Iranian female population.

Materials and Methods

Study design

This case-control study was contained 66 infertile women with PCOS history (patient group) and 44 healthy women without PCOS history (control group), who was attending Rooya Infertility Treatment Center of the Academic Center For Education, Culture, and Research (ACECR), Qom, Iran between January 2019 and October 2019. The Code of Ethics was IR.IAU.QOM.REC.1399.020.

Patients with PCOS were diagnosed according to the 2003 Rotterdam Criteria (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004 (14, 15). Also, healthy women were invited to this study from whom having regular menstrual cycles that referred to our clinic because of other reasons such as a tubular problem or male factor infertility. Exclusion criteria included hyperinsulinemia, insulin resistance, another characteristic of hyperandrogenism, and obesity.

Demographics and clinical features assessment

All participants provided written informed consent and were asked not to change their daily physical activity and diet and not to take any new or additional pharmacotherapy within our research. Demographic information was self-reported and included height and body weight and body mass index (BMI) was calculated using the formula weight (kg)/ height^2 (cm^2). Infertility is defined as failure to establish a clinical pregnancy after 12 months of regular and unprotected sexual intercourse. For both groups, blood samples were taken on the morning of the second or third day of menstruation after overnight fasting of at least 12 hours. The peripheral blood sample was instantly centrifuged for 10 minutes at 3000 rpm (EBA20, Hettich, UK). To assess the hormonal profile of all participants, serum levels of different hormones were measured by enzyme-linked immunosorbent assay (ELISA) commercial kits including follicle-stimulating hormone (FSH, DE1288), luteinizing hormone (LH, DE1289), prolactin (PRL, DE1291), estradiol (E2, LOT 1007843740), and Anti Mullerian Hormone (AMH, LOT 001-50-N488) .These materials had been provided from Demeditec Diagnostics GmbH, Germany.

Single nucleotide polymorphisms genotyping

SNPs of the *THADA* gene with minor allele frequency (MAF)>0.05 were achieved from earlier published polymorphisms related to PCOS in the Hap Map Asian population (10, 13) and were applied to an initial screening. Peripheral blood (3 ml) was collected into EDTA-containing tubes and stored at -70°C until DNA extraction.

Using GeneAll Exgene TM Kit (105-101, GeneAll, South Korean), the DNA extraction process was performed in accordance with the relevant instructions. DNA purity and quantity were evaluated by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) at 260/280 nm. The quality of bands from DNA extracted was studied by agarose electrophoresis. Genotypes were identified by the amplification-refractory mutation system (ARMS) technique. To amplify the gene in specific SNPs, specific primers were designed using PRIMER1, a primer designed for tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR). The characteristics of the primers, along with the length of the reproduced piece, are given in Table 1. Genotyping of rs13429458 (AA/AC/CC) and rs12478601 (CC/CT/TT) were performed by PCR with a sequence-specific primer (Table 1). The polymerase chain reaction was selected to amplify genomic DNA in a total a volume of 20 µl reaction system. The polymerase chain reaction was selected to amplify genomic DNA with a volume of 20 µl reaction system: 2x Master Mix RED (Cat number: A190303, Ampliqon, Denmark) 10 µl, 1 µL of each primer (5 pmol/µL), 100 ng Genomic DNA and add ddH2O (Cat number: DW8505, Sina clon, Iran) up 20 µL. PCR was conducted as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles consisting of 95°C denaturations for 30 seconds and 58°C annealing for the 40 seconds, and 72°C extension 40 seconds with a final extension of 5 minutes at 72°C. The amplification products were electrophoresed in 2% agarose gel at a voltage of 80 w for 45 minutes, also visualized under UV light using Ethidium bromide.

Table 1: Primer sequences of single nucleotide polymorphisms (rs13429458, and rs12478601)

Primer sequences	Single nucleotide polymorphisms	Product size (bp)
	rs13429458	
Inner primer (A allele)	F:GCTGTGCAAAGTTAGAAGATGAAGCA	208
Inner primer (C allele)	R:GGCAGGGTATAGGTGTATGTAATCAGTCTG	286
Outer primer (5'-3')	F:TCAGCGGTATGATTTCGTAGTGGTTATT	438
Outer primer (5'-3')	R:AAGACTTGAAGGCAATGTGATTCTTCTC	
	rs12478601	
Inner primer (C allele)	F:CATTCCTGCTGGTCTTGGTTAGTACAAC	180
Inner primer (T allele)	R: AAAGCCCGGGTCCTAACATTTTATTTAA	220
Outer primer (5'-3')	F:CCAGTAAAAGACAAACATATTGGGCTGT	343
Outer primer (5'-3')	R: TCCAACTCCAGAATGTGTGCTATTGTAG	

Dataset collection

The data on the human *THADA* gene were collected from Entrez Gene on National Center for Biological Information (NCBI) website. The SNP information (SNP ID) of this gene was received from the NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp/).

Data analysis using different bioinformatics tools

The impacts of SNPs were predicted by specific online tools and servers such as SNPNEXUS (https://www.snp-nexus.org), UTRscan server (16), HSF (http://www.umd.be/HSF3/), Repeat Masker (17) and RNAsnp Web Server (https://rth.dk/resources/rnasnp/).

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was investigated through a goodness-of-fit χ^2 test to compare observed and expected genotype frequencies between case and control groups. Differences in demographic characteristics between both groups were assessed via the Chi-square test (for categorical variables) or the Student's t test (for continuous variables). Four unconditional logistic regression genetics models (dominant, codominant, recessive, and over dominant models) were utilized to assess the relation between the SNPs and POCS through SPSS 22.0 (SPSS, Chicago, IL, USA). All P values stated here were two-tailed, and P values below 0.05 were regarded statistically significant. Also, 95% confidence intervals (CIs) and Odds ratios (ORs) were estimated by unconditional logistic regression analyses. For the multiple testing correction for the 2 SNPs tested through clinical features repeatedly, we applied a Bonferroni's correction, which P<4.00×10-3 were regarded significant.

Results

Demographic clinical characteristics

The mean age of the case group was 30.38 ± 5.086 years and the control group was 31.7 ± 5.262 years. The study of Infertility duration and BMI showed that there was a statistically significant difference means in both groups (P<0.05, Table 2).

Table 2: Comparison of baseline characteristics of study subjects in the PCOS and control groups

Characteristics	PCOS group	Control group	P value
Age (Y)	30.38 ± 5.086	31.7 ± 5.262	0.202
Infertility duration (Y)	4.1 ± 0.2	1.1 ± 0.2	0.009*
BMI (Kg/m^2)	27.7 ± 4.841	25.71 ± 1.899	0.003*
Smoking			0.732
Yes	4	2	
No	62	42	
Family history of diabetes			0.09
Yes	39	16	
No	27	28	

BMI; Body mass index and PCOS; Polycystic ovarian syndrome.

Genotyping by tetra-primer ARMS-PCR

The tetra-primer ARMS-PCR method was successfully applied to genotyping selected SNPs (Fig.1).

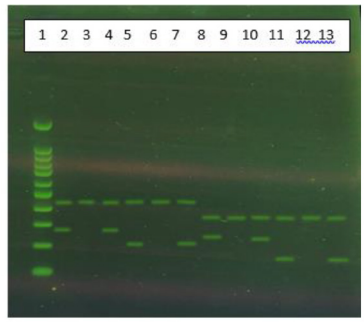

Fig.1: Results of tetra-primer ARM-PCR for locus rs12478601 polymorphism and locus rs13429458 polymorphism.1; Marker 100 bp, 2, 3; Homozygous person CC rs13429458, 4, 5; Heterozygous person CA rs13429458, 6, 7; Homozygous person AA rs13429458, 8, 9; Homozygous person TT rs12478601, 10, 11; Heterozygous person CT rs12478601, and 12, 13; Homozygous person CC rs12478601.

Comparison of hormonal profile

The biochemical and endocrine characteristics of control and case groups are presented in Table 3. The Mean values of LH, LH/FSH, AMH, Estradiol, Prolactin, and fasting blood sugar had a significant increase in the case group (P<0.05), while the mean value of FSH had a significant decrease in the case group compared with the control group (P<0.05).

Table 3: Comparison of hormonal factors between case and control groups

Hormonal factors	PCOS group	Control group	P value
LH (mIU/ml)	6.67 ± 1.08	4.88 ± 1.224	0.000
FSH (mIU/ml)	4.46 ± 1.097	6.97 ± 1.903	0.025
LH/FSH	1.05 ± 0.218	0.717 ± 0.193	0.000
AMH (ng/ml)	6.2 ± 1.739	1.73 ± 0.496	0.000
Estradiol (pg/ml)	23.28 ± 2.028	20.72 ± 1.641	0.000
Prolactin (ng/ml)	29.48 ± 4.831	20.63 ± 3.141	0.000
FBS (mg %)	93.52 ± 8.319	89.64 ± 2.918	0.001

PCOS; Polycystic ovarian syndrome, FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, PRL; Prolactin, AMH; Anti Mullerian hormone, and FBS; Fasting blood sugar.

Comparison of genotype distributions and allele frequencies of SNPs rs13429458 and rs12478601

Genotype distributions and allele frequencies of SNPs, rs13429458 and rs12478601, in the two groups had been presented in Tables 4 and 5. Genotypic and OR were analyzed to evaluate the correlation between *THADA* gene polymorphisms and PCOS. Our results showed that rs13429458 did not significantly associated with PCOS risk in the control and PCOS groups. A second significant SNP (rs12478601) was determined by main model analysis (OR: 2.823, 95% CI: 1.22-6.533, P=0.014) and codominant model analysis (OR: 2.429, 95% CI: 0.998-5.913, P=0.032). At the allelic level, there was a significant difference between. The mutant allele frequency (allele T) increased in comparison to the control group (P=0.001). Subjects with the CT genotype or the TT genotype of *THADA* rs12478601 were at enhanced risk of undergoing PCOS.

Table 4: Association of rs13429458 with PCOS risk based on logistic tests

Genotype	PCOS group n (%)	Control group n (%)	P value	Adjusted OR	95% CI
AA	32 (48.5)	26 (59.1)		1	
AC	26 (39.4)	12 (27.3)	0.42	0.568	0.241-1.339
CC	8 (12.1)	6 (13.6)		0.923	0.284-2.999
Allele					
A	90 (68.2)	64 (72.7)	0.471	0.804	0.443-1.457
C	42 (31.8)	24 (27.3)		1.244	0.686-2.257
Dominant					
AA	32 (47.1)	26 (59.1)	0.275	1	
AC+CC	34 (52.9)	18 (40.9)		0.652	0.301-1.408
Recessive					
CC	8 (12.5)	6 (13.7)	0.815	1	
AC+AA	58 (87.5)	38 (86.3)		0.847	0.281-2.717
Codominant					
AA	32 (48.5)	26 (59.1)		1	
AC	26 (39.4)	12 (27.3)	0.42	0.568	0.241-1.339
CC	8 (12.1)	6 (13.6)		0.923	0.284-2.999
Over dominant					
AA+CC	40 (60.1)	32 (72.8)	0.19	1	
AC	26 (39.9)	12 (27.2)		0.577	0.252-1.319

OR; Odds ratio, CI; Confidence interval, and PCOS; Polycystic ovarian syndrome.

Table 5: Association of rs12478601 with PCOS risk based on logistic tests

Genotype	PCOS group n (%)	Control group n (%)	P value	Adjusted OR	95% CI
CC	14 (21.2)	19 (43.2)		2.429	0.998-5.913
CT	34 (51.5)	19 (43.2)	0.032	1	
TT	18 (27.3)	6 (13.6)		0.596	0.202-1.758
Allele					
C	62 (47)	57 (69.5)	0.001	0.388	0.217-0.695
T	70 (53)	25 (30.5)		2.574	1.439-0.604
Dominant					
CC	14 (21.2)	19 (43.2)	0.014	2.823	1.22-6.533
CT+TT	52 (78.8)	25 (53.8)		1	
Recessive					
TT	18 (28)	6 (13.7)	0.752	1.188	0.409-3.447
CT+CC	48 (72)	38 (86.3)		1	
Codominant					
CC	14 (14)	19 (43.2)		2.429	0.998-5.913
CT	34 (34)	19 (43.2)	0.032	1	
TT	18 (18)	6 (13.6)		0.596	0.202-1.758
Over dominant					
TT+CC	32 (32)	25 (56.8)	0.752	1	
CT	34 (34)	19 (43.2)		1.188	0.409-3.447

OR; Odds ratio, CI; Confidence interval, and PCOS; Polycystic ovarian syndrome.

Results of in silico tools

Data sets produced by ENCODE indicated that rs12478601 SNP is located at the H3K27Ac mark on seven cell lines. The variation occurs in the intronic position, which could lead to the alteration of an exonic splicing silencers (ESSs) element. UTRScan demonstrated that this region is located Upstream Open Reading Frame (uORF) that alteration can result in reducing translational efficiency. This SNP is located in the middle of the intron and most likely (score: 66.93) create or destroy an ESS but making it hard to predict how this SNP might impact splicing. uORFs are essential gene expression regulatory elements. Besides, data have shown how uORF-mediated translational control can affect cell chance decisions.

Discussion

PCOS, a heterogeneous disorder, is considered a genetic base disease based on its distribution between twins and the accumulation of familial cases (18). A strong genetic basis for PCOS has been developed using multidisciplinary approaches including twin studies, transplants, candidate genes, which have paved the way for researchers to unravel the genetic underpinnings of PCOS etiology (19). *THADA* plays a role in diabetes and insulin sensitivity by observation of its altered methylation in the pancreatic islets of T2D patients, and the association of polymorphisms with altered B cell apoptosis and susceptibility to diabetes has been confirmed (20). Some of the SNP variations might influence mRNA expression and or alternative pre-mRNA splicing. Acetylation in K27 on histone H3 leads to open chromatin conformation which significantly increases gene expression (21). The results of the silica tool based on data sets produced by ENCODE indicated that this rs12478601 SNP is located at the H3K27Ac mark on seven cell lines. The variation occurs in the intronic position, which could lead to the alteration of an ESSs site or element (potential alteration of splicing). UTRScan demonstrated that this region is located uORF that its alteration can result in reducing translational efficiency. This SNP is located in the middle of the intron and most likely (score: 66.93) create or destroy an ESS. But it is difficult to predict how this SNP might affect splicing. uORFs are essential gene expression regulatory elements. Besides, data have shown how uORF-mediated translational control can affect cell chance decisions.

Previously, it was reported that these two SNPs (rs13429458 and rs12478601) of *THADA* are associated with PCOS (19). In this study, we investigated the polymorphisms of the *THADA* gene in some Iranian women population with PCOS for the first time. We found that rs12478601 is significantly associated with increased susceptibility to PCOS, while, rs13429458 was not associated with PCOS. Similarly, Goodarzi et al. (10), in their European cohort studies, identified no correlation between rs13429458 and risk of PCOS. Briefly, we identified a significant relationship between increased PCOS risk and the rs12478601 "T" allele of the *THADA* gene in some Iranian populations. Our results demonstrated that patients with the CT and TT genotype of *THADA* rs12478601 were at high risk of undergoing PCOS.

It has been presented those environmental factors are very important in the pathogenesis of PCOS. Obesity seems to be related to PCOS (22). For instance, in the United States, more than half of the patients with PCOS have overweight or obese. Increased adiposity is related to many dysfunctions of sex steroid metabolism, that may result in, increased androgen production and decreased SHBG (23). The impacts of BMI on PCOS were assessed in the study. The results showed that the BMI in the case group (27.7 ± 4.841 kg/m^2) was significantly higher than in the control group 25.71 ± 1.899 kg/m^2), and the risk of PCOS was even higher among obese women. Previous studies are consistent our finding. Our results showed that correlation between BMI and PCO presented similar to Greece and Spain population which is in agreement with that reported by Miazgowski et al. (24) and Li et al. (25), and Skiba et al. (26). PCOS is also related to impaired glucose tolerance (IGT) and increased insulin resistance and BMI (27). Women with PCOS had an increased prevalence of IGT, diabetic Mellitus, metabolic syndrome (28, 29). Our study also showed that in the PCOS women in the case group, FSH, LH, and LH/FSH ratio, and FBS had a significant increase in comparison with the control group. Quantitative trait analysis showed that there was a relationship between the rs2478601 genotype and enhanced levels of LH as well as a higher LH/FSH ratio in the PCOS patients. In the control group, LH, and LH/FSH ratios were detected to be increased in those with the rs2478601 genotype. In summary, we identified a significant association between increased PCOS risk and the rs12478601 "T" allele of the *THADA* gene in some Iranian populations. There is a need for more functional studies to confirm the correlation between the *THADA* gene and PCOS pathogenesis, especially with respect to different ethnicities. We predict that identification and characterization of additional PCOS susceptibility genes will ultimately provide more efficient strategies for the diagnosis, prevention, and treatment of PCOS in genetically heterogeneous populations.

Conclusion

These results showed that there is a relation between rs12478601 of the *THADA* gene and susceptibility to polycystic ovary syndrome, and allele T is a high-risk allele in this aim.

Acknowledgements

We would like to thank the Academic Center for Education, Culture and Research, Qom Branch, for financial supporting this research project. There is no conflict of interest in this study.

Authors' Contributions

L.N., R.J., K.R., M.Kh., N.K.; Study design, data collection and, statistical analysis. L.N., R.J.; Sample

collection. L.N., R.J., N.K.; Data interpretation and conclusion. N.K.; Conducted molecular experiments and ARMS-PCR analysis. All authors read and approved the final manuscript.

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