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Association of *FOXP3* gene polymorphisms with risk of preeclampsia in Lur population of Iran

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Abstract

Introduction: Regulatory T lymphocytes have an effective role in induction of immune tolerance and angiogenesis during pregnancy. Differentiation and replication of these lymphocytes from other T cells is controlled by FOXP3 transcriptional factor. *FOXP3* gene is polymorphic.
Objectives: This study was designed to investigate the role *FOXP3* common polymorphisms in susceptibility to preeclampsia in Lur populations of Lorestan province of Iran.
Patients and Methods: This study was conducted on three polymorphisms rs2232365 A/G, rs3761548 C/A and rs5902434 del/ATT using polymerase chain reaction with sequence specific primers (PCR SSP). A total of 100 participants were subjected to be studied. Data analysis was done using Fisher's exact test and multivariate logistic regression.
Results: In rs2232365 polymorphism, AA genotype was a risk factor ($P=0.002$) and AG genotype was a protecting factor ($P<0.001$). In polymorphism of rs3761548, CA genotype was a risk factor ($P=0.036$) and AA genotype was a protecting factor ($P=0.015$). In polymorphism rs5902434, del/del genotype was a risk factor ($P=0.013$) and del/ATT genotype was a protecting factor ($P<0.001$). After adjusting the effects of genotypes, CA genotype of rs3761584 polymorphism was considered as an attributable risk factor ($P=0.040$; odds ratio [OR] =4.19).
Conclusion: The present population showed a unique association for the role of *FOXP3* polymorphism in susceptibility to preeclampsia in comparison to previous studies.

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Introduction

Preeclampsia is a pregnancy specific syndrome seen in about 5% of all pregnancies. Preeclampsia is an example of pregnancy hypertensive disorders which leads to maternal and fetal mortality and morbidity. Clinically, preeclampsia is called as a new onset hypertension after gestational age of 20 weeks along with a sign of organ damage which was previously defined as proteinuria. If there was no proteinuria or evidence of organ damage it is called gestational hypertension. In addition, chronic hypertension can be superimposed by preeclampsia (1). However, there are some unusual manifestations such as earlier hypertension or lack of hypertension in rare cases (2). The etiology of preeclampsia is not clear; however its pathophysiology has an immunological basis (3).

Key point

Polymorphism of *FOXP3* gene affects the risk of preeclampsia. This association is ethnicity dependent which may be due to haplotype effects. Therefore, from the viewpoint of medical anthropology these polymorphisms should be investigated in other diseases and populations to find immunological signature of ethnicities.

Accordingly, pregnancy is a semi-allograft transplantation. In such conditions, we need immune tolerance and induction of angiogenesis for a normal pregnancy. These functions are triggered by natural killer cells and regulatory T lymphocytes (4,5). It has been observed that reduction in population of regulatory T cells increases the risk of pregnancy complications (6). Regulatory T cells are originated from T helper-0 ($CD4^+$). T

helper-0 is mainly differentiated to T helper-1, T helper-2, T helper-17 and regulatory T cells. Differentiation of regulatory T cells is initiated by transforming growth factor beta (TGF-β). TGF-β results in the intracellular reactions which result in expression of a transcriptional factor named forkhead box protein 3 (FOXP3) from *FOXP3* gene (7). Like many other genes, *FOXP3* is polymorphic. This gene is located on chromosome X (8). Previously, induction of FOXP3 was proposed for treatment of infertility (9). Different hormones and vitamin D3 can induce the expression of FOXP3 (10, 11), and therefore, vitamin D deficiency increases risk of pregnancy complications (12).

FOXP3 gene has some common and uncommon polymorphisms. Previously, Song et al considered the three common polymorphisms of rs2232365 -924 A/G, rs3761548 -3297 C/A and rs5902434 -6054 del/ATT with risk of vitiligo (as an immune system related disease) in a Chinese population. They used logistic regression model and found that all of the above loci might influent the risk depending on the number of risk alleles from 0 to 6 (13). Therefore, these loci may be associated with risk of other diseases of immune system like preeclampsia.

Objectives

Single nucleotide polymorphisms (SNPs) of functional genes affect the susceptibility to different diseases. According to the critical role of FOXP3 in the function of regulatory T cells, the present study was aimed to investigate the association of *FOXP3* SNPs with risk of preeclampsia in Lur population of Iran.

Patients and Methods

Study design

A genetic association study was performed with a case-control design. Genetic epidemiological approaches were used for interpretation of the data.

Study population

The sample size was calculated as 100 participants (50 per group) according to sample size calculation formula for comparison of proportions. During laboratory investigation, 20 samples were excluded because of lack of good quality DNA. The case groups were women with current or previous history of preeclampsia according to American Community of Obstetrics and Gynecology (ACOG) criteria including blood pressure >140/90 mm Hg with onset after 20 weeks of gestational age, and having proteinuria (1+ in dipstick or 300 mg/24 h) or evidence of organ damage instead. The severity of preeclampsia was not regarded. The exclusion criteria were having autoimmune disease or any history of pregnancy complication other than preeclampsia. The control group included women with history of at least two normal pregnancies without history of pregnancy complications. The samples were taken in Asali hospital, Khorramabad, Iran during 2018 using convenient sampling.

Bioinformatics

Among the common polymorphisms of *FOXP3*, three upstream SNPs including rs2232365 -924 A/G, rs3761548 -3297 C/A and rs5902434 -6054 del/ATT were selected. The details of these SNPs are available at www.snpedia.com. Sequence specific primers were used according to previous literature (Table 1) (13-15). The alleles left sided of the slashes were considered as wild type alleles.

Laboratory investigation

From each participant, 2 mL of peripheral blood was taken in EDTA containing tubes. The samples were kept at -20°C till DNA extraction. DNA was extracted by sedimentation method using DNA extraction kit (Maxcell, Iran). Nanodrop spectrophotometer was used for calculating the concentration of DNA (which was 100-300 ng/µL). The extracted DNA was kept at -20°C till the reactions.

Polymerase chain reaction with sequence specific primers (PCR SSP) was used. For primary setup, PCR was performed according to thermal cycling protocol of Song et al for these primers (13). The PCR products were studied on agarose gel electrophoresis (Figure 1). After primary setup, high resolution melting master mix was used for reactions using real-time PCR. The PCR products were run on agarose gel, and melting curves were also studied. Each investigated allele for each sample was recorded as positive or negative.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. Informed consent was taken from each participant. The personal data of the participants were kept in the hospital and deleted from the researcher archive. The present study was approved in the ethics committee of Lorestan University of Medical Sciences with registration number IR.LUMS.REC.1396.369. This study was a dissertation (M.D. Thesis) by Soheila Akbari at this

Table 1. Sequence specific primers used in the study

SNP	Forward primers	Reverse primers	Product size
rs2232365	CCCAGCTCAAGAGACCCCA CCAGCTCAAGAGACCCCG	GGGCTAGTGAGGAGGCTATTGTAAC GCTATTGTAACAGTCCTGGCAAGTG	442 427
rs3761548	CTGGCTCTCTCCCCAACTGA TGGCTCTCTCCCCAACTGC	ACAGAGCCCATCATCAGACTCTCTA ACAGAGCCCATCATCAGACTCTCTA	334 333
rs5902434	ACCTTTAAGTCTTCTGCCATTTATTCTATTATTT CCTTTAAGTCTTCTGCCATTTATTCTATTATTA	TGATTATCAGCGCACACACTCAT TGATTATCAGCGCACACACTCAT	358 356

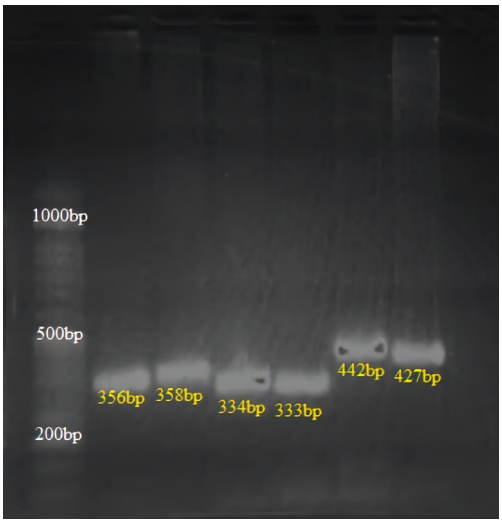

Figure 1. PCR products on agarose gel. The product sizes are according to table 1.

university.

Analysis of data

Genotyping from the raw PCR data was performed using Excel 2013 software (Microsoft, US). Genotypes between the groups were compared using Fisher's exact test in 2 by 2 contingency tables. In order to control haplotype effect, multivariate logistic regression was used, in which all the 3 SNPs were the three independent variables. For each SNP, the most ineffective genotype (according to Fisher's exact test) was considered as the baseline genotype for logistic regression with odds ratio (OR) =1, and the second and third genotypes of each SNP were the risk and protective genotypes, respectively (according to Fisher's exact test). Inferential statistics were performed using Stata 14 software (StataCorp LLC, US).

Results

All the genotypes of all polymorphisms were detected in the present ethnicity. For polymorphism of rs2232365, frequency of the wild type allele (A) was more than 50%. Frequency of homozygote genotypes was low in the control group. For polymorphism of rs3761548, frequency of the wild type allele (C) was less than 50%. Frequency of the mutant genotype AA was more in the control group. For polymorphism of rs5902434, frequency of the wild type allele (del) was more than 50%. Frequency of homozygote genotypes was low in the control group. Since the locus of *FOXP3* was on chromosome X, it was not possible to calculate Hardy-Weinberg equilibrium with the conventional method. All the descriptive results mentioned above are without considering haplotype effect and confounding effect of the polymorphisms on each other (Table 2).

Frequency of genotypes was compared between groups. For polymorphism of rs2232365, wild homozygote genotype AA was a risk factor ($P=0.002$) and heterozygote genotype AG was a protecting factor ($P<0.001$). No significant result was observed for alleles. For polymorphism of rs3761548, mutant genotype AA was a protecting factor ($P=0.015$) and genotype CA was a risk factor ($P=0.036$). The mutant type allele A was a protecting factor ($P=0.048$). For polymorphism rs5902434, wild homozygote genotype del/del was a risk factor ($P=0.013$) and heterozygote genotype del/ATT was a protecting factor ($P<0.001$). No significant result was observed for alleles (Table 3).

To find attributable genotypes and controlling haplotype effects, multivariate logistic regression was used. After running analysis, CA genotype from polymorphism of rs3761548 was found as the attributable genotype which was a risk factor for preeclampsia in this population ($P=0.040$; OR= 4.10 [in comparison to CC genotype] with 95% confidence interval [CI]: 1.06-16.48). In addition, genotype AA from polymorphism rs2232365 showed a positive trend toward being a risk factor for preeclampsia ($P=0.056$; OR = 8.34 with 95% CI: 0.96-73.63). No co-linearity was observed between the polymorphisms after choosing baseline genotype for each. A significant

Table 2. Descriptive data of the distribution of genotypes and alleles

SNP	Genotype	Group			Allele	Group		
		Control	Patient	Total		Control	Patient	Total
rs2232365	AA	1 (3.33)	16 (32.0)	17 (21.2)	A	29 (48.3)	56 (56.0)	85 (53.1)
	AG	27 (90.0)	24 (48.0)	51 (63.7)	G	31 (51.6)	44 (44.0)	75 (46.8)
	GG	2 (6.66)	10 (20.0)	12 (15.0)	Total	60 (100)	100 (100)	160 (100)
	Total	30 (100)	50 (100)	80 (100)				
rs3761548	AA	16 (53.3)	12 (24.0)	28 (35.0)	A	40 (66.6)	50 (50.0)	90 (56.2)
	CA	8 (26.6)	26 (52.0)	34 (42.5)	C	20 (33.3)	50 (50.0)	70 (43.7)
	CC	6 (20.0)	12 (24.0)	18 (22.5)	Total	60 (100)	100 (100)	160 (100)
	Total	30 (100)	50 (100)	80 (100)				
rs5902434	ATT/ATT	2 (6.66)	12 (24.0)	14 (17.5)	ATT	28 (46.6)	42 (42.0)	70 (43.7)
	del/ATT	24 (80.0)	18 (36.0)	42 (52.5)	del	32 (53.3)	58 (58.0)	90 (56.2)
	del/del	4 (13.3)	20 (40.0)	24 (30.0)	Total	60 (100)	100 (100)	160 (100)
	Total	30 (100)	50 (100)	80 (100)				

SNP, single nucleotide polymorphism.

Table 3. Association of genotypes and alleles with risk of preeclampsia investigated by Fisher exact test

SNP	Genotype/allele	Effect direction	P value
rs2232365	AA	Risk	0.002**
	AG	Protective	<0.001***
	GG	NS	0.194
	G	NS	0.413
rs3761548	CC	NS	0.786
	CA	Risk	0.036*
	AA	Protective	0.015*
	A	Protective	0.048*
rs5902434	del/del	Risk	0.013*
	del/ATT	Protective	0<0.001***
	ATT/ATT	NS	0.068
	ATT	NS	0.622

SNP, single nucleotide polymorphism.

* Significant at $P<0.05$; ** Significant at $P<0.01$; *** Significant at $P<0.001$.

Table 4. Multivariate logistic regression for the effect of polymorphisms

SNP	Genotype	Odds ratio	95% CI	*P* value
rs2232365	GG	1	Reference	
	AA	8.34	0.94-73.63	0.056
	AG#	0.86	0.19-3.75	0.843
rs3761548	CC	1	Reference	
	CA	4.19	1.06-16.48	0.040*
	AA#	1.32	0.36-4.78	0.665
rs5902434	ATT/ATT	1	Reference	
	del/del	2.05	0.44-9.46	0.355
	del/ATT#	0.38	0.06-2.23	0.286

SNP, single nucleotide polymorphism.

* Significant at $P<0.05$; # Significant interaction at $P<0.05$.

Note: The reference genotypes are the genotypes without effect direction according to Table 3.

interaction was found among the protecting genotypes of the three polymorphisms ($P=0.008$; OR = 0.187 with 95% CI 0.054-0.643) (Table 4).

Discussion

The present genetic association study was designed to show the role of *FOXP3* polymorphisms with susceptibility to preeclampsia as well as the effect of our ethnicity on this association. The unique results of this study in comparison to the previous ones showed the effect of ethnicity which may be due to haplotype effect. Haplotype effects can influent effects of individual genotypes (16, 17).

From the descriptive point of view, in two polymorphisms of rs2232365 and rs5902434 wild type alleles were most prevalent. For these two polymorphisms, frequency of homozygote genotypes was low. It shows that distribution of the genotypes of *FOXP3* polymorphisms is ethnicity dependent.

According to the analyses of 2 by 2 tables, all three polymorphisms showed significant associations in susceptibility to preeclampsia. Controversial results of 2 by 2 tables and multivariate logistic regression shows that the effects of the attributable genotypes may be covered by other genotypes due to ethnicity related haplotypes. Therefore, the results of 2 by 2 tables are more reliable for in clinics whereas multivariate logistic regression analysis is more reliable to find cause-effect relations.

As we mentioned, unique results were found in comparison to the previous studies. Chen et al investigated the role of rs3761548 and rs5902434 polymorphisms in Han population of China (18). They found no significant association for the first polymorphism whereas for the second polymorphism they found del/del genotype as a protecting factor. In another study, they investigated rs2232365 polymorphism sine no significant association was observed (19). Jahan et al in India investigated the role of rs3761548 polymorphism. They found CA and CC genotypes act as risk factor s(20). Norouzian et al in Bandar Abbas city of Iran investigated the role of rs2232365 and rs3761548 polymorphisms, while no significant association was observed (21). Gholami et al in Tehran investigated the role of rs2232365 and rs3761548 polymorphisms. For the first polymorphism they found AG genotype as a risk factor and for the second polymorphism they found AA genotype as a protecting factor (22). Although our study had been performed in Iran, however Lur population of Iran showed unique results even in comparison to other Iranian populations.

Conclusion

The present study showed a unique association for the polymorphisms of *FOXP3* with susceptibility to preeclampsia in our ethnicity. From the viewpoint of basic medical sciences, immunological basis of preeclampsia was approved for another time. The given data cannot be representative of the gene pool of total Iranian population without comparison to other ethnic groups within the same study by using the same methods.

Limitations of the study

Effects of ethnicity related haplotypes might musk the effects of individual genotypes. Using multivariate logistic regression helped us to solve this problem. However, more powerful studies are suggested in other populations.

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This work is a research project of SA with the same title. This project was used as the MD thesis of BK with ethics registration number IR.LUMS.REC.1396.369.

Authors' contribution

SA; sample taking and clinical supervision. FS; design and conceptualization. BK; search, writing and sample taking. SAYA; primary draft and analysis. SA; laboratory study. SERA; laboratory supervision and approval. All authors read and sign the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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