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ARAŞTIRMA / RESEARCH

Evaluation of single nucleotide polymorphisms of angiotensin II type 2 receptor (AGTR2) gene and interleukin 4 (IL-4) gene for their contribution to the risk of preeclampsia in Turkish population

Anjiyotensin II tip 2 reseptör geni (AGTR2) ve interlökin 4 (IL-4) genindeki tek nükleotid polimorfizmlerinin preeklampsi riskine katkılarının Türk populasyonunda incelenmesi

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Abstract

Purpose: Preeclampsia, specific to human pregnancies, is a serious disorder, which occurs approximately in 6-10% of all pregnancies. It is a complex disorder, in which immune and genetic factors also take part. The aim of the current study was toinvestigate whether there is an association between AGTR2 A1675G and IL4 -590 (C>T) polymorphisms and preeclampsia.

Material and Methods: Genomic DNA was extracted from the peripheral venous blood of 131 preeclamptic and 86 normotensive pregnant women. The AGTR2 and IL-4 polymorphisms were genotyped by using a polymerase chain reaction assay.

Results: As for AGTR2 gene 1675 polymorphism, there was not any significant difference in terms of genotype frequencies but there was a significant difference in terms of allele frequencies. As for IL-4 gene -590 polymorphism, there was not any significant difference in terms of genotype and allele frequencies.

Conclusions: AGTR2, GG genotype and IL-4, TT genotype were found significantly higher in preeclamptic women than normotensive pregnant women.These genotypes might be a susceptibility risk factor for preeclampsia but these findings need to be tested in a larger sample size.

Key words: Preeclampsia, AGT2R, IL-4, polymorphism.

Öz

Amaç: Preeklampsi, tüm gebeliklerin yaklaşık %6-10' unda görülen ve gebe kadınlara özgü ciddi bir hastalıktır. İmmün ve genetik faktörlerin de katkıda bulunduğu kompleks bir hastalıktır. Bu çalışmada amacımız, AGTR2A1675G ve IL4 -590 (C>T) polimorfizmleri ve preeklampsi arasında bir ilişki olup olmadığını incelemekti.

Gereç ve Yöntem: Yüz otuzbir preeklampsili ve 86 normal gebe kadından alınan periferik kandan, genomic DNA izole edildi. AGTR2 ve IL-4 polimorfizmleri polimeraz zincir reaksiyonu kullanılarak genotiplendirildi.

Bulgular: AGTR2 geni 1675 polimorfizminin genotip frekansı açısından önemli bir fark bulunamadı fakat allel frekansları açısından önemli bir fark bulunmuştur. IL-4 geni -590 polimorfizmi genotip ve allel frekansları açısından önemli bir fark bulunamamıştır.

Sonuç: AGTR2, GG genotipi ve IL-4, TT genotipi preeklampsili kadınlarda normal gebe kadınlara oranla önemli derecede yüksek bulundu. Bu genotipe sahip olma, preeklampsi meydana gelmesi açısından bir risk faktörü olabilir ancak bu bulgular daha fazla örnekte test edilmeye ihtiyaç duymaktadır.

Anahtar kelimeler: Preeklampsi, AGTR2, IL-4, polimorfizm.

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INTRODUCTION

Preeclampsia (PE) is a disease of pregnancy characterized by the new onset of hypertension and proteinuria after the 20th week of gestation. It has been estimated that 6-10% of pregnancies world wide are complicated with this disorder resulting in substantial maternal and fetal morbidity and mortality[1,2]. It has been reported that preeclampsia risk during the first pregnancy is 4.1%, but around 1.7% in later pregnancies[3]. The pathogenesis of pre-eclampsia has remained enigmata. The likelihood of there being a single pre-eclampsia gene is execissevely distant. It seems more likely that presence of polymorphism have come together in various different combinations in the same individual. Moreover these polymorphisms are accompanied with enviromental factors and PE phenotype is emerged[4]. Current genetic studies up to today have focused on a variety of genes involved in thrombophilia, oxidative stress and other metabolic factors, including those related to the control of hypertension[3,4].

The renin-angiotensin system (RAS) plays an important homeostatic role in blood pressure regulation, water and salt balance and in the pathophysiology of PE. RAS components are expressed in uteroplasenta. One of them is the Angiotensin II Receptor Type 2 (AGTR2), subtype of angiotensin II[3,5,6,7]. The AGTR2 is widely expressed during fetal development. As a proapoptotic agent AGTR2 expression increases vessel diameter and inhibits cell proliferation[8,9]. There are only few reports on the association between PE and AGTR2 gene polymorphisms in the literature.

There is balance between active and supressive mechanisms of the immune system during implantation and plasental development. This balance is checked by cytokines at the uteroplasental interface. Inflamatuar cytokines are produced by T-helper 1 cells and T-helper 2 cells. Cytokines produced by T-helper 2 cells, such as IL-4, IL-5, IL-6, and IL-10, may downregulate cellular immunity and induce placental growth[11,12,13,14]. Some studies have reported that plasma *IL-4* level were found higher in preeclamptic women than normotensive pregnant controls[11,15]. Although many studies repeat that there is a relationship between PE, immun system and RAS components in the literature, there are only few studies on *IL-4* -590 C>T and *AGTR2*1675 A>G polymorphisms.

Our aim is to shed light on the controversial role of these polymorphisms by performing a case-control study with preeclamptic and normotensive pregnant women. We compared the distribution of genotypes and allele ferquencies for AGTR2 1675 A>G and IL-4 -590 C>T polymorphisms.

MATERIAL AND METHODS

Ethical committee approval was obtained from the Ethics Committee of Cukurova University Hospital, all patients gave their informed consent before peripheral blood samples were taken.

Sampling

A total of 217 subjects, including 131 preeclamptic and 86 healthy pregnant women, were recruited at the Department of Obstetrics and Gynecology at Hospital of Cukurova University between September 2011 and August 2012. Subjects in the control group were normotensive pregnant women, who delivered a healthy neonate at term (37 weeks of gestation) without proteinuria, or antenatal medical or obstetric complications; subjects in the case group were pregnant women with hypertension and proteinuria (blood pressure values >140/ 90mmHg on two measurements at least 6 h apart; 24 h urinary protein >0.3 g) after the 20th week of pregnancy[17]. Exclusion criteria for both groups were listed as diabetes, chronic hypertension, chronic renal disease, pregnancies with malformed fetuses or infections, twin pregnancies, thyroid disease, and chronic infectious diseases.

Genotypying and RFLP

Peripheral venous blood samples were collected in tubes containing EDTA and stored at 4 degrees Celsius until the time of processing. Maternal genomic DNA was isolated from peripheral venous blood leukocytes using standard salting out method as previously described[16].

The A1675G (NM_000686.4: c.-95-29G> A) polymorphism in the 310-bp DNA fragment intron1- exon2 junction of the *AGTR2* gene was amplified by Polymerase Chain Reaction (PCR), using forward primer 5'-AGAGATCTGGTGCTATTACG-3' and reverse primer 5'-CACTTGAAGACTTACTGGTTG- 3' [4]. PCR amplification reaction mixture was carried out

in a total volume of 25 µL, using 200 ng genomic DNA, 25mM dNTPs, 10 pmol of each primer, 1U Taq DNA polymerase , and 2.5 µL Buffer. PCR conditions comprise an initial denaturing step at 95 ºC for 5 min followed by 35 cycles of denaturation at 94 ºC for 45 s, primer annealing at 55 ºC for 1 min, and extension at 72 ºC for 1 min, with a final 7 min extension at 72 ºC[10]. Approximately 10 µL PCR product was digested at 37 ºC for 3 h with 5U HYP 188 III (New England Biolabs), which only cleaves the G allele[10].

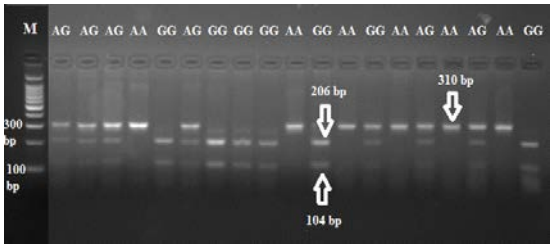

Figure 1. PCR-RFLP results of A1675G polymorphism. PCR product (310 bp), GA (310/206/104 bp), AA (310 bp), GG (206/104 bp), M marker (100 bp).

The –590 (C>T) (NM_000589.3: c.-589C> T) polymorphism in the a 196-bp DNA fragment at the 5'-UTR of *IL-4* gene was performed by Polymerase Chain Reaction (PCR), using forward primer 5'-TAAACTTGGGAGAACATGGT-3' and reverse primer 5'-TGGGGAAAGATAGAGTAATA-3'. The PCR product (196 bp) was digested with Ava II (New England Biolabs) at 37 °C for overnight (16-18 hours)[27].

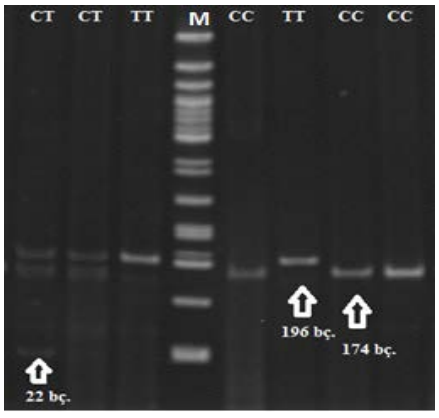

Fig.2. PCR-RFLP results of -590 C>T polymorphism. PCR product (196 bp), CT (196/174/22 bp), CC (174/22 bp), TT (196 bp), M marker (50 bp)

Genotype was assessed following 2% agarose gel electrophoresis and visualised on a transilluminator. The *AGTR2* A allele yielded a single 310- bp fragment while *AGTR2* G allele yielded two fragments of 104 and 206-bp. Heterozygotes yielded three fragments of 310, 206, and 104-bp (Figure 1). The *IL-4* T allele yielded a single 196 bp fragment while C allele gave two fragments of 174 and 22 bp. Heterozygotes yielded three fragments of 196, 174 and 22 bp (Figure 2).

Statistical analysis

Statistical evaluation of the clinical data was performed using Student's t-test. Differences in the distribution of alleles and genotypes between case-control groups were assessed by the Pearson chi-square test with SPSS 11.5 software and the level of statistical significance was defined as $p<0.05$. Odd ratio (OR) and 95% confidence interval (95% CI) were calculated to estimate the associations between different genotypes in PE patients and healthy controls. The interaction between the polymorphisms of *AGTR2* with *IL4* was determined using logistic regression model.

RESULTS

Clinical features

Table 1 represents the clinical features of the study population. Gestational age at birth was higher in all cases compared to the controls. As expected, systolic BP and diastolic BP were slightly higher in women with PE compared to the controls. In this study, there was a statistically significant difference in terms of systolic BP and diastolic BP between control and preeclamptics ($p<0.05$).

Allele and genotype frequencies in the PE and control groups

The allele and genotype frequencies of the *AGTR2* gene A1675G and *IL-4* gene -590 C>T polymorphisms in PE patients and healthy controls are reported in Table 2.

The frequencies of the A and G alleles of the 1675 loci were 54.2% (n=141) and 45.8% (n=119) in the preeclamptic group, respectively, and were 64.5% (n=111) and 35.5% (n=61) in the unrelated control group, respectively. The frequencies of the 1675 AA, AG and GG genotypes were 30% (n=39), 48.5% (n=63) and 21.5% (n=28) in the patient group, respectively, and were 39.5% (n=34), 50%

(n=43) and 10% (n=9) in the unrelated control group, respectively. Significant differences were not observed among cases and controls in relation to genotype frequencies for the A1675G polymorphism (p>0.05). On the contrary, A allel was found significantly different in preeclamptic women than normotensive pregnant women (p=0.033) where there was a slight difference in allele frequencies (p=0.033). There was a higher frequency of recessive genotypes, AG+GG in PE patients compared to controls, but this difference was not statistically significant (%70, %60,5 respectively; p=0.147, OR=1.53; 95%Cl, 0.86-2.70).

Table 1. Clinical features of population group

	Cases (n=131)	Controls (n=86)	P value
Age (years)	30.13±0.71	28.36±1.66	> 0.05
BMI (kg/m^2)	31.15±1.22	28.07±0.53	> 0.05
Gestational Age (weeks)	33.54±0.49	29.63±2.32	> 0.05
Systolic BP (mmHg)	151.22±1.69	120.81±16.77	< 0.05
Diastolic BP (mmHg)	95.46±1.13	75.36±2.55	< 0.05

BMI: Body mass index, BP: Blood pressure

The frequencies of the C and T alleles of the -590 loci were 82.8% (n=212) and 17.2% (n=44) in the preeclamptic group, respectively, and were 86.4% (n=147) and 13.6% (n=23) in the unrelated control group, respectively. The frequencies of the 1675 CC, CT and TT genotypes were 69.5% (n=89), 26.6% (n=34) and 3.9% (n=5) in the patient group, respectively, and 74.1% (n=63), 24.7% (n=21) and 1.2% (n=1) in the unrelated control group, respectively. Significant differences were not observed among cases and controls in relation to genotype and allele frequencies for the -590 C>T polymorphism (p>0.05). There was a higher frequency of recessive genotypes, CT+TT in PE patients compared to controls, but this difference was not statistically significant (30.4%, 25.8% respectively, p=0.468, OR= 1.26 95%Cl, 0.68-2.32).

DISCUSSION

Strong evidence support a crucial role for genetic factors in susceptibility or resistance to PE. To determine whether genetic factors are associated with susseptibility or resistance to PE has been studied using various methods like twin and family studies, case-control studies, candidate gene approches and genome-wide linkage analyses[18].

Etiology and pathophysiology of preeclampsia is still unknown and based on heterogeneous reasons, It has major immun factors and genetic component which will contribute to determine the etiology of the disease[19]. In the present study, we investigated the genotype distribution and allele frequencies of IL-4,which is one of the cytokines occuring in the feto-maternal period and *AGTR2*, which is one of the vasoactive elements of RAS.

The *AGTR2* gene consists of three exons and two introns which is located on the chromosome X at locus q23–26[20]. Until now, a total of 241 SNPs were identified in *AGTR2* gene, among which 110 were found active in human. In these SNPs, the most common studied were the A1675G (NM_000686.4: c.-95-29G> A) polymorphism which lies in the first intron near the important region for gene transcription activity[24]. Moreover, this polymorphism described previously in the other studies as position -1332 and 2041[20,21,23].

In the present study, *AGTR2* gene A1675G polymorphism, GG genotype was found significanly higher in preeclamptic women than healthy control pregnant women (21.5% and 10.5% respectively, OR= 2.71). *AGTR2* gene c.-95-29G> A polymorphism genotype distrubution and allele frequencies in different studies are given in Table 3. Akbar and et. al. studied *AGTR2* gene 1675 polymorphism for three different ethnic populations (Afro-Caribbean, Asian, Caucasian). They also found that *AGTR2* gene 1675 polymorphism, GG genotype, was significantly higher in preeclamptic women than normotensive pregnant women in Afro-Caribbean population (p=0.004), similar to our findings stating GG genotype was higher in preeclamptic women in Caucasian population which was lower in Asian population[10].

Recently, Li and et. al. evaluate the relationship between the *AGTR2*, A1675G polymorphism and PE risk, in order to extensively predicted by conducting a meta-analysis. These data suggest that the pregnant women with GG genotype might have increased risk for preeclampsia[24]. In this study, G allele of NM_000686.4: c.-95-29G>A polymorphism were found statistically significant in preeclamptic women (p=0.033). Our data was consistent with the results of Rahimi et. al. and Akbar et. al. On Afro-Caribbean and Asian populations, respectively, however was opposite of

the findings of Zhou et. al., Plummer et. al. and Akbar et. al.,who studied the Caucasian population[10,21,22,23]. In light of all these data, having G allele or GG genotype considered to be a susceptibility risk factor for preeclampsia but these findings need to be tested in a larger sample size.

Table 2. Genotype and Allele frequencies of AGTR2, A1675G and IL-4 C590T polymorphisms

	Preeclampsia (n=131)	Control (n=86)	P value	X^2	OR (%95Cl)
AGTR2 (A1675G)					
AA n (%)	39 (30)	34 (39.5)	0.07	5.122	*Ref*
AG n (%)	63 (48.5)	43 (50)			1.28 (0.70-2.33)
GG n (%)	28 (21.5)	9 (10.5)			2.71 (1.12-6.54)
Recessive					
AG+GG/AA n(%)	91 (70)	52 (60.5)	0.147		1.53 (0.86-2.70)
Dominant					
AG+AA/GG n(%)	102 (78.4)	77 (59.2)			
Allele					
A n(%)	141 (54.2)	111 (64.5)	0.033	4.522	*Ref*
G n(%)	119 (45.8)	61 (35.5)			0.65 (0.44-0.97)
IL-4 590 (C>T)					
CC n(%)	89 (69.5)	63 (74.1)	0.456	1.570	*Ref*
CT n(%)	34 (26.6)	21 (24.7)			1.15 (0.61-2.16)
TT n(%)	5 (3.9)	1 (1.2)			3.54 (0.40-31.03)
Recessive					
CT+TT/CC n(%)	39 (30.4)	22 (25.8)	0.468		1.26 (0.68-2.32)
Dominant					
CT+CC/TT n(%)	123 (96)	84 (98)			
Allele					
C n(%)	212 (82.8)	147 (86.4)	0.310	1.031	*Ref*
T n(%)	44 (17.2)	23 (13.6)			0.75 (0.44-1.3)

Data are presented as n (%). Ref: referent; OR: odds ratio.

Table 3.The genotype distrubutions and allele frequencies in different studies for AGTR2 gene A1675G polymorphism.

	Preeclampsia						Control					
		Genotype			Allele			Genotype			Allele	
Study	n	AA n(%)	GG n(%)	AG n(%)	A n(%)	G n(%)	n	AA n(%)	GG n(%)	AG n(%)	A n(%)	G n(%)
Our Study	130	39 (30)	28 (21.5)	63 (48.5)	141 (54.2)	119 (48.5)	86	34 (39.5)	9 (10.5)	43 (50)	111 (64.5)	61 (35.5)
Rahimi (19)	155	32 (20.6)	25 (16.1)	98 (63.2)	162 (52.3)	148 (47.7)	97	38 (39.2)	9 (9.3)	50 (51.5)	126 (64.9)	68 (35.1)
Zhou (20)	119	24 (20.2)	34 (28.6)	61 (51.3)	109 (45.7)	129 (54.3)	1084	277 (25.6)	263 (24.3)	544 (50.2)	1098 (50.6)	1070 (49.4)
Akbar (10)	67	9 (13.4)	33 (49.3)	25 (37.3)	43 (32.1)	91 (67.9)	119	14 (11.8)	32 (26.9)	73 (61.3)	101 (42.4)	137 (57.6)
Akbar (10)	47	7 (14.9)	13 (27.7)	27 (57.4)	41 (43.6)	53 (56.4)	118	28 (23.5)	17 (14.8)	73 (61.7)	129 (54.7)	107 (45.3)
Akbar (10)	122	30 (24.6)	28 (23)	64 (52.5)	124 (50.8)	120 (49.2)	189	42 (22.2)	48 (25.4)	99 (52.4)	183 (48.4)	195 (51.6)
Plummer (21)	98	29 (29.6)	19 (19.4)	50 (51)	108 (55.1)	88 (44.9)	118	28 (23.7)	26 (22)	64 (54.3)	120 (50.8)	116 (49.2)

The *IL-4* gene is located on the 5q31-33 in humans[25]. The c.-589C> T polymorphism is located in the promoter of the *IL-4* gene which is relevant functionally with the *IL-4* levels. The *IL-4*c.-590 C>T promoter variant associated to enhanced transcriptional activity and with increased total serum immunoglobulin E[26]. Here,*IL-4* gene c.-589C> T polymorphism between normotensive and

preeclamptic womens revealed no significant difference in the genotype and allele frequencies distribution (respectively, p=0.456 and p=0.310) but the pregnant women with TT genotype have 3.54 times susceptibilty to PE. Concordantly, Fraser and et al. concluded that preganant women with the TT genotype are at higher risk of preeclampsia in the UK population. These data suggest that *IL-4* gene -590 TT genotype may have an impact adverse on plasentation therefore cause on increase in maternal susceptibility to PE[27]. The finding of the association between c.-589C> T SNPs and PE risk was inconsistent with previously reported results conducted in Taiwanese women which did not find an association between *IL-4* gene -590 TT genotype and preeclampsia[11]. These data support the importance of *IL-4* for the identification of susceptibility to PE and open new perspectives for the development of PE. Given the limitations mentioned above well designed studies with larger sample size are required to validate the risk identified in our study.

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