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Polymorphism in the lymphotoxin-alpha gene, position +252 (rs909253), is not associated with preeclampsia development in Brazilian women

Polimorfismo no gene da linfotoxina alfa, posição +252 (rs909253), não está associado com o desenvolvimento de pré-eclâmpsia em mulheres brasileiras

Original Article

Keywords
Hypertension, pregnancy-induced
Lymphotoxin-alpha
Polymorphism, genetic
Women's healthy

Palavras-chave
Hipertensão induzida pela gravidez
Linfotoxina-alfa
Polimorfismo genético
Saúde da mulher

Abstract

PURPOSE: To investigate the frequencies of polymorphic allele and genotypes for the LT-α gene, position +252 (rs909253), in Brazilian women with preeclampsia. **METHODS:** This is a case-control study, in which 30 women with preeclampsia, classified according to the criteria of the National High Blood Pressure Education Program, and 115 women in the control group, with at least two healthy pregnancies, were selected. Peripheral blood was collected, and DNA was extracted, followed by genotyping, using specific primers and restriction analysis. The genotypes obtained were AA, AG and GG. Statistical analysis was performed using the χ^2 association test. The Hardy-Weinberg Equilibrium was tested using the Haploview Program. **RESULTS:** The results showed no association between genotypes and preeclampsia development (χ^2=2.0; p=0.4). When the AG and GG genotypes were grouped according to allele G presence or absence (genotype AA), the data showed that the presence of allele G was not significantly different between cases (women with preeclampsia) and controls (χ^2=0.0; p=1.0). The LT-α gene polymorphism, position +252 (rs909253), seems not to be an important candidate for the development of preeclampsia. Other inflammatory genes should be researched, and studies involving gene-environment interactions should be performed, in order to reach a better understanding of the etiology of the preeclampsia.

Resumo

OBJETIVO: Investigar as frequências do alelo polimórfico e genótipos para o gene da LT-α, posição +252 (rs909253), em mulheres brasileiras com pré-eclâmpsia. **MÉTODOS:** Trata-se de um estudo caso-controle, em que 30 mulheres com pré-eclâmpsia, classificadas de acordo com os critérios do *National High Blood Pressure Education Program*, e 115 mulheres do grupo controle, com pelo menos duas gestações saudáveis, foram selecionadas. Amostra de sangue periférico foi colhida, e o DNA foi extraído, seguido pela genotipagem, usando iniciadores específicos e análise de restrição. Os genótipos obtidos foram AA, AG e GG. A análise estatística foi realizada utilizando-se o teste de associação χ^2. O Equilíbrio de Hardy-Weinberg foi testado com o auxílio do programa Haploview. **RESULTADOS:** Os resultados não mostraram associação entre os genótipos e o desenvolvimento de pré-eclâmpsia (χ^2=2,0; p=0,4). Quando os genótipos AG e GG foram agrupados de acordo com a presença do alelo G ou ausência (genótipo AA), os dados mostraram que a presença do alelo G não foi significativamente diferente entre casos (mulheres com pré-eclâmpsia) e controles (χ^2=0,0; p=1,0). O polimorfismo no gene LT-α, posição +252 (rs909253), parece não ser um importante candidato para o desenvolvimento de pré-eclâmpsia. Outros genes inflamatórios devem ser pesquisados, e estudos envolvendo interações gene-ambiente devem ser realizados para que se possa alcançar um melhor entendimento da etiologia da pré-eclâmpsia.

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Received
07/17/2015

Accepted with modifications
08/18/2015

DOI: 10.1590/S0100-720320150005454

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Conflict of interests: none.

Introduction

Preeclampsia (PE) is a pregnancy-specific syndrome characterized by new-onset hypertension and proteinuria after 20th gestational week[1]. It is a major obstetric problem, leading to substantial maternal and perinatal morbidity and mortality worldwide, especially in developing countries[2]. The clinical spectrum of PE ranges from mild to severe[3]. The established risk factors include primiparity, previous preeclampsia, maternal pre-pregnancy high body mass index, multiple gestations, extremes of maternal age, and familial aggregation[3,4]. Family history of PE suggests a genetic predisposition[5,6]. It is widely accepted that PE does not follow a Mendelian inheritance, except in a few families[6]. Instead, PE is the result of complex interactions between the maternal and fetal genotypes and environment factors[7]. Although the disease has been recognized ever since antiquity to this date, the understanding of its causes is still limited, and PE is known as "the disease of theories"[3]. These theories are often ascribed to two opposing schools of thought: on one side, the ischemia-reperfusion leads to oxidative stress and vascular disease, and, on the other, PE is seen as a maternal-paternal immune maladaptation[8]. A normal pregnancy is accompanied by an inflammatory response, which is exaggerated in the case of PE, and it has been postulated that endothelial dysfunction can be part of this exacerbated phenomenon[9]. The exaggerated inflammatory response to pregnancy could be a condition occurring in genetically predisposed women[10]. Studies on PE have focused predominantly on genes of the renin-angiotensin, coagulation, and angiogenic pathways[6]. Genetic variants associated with maladaptation of the maternal immune response mediated by cytokines and components of the innate immune system have been targeted in recent investigations of genetic markers for detection of PE risk and severity involving primarily Caucasian populations[11]. Considering all these aspects, we could hypothesize that a variation in a gene that codifies an important inflammatory cytokine could be involved in the genesis of PE. Therefore, we chose the lymphotoxin-alpha (LT-α) gene as a candidate gene for this disease. The TNF and LTA genes are arranged in tandem within the major histocompatibility complex class III region on the short arm of chromosome 6[12,13]. LT-α RNA was detected in both macrophage and non-macrophage populations in term decidua[14]. LT-α is a member of the tumor necrosis factor (TNF) superfamily that was originally thought to be functionally redundant to TNF, but these proteins were later found to have independent roles in driving lymphoid organogenesis[15]. LT-α regulates lymphoid organs in ontogeny by its production by lymphoid tissue inducer cells acting on stromal lymphoid tissue organizer cells by means of their induction of lymphoid chemokines and endothelial adhesion molecules during development. In the adult, LT maintains lymphoid organs through its production by T cells, B cells, and dendritic cells[16]. There are so far only few studies in the literature about LT-α gene polymorphisms and development of PE[12,13,17], none of them including the Brazilian population. The aim of this study was to investigate the frequencies of polymorphic alleles and genotypes for the LT-α gene, position +252 (rs909253), in Brazilian women with preeclampsia as compared to a control group. We hypothesized that patients that are homozygous for an LT-α gene polymorphism might be genetically predisposed to develop preeclampsia.

Methods

This was a case-control study. Women who volunteered for the study (n=145) (age range 18–75; mean 37.6±12.34 years) were recruited at the Gynecology and Obstetrics Clinic of the Hospital das Clínicas at Universidade Federal do Triângulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil. The sampling period was 2008 to 2014. All participants received an explanation about the research, after which they signed a written informed consent, previously approved by the UFTM Ethics Committee (Protocol number 1115-08). The study was conducted in accordance with the principles described in the WMA Declaration of Helsinki (2013).

The sample consisted of two groups: the patient group (n=30) (age range 18–37; mean 26.36±6.5), comprising pregnant women with preeclampsia diagnosed in accordance with the Report of the American National High Blood Pressure Education Program[2], and the control group (n=115), comprising women without a history of preeclampsia or any other hypertensive episode during pregnancy (age range 18–75; mean 40.6±11.8), not necessarily pregnant at the time of the study. From each woman, 20 mL of peripheral blood were collected.

The mass of red and white blood cells was submitted to osmotic lysis with Tris-EDTA lysis buffer (20:5) consisting of 1 M Tris-HCl and 0.5 M EDTA, pH 8. The samples were centrifuged at least three times at 13,346 g for 15 min. at controlled room temperature (approximately 27ºC). DNA was extracted using the phenol-chloroform method[18]. The samples were then resuspended in water, and the DNA was analyzed by electrophoresis on 1% agarose gel.

The presence of a polymorphism in the LT-α gene, position +252 (rs909253), was verified by PCR amplification followed by digestion with the appropriate restriction enzyme, as described previously[19]. PCR was carried out in a total volume of 30 µL containing 16 µL sterile and filtered Milli-Q water, 3.0 µL buffer without $MgCl_2$ (Invitrogen), 1.8 µL 50 mM $MgCl_2$ (Invitrogem), 2.0 µL 10 mMdNTPs (Invitrogen), 1.0 µL of each primer (forward primer: CTC CTG CAC CTG CCT GGA TC; reverse primer: GAA GAG ACG TTC AGG TGG TGT CAT) (Invitrogen), 0.20 µL (500 units, 5 U/mL) Taq DNA platinum polymerase (Invitrogen), and 2.0 mL genomic DNA at a concentration

of 20 mg/mL. The following PCR conditions were used: 5 min. at 95ºC for initial denaturation and 35 cycles at 95ºC for 1 min. (denaturation), 60ºC for 1 min. (primer annealing), and 72ºC for 1 min. (extension), followed by a final extension step at 72ºC for 5 min.

Next, RFLP was performed using 0.1 µL NcoI (10 U/µL) (New England Biolabs), 1.5 µL REACT 3 buffer provided with the restriction enzyme, 10.5 µL sterile and filtered Milli-Q water, and 3.0 µL of the PCR product, in a final volume of 12 µL. The samples were incubated overnight at controlled temperature (approximately 37ºC). The digestion products were analyzed on a 10% polyacrylamide gel. The LTA + 252A allele produces one fragment of 368 bp (base pair), whereas the LTA + 252G allele produces two fragments (235 and 133 bp, respectively). The heterozygous genotype presents three fragments: 368, 235 and 133 bp, respectively.

The Hardy-Weinberg Equilibrium (HWE) was analysed using the χ^2 test with the Haploview 4.2 program. A statistical power of 80.7% was tested using the G* Power program 3.1.9.2. Furthermore, a post hoc test with the total sample (n=145) was performed, with an effect size of 0.26 and an alpha significance level of 0.05. Genotype and allele frequencies were analyzed statistically by the χ^2 test. The level of significance was set at 5% (p=0.05).

Results

A total of 145 women were included in the study — 115 (79,3%) in the control group and 30 (20,7%) in the preeclampsia patient group. The control group, with an age range of 40.6±11.8, was composed of women with at least two healthy children and hypertensive episode during the pregnancies, but who were not necessarily pregnant at the time of blood collection. The preeclampsia group, with an age range of 26.3±6.5, was composed of pregnant women with preeclampsia diagnosed according to the Report of the American National High Blood Pressure Education Program[2]. In the control group, the average number of pregnancies was 3.1±1.6, while in the preeclampsia group it was 1.7±1.0. With regard to the HWE, the control group was not in HWE (p<0.001), whereas the preeclampsia group was in HWE (p=1.0).

The frequencies of genotypes AA, AG, GG in the control and preeclampsia groups were analyzed and compared. Table 1 shows the distribution of the LT-α genotypes at position +252 (rs909253). No association was observed between genotypes and preeclampsia development (χ^2=2.0; p=0.4). When the AG and GG genotypes were grouped as allele-G-present and the genotype AA as allele-G-absent, the results showed the presence of allele G was not significantly different between cases (women with preeclampsia) and controls (χ^2=0.0; p=1.0) (Table 2). An odds ratio of 1.0 was observed for G presence (95%CI 0.4–2.2), which was statistically not significant.

Table 1. *LT-a* genotypes, position +252, found in women with and without preeclampsia

Genotypes	Phenotype						p-value (χ^2)
	Control group		Preeclampsia		Total		
	n	%	n	%	n	%	
AA	65	79.3	17	20.7	82	56.6	0.4
AG	32	74.4	11	25.6	43	29.6	
GG	18	90.0	02	10.0	20	13.8	
Total	115	79.3	30	20.7	145	100	

Table 2. Allele G presence in women with and without preeclampsia

Presence of G	Phenotype				Total (100%)	p-value (χ^2)
	Control group		Preeclampsia			
	n	%	n	%		
G present	65	79.3	17	20.7	82	1.0
G absent	50	79.4	13	20.6	63	
Total	115	79.3	30	20.7	145	

Discussion

To our best knowledge, this is the first study using LT-α gene polymorphisms, position +252 (rs909253), in Brazilian women with preeclampsia. However, there are previous studies[12,13,17] about the same issue in different populations.

The first one was performed in a Dutch population and is a familial, case-control study. The authors did not find any evidence of an association or linkage with familial preeclampsia[12]. Another study was performed in North American women (USA) with severe preeclampsia. It was a case-control study that analyzed the maternal and fetal genotype[13], and did not observe an association with the polymorphism studied either. The third study genotyped 503 tagSNPs in 40 genes related to inflammation and found that LT-α/TNF were associated with PE in European Americans[17]. However, in this report, it was analyzed another LT-αgene polymorphism (rs2229094)[17] instead of the one studied by us. Although there are some differences between the studies cited and ours, they all agree that there is no association between the respective polymorphism studied and the development of PE.

It is difficult to compare our results with those presented by Lachmeijer et al.[12] because the strategy used was quite different. We performed a case-control study focusing a single polymorphism, while they analyzed a TNF-I haplotype. The LT-α gene is located near the TNF gene, and the results presented by those authors include the LT-α gene, but in association with TNF (haplotype). Thus, although they suggest the absence of association with PE, we cannot say whose effect it is — of the TNF or of the LT-α gene.

Comparing our results with those of Livingston et al.[13], we noticed some differences such as the selection of women with severe PE. In the current study, we did not categorize the PE according to its severity. We included women with

mild to severe PE. Although this is an important issue to consider, their results did not differ from ours. Another point to take into account is that the populations were different in all the studies cited. It seems, however, that this did not influence the association with PE.

On the other hand, Harmon et al.[17] detected an association between an LT-α gene polymorphism and PE development, but the polymorphism analysed by them was different from the one analyzed here.

LT-α is related to the presence of a strong inflammatory profile and the induction of the generation of lymphoid chemokines and endothelial adhesion molecules during development. Although it would be expected that genetic variations in LT-α gene could contribute to the appearance of different phenotypes, our results were unable to show any difference between the two groups. Besides, we did not study other polymorphisms in the same gene that could contribute to PE development in this population. We are aware of the fact that our study suffers from some notable limitations, such as the lack of fetal DNA and the absence of data on the severity of the disease and the timing of clinical onset. These additional pieces of information may be important to detect some influence of this polymorphism in the development of PE.

PE is a heterogeneous disease and may encompass a number of distinct phenotypes with shared clinical presentation and with multiple manifestations. Its etiology is certain to be equally as complex. It is unlikely that a single causative gene exists. Rather, multiple maternal and fetal genes probably interact with each other and with the environment to influence the disease outcome[17]. The susceptibility to PE may also be the result of interactions between genetic susceptibility and exposure to environmental factors such as smoking, infections, nutrition or environmental toxicants[17]. Although the LT-α gene polymorphism, position +252 (rs909253), seems not to be a major candidate for the development of PE, other inflammatory genes should be investigated, and studies involving gene-environment interactions should be performed in order to reach a better understanding of the etiology of PE.

Acknowledgments

To Prof. Dr. Virmondes Rodrigues Jr. for providing some reagents, and to *Fundação de Amparo à Pesquisa do Estado de Minas Gerais* (FAPEMIG) NºCBB\APQ000838-11 and *Universidade Federal do Triângulo Mineiro* (UFTM) for financial support.

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