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ARTICLE

Lack of association between the prothrombin rs1799963 polymorphism and juvenile myoclonic epilepsy

Ausência de associação entre o polimorfismo G20210A (rs1799963) da protrombina e epilepsia mioclônica juvenil

João Paulo Lopes Born[1], Bruna Priscila dos Santos[1], Rodrigo Secolin[2], Fernando Tenório Gameleira[3], Tiago Gomes de Andrade[3], Luciana Cláudia Herculano Machado[4], Lívia Leite Góes Gitaí[5], Daniel Leite Góes Gitaí[6]

ABSTRACT

Juvenile myoclonic epilepsy (JME) accounts for 26% of generalized idiopathic epileptic syndromes. The highest levels of thrombin activity are closely involved in the development of neurological diseases, including epilepsy. The prothrombin c.20210G>A (rs1799963) variation, which alters prothrombin mRNA stability, is associated with high plasma prothrombin levels. **Objective:** The present study was designed to investigate whether the SNP rs1799963 is a risk factor for JME in the northeastern Brazilian population. **Results:** The polymorphism was genotyped in 207 controls and 123 patients using polymerase chain reaction-restriction fragment length polymorphism method. No significant differences were observed in the genotype and allele frequencies of this polymorphism between cases and controls. **Conclusion:** These results present no evidence for an association of rs1799963 with JME. Further studies including other types of epilepsy are required to investigate the involvement of prothrombin gene in the genetic susceptibility to chronic seizure.

Keywords: polymorphism, prothrombin, juvenile myoclonic epilepsy.

RESUMO

Epilepsia mioclônica juvenil (EMJ) representa 26% das síndromes epilépticas idiopáticas generalizadas. Níveis elevados de atividade da trombina estão intimamente envolvidos no desenvolvimento de distúrbios neurológicos, incluindo epilepsia. A variante c.20210G>A (rs1799963) do gene de protrombina, que altera a estabilidade do RNAm, está associada com altos níveis de protrombina no plasma. **Objetivo:** Investigar se o SNP rs1799963 é um fator de risco para EMJ em uma amostra da população do nordeste brasileiro. **Resultados:** O polimorfismo foi genotipado em 123 pacientes e 207 controles usando a reação de polimerase em cadeia com restrição de polimorfismo. Não observamos diferença significativa nas frequências alélicas e genotípicas deste polimorfismo, entre as populações de pacientes e controle. **Conclusão:** Estes resultados não demonstram evidências para uma associação do polimorfismo rs1799963 com EMJ. Estudos posteriores, incluindo outros tipos de epilepsia, são necessários para investigar o envolvimento do gene protrombina na susceptibilidade genética a crises crônicas.

Palavras-chave: polimorfismo, protrombina, epilepsia mioclônica juvenil.

Juvenile myoclonic epilepsy (JME) is a subtype of common idiopathic generalized epilepsy (IGE) and accounts for 10% of all forms of epilepsy and up to 26% of IGE. Onset is at puberty with equal sex ratio and it is characterized by myoclonic jerks, occasional generalized tonic-clonic seizures, and sometimes absence seizures[1]. It is also highly drug-dependent, since a

[1]Universidade Federal de Alagoas, Instituto de Ciências Biológicas e da Saúde, Maceió AL, Brazil;

[2]Universidade de Campinas, Departamento de Genética Médica, Campinas SP, Brazil;

[3]Universidade Federal de Alagoas, Arapiraca AL, Brazil;

[4]Universidade Federal de Alagoas, Instituto de Ciências Biológicas e da Saúde, Departamento de Histologia, Maceió AL, Brazil;

[5]Universidade Federal de Alagoas, Faculdade de Medicina, Maceió AL, Brazil;

[6]Universidade Federal de Alagoas, Instituto de Ciências Biológicas e da Saúde, Departamento de Biologia Celular Molecular e Genética, Maceió AL, Brazil.

Correspondence: Daniel L. G. Gitai; Av. Lourival Melo Mota, s/n, Cidade Universitária, Universidade Federal de Alagoas, Instituto de Ciências Biológicas e da Saúde; 57072-900 Maceió AL, Brasil; E-mail: danielgitai@gmail.com

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frequent recurrence is reported after antiepileptic drugs (AED) discontinuation[2]. Genetic factors are known to play an important role in the etiology of JME. Despite the existence of rare mutations responsible for some familial forms inherited in a mendelian pattern, the genetics of JME is complex and probably reflect the simultaneous involvement of multiple genes with minor effect and environmental factors[3]. The identification of these susceptibility genes is a great challenge[4]. For this purpose, an experimental approach that has been widely used is genetic association studies directed to candidate genes selected according to their molecular function.

Evidences support a role for serine proteases in the sequence of events that can lead to neuropathological situations[5]. Thrombin, a serine protease essential in the coagulation cascade is involved in the production of seizures[6]. It was demonstrated that active thrombin injected into rat brains resulted in both electrographic and clinical seizures[7]. It was proposed that thrombin, acting on its receptor, protease-activated receptor 1 (PAR1), has marked effects on the production of long-term potentiation (LTP) in responses to afferent stimulation and that it enhances the sensitivity to epileptic seizures in brain slices[6]. Thrombin may triggers the generation of epileptic seizures by reducing the inhibitory and increasing the excitatory tone in CA3 neurons[5,8,9]. The brain can be exposed to thrombin as a result of increased permeability of the blood-brain barrier that takes place during severe epilepsy. Moreover, at a brain with a intact BBB as supposed for idiophatic epilepsy, thrombin can be generated locally from prothrombin which was shown to be expressed in rat and human brains[10,11]. In support of this, patients with neurological disorders present the levels of local prothrombin, thrombin and PAR1 increased on astrocytes and neurons[12].

The SNP rs1799963 leads to the c.20210G>A variation in the 3´-UTR region of prothrombin gene, which is a bifunctional polymorphism that alters prothrombin mRNA stability and processing[13]. Three-fold more prothrombin protein and mRNA were produced in NIH-3T3 cells transfected with the prothrombin cDNAs containing the rs1799963*A allele compared to cells with rs1799963*G[13]. In fact, this polymorphism is associated with high plasma prothrombin levels and with an increased risk of thrombosis and other disease conditions[14,15]. These physiological attributes make the protrombin an interesting candidate gene for investigation in an IGE syndrome such as JME. Through this case/control design, we intended to investigate whether SNP rs1799963 show association with JME in one northeastern Brazilian population.

METHOD

Patients and controls

This study included 123 unrelated Brazilian patients with JME and 207 normal control subjects. The study was approved by the Ethics Committee of the Federal University of Alagoas, Brazil (no. 0900-2005-11). The subjects signed informed consent before blood tests were performed. Cases were matched with controls according to age, sex, ethnicity, and geographic location of origin. Individuals with a history of epileptic seizures or neuropsychiatric disorders were excluded from the control sample. All patients were recruited from the state of Alagoas in northeastern Brazil. The probands were unambiguously diagnosed cases of JME with classification based on the published criteria of the Commission on Classification and Terminology of the International League Against Epilepsy[16]. All patients were submitted to electroencephalography analysis and only those with generalized spike wave were included in this study.

Genetic analysis

The SNP rs1799963 was detected by the use of the protocols described by Kruse et al.[17], with modification. Briefly, DNA was extracted from peripheral blood leucocytes using FlexiGene DNA Kit (Qiagen). A total of 50 ng of genomic DNA was mixed with 5 pmol of each polymerase chain reaction (PCR) primer in a total volume of 25 μL containing 10 mM Tris-hydrochloride, pH 8.8; 50 mM potassium chloride; 0.8% Nonidet P40; 1.5 mM magnesium chloride; 0.2 mM each deoxyribonucleotide triphosphate; and 0.5 of DNA polymerase (Fermentas Life Sciences). Following primers: 5` CAATAAAAGTGACTCTCATC 3´ (forward, underlined T indicates change to create TaqI site) and 5´ AGGTGGTGGATTCTTAAGTC 3` (reverse) were used to obtain PCR products of 118 bp size (Gene-Bank accession no. ref|NT_009237.18). The reaction mixture was cycled as follows in a DNA thermal cycler (BioCycler, MJ96G model): inicial denaturation step at 94ºC for 3 min, 35 cycles at 94ºC for 1 min, 55ºC for 1 min and 72ºC for 1 min, and a final extension at 72ºC for 7 min. A 12 μL aliquot of the amplicon was then submitted to restriction reaction at 65°C for 6 h with Taq I Restriction Enzyme (New England Biolabs, UK; cat. no. R0149L). Digestion products were separated by electrophoresis on 2.5% agarose gels and were visualized by ultraviolet light after staining with ethidium bromide. In this assay, the rs1799963*G allele was digested, whereas the rs1799963*A was not. Digestion of the wild-type PCR product gave a fragment of 98 bp, and digestion of the variant PCR product gave a fragment of 118 bp.

Statistical analysis

All descriptive and statistical analysis was performed using SNPStats[18]. Allele and genotype frequencies were calculated by counting and Hardy-Weinberg equilibrium was estimated using Chi-square test. Genetic association analysis was performed using logistic regression analysis, including odds ratio (OR) with the 95% confidence interval (95%CI). We then estimated the OR adjusted by those clinical variables that were selected in the general linear model analysis with a logistic regression stepwise procedure. The selected clinical variables evaluated were sex, pharmacological treatment and type of seizure. A priori statistical power analysis

was performed by GPOWER 3.1.7 software[19] using the following parameters: logistic regression test; two-tail; OR = 1.5; nominal significance level $\alpha = 0.05$; n = 330.

RESULTS

The mean age at onset of the JME probands was 13 years (standard deviation (SD) = 4.0), of which 65% were females. The triad of myoclonus, absences, and generalized tonic-clonic seizures was observed in 41%, the combination of myoclonus and generalized tonic-clonic seizures in 50%, the combination of myoclonus and absence was observed in 8%, and the myoclonus alone in 1% of patients. Of the patients, 58% were receiving monotherapy treatment with sodium valproate, 7% with Phenobarbital and 5% with lamotrigine, carbamazepine or fenitoine. The other 30% of patients were receiving polytherapy treatment. The populations studied had the following ethnic distribution: among patients 23% were Caucasians, 73.7% were Mulatto and 3.3% were African descent; among controls 30.3% were Caucasians, 62.2% were Mulatto and 7.5 % were of African descent.

The genotype distribution did not deviate significantly from that expected by Hardy-Weinberg equilibrium (p = 1.0). Duplicated genotyping of 20% of samples revealed 100% of genotyping concordance. According Table, the proportions of rs1799963*GG and rs1799963GA were 97.6% and 2.4% in JME patients, and 97.6% and 2.4% in control group, respectively. None individual was genotyped as rs1799963*AA. The allele frequencies of rs1799963*G and rs1799963*A were 99% and 1% in JME patients, and 99% and 1% in control group, respectively. Logistic regression results did not showed significant association signal between rs1799963 and JME phenotype (p = 0.99) even when odds ratio was adjusted by clinical variables (data not shown). This study showed a statistical power of 82.81% to detect association of SNP rs1799963 as susceptibility for JME.

Table. Genotype frequencies of G20210A polymorphism in the controls and JME patients.

Genotype	Control (207)	JME (123)	OR (95%CI)	p-value
GG	202	120	1.00	0.99
GA	5	3	1.01 (0.24-4.30)	

The p-values were calculated from logistic regression analysis adjusting ethnicity and sex. JME: Juvenile myoclonic epilepsy; OR: Odds ratio; CI: Confidence interval.

DISCUSSION

To our knowledge this is the first association study between the SNP rs1799963 of the prothrombin gene and epilepsy. Patients with JME, the most common subtype of idiopathic generalized epilepsy which presents a strong influence of the genetic component, were selected. We studied rs1799963 in JME because it has been functionally related to altered levels of products (RNA and proteins) of the prothrombin gene and thrombin protein[13,14]. The highest levels of thrombin activity are closely involved in the development of neurological diseases, including epilepsy[9]. However, our data did not show a significant difference in the genotype and allele frequencies of this polymorphism between cases and controls, suggesting that there is no association of rs1799963 with JME in this Brazilian sample.

Although the sample size used in this analysis, statistical power showed that this sample is enough to detect association with JME Additionaly, our study included only the patients with JME rather than analyzing a clinically heterogeneous population with several epilepsy syndromes. This approach might have minimized possible bias from the limited sample size, which is a common problem in genetic association studies[20,21]. However, replication studies in independent sample are needed in order to strength our findings.

Our data also contributes to the investigation of the frequency of rs1799963 polymorphism in Brazilian population. Considering the general population, including both patient and control individuals, the prevalence of heterozygous carriers were 0.6% and 1.8% among Caucasian and Mulatto individuals, respectively. The mutant allele rs1799963*A was not detected in Afro-descendants individuals. These findings are in agreement with other reports showing that the prothrombin polymorphism varies among different ethnic groups, presenting a very low frequency in Afro-descendants (0.3%)[22].

The frequencies of genotypes (98% for rs1799963*GG and 2% for rs1799963*GA) and alleles (99% for rs1799963*G and 1% for rs1799963*A) in northeastern Brazilian population (patients and control) observed in this study are consistent with results in other Brazilian subpopulations reported in previous studies. In fact, in general Brazilian population the mutant allele frequency ranged between 0.7%-3.6% (rs1799963*A)[23,24,25]. Brazilian population has an extremely heterogeneous ethnic composition, unevenly distributed across the country with variable degrees of admixtures[25]. This heterogeneity may also explain the allele frequency spectrum observed when different reports are considered. Various studies showed that the carrier frequency of the prothrombin rs1799963 is different among populations who immigrated to Brazil. In healthy Southern Europeans it was around 3%[26], nearly twice as high as the data obtained from Northern European populations[27,28]. In contrast, rs1799963 was found to be rare in African Americans and totally absent among Japanese and Koreans[29,30]. Knowing the frequency of prothrombin polymorphism in Brazilian miscigenated population and subgroups may have clinical and epidemiological implications.

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