

Relationship between Methylenetetrahydrofolate Reductase (C677T), Factor V Leiden (G1691A), Prothrombin Mutation (G20210A) and Severe Preeclampsia in a Brazilian Population

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Abstract

Objectives: To better understand the etiologic factors that can influence preeclampsia, we investigated hereditary factors for thrombosis, FV Leiden, F II 20210A mutation and the polymorphism C677T of the MTHFR, as singly and as in association, in a group of women from Ceará state-Northeast Brazil with severe preeclampsia. **Material and Methods:** We conducted a case-control study. 101 cases of severe preeclampsia were recruited from School Maternity Assis Chateaubriand, a reference Maternity of State of Ceará, Brazil, from December 2009 to December 2010. For clinical correlations, women were interrogated about fetal low weight (less than 2500 grams) and fetal loss in previous pregnancies. Low weight, fetal loss and necessity of neonatal intensive care unit (neonatal ICU) related to current pregnancy were registered. 245 healthy voluntary women were recruited from the blood bank donors to verify the frequency of FV Leiden, FII and MTHFR as a control group. The study was approved by the Committee on Ethics in Human Research of Maternidade Escola Assis Chateaubriand and all individuals gave their informed consent to participate in the study. Standard veinipuncture, with EDTA as anticoagulant, was used to collect blood samples. Genomic DNA was extracted, soon after, using a salting out method. Agarose

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gel electrophoresis with ethidium bromide staining was performed to ensure the quality of DNA extraction. Results: The frequencies of FV Leiden and FII mutation carrier were 0.99% (1/101) for both factors in the preeclampsia patients and 1.86% (4/214) and 0.93% (2/214), respectively, in control group. All mutates were heterozygous and concomitance of both mutations was not found. The genotype distribution of the MTHFR C677T in the patients and controls frequencies was in Hardy-Weinberg equilibrium ($p \leq 0.05$). No statistical difference was observed between cases and controls in MTHFR genotypes or alleles. Conclusions: The FV Leiden, FII G20210A mutation and MTHFR C677T were not risks for preeclampsia development. FV Leiden and FII G20210A mutations had low frequency in the population studied, which may justify the absence of association.

Keywords

Preeclampsia, Thrombophilia, Neonatal Outcomes, FV Leiden, Prothrombin, MTHFR

1. Introduction

Preeclampsia, an important cause of maternal and fetal morbidity and mortality, is a pregnancy-specific syndrome defined as elevated blood pressure and proteinuria. The etiology of preeclampsia is unknown, but genetic and immunologic factors and abnormal placentation have been proposed to play a causative role [1] [2].

Severe preeclampsia has been shown to be associated with thrombophilia and abnormal placentation increases tendency toward thrombosis influenced by a genetic risk factor [3]-[5]. Therefore, the correlation between genetic risk factor for venous thrombosis and preeclampsia is the focus of some investigations that ask for the causative causes of the preeclampsia [6]-[8]. The hereditary factors frequently associated to thrombophilia are the mutation G1591A in gene of Factor V (Factor V Leiden—FV Leiden), the mutation G20210A in gene of prothrombin (FII) and the polymorphism C677T of methylenetetrahydrofolate reductase (MTHFR) [9].

The FV Leiden variant arises as a result of a point mutation at nucleotide position 1691, resulting in an arginine to a glutamine substitution at position 506 that reduces its sensitivity to inactivation by activated protein C. FV Leiden has been associated with familial thrombophilia and indeed, is the commonest inherited risk factor for venous thrombosis [10] [11].

A G to A transition at nucleotide position 20210 in the 3'-untranslated region of the prothrombin/FactorII (FII) gene is associated with higher plasma prothrombin concentrations, augmented thrombin generation and increased risk of thrombotic disease [10] [11]. This mutation has a wide range of frequency, varying from 0% to 14% depending on the ethnic origin [12].

The C677T MTHFR polymorphism is responsible for a decreased MTHFR activity and associated with increased plasma homocysteine concentrations that are induced for a folate metabolism disturbance [13] [14]. Hyperhomocystenemia has been implicated in thrombophilia and preeclampsia [15] [16].

Previous studies results are contradictory to show association of these factors and preeclampsia [17] [18]. Different prevalence of these mutations and polymorphism in different populations depending on ethnic background could explain this contradiction.

Preeclampsia is a disorder found in a large number of pregnant women in our clinical routine. To better understand the etiologic factors that can influence the syndrome, we investigated hereditary factors for thrombosis, FV Leiden, F II 20210A mutation and the polymorphism C677T of the MTHFR, as independent and as in association, in a group of women from Ceará state (Northeast Brazil) with severe preeclampsia. Also, the combination of these parameters and fetal clinical history of low weight, fetal loss and necessity of neonatal intensive care unit (neonatal ICU) were analyzed.

2. Material and Methods

2.1. Subjects

We conducted a case-control study with women who had severe preeclampsia recruited from Maternidade Escola Assis Chateaubriand, a reference Maternity of State of Ceará, Brazil, from December 2009 to December 2010. We identified 101 cases of severe preeclampsia according to the criteria proposed by American College of Obstetricians and Gynecologists, 2002 [19].

Preeclampsia was defined as a blood systolic pressure of 140 mm Hg or higher, or blood diastolic pressure of 90 mm Hg or higher after 20 weeks of gestation in a woman with previously normal blood pressure, and proteinuria of 0.3 g or more of protein in a 24-hour urine collection (1+ or greater on a urine dipstick test). The severe preeclampsia was defined as a blood pressure $\geq 160/110$ mm Hg, or urinary protein excretion ≥ 5 g (3+ or greater on urine dipstick testing), a platelet count of $<100,000 \text{ mm}^{-3}$ in at least two samples; oliguria (<500 mL per day); persistent and severe symptoms as: altered mental status, headaches, blurred vision or blindness; presence of multiorgan involvement such as pulmonary edema. Also, the combination of hemolysis, abnormal liver enzymes associated with persistent epigastric or upper right quadrant pain [19].

For clinical correlations, women were interrogated about fetal low weight (less than 2500 grams) and fetal loss in previous pregnancies. Low weight, fetal loss and necessity of neonatal intensive care unit (neonatal ICU) related to current pregnancy were registered.

A number of 246 healthy voluntary women were recruited from the blood bank donors to verify the frequency of FV Leiden, FII and MTHFR as a control group. None of them had experienced any past or current thrombotic events or had a family history of venous or arterial thrombosis.

The study was approved by Human Research Ethics Committee of Maternidade Escola Assis Chateaubriand and all individuals gave their informed consent to participate in the study.

Standard venipuncture, with EDTA as anticoagulant, was used to collect blood samples. Genomic DNA was extracted, soon after, using a salting out method. Agarose gel electrophoresis with ethidium bromide staining was performed to ensure the quality of DNA extraction.

2.2. Genotype Analysis

The genotyping was performed by polymerase chain reaction restriction fragment length polymorphism technique (PCR-RFLP) for all the genes analyzed.

Factor V Leiden: amplification of a 220 pb DNA fragment of the FV gene surrounding nucleotide 1691 was done using primers and conditions as described by Bertina *et al.* [20]. Subsequently, 10 μL of the PCR product was digested with 2.5 U of MNLI to define the genotyping. This enzyme cleaves the normal allele in two sites, creating 3 fragments with 116 pb, 67 pb and 37 bp. Mutate allele abolishes one site resulting in two fragments of 153 pb and 67 pb. Heterozygous individuals had both normal and mutant digestion products.

Prothrombin (FII) G20210A mutation: The analysis was performed according to Poort *et al.* [21]. The amplified PCR product containing 345 pb was digested with HindIII according with the manufactured instruction. The wild type allele remains intact while in the mutated allele a fragment of 332 pb is observed.

MTHFRC677T: The fragment of 198pb from exon 4 of the gene was amplified using primers sequence and conditions described by Froost *et al.* [22]. The restriction endonuclease HinfI was used to digest the 10 μL PCR product to determinate the genotype. The C to T transition at the nucleotide 677 (valine variant) creates a new HinfI site which generates fragments of 175 pb and 23 pb.

PCR products for all fragments were verified in agarose gel at 2% ethidium bromide stained and the fragments digested were visualized by silver stained 7% polyacrylamide gel.

Quality control samples were included in all laboratory analysis and the confirmation of the mutation was done for both FV Leiden and FII. Random samples were reanalyzed for laboratory procedures control.

2.3. Statistical Analysis

Differences between genotypes and clinical parameters were calculated with χ^2 test. Relative risks were estimated by odds ratio and p value <0.05 were considered statistically significant for all analysis. All confidence intervals were calculated at the 95% level. The analyses were performed with SPSS software (version 10.0).

3. Results

The frequencies of FV Leiden and FII mutation carrier were 0.99% (1/101) for both factors in the preeclampsia patients and 1.86% (4/214) and 0.93% (2/214), respectively, in control group. All mutates were heterozygous and concomitance of both mutations were not found. **Table 1** shows the characteristics of mutated patients.

The genotype distribution of the MTHFR C677T in the patients and controls are presented in **Table 2**. All genotypes frequencies were in Hardy-Weinberg equilibrium ($p \leq 0.05$). No statistical difference was observed between cases and controls in MTHFR genotypes or alleles.

Table 3 shows the statistical analysis of the association among the neonatal characteristics and maternal genotype. The genotype TT was significantly associated with previous low weight and neonatal ICU both increasing risk. The genotype CC was significantly associated with neonatal ICU as a protection factor.

4. Discussion

The low frequency of FV and FII mutations in women with severe preeclampsia (0.99%, only in heterozygosis) does not show an evident relationship with the development of the disease. These results suggest that thrombophilic predisposing mutations Factor V Leiden and FII G20210A don't increase risk of preeclampsia in this Brazilian population.

Similar results were previously reported by Dalmaz *et al.* [23] in Brazilian women, in which no significant association was found between preeclampsia and FV Leiden. However, the association between preeclampsia and FV Leiden varies around the world [2]-[4] [8]. Like this study, any association was found in Netherlands and in English population [24] [25]. However, Hoffman *et al.* [15] related an increase from three to five times in preeclampsia incidence in carriers of FV Leiden. Also, Dizon-Townson *et al.* [25] showed that women with FV Leiden mutation are predisposed to severe preeclampsia.

Conflicting data were also reported for FII mutation. Studying an Italian severe preeclamptic women group, Mello *et al.* [26] found a frequency of 10.8% of FII G20210A +/- in preeclamptic women, with a significant association. Kupferminc *et al.* [27] found a frequency of 8.0% FII G20210A +/- in Israelipopulation. This association was not significant in spite of the high frequency ($p = 0.14$; OR = 2.6; 95% CI = 0.7 - 10.2). Using a multiple logistic regression analysis, the prothrombin mutation was significantly associated with preeclampsia ($p = 0.03$; OR = 4.5; 95% CI = 1.12 - 19). Unlike these studies, our data didn't show any association between preeclampsia and FII mutation. Also, studying FII mutation in a Brazilian population, Dalmaz *et al.* [23] found a

Table 1. Characteristics of FV Leiden and FII mutated patients.

	FATOR II mutation	FATOR V mutation
Age	33 years old	36 years old
Pregnancies	9	6
Births	3	6
Abortions	6	0
Oral contraceptive use	Yes, for 6 years.	Yes, for 3 months.
Familiar obstetric complications	No obstetric complications.	Sister had prematurity.
Previous obstetric complications	Severe preeclampsia in first pregnancy.	No obstetric complications.
Comorbidities in pregnancy	Severe preeclampsia.	Severe preeclampsia + chronic hypertension
Neonatal weight	2820 g	1825 g
Time at birth	38 weeks	37 weeks and 6 days
Neonatal comorbidities	No	No

Table 2. Frequencies of MTHFR genotypes and alleles in women with preeclampsia and controls.

Genotype/Allele	Preeclamptic group n = 101	Controls n = 245	p	Odds ratio (95% CI)
CC	46 (45.54%)	115 (46.93%)	0.813	0.95 (0.58 - 1.25)
CT	47 (46.53%)	112 (45.71%)	0.889	1.03 (0.63 - 1.69)
TT	8 (7.92%)	18 (7.34%)	0.853	1.08 (0.42 - 2.75)
C	139 (68.81%)	342 (69.79%)	0.798	0.95 (0.66 - 1.38)
T	63 (31.18%)	148 (30.20%)	0.798	1.05 (0.72 - 1.52)

Table 3. Analysis of the interaction among MTHFR genotypes and alleles in women with preeclampsia and neonatal characteristics.

		Previous Neonatal Low Weight (<2500 g)	Previous Neonatal Loss	Neonatal ICU	Neonatal Low Weight (<2500 g)	Neonatal Loss
CC	OR	0.48	1.20 ^{***}	0.33	0.44	0.00 ^{***}
	(95% CI)	0.09 - 2.24	0.00 - 45.41	0.09 - 1.08	1.77 - 1.12	0.00 - 1.09
	P	0.339	1.000	0.039*	0.057 ^{**}	0.248
CT	OR	0.74	0.00	1.53	2.47	0.00
	(95% CI)	0.16 - 3.26	0.00 - 4.93	0.52 - 4.56	0.59 - 3.67	0.00 - 2.68
	P	0.747	0.499	0.396	0.357	0.593
TT	OR	8.70	13.14 ^{***}	4.81	3.68	6.50
	(95% CI)	1.26 - 59.98	0.00 - 555.6	0.89 - 26.43	0.70 - 21.12	0.00 - 114.1
	P	0.022*	0.152	0.047*	0.114	0.221
C	OR	0.54	0.86	0.55	0.66	0.01
	(95% CI)	0.23 - 1.27	0.11 - 5.58	0.29 - 1.03	0.39 - 1.11	0.00 - 0.03
	P	0.484	0.033*	0.460	0.791	0.283
T	OR	1.34	5.64	1.27	0.92	1.72
	(95% CI)	0.54 - 3.26	0.65 - 41.21	0.64 - 2.54	0.49 - 1.74	0.57 - 5.09
	P	0.126	0.861	0.045*	0.098**	0.000*

*Represents statistically significant association (p value < 0.05); **Represents a trend to significant association (p value next to 0.05); *** One or more variants for Odds Ratio calculating were zero.

frequency of 4% in heterozygosis and 1.3% in homozygosis (control group: 0.7% in heterozygosis), but no significant associations were found both for GA or AA genotypes (OR = 8.11; 95% CI = 0.89 - 73.92).

The possible explanations for the diversity of results among the literature reports related to FV Leiden and FII mutation could be due the ethnic difference of the studied groups, or to the fact that some authors only study severe preeclampsia, when other includes preeclampsia and eclampsia.

The frequency of MTHFR TT (7.92%) in preeclampsia observed in our study is similar to results from several countries. The difference between our data and the other Brazilian study [28] could be due to the low frequency of the MTHFR TT genotype in the population studied (7.34% and 12.4% respectively). In fact, Brazilian population is heterogeneous, formatted by European, African, Asian, as well Indian groups, moreover, with differences in its composition between regions of the country, so the difference found could be due to the differences of ethnic composition. That point is reinforced by the finding of individuals homozygous for FII mutation in Dalmaz study [23]. In the same study, it is found a high incidence (22.7%) of the TT genotype for the MTHFR in relation to results found in our study (7.92%).

Worldwide variations in incidence of these thrombophilic genetic factors highlight the importance of screening in different ethnic populations.

Despite of any association between preeclampsia and MTHFR genotype, we found that MTHFR TT increased the risk for neonatal low weight, neonatal loss and necessity of neonatal intensive care unit. There are few studies relating MTHFT polymorphism to neonatal complications in preeclamptic women. The majority of articles have studied this relation in healthy women. Infante-Rivard *et al.* [29] who were studying healthy women found that there was no relationship between the MTHFR (677) T allele and the risk of intrauterine growth restriction (CT: OR = 0.84, 95% CI 0.63 - 1.12) (TT: OR = 1.17, 95% CI 0.72 - 1.92). On the other hand, Martinelli *et al.* [30] who were studying the association between unexplained or gestational-hypertension-associated fetal growth restriction and MTHFR polymorphism, found a 677 TT genotype frequency of 17 (27.8%), a similar result to our study, and a significant association.

Folate is an essential nutrient required for the provision of one-carbon sources in biosynthetic processes [31]. It is known that is essential for cell division and growth [32] and deficiencies in folate impair fetal development [33]. It has been reported that folate deficiency during pregnancy increases the risk of the development of neur-

al-tube defects in the infant. The allele T in 677 MTHFR has reduced enzyme activity. In this way, it could harm fetal conditions in women with preeclampsia, a pathologic condition that independently harms intrauterine homeostasis [34] [35].

5. Conclusion

In Summary, in our study we found that FV Leiden, FII G20210A mutation and MTHFR C677T didn't increase risk for preeclampsia development. FV Leiden and FII G20210A mutations had low frequency in the population studied, which may justify the absence of association. Also, the polymorphism of C677T was not associated to preeclampsia. Other genetic and environmental risk factors should contribute to the development of preeclampsia in the population studied. On the other hand, the polymorphism of the C677T in women with preeclampsia was relevant to fetal development, since the TT genotype was associated with low weight and neonatal ICU.

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