ANALYSIS OF FACTOR V LEIDEN IN WOMEN WITH FETAL LOSS USING SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP PROCEDURE)

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INTRODUCTION

Fetal loss represents a problem in obstetric complications caused by inherited and environmental factors. Inherited thrombophilia plays an important role in the pathogenesis of fetal loss because of the thrombotic events that occur in the placenta.1

Factor V Leiden represents the most common inherited risk factor for thrombophilia. The prevalence is 3-8% in European different populations. There is a considerable difference between European countries with a high prevalence in Greece and Sweden (10-15%) and a low prevalence in Italy and Spain (2-3%).2

In the meantime this polymorphism is rare in Asian, African and indigenous Australian populations.3

Factor V Leiden represents a single base pair substitution, G → A at nucleotide 1691 (G1691A) in the DNA of factor V Leiden. The substitution results in a change of the amino acid glutamic acid (Glu) to alanine (Ala) at position 506 of the factor V protein. The factor V Leiden mutation leads to a decrease in the activation of the antithrombin III.4

The factor V Leiden mutation is associated with an increased risk of deep venous thrombosis and pulmonary embolism.5 The factor V Leiden mutation also increases the risk of fetal loss.6

The objective of this study was to determine the prevalence of factor V Leiden mutation in women with fetal loss and controls. The study was conducted at the University of Medicine and Pharmacy, Cluj-Napoca, Romania.

METHODS

We tested for factor V Leiden mutation 56 women with fetal loss in different trimester of pregnancy (mean age, 29.42 ± 3.24 years, median 29) compared with 42 control women with at least one normal pregnancy (mean age, 28.96 ± 3.59 years, median 29). Patients selection: We tested for factor V Leiden mutation 56 women with fetal loss in different trimester of pregnancy (mean age, 29.42 ± 3.24 years, median 29), compared with 42 control women with at least one normal pregnancy (mean age, 28.96 ± 3.59 years, median 29). Patients with malignancies, inflammatory diseases, hormone replacement therapy, and antithrombotic therapy were excluded from the study. The study was approved by the institutional ethics committee.

RESULTS

EIGHTEEN OF 56 WOMEN WITH FETAL LOSS AND FOUR OF 42 CONTROLS WERE POSITIVE FOR FACTOR V LEIDEN MUTATION (7.14% versus 9.52%, odds ratio (OR) 4.5, 95% CI [1.39-14.54], p < 0.01). Fourteen of 56 women with fetal loss and one of 42 controls were heterozygous for factor V Leiden mutation (25% versus 2.38%, odds ratio (OR) 13.67, 95% CI [1.71-108.73], p < 0.01). Four of 56 women with fetal loss and 3 of 42 controls were homozygous for factor V Leiden mutation (32.14% versus 9.52%, odds ratio (OR) 4.5, 95% CI [1.39-14.54], p < 0.01). Fourteen of 56 women with fetal loss and one of 42 controls were heterozygous for factor V Leiden mutation (25% versus 2.38%, odds ratio (OR) 13.67, 95% CI [1.71-108.73], p < 0.01). Four of 56 women with fetal loss and 3 of 42 controls were homozygous for factor V Leiden mutation (32.14% versus 9.52%, odds ratio (OR) 4.5, 95% CI [1.39-14.54], p < 0.01).

CONCLUSIONS

Our study shows that there is an association between factor V Leiden mutation and the risk for fetal loss. Diagnosis of factor V Leiden is recommended in women with unexplained fetal loss. PCR-SSCP results were the same with those obtained by PCR-RFLP analysis. Moreover, PCR-SSCP procedure is less expensive than PCR-RFLP analysis because it eliminates the restriction of endonuclease enzymes.

Key Words: fetal loss, pregnancy, factor V Leiden mutation, inherited thrombophilia, hypercoagulable state

ABSTRACT

Background: Factor V Leiden represents the most common inherited risk factor for thrombophilia. Objectives: Evaluation of factor V Leiden mutation in women with fetal loss. Patients selection: We tested for factor V Leiden mutation 56 women with fetal loss in different trimester of pregnancy (mean ± SD age, 29.42 ± 3.24 years, median 30), compared with 42 control women with at least one normal pregnancy (mean ± SD age, 28.97 ± 3.59 years, median 29). Methods: Prevalence of factor V Leiden mutation is made by single strand conformation polymorphism (SSCP) procedure, with genetic confirmation made by DNA test involved RFLP analysis of the factor V gene (F5 gene). Results: Eighteen of 56 women with fetal loss and 4 of 42 controls were positive for factor V Leiden mutation (32.14% versus 9.52%, odds ratio (OR) 4.5, 95% CI [1.39-14.54], p < 0.01). Fourteen of 56 women with fetal loss and one of 42 controls were heterozygous for factor V Leiden mutation (25% versus 2.38%, odds ratio (OR) 13.67, 95% CI [1.71-108.73], p < 0.01). Four of 56 women with fetal loss and 3 of 42 controls were homozygous for factor V Leiden mutation (7.14% versus 1.71%, odds ratio (OR) 1, 95% CI [0.21-4.72], p=0.69). Conclusions: Our study shows that there is an association between factor V Leiden mutation and the risk for fetal loss. Diagnosis of factor V Leiden is recommended in women with unexplained fetal loss. PCR-SSCP results were the same with those obtained by PCR-RFLP analysis. Moreover, PCR-SSCP procedure is less expensive than PCR-RFLP analysis because it eliminates the restriction of endonuclease enzymes.

Key Words: fetal loss, pregnancy, factor V Leiden mutation, inherited thrombophilia, hypercoagulable state

REFERENCES

1. Inherited thrombophilia plays an important role in the pathogenesis of fetal loss because of the thrombotic events that occur in the placenta. Factor V Leiden represents the most common inherited risk factor for thrombophilia. The prevalence is 3-8% in European different populations. There is a considerable difference between European countries with a high prevalence in Greece and Sweden (10-15%) and a low prevalence in Italy and Spain (2-3%). In the meantime this polymorphism is rare in Asian, African and indigenous Australian populations.

2. Factor V Leiden represents a single base pair substitution, G → A at nucleotide 1691 (G1691A) in
the gene encoding coagulation factor V that predicts a single amino acid replacement Arg to Gln at position 506 (Arg506Gln) in the factor V molecule. This point mutation is associated with increase thrombin generation and a hypercoagulable state. Individuals heterozygous for factor V Leiden mutation have a 7 fold increased risk for venous thrombosis while individuals homozygous have a 80-100 fold increased risk.\textsuperscript{4,6}

Presence of factor V Leiden mutation was confirmed in 20 to 46% of women with venous thrombosis during pregnancy and in 32% of women with fetal loss.\textsuperscript{7-11}

The aim of this study was to test for factor V Leiden mutation women with obstetric complications such as repeated fetal loss, compared with control women with at least one normal pregnancy. In the meantime we also attempted to compare the prevalence of factor V Leiden mutation in women with first trimester unexplained fetal loss with the prevalence of the same polymorphism in women with second and third trimester fetal loss.

\textbf{MATERIALS AND METHODS}

\textbf{Patients' selection}

\textbf{Women with fetal loss}

Between September 2001 and March 2002, we studied 56 consecutive women with recurrent fetal loss with the mean age of 29.42 ± 3.24 years (median 30).

All were healthy early in pregnancy, except 3 (5.35%) who had a personal history of thromboembolic events. Another three (5.35%) women had familial history of thromboembolic events. This group comprised 14 (25%) women with fetal loss in first trimester of pregnancy and 42 (75%) women with fetal loss in the second and third trimester of pregnancy, or combined fetal loss. Fetal loss in the first trimester of pregnancy was defined as 7-12 weeks of gestation; fetal loss in the second trimester of pregnancy was 13-24 weeks of gestation; fetal loss in the third trimester of pregnancy meant more than 24 weeks of gestation. Eighteen (32.14%) women used oral contraceptives and 26 (46.42%) women were smokers. Three (5.35%) women consumed alcohol prior to their pregnancies.

\textbf{Control group}

We also studied 42 women with at least one normal pregnancy, with no personal or familial history of thromboembolic events. The mean age for women in the control group was 28.97 ± 3.59 years (median 29). Three (7.14%) women used oral contraceptives and 2 (4.76%) women were smokers.

This study was approved by the Ethics Committee and informed consent was obtained from each woman.

\textbf{Methods}

The diagnosis for factor V Leiden mutation involved:

a). Single strand conformation polymorphism (SSCP) techniques;\textsuperscript{13}

b). DNA test involved RFLP analysis of the factor V gene.\textsuperscript{14}

\textbf{DNA amplification of the 223bp fragment}

The polymerase chain reaction was performed by the methods described by Ridker with minor modifications.\textsuperscript{14,15}

\textbf{SSCP reaction}

The SSCP reaction was performed by the methods described by Jeunemaitre with minor modifications.\textsuperscript{13}

After completion, 1 µl of PCR product was incubated with 1 µl of formamide and after denaturation at 94°C for 5 minutes, the mixture was loaded on 5% polyacrilamide gels containing 0.5 XTBE (1 XTBE= 90mM Tris-borate [pH 7.8], 2mM EDTA). The samples were electrophoresed at room temperature for 4 hours. (Fig. 1)

\textbf{Enzymatic digestion of the 223bp amplification product}

In order to confirm the results obtained by SSCP techniques, the 223bp amplification product was digested with MnlI restriction enzyme using the method described by Ridker with minor modifications.\textsuperscript{14,15} (Fig. 2)
Figure 2. Enzymatic digestion of the 223bp fragment. Lane 1 - pBRHaeIIIDigest DNA molecular marker; Lane 2 - 223bp amplified fragment; Lane 3,4 - heterozygous patient for factor V Leiden mutation: 141, 104 and 82bp fragments; Lane 5,7 - homozygous negative patient for factor V Leiden mutation: 104 and 82bp fragments; Lane 6 - homozygous positive patient for factor V Leiden mutation: 141 and 82bp fragments.

This method is based on the fact that factor V Leiden destroys an MnlI restriction endonuclease recognition site.

These two methods allow the distinguishing of heterozygotes from homozygotes and from normal. All the reagents were from Sigma, except the restriction enzyme which was from New England Biolabs.

Statistics

In order to estimate the relative risks for pregnancy loss in patients with factor V Leiden mutation odds ratio (OR) and 95% confidence intervals (95% CI) were calculated. χ2 test was taken to indicate statistical significance, that means p values < 0.05.

Table 1. The mean characteristics and the genotypes of the 2 study groups: women with fetal loss and women with normal pregnancy; FL- fetal loss; NP- normal pregnancy.

<table>
<thead>
<tr>
<th>Characteristics of women</th>
<th>Women with FL</th>
<th>Women with NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr) mean ± SD</td>
<td>29.42 ± 3.24</td>
<td>28.97 ± 3.59</td>
</tr>
<tr>
<td>Median ± SD</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>FL in early pregnancy No. (%)</td>
<td>14 (25)</td>
<td>-</td>
</tr>
<tr>
<td>FL in late pregnancy No. (%)</td>
<td>42 (75)</td>
<td>-</td>
</tr>
<tr>
<td>Oral contraceptive used No. (%)</td>
<td>18 (32.14)</td>
<td>3 (7.14)</td>
</tr>
<tr>
<td>Smoking status No. (%)</td>
<td>26 (46.42)</td>
<td>2 (4.76)</td>
</tr>
<tr>
<td>Alcohol consumption No. (%)</td>
<td>3 (5.35)</td>
<td>-</td>
</tr>
<tr>
<td>Family history of thrombosis No. (%)</td>
<td>5 (9.22)</td>
<td>-</td>
</tr>
<tr>
<td>Preeclampsia No. (%)</td>
<td>1 (1.78%)</td>
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Thirty-two percent (18/56) of women with fetal loss had factor V Leiden mutation compared with 9.52% (4/42) women with at least one normal pregnancy (OR 4.5, 95% CI 1.39-14.54, p < 0.01). We found 25% (14/56) women with fetal loss and 2.38% (1/42) women with normal pregnancies heterozygous for factor V Leiden mutation (OR 13.67, 95% CI 1.71-108.73, p < 0.01). The frequency of homozygous was 7.14% (4/56) in the group of women with fetal loss and 7.14% (3/42) in the group of women with normal pregnancies (OR 1, 95% CI 0.21-4.72, p = 0.69). (Table 2)

Table 2. Distribution of factor V Leiden mutation in women with fetal loss and statistical significance for the association between factor V Leiden mutation and fetal loss.

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<td>Factor V Leiden, No. (%)</td>
<td>18/56 (32.14%)</td>
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<td>1691AG, No. (%)</td>
<td>14/56 (25%)</td>
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<td>&lt; 0.01</td>
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<td>1691AA, No. (%)</td>
<td>4/56 (7.14%)</td>
<td>3/42 (7.14%)</td>
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<td>0.19</td>
<td>0.08</td>
<td>2.69 [1.08-6.63]</td>
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RESULTS

In order to identify the factor V Leiden mutation in women with obstetric complication - women with fetal loss - we have studied 56 women with fetal loss in different trimesters of pregnancy.

We tried to compare the results with those obtained in 42 women with at least one normal pregnancy. The mean characteristics of these 2 groups are presented in Table 1.

All the women from the group with fetal loss were normotensive, except one female patient, who had preeclampsia in a previous pregnancy. Preeclampsia was diagnosed on the basis of systolic and diastolic blood pressure higher than 140/90mmHg.

Table 1: The mean characteristics and the genotypes of the 2 study groups: women with fetal loss and women with normal pregnancy; FL- fetal loss; NP- normal pregnancy.

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CONCLUSIONS

Pregnancy is a hypercoagulable state because of the increased levels of procoagulant factors and decreased levels of natural anticoagulants. During pregnancy there is a decrease in fibrinolysis. These haemostatic changes in pregnancy favor excessive blood coagulation thus a hypercoagulable state.16

There are some obstetric complications associated with a hypercoagulable state, such unexplained fetal loss. Pathologic features of fetal loss comprise a deficit of implantation in the first trimester of pregnancy or a thrombotic event in the placenta that probably occurs in the second and third trimester of pregnancy.16

Several mutations in some proteins involved in coagulation process were associated with thrombo-
embolic events that can appear in recurrent unexplained fetal loss.\textsuperscript{17-21} The most important is resistance to activated protein C (APC\textsubscript{r}) caused by factor V Leiden mutation. The association between resistance to activated protein C and factor V Leiden mutation appears in 90\% of cases.\textsuperscript{22-25}

A large number of studies found a high prevalence of factor V Leiden in women with unexplained recurrent fetal loss, up to 30\% compared with 1 to 10\% of controls.\textsuperscript{31,18,21,22,24-30} For example, in 1997, Brenner et al showed an association between factor V Leiden mutation and vascular placental insufficiency that caused fetal loss.\textsuperscript{31} Compared with controls (4.1\%), fetal loss were more often associated with heterozygous factor V Leiden mutation (10.1\%). The same results were obtained by Murphy et al in 2000: fetal loss occurs in 11\% of women with factor V Leiden mutation (in a heterozygous or homozygous form) compared with only 4.2\% in women with normal genotype.\textsuperscript{32} Brenner found 32\% women with fetal loss positive for factor V Leiden mutation and Grandone found 16.28\% women with fetal loss positive for the same polymorphism.\textsuperscript{11,22} Finan (2002) identified factor V Leiden mutation in 40.91\% women with first trimester pregnancy loss.\textsuperscript{33}

In our study we identified factor V Leiden mutation in 32.14\% women with fetal loss in a different trimester of pregnancy compared with only 9.52\% women with at least one normal pregnancy. The relative risk was 4.5, 95\% confidence intervals [1.39-14.54], p<0.01, that means that the association between factor V Leiden mutation and fetal loss was statistically significant. The results are in agreement with those found also by Brenner et al in 1999.\textsuperscript{11}

In this preliminary study, women with fetal loss were more often heterozygous (25\%) than women with normal pregnancies (2.38\%) (OR 13.67, 95\% confidence intervals 1.39-14.54), p<0.01, that means the association between factor V Leiden mutation and fetal loss was statistically significant. The frequency of the normal G1691 allele was 0.80 in the group of women with fetal loss compared with 0.91 in the group of women with at least one normal pregnancy; the frequency of the mutated A1691 allele was 0.19 in the group of women with fetal loss compared with 0.08 in the group of women with at least one normal pregnancy.

In our preliminary study we investigated for factor V Leiden mutation 14 (25\%) women with fetal loss in first trimester of pregnancy and 42 (75\%) women with fetal loss in the late pregnancy (fetal loss in second, third or combined trimester of pregnancy). There are several studies which showed that factor V Leiden mutation represents a strong risk factor for losses in the second or third trimester of pregnancy than for losses in the first trimester of pregnancy. Thus, Grandone et al in 1999 and Tormene et al in 1999 showed that women with thrombophilia such as factor V Leiden mutation had a high risk of fetal loss in the second and third trimester of pregnancy.\textsuperscript{22,25}

In our study we found that 16 women with fetal loss in the second or third trimester of pregnancy, compared with 2 women with fetal loss in the first trimester of pregnancy positive for factor V Leiden mutation, that means 28.57\%, respectively 3.57\% (OR 10.8, 95\% confidence intervals [2.34-49.66], p<0.01). The result confirms the observation that women with factor V Leiden mutation have a higher risk of developing fetal loss in the second or third trimester of pregnancy than in the first trimester of pregnancy.

Familial history of thrombotic events was found in 3 (5.35\%) women with fetal loss. Concerning the SSCP techniques, that we used to identify factor V Leiden mutation, we can say that this represents a modern method to check for point mutation. The results obtained by PCR-SSCP techniques were the same with those obtained by PCR-RFLP analysis. Moreover, PCR-SSCP procedure is less expensive than PCR-RFLP analysis because it eliminates the use of restriction enzymes which are very expensive. This method can be used in the routine detection of factor V Leiden mutation and of other point mutations.

Our results suggest that heterozygosity or homozygosity for factor V Leiden mutation represents a risk factor for fetal loss, especially for fetal loss in the second or third trimester of pregnancy, but to confirm this we have to study more cases of fetal loss.

The diagnosis of factor V Leiden is recommended in women with unexplained fetal loss and in women with a familial history of thrombosis. On the other hand women that carry factor V Leiden mutation but are asymptomatic, should be warned about potential thrombotic complications (such as fetal loss) and counseled about the risks and benefits of anticoagulation during pregnancy.\textsuperscript{34} Women with factor V Leiden mutation and a history of thrombosis event should receive prophylactic anticoagulation with unfractionated low molecular weight heparin during pregnancy and for at least 6 weeks postpartum.\textsuperscript{35}
REFERENCES