Abstract

Objective: In the present study, we have aimed to determine the frequency of common inherited thrombophilias among women with pre eclampsia, intrauterine growth retardation, placental abruption, recurrent pregnancy loss, and stillbirth.

Materials and Methods: Sixty women with complicated pregnancies and as a control group 53 normal pregnant women were included in the study. Women with complicated pregnancies consist of pre eclampsia (n=21), intrauterine growth restriction (n=12), intrauterine fetal death (n=12), placental abruption (n=5) and recurrent pregnancy loss (n=10). Genotype analysis for factor V Leiden mutation, prothrombin mutation (PT 20210G/A), and methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism were performed by real-time online polymerase chain reaction.

Results: The frequency of Factor V Leiden mutation was found statistically higher in the complicated pregnancy group, compared to normal group (23.3% vs 7.5%) (p=0.04). On the other hand, no difference was detected on the heterozygous MTHFR frequencies between the two groups. However, 9% of the women with complicated pregnancies had homozygous mutation and no woman was homozygous for MTHFR in the control group. PT gene mutation was found in only one patient from the control group.

Discussion: Factor V Leiden mutation and homozygosity for the MTHFR polymorphism, rather than its heterozygosity, might be involved in the pathogenesis of adverse pregnancy outcome associated with placental vasculopathy.

Keywords: hereditary thrombophilias, adverse pregnancy outcome

Özet

Kötü Gebelik Sonuçları Olan Kadınlarda Faktör V Leiden (G1691A), Protrombin (G20210A) ve Metilentetrahidrofolat Redüktaz (C677T) Gen Mutasyonlarının Sıklığı

Amaç: Bu çalışmada amaç, kalıtsal trombofililerin pre eklampsi, intrauterin gelişme kısıtlığı, ablasiyo plasenta, tekrarlanan gebelik kayıpları ve ölüm doğumları olan gebelikleri araştırırmaktır.

Materyal ve Metot: Çalışmaya komplikasyonlu gebelik olan 60 hasta ve kontrol grubu olarak da 53 normal gebeliğe dahil edildi. Komplikasyonlu gebelik grupları olan 21 pre eklampisi, 12 intrauterin gelişim kısıtlığı, 12 intrauterin fetal ölüm, 5 plasental abruption ve 10 tekrarlayan gebelik kaybı ve ölüm doğumları alt gruptan oluşmuştur. Faktör V Leiden mutasyonu, protrombin mutasyonu (PT 20210G/A) ve methylenetetrahydrofolat reductaz (MTHFR) C677T polimorfizminin genotip analizleri real-time online polimeraz zincir reaksiyonu ile araştırılmıştır.

Sonuç: Faktör V Leiden mutasyonu, komplikasyonlu gebelik grubunda normal gebelikte iki kat daha yüksek oranda bulunmuştur (%23-7,5) (p=0.04). Diğer taraftan, heterozigot MTHFR sıklığı açısından her iki grup arasında farklılık bulunmamıştır.
Introduction

Three important risk factors for inherited thrombophilias have been discovered: A transition of guanine to adenine in nucleotide 1691 of the factor V gene (FV Leiden) that causes resistance to activated protein C (1), a transition of guanine to adenine at nucleotide 20210 in the prothrombine gene (PT G20210A) that is associated with higher plasma levels of prothrombin (2), and a transition of cytosine to thymine at nucleotide 677 in the gene encoding methylenetetrahydrofolate reductase (MTHFR) (3). Mutations in factor V and prothrombine genes result in an increased susceptibility to develop venous thrombosis. Hyperhomocysteinemia, which is associated with a polymorphism in the gene for methylenetetrahydrofolate reductase, is a risk factor for venous and arterial thrombosis (4). Collectively, heritable thrombophilias are present in at least 15% of Western populations and underlie approximately 50% of episodes of venous thromboembolism in pregnancy (5).

Serious obstetric complications, including recurrent pregnancy loss, fetal growth retardation, preeclampsia, and placental abruption, occur in 1% to 5% of pregnant women and may involve impaired placental perfusion (6). Abnormal placental development that results in vascular insufficiency plays an important role in the pathophysiology of these conditions. Placental vascular insufficiency may be caused by immunological factors that lead to abnormal placentation, or by vasculopathy associated with chronic hypertension or diabetes mellitus (7,8). Recently, several studies have shown that inherited and acquired thrombophilias markedly increase the risk of venous thrombosis during pregnancy and may predispose to gestational vascular complications, which are associated with poor pregnancy outcome (9-11). However, all investigators did not confirm the association between the thrombophilias and adverse pregnancy outcome (12-14).

For this reason, in the present study we have aimed to make our own contribution to the investigation and also to estimate the prevalence of FV Leiden, PT and MTHFR gene mutations and polymorphism in women who have thrombotic damage in their placental bed and in normal pregnant women.

Materials and Methods

In this study, we examined 60 women with complicated pregnancies and 53 women with normal pregnancies, as a control, during the period between January 2002 and January 2004. Women with complicated pregnancies consist of preeclampsia (n=21), intrauterine growth restriction (IUGR) (n=12), intrauterine fetal death (IUFD) (n=12), placental abruption (n=5) and recurrent pregnancy loss (RPL) (n=10). All women had attended labor in our clinic. Preeclampsia was diagnosed in the presence of both hypertension (diastolic blood pressure 90 mmHg or greater in two consecutive measurements 4 h or more apart) and significant proteinuria (≥300 mg in 24 h urine collection), after the twentieth week of gestation, in a previously normotensive and non-proteinuric woman. Abruptio placenta was diagnosed when patients had vaginal bleeding with or without uterine tenderness and fetal distress, shock or maternal coagulopathy, and also by the examination of the maternal side of the placenta. Intrauterine growth restriction was diagnosed when fetal growth was below the 10th centile. Recurrent pregnancy loss was diagnosed as three or more consecutive spontaneous miscarriages before 20 weeks of gestation. Women with anatomic, autoimmune, hormonal, chromosomal abnormalities, and anti-phospholipid antibody syndrome were excluded. Losses after 20 weeks are considered stillbirths or IUFD.

Normal pregnant women were followed as outpatients until parturition. Before inclusion in the study, they underwent physical examination, blood pressure measurement, and hematological and urinary examination to exclude chronic hypertension, chronic nephropathy and other major systemic diseases. None of the women included in the study had previous thromboembolic diseases or familial history. The maternal age, gestational week at delivery and the birthweight were recorded.

All patients, constituting either the study or control group were examined for the Factor V Leiden or prothrombin G20210A mutations. In addition, the C677T polymorphism in the MTHFR gene was examined in 44 out of 60 women with complicated pregnancies and in 24 out of 53 normal pregnant women. For DNA isolation, peripheral blood was collected in EDTA tubes after delivery and with the consent of all participating women. Genotype analysis was performed for the factor V Leiden mutation, prothrombin mutation (G20210A), and methylenetetrahydrofolate reductase (C677T) polymorphism by real-time online polymerase chain reaction.

Factor V Leiden (G1691A) and prothrombin (G20210A) mutations

Genomic DNA was extracted from peripheral leukocytes of the subjects using the High Pure PCR Template Preparation
Kit (Roche Applied Science; Mannheim, Germany). All patients were tested for the presence of the Factor V Leiden and prothrombin G20210A mutations on the Lightcycler™ system using the commercial LightCycler Factor V Leiden (G1691A) and Prothrombin (G20210A) Mutation Detection Kits, respectively (Roche Diagnostics; Mannheim, Germany).

Genotyping of the different alleles for the Factor V Leiden mutation was done according to the specific melting temperature (Tm) of the resulting amplicons. Wildtype genotype with two copies of the G allele (G/G) show a single melting peak at 65°C, mutant genotype with two copies of the A allele (A/A) also show a single melting peak but at 57°C, and heterozygous genotype with both alleles (G/A) show two melting peaks at 65°C and 57°C in this analysis.

Specific melting temperature (Tm) of the resulting amplicons identified different alleles of the prothrombin (G20210A) mutation. Wildtype genotype with two copies of the G allele (G/G) show a single melting peak at 59°C, mutant genotype with two copies of the A allele (A/A) also show a single melting peak but at 49°C, and heterozygous genotype with both alleles (G/A) show two melting peaks at 59°C and 49°C in this analysis.

MTHFR (C677T)
For the detection of the C677T polymorphism at the MTHFR gene, specific primer probes were used together with the LightCycler-DNA Master Hybridization Probes Kit (Roche Applied Science; Mannheim, Germany). Experiments were carried out on the LightCycler™ system (Roche Applied Science; Mannheim, Germany) according to the protocol of Charalampos Aslandis and Gerd Schmitz (Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany). Specific melting temperature (Tm) of the resulting amplicons identified polymorphic alleles. Individuals with two copies of the C allele (C/C) show a single melting peak at 63.1°C, individuals with two copies of the T allele (T/T) also show a single melting peak but at 54.6°C, and individuals with both alleles (C/T) show two melting peaks at 54.6°C and 63.1°C in this analysis.

Statistical analyses
The statistical differences between age, week of gestation at delivery and birthweight of both study and control groups were analyzed using student t-test. Chi-square test was applied to detect the statistical differences of genetic mutations between complicated and normal pregnancies.

All statistical analyses were performed using Sigmastat for Windows, version 3.0 (Jandel Scientific Corporation; San Rafael, CA). Data are presented as the mean ±SD. Differences were considered to be significant at p<0.05.

Results
Birth weight and week of gestation at delivery were significantly lower in the complicated pregnancy group, compared to that of the normal pregnant group as expected. The clinical characteristics of complicated and normal pregnancies included in the study are shown in Table 1.

Only one woman in the control group carried the prothrombin mutation, whereas no patient had this mutation in the complicated pregnancy group.

In the complicated pregnancy group, the FV Leiden mutation was found in 14 women (13 heterozygous, 1 homozygous), and in 4 women (3 heterozygous, 1 homozygous) from the control group. The difference was found statistically significant (p=0.04). Preeclampsia was the major subgroup of complicated pregnancy group. FV Leiden mutation was detected in 8 (7 heterozygous, 1 homozygous) out of 21 women (38%) with preeclampsia. However, 7 women with preeclampsia had also developed IUGR. The prevalence of FV Leiden mutation in complicated and normal pregnancies are summarized in Table 2.

### Table 1. Clinical characteristics of women who were examined for FVL, PT genes mutations and MTHFR gene polymorphism

<table>
<thead>
<tr>
<th>Characteristics (FVL and PT)</th>
<th>Complicated pregnancies (n=60)</th>
<th>Normal pregnancies (n=53)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year, mean ±SD) (range)</td>
<td>29.1±5.2 (18-40)</td>
<td>28.0±4.8 (19-37)</td>
<td>NS</td>
</tr>
<tr>
<td>Week of gestation at delivery (mean ±SD)</td>
<td>33.5±5.7</td>
<td>38.4±0.9</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Birthweight (g, mean ±SD)</td>
<td>2100±1158</td>
<td>3344±457</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Characteristics (MTHFR)</th>
<th>Complicated pregnancies (n=44)</th>
<th>Normal pregnancies (n=24)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year, mean ±SD) (range)</td>
<td>28.7±4.4 (20-38)</td>
<td>28.5±4.7 (19-35)</td>
<td>NS</td>
</tr>
<tr>
<td>Week of gestation at delivery (mean ±SD)</td>
<td>33.6±5.1</td>
<td>38.5±1.1</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Birthweight (g, mean ±SD)</td>
<td>2249±1567</td>
<td>3275±477</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>
Heterozygous MTHFR gene polymorphism was observed in 23 women (52.2%) from the complicated group and in 13 women (54.1%) from the normal group. The difference was not significantly different between the two groups; however, in the complicated pregnancy group 4 women (9%) were homozygous for MTHFR and no women was homozygous for MTHFR in the control group. The prevalence of the MTHFR polymorphism in complicated and normal pregnancies are summarized in Table 3.

On the other hand, 4 women (6.6%) with complicated pregnancy had both FV Leiden mutation and MTHFR polymorphism: one woman with preeclampsia, one woman with abruptio placenta and one woman with RPL were heterozygous for both mutations, and one woman with abruptio placenta was heterozygous for FV Leiden and homozygous for MTHFR.

Discussion

Successful pregnancy outcome is dependent upon trophoblast invasion into the uterine vasculature and on the development and maintenance of an adequate uteroplacental circulation in the mother. Inadequate invasion of the maternal circulation by the trophoblast and damage to the maternal vessels supplying the placenta lead to impaired flow and thrombotic changes in the vessel wall, which are implicated in pregnancy complications including miscarriage, IUGR, preeclampsia with fetal compromise, placental abruption and stillbirth. There is now accumulating data for a role of thrombotic mechanism in the development of these conditions (5,6). In other words, all these conditions might be associated with thrombotic damage in the placental bed. Since our series does not include adequate number of case in each subgroup, we decided to collect all cases associated with abnormal placental vasculature under a common title: complicated pregnancies.

Although preeclampsia, IUGR and placental abruption are though to involve impaired placent perfusion, their association with thrombophilia remains controversial, with conflicting results from different studies. The reasons underlying differences in results with regard to the association between thrombophilia and pregnancy complications are unclear. However, it may reflect different diagnostic criteria, small sample size and reported bias as many studies had relatively low levels of heterozygosity for gene mutations in the control group studied.

There have been reports of both heritable and acquired thrombophilias being associated with preeclampsia, IUGR, placental abruption and RPL. Several case-control studies found at least one thrombophilic defect in 40% to 72% of women with preeclampsia compared with 8% to 20% of control women with normal pregnancies (15-17). However, several studies found a significantly higher prevalence of Factor V Leiden in women with preeclampsia (8-26%) compared to normal pregnant women (2-10%) with Odd Ratios ranging from 2 to 6 (18-20).

<table>
<thead>
<tr>
<th>Table 2. Prevalence of Factor V Leiden in normal pregnant women and in those with complicated pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Complicated pregnancy</strong> (n=44)</td>
</tr>
<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>Preeclampsia (n=15)</td>
</tr>
<tr>
<td>IUGR (n=12)</td>
</tr>
<tr>
<td>IUMF (n=8)</td>
</tr>
<tr>
<td>Abruptio pl (n=4)</td>
</tr>
<tr>
<td>RPL (n=6)</td>
</tr>
<tr>
<td>Normal pregnancy (n=24)</td>
</tr>
</tbody>
</table>

*p=0.04 (complicated vs. normal pregnant women)

<table>
<thead>
<tr>
<th>Table 3. Prevalance of MTHFR in normal pregnant women and in those with complicated pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Complicated pregnancy</strong> (n=44)</td>
</tr>
<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>Preeclampsia (n=15)</td>
</tr>
<tr>
<td>IUGR (n=12)</td>
</tr>
<tr>
<td>IUMF (n=8)</td>
</tr>
<tr>
<td>Abruptio pl (n=4)</td>
</tr>
<tr>
<td>RPL (n=6)</td>
</tr>
<tr>
<td>Normal pregnancy (n=24)</td>
</tr>
</tbody>
</table>

198
In contrast, there are also several studies, which reported no association of Factor V Leiden with preeclampsia (21-23). In our series, women with preeclampsia constitute the major subgroup of women with complicated pregnancies; we found Factor V Leiden mutation in 8 out of 21 women (38%). Therefore, our results support the view that this mutation might contribute to the pathogenesis of preeclampsia.

A few studies suggested that the homozygous MTHFR polymorphism confers a 2-to 3-fold increased risk for preeclampsia (24-25). However, most of the studies found no association between MTHFR and preeclampsia (26-29). These results suggest that the increased folate levels achieved with prenatal vitamins may affect maternal homocysteine levels more than the MTHFR genotype. In the present study, the prevalence of the MTHFR C677T polymorphism was 46% among the preeclamptic subgroup and the difference was not significant compared to the normal pregnant group. Moreover one woman in the preeclamptic subgroup (4.7%) found to be a carrier for both Factor V Leiden mutation and MTHFR polymorphism.

Recurrent pregnancy loss (RPL) affects 1-3% of women of reproductive age. Anatomic, autoimmune, hormonal, chromosomal abnormalities, and antiphospholipid antibody syndrome are the main implicated causes for recurrent pregnancy loss. In recent years, a large number of case-control studies found a high prevalence of FV Leiden mutation in women with unexplained RPL (up to 30%) compared with 1-10% of control subjects (30-32). In addition, three retrospective cohort studies found that Factor V Leiden carriers have a 2-fold increased risk of fetal loss (33-35). Furthermore, it was reported that women with homozygous mutation had a 2-fold higher risk than heterozygous carriers (35). On the other hand, although a few studies suggested that a homozygous MTHFR polymorphism increased the risk of RPL and placental vasculopathy (36,37), the majority found no significant association (38-40). A meta-analysis, including 1818 women, showed that there was no association between the MTHFR polymorphism and RPL (41). In the present study, although a small number of cases were investigated, heterozygous FV Leiden mutation was detected in 30% of patients with RPL. This finding is consistent with the majority of the studies, which implicate a role of the FV Leiden mutation in the thrombotic pathogenesis of RPL. It has also been reported that women with combined thrombophilia have the highest risk of fetal loss (30,34,42).

Available data on the risk of fetal growth retardation are more limited, but also conflicting. Thrombophilic defects were found in 60% to 70% of women with a history of fetal growth retardation compared with 13% to 18% of those with normal pregnancies suggesting a 4-to 5-fold increased risk (15,43). Factor V Leiden mutation was found in 8% to 35% of women with IUGR compared with 2% to 4% of control women (15,43). However, a larger case-control study found no significant association between maternal or fetal thrombophilia and fetal growth retardation (44). It has also been reported that homozygosity for the MTHFR polymorphism also confers no increased risk, except in the subgroup of women who did not take multivitamins during the third trimester (45). In our series, Factor V Leiden mutation and MTHFR gene polymorphism was found in 8.3% and 83.3% of women with IUGR, respectively. Because of the fact that the heterozygous MTHFR gene polymorphism is also very common in the control group (54.1%), we do not believe that heterozygosity for this polymorphism has a negative effect on the adverse pregnancy outcome. However, homozygosity for MTHFR might contribute to the pathogenesis of IUGR.

There were even fewer studies evaluating the association of thrombophilia with placental abruption, and most included only a small number of cases. At least one thrombophilic disorder was found in 70% of women with placental abruption compared with 18% of women with uncomplicated pregnancies (15). Several studies reported a significantly higher prevalence of the Factor V Leiden mutation in women with placental abruption (22%-30%) compared with 3% to 6% of the controls (15,46,47). Hyperhomocysteinemia was associated with a 2-to 8-fold increased risk for placental vasculopathy (defined as placental abruption or infarction) and was found in 31% of women with this complication (37,46). In the present study the placental abruption subgroup consists of a small number of cases. Therefore, it limits our consideration about this condition. However, heterozygous Factor V Leiden mutation and homozygous MTHFR gene polymorphism were found in 33% and 25% of women with placental abruption, respectively, which might suggest an association of thrombophilias and placental abruption.

In a general population based-study performed in Turkey, Sazcet al. reported that the frequencies of the heterozygous (C677T) and homozygous (T677T) MTHFR gene polymorphism were 42.9% and 9.6%, respectively (48). In our series, the frequency of the MTHFR polymorphism was 54.1% and not significantly different from the complicated group. However, we found 4 women homozygous for the MTHFR polymorphism (9%) in the complicated group. These findings suggest that homozygosity, rather than heterozygosity for the MTHFR polymorphism might be involved in the pathogenesis of adverse pregnancy outcome associated with placental vasculopathy.

Similar to the Factor V Leiden mutation and MTHFR gene polymorphism, the PT gene mutation was also investigated on the pathogenesis of adverse pregnancy outcome, and these results were also conflicting. The prothrombin gene mutation was found in 7% to 11% of women with preeclampsia compared with 1% to 4% of those with normal pregnancies, suggesting a 2-to 7-fold increase in risk (24,25). However, the majority of studies found no significant association (12,15,49). In a meta-analysis, including 2087 women, the prothrombin gene mutation was associated with a 2-to 3-fold increased risk of recurrent pregnancy loss (41). Similarly, it has been reported that this mutation is significantly higher in women with placental abruption compared to normal pregnancies (15,47).
Ayıldız et al. investigated the prevalence of the PT G20210A gene mutation in patients with venous thrombosis and in the healthy population in the southeast region of Turkey (50). This mutation was found to be 6.5% in the venous thrombosis group and 1.2% in the healthy group. Interestingly we found the PT mutation in only one woman from the control group (1.9%), and no woman from the complicated pregnancy group carried this mutation. Ethnic and regional variations might explain this difference. In our series, this mutation does not seem to be involved in the pathogenesis of complicated pregnancies.

In conclusion, although the case number is not adequate to make definite comments, our results suggest that the Factor V Leiden mutation might contribute especially to the pathogenesis of preeclampsia. Homozygosity for the MTHFR polymorphism, rather than its heterozygosity might be involved in the pathogenesis of adverse pregnancy outcome associated with placental vasculopathy. However, further studies with larger series are needed to clarify this issue.

References


