Thrombophilic Status of Extracted Fetal Tissues of Spontaneously Aborted Embroys

Spontan Abortus Embriyolarından Ekstrakte Edilen Fetal Dokuların Trombofilik Durumu

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ABSTRACT

Objective: The reports about Factor V (FV) Leiden, Factor II (FII) G20210A and Methylenetetrahydrofolate reductase (MTHFR) C677T gene mutations of parents and fetal viability are frequently encountered in the literature, despite the fetal side of thrombophilia is scant. To clarify the three common thrombophilic gene mutations of the spontaneously aborted embryos, an accurate algorithm was followed to extract the fetal tissues and then the mutations were searched.
Material and Methods:70 spontaneous abortion materials were included to the study and all were karyotyped. Cytogenetically abnormals were excluded from the study. To extract the fetal tissues, amplifications of sex determination region of chromosome-Y (SRY) gene and genotypings were performed, respectively. Extracted fetal tissues of spontaneous aborted embroys and parents were screened for the thrombophilic gene mutations via electronic microarray.
Results: After excluding chromosomally abnormal and maternally contaminated ones totally ten fetal tissues were screened for the FII G20210A, FV Leiden and MTHFR C677T gene mutations, and two carry F II G20210A and F Leiden heterozygote mutations, and six carry heterozygote forms of MTHFR C677T.
Conclusion: The present study performed on the limited number of abortion materials, has a value for distinguishing the fetal tissues before analyzing the three common mutation of thrombophilic genes which make the results are very substantial.

Key Words: Fetal thrombophilia , Spontaneous abortion, SRY gene

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ÖZET

Amaç: Ebeveynlerdeki Faktör V (FV) Leiden, Faktör II (FII) G20210A ve Metilen Tetrahidrofolat redüktaz (MTHFR) C677T gen mutasyonları ve fetal tutunma ile ilgili bilgiler literatürde sıklıkla karşılaşılmasına rağmen, trombofilinin fetal yönü üzerine yapılan çalışmalar oldukça sınırlıdır. Bu üç sıklıkla rastlanılan gen mutasyonunu spontan abort embriyolarında netleştirmek için fetal dokuları ekstrakte edebilmek maksadıyla doğru bir algoritma takip edildi ve mutasyon taraması gerçekleştirildi.
Yöntem: Toplam 70 spontan abortus materyali çalışmaya dâhil edildi ve hepsinin karyotip analizi yapıldı. Sitogenetik olarak anormal olanlar çalışmadan çıkartıldı. Fetal dokuları ekstrakte edebilmek için Y kromozomu üzerindeki SRY gen bölgesi amplifikasyonu ve genotiplendirme işlemleri ayrı ayrı yapıldı. Spontan abort embriyolarının ekstrakte edilen fetal dokuları elektronik mikroarray kullanılarak trombofilik gen mutasyonları açısından tarandı.
Bulgular: Kromozom anomalisi ve maternal hücre kontaminasyonu tespit edilenler çalışma grubundan çıkartıldıktan sonra 10 fetal dokuda FV Leiden, FII G20210A ve MTHFR C677T gen mutasyonlarının taraması gerçekleştirildi, iki tanesinin FII G20210A ve FV Leiden heterozigot mutasyon taşıdığı ve altı tanesinin de MTHFR C677T heterozigot mutasyon taşıdığı tespit edildi.
Sonuç: Sınırlı abort materyalinde yapılan bu çalışmada ilgili üç trombofilik gen mutasyonunun analizinden önce fetal dokuların ayırt edilmesi elde edilen sonuçları değerli kılmaktadır.

Anahtar Sözcükler: Fetal trombofili, spontan abort, SRY geni

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INTRODUCTION

The majority of the spontaneous abortions occur during the first trimester and over 50% of these are detected chromosomally abnormal (1,2). Thrombophilia can predispose an individual to thromboembolism and this condition could have been a significant role of the production of the spontaneous abortions (3). Although several studies are available concerning the relationship between thrombophilic pattern of the parents and abortions, thrombophilia mutations of the extracted fetal tissues of spontaneously aborted embroys are very limited.

Factor V (FV) Leiden, Factor II (FII) G20210A and Methylenetetrahydrofolate reductase (MTHFR) C677T gene alterations are the common mutations of the thrombophilia. Detecting these mutations on the abortion materials have difficulties because of the maternal decidual cell contamination (MCC) of the pregnancy loss tissues and additional techniques are necessary to extract the fetal tissues (4-6). In this present study after excluding abnormally karyotyped ones, cytogenetically normal females materials were searched to detect the very tiny component of chromosome Y by using amplifications of sex determination region of chromosome-Y (SRY) gene. Then if negative, genotyping procedures were used to ensure the origin of the materials. The details of the procedures were presented in our previous paper publicated on 2010 (7).

The study give us the results of the electronic microarray screening of the FV Leiden, FII G20210A and MTHFR C677T gene mutations of the spontaneously aborted materials XY karyotyped ones and additionally XX karyotyped ones which were confirmed via molecular genetic techniques.

METHODS

Tissue culture and chromosome analysis

A total of 70 spontaneous abortions which occurred between the fifth and twentieth weeks of pregnancies (singleton gestations) were referred for cytogenetic evaluation. Written informed consent forms were obtained from all participants and the study was approved by the ethics committee of Gazi University. Materials were transferred immediately to the laboratory in a sterile culture medium. After separating the chorionic villi in small pieces in sterile condition, long-term tissue cultures were set up using a slightly modified procedure of Verma and Babu (8). Karyotyping was performed by using Giemsa-trypsin banding (GTG) and five metaphase spreads were analyzed; fifteen metaphase spreads were counted for each one of the specimens which were cultivated for a period shorter than two weeks from two separate primer cell cultures (9). Some parts of the materials were stored at -20°C for DNA isolations.

DNA isolation

The abortion materials which were stored in -20°C for molecular studies were placed in 1000 µl of lysis buffer, 50 µl SDS and 20 µl Proteinase K (20 mg/ml) for overnight at 37°C. A 750 µl of ammonium acetate was added and agitated 20 times. Following incubation for 10 min at room temperature centrifugation at 3500 rpm for 15 min was done. The supernatant was separated to a new tube and absolute alcohol was added. DNA was taken to the tubes that contain 50 µl TE. After dissolving the DNA at room temperature for one day, it was stored at -20°. Genomic DNAs were isolated from paternal and maternal peripheral venous blood cells by using proteinase K digestion using salt extraction method according to the slightly modified procedure of Miller et al (10).

SRY Amplification and Genotyping

The primers of SRY gene and G6PDH gene as an internal control were used in PCR reaction. DNAs were amplified in three step cycles: denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, extension at 72°C for 30 sec. After 35 cycles, the DNAs were given a final extension step at 72°C for 5 min. By using high-polymorphic microsatellite DNA markers, chorionic villi DNAs and maternal and paternal DNAs were evaluated. Totally four different DNA markers including chromosome 10, 15 and X (D15S999, D10S1714, DXS987 and DXS1058) were used. The information about sequences and amplification conditions were obtained from Genome Data Base. Amplified PCR products were visualized on a 2% agarose gel by staining ethidium bromide. These products were separated in 167 bp, 193 bp, 206-244 bp and 273-285 bp respectively by 6% denatured polyacrylamide gel electrophoresis. The DNAs of the fetuses and their related parents were loaded to electronic microarray for mutation screening. Electronic microarray is a reliable method that is based on hybridization between molecules on the surface of streptavidin-coated chip and DNA molecules that are labelled with biotin. Finally the analyzes are done according to the signals.

RESULTS

Of the 70 spontaneous abortion materials, chromosomal abnormalities were identified in 26 (37.1%); 3 of them were structural (11.5%), 23 of them were numerical (88.5%) aberrations. The cytogenetic analyses of the 6 abortion materials were revealed as male karyotypes and screened directly for the FII G20210A, FV Leiden and MTHFR C677T gene mutations. SRY gene amplifications via PCR were performed on the 38 XX karyotyped abortion materials and a part of SRY gene was observed on the 16 of them (42.1% of the materials were maternally contaminated) as shown in figure 1. The genotypings were performed on the remaining twenty-two materials and only four of them were evaluated as chorionic villi (81.8% of the materials were maternally contaminated). Finally the MCC (-) four chorionic villi materials were screened for the mutations and the results are shown on table 1. No homozygous mutant abortion materials were detected in terms of FV Leiden, MTHFR C677T and FII G20210A. The strongest association between thrombophilic patterns and spontaneously aborted materials were observed in MTHFR C677T. Six of ten materials were detected heterozygous carrier for this gene and the results are shown on table 2. Combined thrombophilia was found in 2 of them; one of them was both MTHFR C677T and FII G20210A, the other is both MTHFR C677T and FV Leiden respectively.

Table 1. The results of the abort materials screened for maternal cell contamination, Factor II G20210A, Factor V Leiden, and MTHFR C677T mutations. G: gravida P: para A: abortion L: living het: heterozygote MI: maternally inherited PI: paternally inherited

No	Maternal age	Gestation weeks	Obstetric history	Karyotype	MCC	Factor II G20210A	Factor V Leiden	MTHFR C677T
1	29	8,3	G2P0A2L0	46,XY	(-)	wt	het/MI	wt
2	33	7,3	G1P0A1L0	46,XY	(-)	wt	wt	het/MI
3	26	7,5	G3P1A2L1	46,XY	(-)	wt	wt	het/MI
4	32	10,2	G3P0A3L0	46,XY	(-)	wt	wt	wt
5	38	15	G2P0A2L0	46,XY	(-)	wt	wt	wt
6	28	6,5	G1P0A1L0	46,XY	(-)	wt	wt	het/MI
7	36	8,1	G2P0A2L0	46,XX	(-)	het./MI	wt	wt
8	35	6,3	G5P2A3L2	46,XX	(-)	het./PI	wt	het/PI
9	32	8	G3P1A2L1	46,XX	(-)	wt	het/MI	het/MI or PI
10	27	8,5	G1P0A1L0	46,XX	(-)	wt	wt	het/MI or PI
11	35	6,10	G2P0A2L0	46,XX	SRY (+)	UD	UD	UD
12	30	7,6	G2P1A1L1	46,XX	SRY (+)	UD	UD	UD
13	25	7,6	G2P0A2L0	46,XX	SRY (+)	UD	UD	UD
14	29	7,4	G6P0A6L0	46,XX	SRY (+)	UD	UD	UD
15	27	7,3	G2P0A2L0	46,XX	SRY (+)	UD	UD	UD
16	37	6	G2P1A1L1	46,XX	SRY (+)	UD	UD	UD
17	29	6	G1P0A1L0	46,XX	SRY (+)	UD	UD	UD
18	29	8,4	G1P0A1L0	46,XX	SRY (+)	UD	UD	UD
19	38	8,2	G1P0A1L0	46,XX	SRY (+)	UD	UD	UD
20	22	9,5	G2P0A2L0	46,XX	SRY (+)	UD	UD	UD
21	36	9	G1P0A1L0	46,XX	SRY (+)	UD	UD	UD
22	34	6	G3P1A1L1 (D&C:1)	46,XX	SRY (+)	UD	UD	UD
23	30	6,6	G1P0A1L0	46,XX	SRY (+)	UD	UD	UD
24	23	7	G1P0A1L0	46,XX	SRY (+)	UD	UD	UD
25	25	8	G2P0A2L0	46,XX	SRY (+)	UD	UD	UD
26	37	9,1	G4P1A4L1	46,XX	SRY (+)	UD	UD	UD
27	36	7	G2P1A1L1	46,XX	(+)/ matDNA	UD	UD	UD
28	29	12,6	G3P0A3L0	46,XX	(+)/ matDNA	UD	UD	UD
29	29	10,4	G1P0A1L0	46,XX	(+)/ matDNA	UD	UD	UD
30	37	8,1	G3P2A1L1	46,XX	(+)/ matDNA	UD	UD	UD
31	31	6,3	G3P1A2L1	46,XX	(+)/ matDNA	UD	UD	UD
32	37	5,6	G4P2A1L2 (D&C:1)	46,XX	(+)/ matDNA	UD	UD	UD
33	41	10,2	G4P0A4L0	46,XX	(+)/ matDNA	UD	UD	UD
34	29	6,5	G1P0A1L0	46,XX	(+)/ matDNA	UD	UD	UD
35	33	6,3	G3P1A2L0	46,XX	(+)/ matDNA	UD	UD	UD
36	29	9,5	G2P1A1L1	46,XX	(+)/ matDNA	UD	UD	UD
37	31	8,4	G1P0A1L0	46,XX	(+)/ matDNA	UD	UD	UD
38	43	6,2	G1P0A1L0	46,XX	(+)/ matDNA	UD	UD	UD
39	32	6	G2P0A2L0	46,XX	(+)/ matDNA	UD	UD	UD
40	41	5,6	G5P0A5L0	46,XX	(+)/ matDNA	UD	UD	UD
41	30	8	G2P0A2L0	46,XX	(+)/ matDNA	UD	UD	UD
42	29	7	G1P0A1L0	46,XX	(+)/ matDNA	UD	UD	UD
43	26	6,5	G1P0A1L0	46,XX	(+)/ matDNA	UD	UD	UD
44	27	10,6	G3P0A3L0	46,XX	(+)/ matDNA	UD	UD	UD

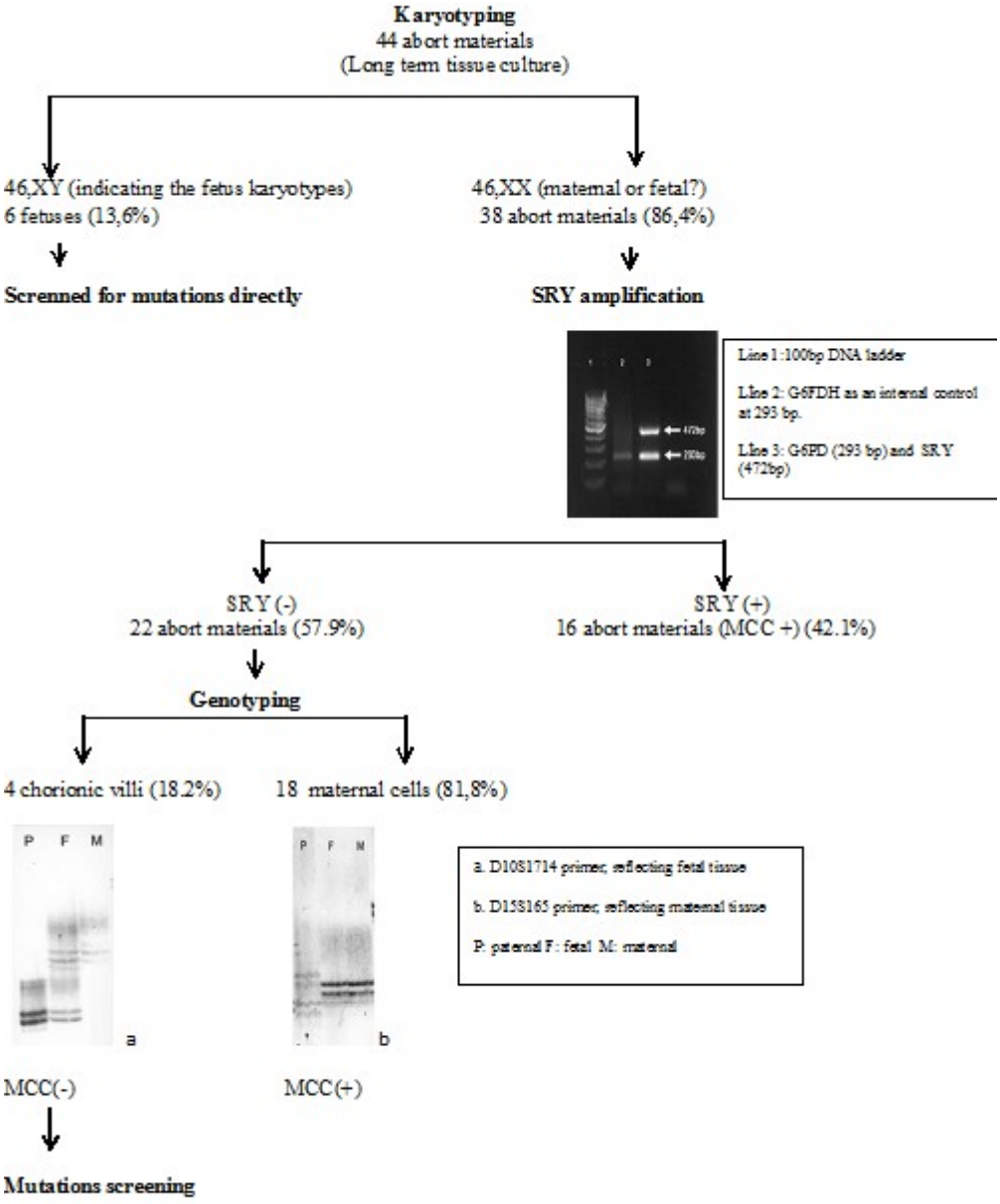

Figure 1. The algorithm followed for excluding maternal cell contamination of the 46, XX karyotyped abortion materials. MCC: Maternal cell contamination

Table 2. The genotypes and allelle frequencies of FII G20210G, FII G20210A, FII A20210A, FV G1691G, FV G1691A, FV A1691A, MTHFR C677C, MTHFR C677T, MTHFR T677T in aborted materials and their parents in the current limited results.

	Fetus (n/%)	Mother (n/%)	Father (n/%)
FII G20210G	8/80	9/90	9/90
FII G20210A	2/20	1/10	1/10
FII A20210A	-/0	-/0	-/0
FV G1691G	8/80	8/80	10/100
FV G1691A	2/20	2/20	-
FV A1691A	-/0	-/0	-/0
MTHFR C677C	4/40	1/10	3/30
MTHFR C677T	6/60	3/30	3/30
MTHFR T677T	-/0	2/20	-/0

DISCUSSION

Venous thromboembolism and pre-eclampsia are the most frequent pregnancy complications. Heritable prothrombotic factors lead to an increased risk of thromboembolism as hypercoagulable state within the fetal circulation could lead to fetal stem vessel thrombosis, placental infarction in the distribution of fetal vessels and spontaneous abortion. By that way these factors play a substantial role in the pathogenesis of spontaneous abortions. Various studies in the recent years have been examined the incidence of specific thrombophilic gene mutations in women with unexplained spontaneous abortion. Some of these studies have been demonstrated an association between thrombophilic gene mutations and abortions (11-13) whereas others have shown the lack of any association (14-16).

It was hypothesized that when the fetus itself has an inherited risk of thrombosis, pregnancy is also more prone to placental infarction at the maternal–fetal interface resulting in an increased risk for intrauterine death but most of the studies have focused on genetic contribution of parents either than the fetuses (17). It is reliable to study with the late pregnancy losses as umbilical cord blood and neonate cord blood collected during the delivery which are the specimens that belong to the fetus. The prevalence of FV Leiden and FII G20210A allele in the umbilical cords of 139 cases (intrauterine exitus) with a gestational age of more than 16 weeks were analyzed.

Fetuses born from an uncomplicated pregnancies were used as control group and the incidence of thrombosis was found higher in the study group, suggesting an important role of abnormal coagulation in placentation (18).

Placental samples of eighty-six patients with pregnancy complicated in the third trimester by idiopathic intrauterine fetal death (IUFD) and 100 healthy pregnant controls were screened for MTHFR C677T, FV Leiden and FII G20210A mutations. It was determined that carrier status of mutant MTHFR C677T must be considered a risk factor for intrauterine fetal demise. As most of the studies have focused their attention on maternal biologic samples to search for the genetic contribution of the mother, they highlight the importance of analyzing the mother–fetus–father triad DNA to screen the thrombophilic mutations in the evaluation of the risk of IUFD (19). Supporting this data in our study six of ten abortion materials were found to be carrying mutant allele of MTHFR C677T in a heterozygote manner.

The umbilical cords from 75 patients with preeclampsia and 92 cords as control group were screened for inherited thrombophilic gene mutations; FV Leiden, MTHFR C677T, and FII G20210A as thrombotic vascular disease may predispose patients to the development of preeclampsia (20). No significant differences between patients with severe preeclampsia and controls in terms of maternal FV Leiden mutation, MTHFR C677T mutation or FII G20210A mutation were determined.

When studying with the early fetal losses, there can be misdiagnosis because of the probability of MCC without the verification of the origins of the materials. Pauer et al., screened FV Leiden mutations on the 139 abortion materials (with the mean gestational age of twelve weeks) and maternal materials (17). It was figured out that there was a tendency towards the pregnancy loss if both fetus and mother carry the same mutation. Based on no information about the verification of the origin of the materials on this report and the others, the results could not be considered completely accurate (12,17,21,22). In our study after confirmation of the fetal tissues in terms of MCC, trombophilic status of the fetal tissues were searched and FV Leiden mutation was detected in heterozygous manner in two of ten materials and they both inherited maternally.

As far as we met in the literature there are only two reports addressing the examination of the maternal tissue contamination by using hyper-polymorphic short tandem repeats. In Zeeteberg et al., study the embryonic tissues were analyzed with microsatellite markers and their haplotypes were compared with the corresponding pattern of their parents (23). Eighty fetal tissue samples from spontaneous abortions that occurred between sixth and twentieth weeks and 125 DNA samples from healthy blood donors as control group were analyzed for MTHFR C677T and A1298C polymorphisms. They found significantly higher frequencies in abortion materials indicating that the MTHFR polymorphisms may have a major impact on fetal survival. Yalcintepe et al., investigated the possible role of multiple inherited thrombophilic gene variations in women with unexplained spontaneous abortions (24). For this aim, they genotyped the F V Leiden, F II G20210A, MTHFR C677T, PAI-1 4G/5G, ACE I/D, eNOS E298D and Apo E E2/E3/E4 mutations in spontaneously aborted fetal materials, and their mothers. 22 abortion materials and their mothers, 22 control subjects with have at least two healthy birth were studied. According to their results combined thrombophilic gene variations may be associated with increased risk for spontaneous abortions. Departure from our study, karyotyping of the fetal tissues was not performed and the chromosome constitution of the fetal tissues were obscure in these two study (23,24).

In the present study it was aimed to study hereditary thrombophilic gene mutations on unmixed spontaneous abortion specimens. For this purpose, efficient algorithms were constructed to exclude the maternal decidual cells. To our knowledge this is the first study that the thrombophilic gene mutations are screened in abort materials after an accurate algorithm. It is find out that MTHFR C677T mutations of the fetus could be more effectual in the formation of abortion. Despite the low number of materials screening for mutation, the parameters are very substantial as they almost reflect the pregnancy loss tissues. As a result a conservative algorithm should be performed to analyze the unmixed materials of the abortions otherwise the results may not be accurate and direct the scientists improperly. There is a need for larger studies to explore the effect of thrombophilic factors in family members for pregnancy losses.

Conflict of interest

No conflict of interest was declared by the authors.

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