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Original Article

The Prevalence of Common Mutations in Thrombophilic Patients in Iranian Population with Recurrent Miscarriage

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Background and Aims: To date, several factors have been reported in recurrent miscarriage. Genetic mutations are the most important causative factors in women. Fetal thrombotic vasculopathy is a new described placental alteration with varying degrees of involvement and often associated with adverse prenatal outcomes. The diagnosis is made histologically and so is postnatal, which makes it a challenge in clinical practice. The aim of the present study is investigation of the common mutations in women with recurrent miscarriage.

Materials and Methods: A cross-sectional study was conducted on 100 women with a history of recurrent miscarriage fetus in 2018. In these patients, several genes such as MTHFR, F2, F5 Leiden, PAI1, F13 and FGB were analyzed by sequencing techniques. The most common mutations in these genes were sequenced and analyzed.

Results: According to statistical results obtained, MTHFR gene (C677T, A1298C) has the highest rate (50 %) of common mutations (p=0.001). After that F2 (G20210A) and F5 Leiden (G1691A) have the highest statistical values (each one 20%). In addition to these genes, there are other unknown mutations which have not been studied in terms of pathogenicity. Other genes have a smaller percentage of aborted fetus infrequently.

Conclusions: Common polymorphisms in the thrombophilic system are likely to result in abortion in these subjects, due to impaired coagulation of the mother and the fetus. Investigating the presence of common mutations and examining their association with other mutations in the thrombophilia as a prognostic in patients with recurrent miscarriage is necessary.

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Introduction

Thrombophilia is an abnormality of blood coagulation which can raise the risk of thrombosis (blood clots in blood vessels) [1]. Such abnormalities can be identified in 50% of people who have an episode of thrombosis (such as deep vein thrombosis in the leg) not provoked by other causes [2]. Fibrinogen, the precursor of fibrin and the end-product of blood coagulation, is an essential component of the hemostatic system [3]. Fibrinogen is converted to fibrin through limited proteolysis by thrombin which exposes polymerization sites to fibrin monomers [4]. Activated factor XIII forms covalent bonds between adjacent fibrin monomers. These cross-links strengthen the fibrin clot and increase its resistance to degradation by the fibrinolysis system [5]. There is no specific treatment for the thrombophilias, but recurrent episodes of thrombosis may be an indication for long term preventative anticoagulation [6]. The most common types of congenital thrombo-philia are those that arise as a result of overactivity of coagulation factors. They are relatively mild and are therefore classified as (type 2) defects [7]. The most common types are factor 5 Leiden (a mutation in the F5 at position 1691) and prothrombin G20210A, a mutation in prothrombin (at position 20210 in the 3' untranslated region of the gene). Thromboembolic events occur as a result of disruption of the balance between fibrinolysis and thrombosis. Thus, it is important to examine the inherited and acquired thrombotic risk factors [8]. In the last five decades, the molecular biases of both coagulation and anticoagulation pathways have been well studied and some hereditary risk factors have been found responsible for venous thromboembolism (VTE) [9]. F5 Leiden (FVL), Prothrombin gene (PT) and methylenetetrahydrofolate (MTHFR) polymorphism are the common molecular biomarkers used in the evaluation of tendency to venous thromboembolism [10]. The mutant F5 molecule is known as F5L and named as F5(R 506 Q) [11]. A single G allele was found as a nucleotide transition at position 20210 in the 3' untranslated region of the prothrombin gene in 18% of selected patients with familial venous thrombosis [12].

MTHFR enzyme plays a significant role in the cellular metabolism of folate and synthesis of nucleotides and is essential in the methyl cycle that converts homocysteine to methionine [13]. To date, two important MTHFR polymorphisms (C677T and A1298C) have been identified. These polymorphisms are related to reducing MTHFR activity [14]. The C677T mutation is known as a point mutation with the substitution of cysteine-thymine at the nucleotide position 677 of the MTHFR gene. Adverse pregnancy outcomes have recently been linked to inherited thrombophilias through extensive studies. However, conclusions regarding their association still remain inconsistent. Normal pregnancy is related with an acquired hypercoagulable state due to increased levels of coagulation factors, decreased levels of anticoagulants and decreased fibrinolytic activity. This

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hypercoagulability may be intensified in women with heritable predisposition to thrombosis, known as thrombophilia and may contribute to various pregnancy complications such as venous thromboembolism (VTE), deep venous thrombosis (DVT), first trimester abortion, mid-trimester abortion, intrauterine fetal death, preeclampsia, placental abruption and fetal growth restriction [15]. The adequate fetomaternal circulating system is essential for normal development and function of the placenta [16]. It is obtained with a mechanism which prevents coagulation of the maternal blood around chorionic villas and fetal blood in them. Normal pathway in coagulation cascade includes the balance between procoagulants, anticoagulant and fibrinolytic components in the blood [17]. Depending on of the type of inherited thrombophilia, there is impaired neutralization of thrombin or failure to control the generation of thrombin. This will cause malfunction of natural anticoagulants that maintain the fluidity of the blood [18]. Coagulation factor (FV) is a large glycoprotein that is produced in the liver and circulates in plasma as a single-chain inactive precursor. After limited proteolysis by thrombin or FXa, FV is converted to its active form (FVa), which acts as an essential non-enzymatic cofactor of FXain prothrombin activation [19].

FVa is inactivated by activated protein C (APC) via proteolytic cleavage at Arg306, Arg506, and Arg679. Although Arg506 is the kinetically preferred APC-cleavage site, cleavage atArg306 is required for complete loss of cofactor activity. Therefore, FVa is mainly inactivated via initial cleavage at Arg506 which yields an intermediate 40% activity followed by slow cleavage at Arg306 [20]. However, FVa can also be inactivated by a single slow cleavage at Arg306 [21].

Recurrent miscarriage is a significant clinical problem of different etiologies [21]. Certain thrombophilic gene mutations have been associated with an increased risk of recurrent miscarriage [22]. Also mutations in Folate-related genes can lead to abnormal chromosomal segregation during meiosis which is the most common cause of recurrent miscarriage. We have developed a multiplex single-base extension reaction assay that allows simultaneous analysis of 10 different mutations in thrombophilia and folate related genes (Factor V Leiden G1691A), (Factor V H1299R), (Factor II G20210A), (Factor XIII V34L), (PAI-I -675 4G/5G), (FGB -455G/A), (MTHFR C677T, MTHFR A1298C), (MTR A2756G, and MTRR A66G) [23]. The purpose of this study is to check the common mutations in women with recurrent miscarriage. Furthermore, the study wants to scrutinze the relationship between common mutations and novel mutations.

Materials and Methods

Subjects and DNA extraction

A cohort of patients (100 women) with a history of two or more miscarriage up to 20 weeks was conducted at our laboratory between 2017 to 2018 in Tehran, Iran. This study included couples with repeated pregnancy losses who had presented themselves during this period for further investigations. Pregnancy was confirmed by a positive human chorionic gonadotropin test using serum or urine in

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combination. In this study, the corresponding genes including F5 Leiden, F2, MTHFR-A, MTHFR-C, PAI 1, FGB and F13 were sequenced with sequencer device. Primers designed in this study are listed in table 1. Informed written consent was taken from each participant and a specific questionnaire was filled out by them. Blood samples were collected using 5 ml syringe. DNA Extraction was performed from ethylenediaminetetraacetic acid blood by using Gene All DNA Extraction kit (Cat No: 105-152, Korea). The quality and quantity of the samples were assessed by DeNovix DS-11 Spectrophotometer (DeNovix Technologies, USA) [24]. DNA extracted after purification (Sigma/Cat. No. 11732 668001) was obtained around 1.82- 1.95 with a suitable concentration. Finally DNA extracted with the final volume 50 µl was stored at -20˚C.

Polymerase chain reaction and Sequencing

Primers for polymerase chain reaction (PCR) were designed by Gene Runner software (version 3.05) and their specificity for PCR was checked by nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [25]. After DNA extraction, the PCR was performed with specific primer (Table 1).

The Optimum annealing temperature of all primers was set at 58-60˚C. PCR reactions were performed in 20 µl total volume by specific primers for 100 samples. The reaction mixture contained 10 pM of each primer, PCR Master mix containing 1 unit of Taq DNA polymerase, 0.2 mM of each of dNTPs, 1.5 mM of MgCl2 and 200 ng of DNA as template. All PCR reaction components were obtained from Fermentas Company. The amplification program was as follows: initial denaturation at 94˚C for 4 min, followed by 35 cycles of denaturation at 94˚C for 25 s, annealing at 58-60˚C for 30 s and extension at 72˚C for 2 min and an additional final extension at 72˚C for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis. The PCR products were run in gel electrophoresis and then were subjected to PCR product gel purification. The purified PCR products were sequenced for finding common mutations.

Table 1. The sequencing of primers that used in this study

Name	Primers	PCR Product (bp)
MTHFR-A-F*	CTTCTACCTGAAGAGCAAGTC	**198**
MTHFR-A-R**	CATGTCCACAGCATGGAG	
MTHFR-C-F	TGAAGGAGAAGGTGTCTGCGGGA	**256**
MTHFR-C-R	AGGACGGTGCGGTGAGAGTG	
F2-F	TCTAGAAACAGTTGCCTGGC	**345**
F2-R	ATAGCACTGGGAGCATTGAGGC	
F5-F	TGCCCAGTGCTTAACAAGACCA	**595**
F5-R	TGTTATCACACTGGTGCTAA	
F13-F	GGCAAAATGTGTTGCTCAAG	**484**
F13-R	AGAACTGGGACAAGGCTCTG	
PAI1-F	CACAGAGAGAGTCTGGACACGTG	**220**
PAI1-R	TGCAGCCAGCCACGTGATTGTCTAG	
FGB-F	TGGGAAATGAAGGAAAATGG	**504**
FGB-R	TGACCTACTCACAAGGCAACC	

*Forward primer
**Reverse primer

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Results

The frequencies were as follows: in the group with preeclampsia 50% were MTHFR gene thus containing homozygous and heterozygous mutation; 30% was found in MTHFR (A1298C) and 20% was identified in MTHFR (C677T). Then F2 (G20210A) and F5 Leiden (G1691A or R506Q) were aligned with 20% frequency. After that, PAI 1(-675 4G/5G) had a low frequency of about 5% and eventually, F13 (V34L or G100T) and FGB (-455 G>A) had the lowest frequency of about 2.5%. According to the results obtained, MTHFR has the highest value for analyzing in recurrent miscarriage fetus. As a result, most women with recurrent miscarriage of the fetus have heterozygous mutations in the MTHFR gene. Moreover, many homozygous mutations in this gene were observed. In the following, common heterozygous mutations in these genes are visible (Table 2 and Fig. 1). In addition, the percentages of heterozygote and homozygote for mutations in MTHFR, F2 and F5 Leiden, being the most frequent among the studied subjects, are illustrated in table 3.

Table 2. Prevalence of common mutations in Iranian patients with miscarriage abortions

Mutations	N = %	P-value	OR (CI 95%)
A1298C (MTHFR)	30	0.001	8.16 (1.62-36.49)
C677T (MTHFR)	20	0.001	7.14 (1.53-33.97)
G20210A (F2)	20	0.6	1.79 (0.16- 36.49)
G1691A or R506Q (F5 Leiden)	20	0.07	2.09 (0.42- 32.86)
(- 675 4G/5G (PAI 1)	5	1	NS*
V34L or G100T (F13)	>2.5	NS	NS
- 455 G>A (FGB)	>2.5	NS	NS

*Not significant

Table 3. The percentages of heterozygote and homozygote for each mutation

Mutations names	Normal (N = %)	Hetrozygous (N = %)	Homozygous (N = %)
A1298C(MTHFR)	60	30	10
C677T (MTHFR)	72	20	8
G20210A (F2)	64	20	16
G1691A or R506Q (F5 Leiden)	48	20	32

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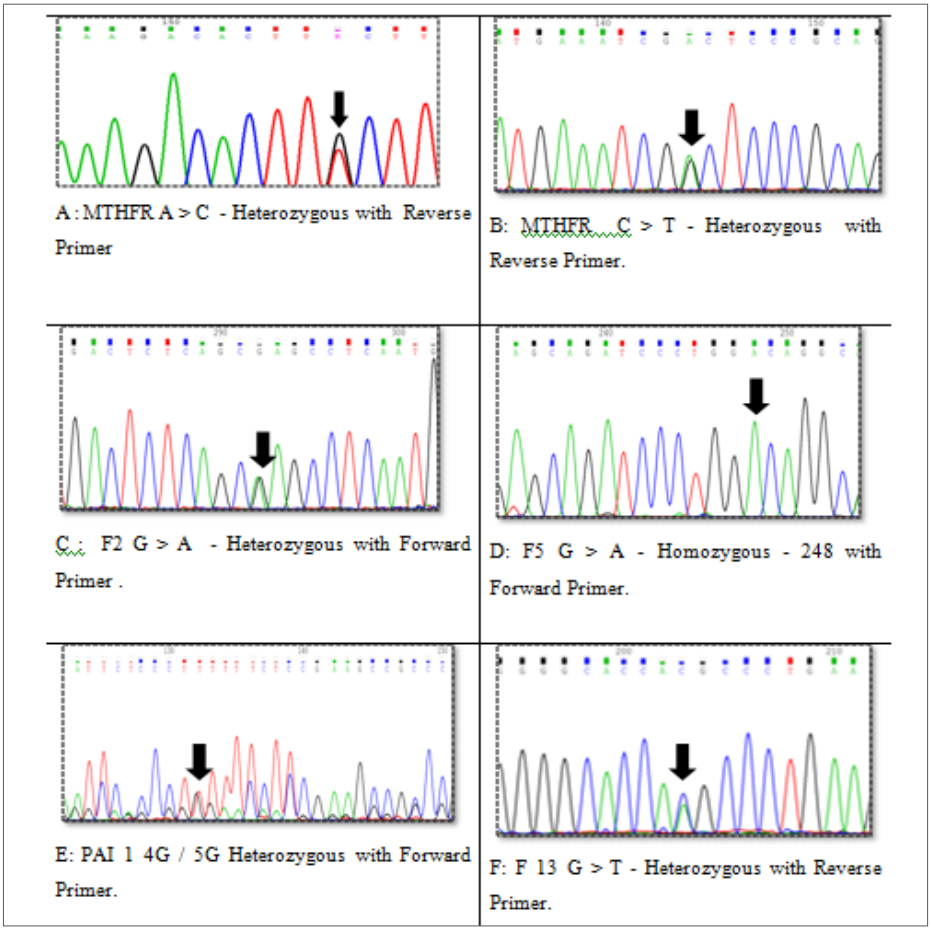

Fig 1. A and B both belong to the results of sequence of the gene MTHFR by reverse primers, which indicate the conversion of A>C and C>T respectively .In both cases, the mutant is heterozygous. In figure C, a mutation in the F2 gene is shown which is marked with a flash of heterozygote mutation. The type of mutation in figure D identified belongs to F5 gene. This mutation is homozygous and G>A. Moreover, E and F respectively belong to the results of sequence of the gene PAI and F13, which indicate the heterozygous mutant.

Discussion

The development of venous thrombosis is generally considered to involve in a combination of locoal regional and systemic prothrombogenic factors [23]. Portomesenteric vein thrombosis (PMVT) is an uncommon but potentially catastrophic clinical complication that may lead to intestinal ischemia and infarction [26]. The etiology of PMVT complication following laparoscopic surgery is likely multifactorial. Some factors that may contribute to the pathogenesis of this condition include undiagnosed thrombophilia, local injury near the portal flow, surgical damage (including direct trauma leading to diminished blood flow), and oral contraceptive use, increased intra-abdominal pressure due to pneumoperitoneum and prolonged time in the reverse trendelenburg position [27]. In patients with thrombo-embolic events, inherited thrombophilia is suspected when the patient has recurrent or life-threatening venous thromboembolism, family history of venous thrombosis, thrombo-embolic events in patients. The highest prevalence of F2

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20210G <A mutation has been reported among the European Caucasus and the Mediterranean population living near Europe [28]. Furthermore, it was reported that the double heterozygotes had thrombosis at a significantly younger age compared to the rest [3]. Other than FV Leiden, another mutation F2 19911A>G found in the same gene has also been described as a risk factor which slightly increases the risk of venous thrombosis when co-existing with the F2 (20210G>A) mutation [5]. Rai and colleagues reported that there is no increase in mutation rates in coagulation factor 5 compared with healthy subjects. In this study, the 20% mutation prevalence in the population studied was related to this mutation [29].

Studies conducted among different racial groups have confirmed varying prevalences of the individuals with F2 20210G>A mutation [8].The highest prevalence of F2 20210G>A mutation has been reported among Caucasians of European origin and Mediterranean populations living close to Europe [11]. Furthermore, the prevalence of the mutation in these populations is higher among patients with thromo-embolic disorders compared with healthy controls [26]. However, in many Asian countries it is reported to be absent in both the healthy controls as well as the patients with thrombo-embolic disorders [21]. In 2009, a study was conducted by Dissanayake et al. to determine the F2 20210G>A mutant allele frequency among healthy controls in the Sri Lankan population. They reported that the mutation was absent among healthy controls across all the three ethnic groups (Sinhala, Tamil and Moor) in Sri Lanka [30]. The present study was undertaken to determine the prevalence of the F2, 20210G>A mutation among Sri Lankan patients with thromboembolic disorders [9, 30]. In contrast to most reports from other Asian populations, we found that the mutation was not entirely absent among these patients as 0.8 % were heterozygotes for the mutation with a mutant allele frequency of 0.4 % [28].

Some of the individual studies have detected an increased risk of pregnancy loss in Indian, Turkish, and the Tunisian population with 1298AC and 1298 CC polymorphisms [31-33]. Mean plasma homocysteine was elevated among 1298AA homozygotes as compared with 1298C allele and was associated with recurrent pregnancy loss [34]. MTHFR 1298 AC genotype allele increased the risk of spontaneous abortion along with MTHFR-C677T [35]. In this study, the frequency of MTHFR gene was 50% so that 30% was found in MTHFR (A1298C) and 20% was identified in MTHFR (C677T). Base on our result, this mutation was higher than the other common mutation in thrombophilia.

Conclusion

In this study, the frequency of mutant allele of the gene MTHFR has a slightly higher percentage than others. According to the findings, mutations in the MTHFR gene in women with abortion that have no physical problems and no disease in holding a fetus in the womb, has been assessed. Concluding from the study, gene MTHFR and F5 are most pathogenic in recurrent miscarriage fetus, so it

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can be referred to as very important genetic mutagenesis. In relation to MTHFR gene mutagenesis and F2, it can be concluded that these two genes may also be involved in recurrent miscarriage of the fetus.

Conflict of Interest

The authors have no financial conflict of interest.

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