Evaluation of GenoFlow Thrombophilia Array Test Kit in Its Detection of Mutations in Factor V Leiden (G1691A), Prothrombin G20210A, *MTHFR* C677T and A1298C in Blood Samples from 113 Turkish Female Patients

Ebru Aytekin, Sezen Guntekin Ergun, Mehmet Ali Ergun, and Ferda E. Percin

Thrombophilia is a heritable blood disease characterized by an increased tendency to form abnormal blood clots that can block blood vessels. In obstetrics and gynecology, it has been shown by a number of reports that a proportion of recurrent miscarriages involve thrombophilia-related mutations, in particular, Factor V G1691A, prothrombin G20210A, and *MTHFR* C677T and A1298C. In this study, we examined the frequency of these four mutations in 113 female Turkish patients who had prior complications in pregnancy, using the DiagCor GenoFlow Thrombophilia Array Test kit. Heterozygous *MTHFR* C677T and A1298C mutations were detected in 46% of the patients, and among these patients, 60% of them carried double heterozygous mutations. In contrast, the heterozygous Factor V G1691A and prothrombin G20210A were detected only in a smaller number of patients, respectively, 13% and 3%. The GenoFlow kit demonstrated 100% concordance with results from Sanger sequencing, which can be translated into sensitivity and specificity both at 100% within this series of patients.

Introduction

THROMBOPHILIA IS AN ABNORMALITY of blood coagulation or formation of blood clots in the blood vessels. It is one of the three main causes of venous thrombosis, along with abnormal blood flow and alternation in the endothelium (Kyrle and Eichinger, 2005). It has been well documented that coagulation Factor V G1691A and prothrombin G20210A are associated with an increased risk of thrombophilia (Lane and Grant, 2000) and venous thrombosis (Reitsma and Rosendaal, 2007). Laboratory screening for thrombophilia has been commonly advocated and is now done on a routine basis in many institutions worldwide (Hyers *et al.*, 2001; Seligsohn and Lubetsky, 2001; Kyrle and Eichinger, 2005). A test for Factor V G1691A has also been recommended by the British Committee for Standards in Hematology (Baglin *et al.*, 2010).

Factor V G1691A is also known as “Factor V Leiden.” It confers a substitution of glutamine at the arginine-506 located within Factor V’s specific cleavage site by activated protein C, which is an anticoagulant protein that cleaves and inactivates procoagulant factors. As a result of such substitution, Factor V can no longer be cleaved by the activated protein C and, in turn, becomes constitutively active, resulting in excessive production of thrombin (Bertina *et al.*, 1994; Segers *et al.*, 2007; Dahlback, 2008).

Prothrombin is the precursor of the serine protease thrombin, which is a key enzyme in blood coagulation. Prothrombin G20210A is also known as Factor II G20210A; it directly confers a dominantly inherited thrombophilia that causes increased plasma prothrombin concentrations (Walker, 2000). The prevalence of the prothrombin G20210A mutation has been reported ranging from 0.7% to 4% in Caucasian populations (Poort *et al.*, 1996; Rosendaal *et al.*, 1998).

Factor V G1691A and prothrombin G20210A are present at different frequencies in different ethnic groups, for example, both variants are less frequently found in the Asian and African populations (Heit *et al.*, 2010; Kenet *et al.*, 2010). According to the US National Institute of Health Genetics Home Reference, between 3% and 8% of the general population with European ancestry carry the heterozygous Factor V G1691A and about 1 in 5000 of them carry a homozygous mutation. In patients with spontaneous venous thrombosis, Factor V G1691A and prothrombin G20210A are the most frequently found mutations (Kyrle and Eichinger, 2005). It has been reported that 12–30% of venous thrombosis patients carry Factor V G1691A (Ridker *et al.*, 1995; Eichinger *et al.*, 2002).

Thrombophilia has been shown to be associated with an increased risk of venous thrombosis during pregnancy according to nine studies consisting of 2,526 pregnant women (Robertson *et al.*, 2005). The heterozygous Factor V G1691A

alone could significantly contribute to a 9-fold increase in the risk of developing venous thrombosis during pregnancy, while it is a 15-fold risk in the case of prothrombin G20210A alone; when both mutations are present, the overall risk of venous thrombosis during pregnancy increased over 100-fold (Gerhardt *et al.*, 2000; Baglin *et al.*, 2010).

5, 10-methylene-tetrahydrofolate reductase (*MTHFR*) catalyzes remethylation of homocysteine to methionine. Homozygous *MTHFR* C677T results in a thermolabile enzyme with reduced activity for the remethylation of homocysteine, causing elevated levels of homocysteine, and has been identified as a risk factor for thrombosis-related cystathionine beta-synthase deficiency in Dutch patients (Kluijtmans *et al.*, 1996) and also for thrombotic stroke in Chinese patients (Li *et al.*, 2003). Such mutation occurs in 10–20% of the general population in Australia and predisposes to mild hyperhomocysteinemia, usually in the setting of suboptimal folate levels (Bezemer *et al.*, 2007).

In pregnant women, *MTHFR* C677T has been shown to predispose thrombosis during the first trimester, together with the presence of Factor V G1691A (Pihusch *et al.*, 2001). For women with a history of recurrent pregnancy loss, homozygous *MTHFR* C677T is more often detected than in those without such history (Goodman *et al.*, 2006). Like C677T, *MTHFR* A1298C has been shown to correlate with a significant increase in the prevalence of thrombosis (Goodman *et al.*, 2006). However, the presence of *MTHFR* A1298C alone is generally not associated with thrombotic ischemic stroke. It is only when both *MTHFR* C677T and A1298C mutations are present, the risk of thrombotic ischemic stroke would increase by 3.39-fold (Szolnoki *et al.*, 2006).

There have been a few commercially available molecular assays in the market for detection of thrombophilia-related mutations. The hybridization-based test has a longer turnaround time. The GenoFlow Thrombophilia Array Test kit was developed using the Flow-through hybridization method aiming to detect both the wild-type and mutant sequences for Factor V, Prothrombin, and *MTHFR*, allowing identification of homozygous or heterozygous mutations in patient samples. The flow-through hybridization system has been reported to be the most efficient method for molecular hybridization. In addition, this method has been reported to have the advantages of being very fast, semiautomated, clean, versatile, and less expensive than traditional hybridization techniques (Xing *et al.*, 2009).

In this study, we examined the mutation status of the four thrombophilia-related genes in blood samples of 113 Turkish female patients and evaluated the reliability of the GenoFlow Thrombophilia Array Test kit in a routine setting of a clinical laboratory.

Materials and Methods

Sample collection

Blood samples were collected with consent from 113 female patients who had been admitted to the Medical Genetics Department of Gazi University, due to infertility or having a complicated obstetric history (Table 1). Five milliliters of peripheral blood of the patients was collected in EDTA tubes and stored at -20°C until DNA isolation.

TABLE 1. THE NUMBER AND HISTORY OF THE PATIENTS ADMITTED TO OUR DEPARTMENT

Patient history	Number of patients
Bad obstetric history	56
Infertility	26
Habitual abortus	17
Venous thrombosis	14
Total	113

DNA extraction

Isolation of blood DNA was performed with the NucleoSpin[®] Blood kit (Macherey-Nagel) according to the protocol. DNA was eluted into 100 μL . The concentration and quality of eluted DNA samples were analyzed by a spectrophotometer (NanoDrop ND 1000).

Real-time polymerase chain reaction

The Factor V G1691A, prothrombin G20210A, and *MTHFR* mutations of C677T and A1298C were amplified and detected by TaqMan probes using a real-time PCR kit (SNP; Biotech). For the procedure, 20.5 μL master mix and 0.3 μL hot start Taq DNA polymerase were added into the polymerase chain reaction (PCR) tube and then 4.5 μL of the patient's DNA was added. The real-time PCR program was performed as including an initial denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 60 s. Allelic discrimination was facilitated by software analysis of the fluorescence data. The homozygous or heterozygous presence of the analyzed mutations was reported.

Polymerase chain reaction

The GenoFlow test was used according to the manufacturer's protocol. PCR was set up with a minimum of 10 ng DNA per reaction extracted from each blood sample. The final reaction volume per reaction was 25 μL , comprising 23 μL Master Mix supplied in the kit, and 2 μL of DNA sample and/or DNase Free Water as the top-up volume. The PCR was conducted according to the following thermal profile: an initial denaturation step at 95°C for 5 min, followed by 42 cycles of denaturation at 95°C for 25 s, annealing at 55°C for 30 s, and extension at 72°C for 8 min. The PCR was completed with the final extension step at 72°C for 8 min.

Genotyping by flow-through hybridization

PCR-amplified DNA samples were first denatured at 95°C for 5 min in a heat block or a thermal cycler and then immediately chilled on ice. The amplicons were genotyped using flow-through hybridization according to the manufacturer's instructions. Positivity was shown by colored dots on the membrane and recorded by scanning of the membrane by a flatbed scanner (Figs. 1 and 2).

Sanger sequencing

All the 113 results from using the DiagCor Thrombophilia Array kit were confirmed by Sanger sequencing, using DNA amplified by single-plex PCR from the original samples. The

FIG. 1. Layout of the wild-type and specific probes on the membrane in the GenoFlow Thrombophilia Array Test kit. HC, hybridization control.

sequencing results were then aligned with the reference gene sequences available on the NCBI database. Sequencing reactions were conducted by using the Genetic Analyzer 3130xl (ABI/Life Technologies), with 300 ng DNA and 3.2 picomoles of primer per reaction, respectively, for Factor V G1691A, prothrombin G20210A, and *MTHFR* C677T and A1298C.

Results

With reference to results from Sanger sequencing, the GenoFlow Human Thrombophilia Array Test Kit accurately detected all four specified thrombophilia-related mutations among the 113 Turkish female patients who had infertility problems or other complications during previous pregnancies, with 100% specificity and sensitivity. *MTHFR* heterozygous C667T and A1298C were most frequently detected among the patients, in which 71% (80/113) of the patients carry either one or both mutations. In contrast, the heterozygous Factor V G1691A was found only in 13% (16/113) of

the patients and only 3% (3/113) for heterozygous prothrombin G20210A. Triple heterozygous mutations of different combinations were found in 3.5% (4/113) of the patients.

Despite such a high percentage of heterozygous double mutation (C667T and A1298C), no double homozygous mutation was detected. Homozygous mutation was only present in a single form (Fig. 3), with *MTHFR* C667 present in 12% of the patients and *MTHFR* A1298C in 8% of the patients. There was only one patient carrying the homozygous Factor V G1691A, and no homozygous mutation in prothrombin G20210A was detected. Finally, 13% (15/113) of the patients did not carry any of the four mutations (Table 2).

Discussion

It is generally recognized that the risk of recurrent miscarriage is reflected by the total number of thrombophilia-related mutations rather than by the status of individual genes; in addition, there are still various nongenetic factors (Coulam *et al.*, 2006). Among the known mutations, Factor V G1691A and prothrombin G20210A appear to have a modest role in the second- and third-trimester fetal loss, and therefore, the current practices of miscarriage risk estimation should continue to include genetic testing for Factor V G1691A or prothrombin G20210A, with reference to prior late pregnancy losses (Bouvier *et al.*, 2014; Connors, 2014).

The prevalence of the FV and prothrombin mutations has been reported as 6.1% and 4.6%, respectively. (Carp *et al.*, 2002). The frequency of mutant alleles in Factor V G1691A and prothrombin G20210A mutations was reported to be 1.12% and 1.68%, respectively (Poursadegh Zonouzi *et al.*, 2013). The homozygosity of *MTHFR* C677T genes in women with RPL and heterozygosity of Factor V Leiden (FVL) have been reported to have a role in recurrent pregnancy loss (Ozdemir *et al.*, 2012).

It has been reported that the homozygous *MTHFR* C677T mutation was detected in 10.8% (29/272) of Turkish female

FIG. 2. A representative set of results by the GenoFlow Thrombophilia Array Test Kit, size of each membrane is 2.7×1.7 cm. (A) Heterozygous mutation of *MTHFR* C677T and Factor V G1691A, the wild type was coherently detected on the upper panel; (B) homozygous mutation of *MTHFR* C677T, no wild-type sequence was detected in the upper panel; (C) an all wild-type result; (D) *MTHFR* double heterozygous mutation at C677T and A1298C, the wild-type sequence was coherently detected on the upper panel; (E) the no-template control for this batch of samples, the positive HC spot signal indicates that the hybridization condition was optimal.

FIG. 3. Frequency of individual thrombophilia-related mutations in the 113 Turkish women. Hom., homozygous; Het., heterozygous.

patients who had recurrent miscarriages; in contrast, the frequency of the Factor V G1691A and prothrombin G20210A mutations were insignificant, relative to a control group of normal people (Yenicesu *et al.*, 2010). Consistent with such finding, using the GenoFlow Thrombophilia Test Kit, we identified that among the 113 Turkish female patients, 12% of them carry homozygous *MTHFR* C677T, while the frequency of Factor V G1691A and prothrombin G20210A was comparatively lower.

Our results are similar to Altintas *et al.*, 2007, which reported that Factor V G1691A and prothrombin G20210A did not have a direct association with recurrent miscarriages during the first trimester of 114 Turkish women, based on a control group of 185 women with normal pregnancies. The GenoFlow Thrombophilia Array Test Kit was designed to detect both the wild-type and mutant alleles of the four thrombophilia-related mutations; with this capability, we report for the first time that 24% of the Turkish female patients carry double mutations of both *MTHFR* C677T and

TABLE 2. A BREAKDOWN OF FREQUENCIES OF THE FOUR THROMBOPHILIA-RELATED MUTATIONS DETECTED BY GENOFLow THROMBOPHILIA ARRAY TEST KIT

Mutation	DiagCor GenoFlow kit	Sanger sequencing
Negative	15	15 (100% matched)
FVL only	2	2 (100% matched)
FII only	0	0 (100% matched)
M677 only	28	28 (100% matched)
M1298 only	24	24 (100% matched)
M677 + M1298	27	27 (100% matched)
FVL + FII	1	1 (100% matched)
FVL + M677	6	6 (100% matched)
FVL + M1298	4	4 (100% matched)
FII + M677	1	1 (100% matched)
FVL + FII + M1298	1	1 (100% matched)
FVL + M677 + M1298	3	3 (100% matched)
FII + M677 + M1298	1	1 (100% matched)
Total	113	113 (100% matched)

FVL: Factor V Leiden, G1691A; FII: Prothrombin G20210A; M677: *MTHFR* C677T; M1298: *MTHFR* A1298C.

M1298A and several with triple mutations. *MTHFR* mutations appear to have a stronger association with pregnancy complications in Turkish women than Factor V G1691A and prothrombin G20210A. It would be worthwhile to conduct more screenings and demographic studies to validate the preliminary relationship.

With respect to the real-time PCR, the GenoFlow test is not only rapid but also an accurate method for patient diagnosis. This test is both practical and easy to interpret the results. Finally, no expensive hardware is needed.

As a conclusion, this study demonstrated that the GenoFlow test is an accurate assay for detection of thrombophilia-related mutations in routine clinical samples as its results were 100% concordant with that by Sanger sequencing in this patient series.

Author Disclosure Statement

GenoFlow Thrombophilia Array Test Kits used in this study were supplied by DiagCor Bioscience, Inc., Ltd., Kowloon Bay, Hong Kong.

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Address correspondence to:
 Mehmet Ali Ergun, MD, PhD
 Department of Medical Genetics
 Gazi University Faculty of Medicine
 Ankara 06510
 Turkey
 E-mail: maliergun@gmail.com