Significance of the use of the ViennaLab “Cardiovascular Disease panel” (CVD) Assay as a reflex test for the “Factor V/II/MTHFR Assay”☆

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Article info

Article history:
Received 11 October 2013
Accepted 13 October 2013

Keywords:
Thrombophilia
CVD
FV
PTH
MTHFR
StripAssay

Abstract

Introduction: Trends toward identifying risk factors of thrombotic complications had become essential as an attempt to prevent and decrease the incidence of the complications. Thrombosis has been associated with predisposing factors like mutations in FV, PTH, MTHFR and other genes. Aim: Evaluate whether the CVD StripAssay has an added value in the screening for more thrombophilia risk factors, which may predispose for the development of cardiovascular diseases and other thrombotic clinical conditions. Methods: We compared the results for 94 patients who were previously tested for Factor V, Factor II and MTHFR gene mutations using the ViennaLab FV-PTH-MTHFR StripAssay, and for whom additional testing for the Cardiovascular Disease panel (CVD StripAssay, ViennaLab) was requested. Results: Using the CVD StripAssay, 66% of patients who had no mutations when tested using the FV-PTH-MTHFR StripAssay or carried a mutation for MTHFR, were found to have additional genes’ SNPs or mutations that are highly associated with a risk of thrombosis as per the available international literature. Conclusion: This observation is of extreme importance in clinical practice for the introduction of the extended CVD panel into routine molecular diagnostic test menus and highlights the importance of genetic

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1. Introduction

The blood coagulation system involves various clotting factors that, under normal conditions, maintain a balanced response to injury. This balance can be shaken under various conditions including obesity, surgery and oral contraceptives intake, leading to thrombosis; however the most important cause remains the mutation in one or more genes that regulate the coagulation process (Yokus et al., 2009). Clinically, the common causes of thrombophilia include stroke, pregnancy complications (for example, miscarriage, intrauterine growth restriction and placental abruption) and venous thromboembolism (VTE) leading to cardiovascular diseases and pulmonary embolism (Kosar et al., 2011).

Trends toward identifying risk factors of thrombotic complications had become essential as an attempt to prevent and decrease the incidence of the complications. Thrombosis has been associated with predisposing entities like Factor V Leiden (FVL), Prothrombin and Methylenetetrahydrofolate reductase (MTHFR) gene mutations, in addition to Protein C and S deficiencies (Thomas, 2001). Factor V Leiden mutation results in activated protein C resistance phenotype, which is in turn correlated with increased risk of venous thromboembolism (Bertina et al., 1995). A study by Taher et al. revealed a high prevalence of FVL mutation among the Lebanese population, with a frequency of 14% among healthy individuals and 40% among patients with deep venous thrombosis (DVT) (Taher et al., 2001). Mutations in the Methylenetetrahydrofolate reductase gene could reduce enzymatic activity and lead to hyperhomocysteinemia, a condition that has been associated with several vascular conditions, in particular, coronary artery disease and deep vein thrombosis (D’Angelo et al., 1997; den Heijer et al., 1996; Graham et al., 1997; Hanson et al., 2001; Klerk et al., 2002; Wilcken et al., 1996).

Prothrombin gene (Factor II) G20210A mutation is an autosomal defect that has been reported as a risk factor for arterial thrombosis with reported increased risks of more than fivefold for thrombotic cerebrovascular disease (Bick, 2003). Among the Lebanese population, the frequencies of the G and A alleles were found to be 99% and 1%, respectively (Sabbagh et al., 2007a). Factor V H1299R (also known as HR2) has been shown to contribute by itself to an increased activated protein C (APC) resistance both in normal and thrombophilic patients, independent of the status of FV Leiden carriership (Bernardi et al., 1997). The coinheritance of HR2 and FV Leiden appears to be associated with severe APC resistance phenotype (Castaman et al., 1997). Previous studies have shown that the presence of HR2 haplotype is associated with increased risk of venous thromboembolism (VTE, 2–3 fold) (Castaman et al., 1997; Margaglione et al., 2002). In Lebanon, HR2 was found in 10.4% of the population (Zaatari et al., 2006) and among FVL negative individuals, VTE patients were 2.7 times more likely to carry HR2 than controls, implying a condition of severe APC resistance phenotype in the presence of both mutations (Otrock et al., 2008).

The prevalence of MTHFR (A1298C) reduces the activity of the Methylenetetrahydrofolate reductase enzyme to a lesser extent than in the case of the C677T polymorphism (van der Put et al., 1998). This reduced enzyme activity leads to hyperhomocysteinemia, which has been associated with several vascular conditions including coronary artery disease and deep vein thrombosis (D’Angelo et al., 1997; den Heijer et al., 1996; Graham et al., 1997; Hanson et al., 2001; Klerk et al., 2002; Wilcken et al., 1996). The frequencies of homozygous and heterozygous MTHFR C677T mutation in Lebanon are 11.04% and 39.73%, respectively (Isma’eel et al., 2006).

Available are various genotyping assays that can identify the presence or absence of a gene mutation or polymorphism that predisposes to thrombosis. The FV-PTH-MTHFR StripAssay (ViennaLab, Austria) covers three mutations: FV G1691A (Leiden), PTH G20210A and MTHFR C677T. The CVD StripAssay (ViennaLab, Austria) covers twelve mutations: FV G1691A (Leiden), FV H1299R (R2), PTH G20210A, Factor XIII V34L, β-Fibrinogen − 455 G-A, PAI-1 4G/5G, GPIIIa L33P (HPA-1), MTHFR C677T, MTHFR A1298C, ACE I/D, Apo B R3500Q and Apo E2/E3/E4.

In this study, we compared the results for all patients who were previously tested for Factor V, Factor II and MTHFR gene mutations using the FV-PTH-MTHFR StripAssay and for whom additional testing for the genetic Cardiovascular Disease panel (CVD StripAssay) was requested. Our aim was to evaluate whether
the CVD StripAssay has an added value as a reflex test in the screening for more thrombophilia risk factors, which may predispose for the development of cardiovascular diseases and other thrombotic clinical conditions, especially for those patients with a negative initial screening result by the conventional Factor V/II/MTHFR assay.

2. Materials and methods

2.1. Study design and subjects

This study was performed at the American University of Beirut Medical Center (AUBMC), a tertiary-care center in Lebanon and involved subjects screened using the FV-PTH-MTHFR StripAssay as well as the CVD panel StripAssay. The use of archived DNA screening results was approved by the Institutional Review Board at AUBMC. The tested DNA was extracted using the Pel-Freez extraction kit (Dynal, USA) and always stored at $-80^\circ$C for later use.

Subjects were tested for Factor V, Prothrombin and MTHFR gene mutations using the FV-PTH-MTHFR StripAssay (ViennaLab, Austria) or additional genes using the CVD StripAssay (ViennaLab, Austria) and the tests’ protocols were followed as described by the manufacturer.

2.2. FV-PTH-MTHFR StripAssay

The FV-PTH-MTHFR StripAssay covers three mutations: FV G1691A (Leiden), PTH G20210A and MTHFR C677T.

2.3. Cardiovascular Disease StripAssay

The CVD StripAssay covers 12 mutations: FV G1691A (Leiden), FV H1299R (R2), PTH G20210A, Factor XIII V34L, β-Fibrinogen $-455$ G-A, PAI-1 4G/5G, GPIIIa L33P (HPA-1), MTHFR C677T, MTHFR A1298C, ACE I/D, Apo B R3500Q and Apo E2/E3/E4.

2.4. Polymerase chain reaction and hybridization

Briefly, in vitro, the different gene sequences were simultaneously amplified and biotin-labeled in a single amplification reaction (multiplexing). The thermocycler program consisted of an initial step of 94 $^\circ$C for 2 min, followed by 30 cycles for FV-PTH-MTHFR and 35 cycles for the CVD assay of 94 $^\circ$C for 15 s, 58 $^\circ$C for 30 s, 72 $^\circ$C for 30 s, and a final extension step of 72 $^\circ$C for 3 min. Finally, the amplification products were selectively hybridized to a test strip which contained allele-specific (corresponding to wild type or mutant) oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences were detected using streptavidin–alkaline phosphatase and color substrates.

2.5. Interpretation of genotyping results

For each polymorphic position, one of three possible patterns may be obtained: normal, heterozygous, or homozygous mutant genotype. For the Apo E isoforms E2, E3 and E4, six possible homozygous and heterozygous Apo E genotypes (E2/2, E3/3, E4/4, E2/3, E2/4, and E3/4) could be obtained.

3. Results

Using the FV-PTH-MTHFR StripAssay and out of the 94 patients, 22 were found normal, 17 were normal for FV and PTH while homozygous for MTHFR, and 55 were normal for FV and PTH while heterozygous for MTHFR (Diagram 1). Among these 94 patients, and using the CVD StripAssay, 62 (65.96%) were found to have additional genes (SNPs or mutations) that are highly associated with a risk of thrombosis including, FV (H1299R), MTHFR (A1298C), PAI-1 4G allele, HPA-1 1b allele, ACE D allele and ApoE E2 genotype.

Among the 22 normal individuals, 4 were found heterozygous for FV H1299R and expressed at least one copy of the MTHFR A1298C gene. Moreover, 4 patients typed “normal” were homozygous for MTHFR A1298C,
and 10 were heterozygous for MTHFR A1298C. Additionally, some of the individuals expressed the ACE D allele, the PAI-1 4G allele and/or ApoE E2 alone or in combination with the above-mentioned mutations.

Out of the 17 patients who were normal for FV and PTH genes mutations while homozygous for MTHFR gene mutations, 3 were also homozygous for ACE D and one was assigned the ApoE E2/E3 genotype.

As for the 55 individuals who were normal for FV and PTH genes mutations while heterozygous for MTHFR gene mutations, 7 were found heterozygous for FV H1299R, and of whom 5 were also heterozygous for MTHFR A1298C. Some of these individuals expressed additional alleles associated with an increased risk of thrombophilia, such as ACE D and ApoE E2. Moreover, 29 individuals were heterozygous for MTHFR A1298C and some of them were also positive for PAI-1 4G, HPA-1 1b, ACE D and ApoE E2.

4. Discussion

Genetic screening for the most common genes in molecular thrombophilia profiling (Factor V, II, and MTHFR) is becoming essential in most clinical practice, however, the more extended genetic panel represented by the Cardiovascular Disease (CVD) profile (or panel) offers a broader spectrum of genes, polymorphisms or mutations, that have been described in the literature as predisposing for thrombotic events.

Among the interesting findings obtained from the CVD panel assay, mutations in factor XIII (FXIII), also known as fibrin stabilizing factor, were found to be associated with protection against some cardiovascular events. The most common mutation is V34L which reduces the clot stability by modifying the structure of the cross-linked fibrin, thus causing spontaneous bleeding. This in turn can be considered a mode of protection against thrombotic conditions (Mahfouz et al., 2008). β-fibrinogen is yet another protein involved in the thrombotic events, where the most common mutation (−455 G > A) is associated with an increased risk of venous thromboembolism (VTE) and atherothrombotic diseases. Among the Lebanese population where the frequencies of the G and A alleles were reported as 77% and 23%, respectively, individuals who were found homozygous or heterozygous for −455A had higher levels of plasma fibrinogen than those who were homozygous for −455G (Shammaa et al., 2008a). Furthermore, polymorphisms in the plasminogen activator inhibitor type-1 (PAI-1) gene, including the 4G/5G polymorphism, contribute to the occurrence of thrombotic events by increasing the level of PAI-1 thus leading to excessive buildup of fibrin (Shammaa et al., 2008b). The data from Lebanon revealed that the frequency of the mutant 4G allele is 40.3%, with a higher rate of heterozygous individuals compared to homozygous, while the 5G allele frequency stands at 59.7% (Shammaa et al., 2008b).
Other important genetic information is obtained through the CVD assay. In addition to transforming angiotensin I to active angiotensin II, the angiotensin-converting enzyme (ACE) cleaves the vasodilator bradykinin, thus leading to decreased production of tissue plasminogen activator. The activity of ACE varies depending on the carried genotype, with the D/D genotype being associated with the highest activity and the I/I with the lowest (Sabbagh et al., 2007b). The D/D genotype is highly associated with VTE and is expected to increase the thrombotic risk about 11 times. Among Lebanese individuals, the I/D genotype is detected in about 45% of the population while the D/D and I/I are present in 39% and 16%, respectively (Sabbagh et al., 2007b).

Apolipoprotein B (ApoB) serves as a ligand of the low density lipoprotein (LDL) receptor and a defect in the gene coding for ApoB, such as the R3500Q mutation, leads to a decrease in the binding activity that in turns causes a delayed clearance of LDL. This subsequently increases the risk of atherosclerosis and hypercholesterolemia (Sabbagh et al., 2007c). The frequency of R3500Q varies geographically, being the highest in Europe. In Lebanon, as per the study performed by Sabbagh et al., the R3500Q mutation was not detected among healthy individuals (Sabbagh et al., 2007c).

Apolipoprotein E (ApoE) is responsible for the regulation of the metabolism and the clearance of chylomicrons, intermediate density lipoprotein and very low density lipoprotein. The presence of a certain ApoE isoform (E2, E3, or E4) can either contribute to the protective role of this molecule (E2) or to increased risk of CVD (E4) (Mahfouz et al., 2013). In Lebanon, the genotype frequencies of E3/E3, E3/E4 and E2/E3 are 68.75%, 16.25% and 13.75%, respectively, and the E2/E4 and E4/E4 were only found in 0.625% of the population (Mahfouz et al., 2006).

According to this study, we observed around 66% increased positivity rate of detection of an “abnormal” finding in genetic profiling in this category of patients. This observation is of extreme importance in clinical practice for the introduction of the extended cardiovascular disease panel into routine molecular diagnostic test menus and highlights the importance of genetic analysis of the implicated genes in the management of patients with a thrombotic episode presentation especially in populations harboring high prevalence of the above-stated polymorphisms and mutations. Based on our experience using the two above-described assays, we highly advocate for the CVD StripAssay as an important reflex test for the available FV-III-MTHFR StripAssay.

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