Molecular Diagnostics of Inherited Thrombosis

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Thrombophilia can best be defined as a disorder of coagulation that contributes to a predisposition towards thrombosis. Although the term thrombophilia has been used to describe arterial thrombosis, its most common usage has been in reference to venous thromboembolism (VTE). Thrombophilia can be a consequence of both acquired and inherited or genetic causes. Acquired causes include conditions such as surgery, cancer, and prolonged immobilization, while genetic causes have been linked to the inherited deficiencies of antithrombin, protein C, and protein S. The identification of the genetic basis of these inherited causes of thrombophilia ushered in a new way of thinking about thrombosis and the importance of its genetic component. Interest in the genetic basis of VTE was accelerated with the subsequent discovery of factor V Leiden, prothrombin G20210A, and MTHFR C677T. These single nucleotide polymorphisms (SNPs) and other genetic variants associated with VTE have become fixtures in the molecular diagnosis of inherited thrombophilia. Because of the large volume of current and anticipated future genetic testing, there has been a push to develop many different genotyping methods which are now used in both clinical and research settings. The identification of new genetic variants that may either directly or indirectly affect coagulation or the anticoagulant pathway, may greatly advance the understanding and clinical management of thrombophilia.

ABBREVIATIONS: APCR = activated protein C resistance; CBS = cystathionine β-synthase; FVL = factor V Leiden; LDL = low density lipoprotein; MTHFR = methylenetetrahydrofolate reductase; MTR = methionine synthase; MTRR = methionine synthase reductase; PCR = polymerase chain reaction; PE = pulmonary embolism; PT = prothrombin; SNP = single nucleotide polymorphism; VTE = venous thromboembolism.

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LEARNING OBJECTIVES

1. Define thrombophilia and explain the difference between acquired and inherited forms.
2. Define the terms multigenic and multifactorial.
3. Identify the three most common inherited protein deficiencies associated with venous thrombosis.
4. List three common genetic single nucleotide polymorphisms (SNPs) tested in the diagnosis of inherited thrombosis.
5. Describe the mechanism by which the Factor V Leiden mutation affects hemostasis.
6. Describe the effect of the MTHFR C677T mutation on homocysteine metabolism.
7. Explain the purpose of polymerase chain reaction (PCR).
8. Compare and contrast laboratory methods used to identify single nucleotide polymorphisms.

Hemostasis is a complex balance of procoagulant and anticoagulant forces that act in concert to physiologically control

bleeding and clotting. The term thrombophilia refers to a disorder in coagulation that shifts hemostasis towards an increased predisposition for thrombosis. Although thrombophilia has been used by some to describe arterial thrombosis, its usage has commonly been synonymous with VTE and both will be used interchangeably in this review.

With an approximate annual incidence of 1 in 1000, venous thromboembolism (VTE) affects approximately 250,000 individuals each year.[1,2] Pulmonary embolism (PE), a complication of VTE, has a high morbidity rate and is a leading cause of hospital in-patient deaths.[3] Because thrombophilia is both multigenic (involving many different genes) and multifactorial (involving the interaction of genetic and acquired factors) it can be difficult to identify causes and predict risk.

Thrombophilia can be divided into two basic categories: acquired and inherited (Table 1). As implied, in acquired thrombophilia the increased risk of thrombosis is significantly reduced or removed when the associated condition no longer exists, such as the discontinuation of hormone replacement therapy.[4] Inherited thrombophilia is a much more intricate puzzle because of the involvement of a wide array of genes and their proteins that either directly or indirectly affect coagulation and the anti-coagulant pathway. Consequently the possibilities for finding genetic variation related to thrombosis are seemingly endless. In most cases, a genetic predisposition alone is not enough to cause thrombosis. Therefore, the assessment of gene-gene and gene-environment interactions is another important piece of the puzzle. Also adding to the challenge are the differences between ethnicities. For example, the factor V Leiden polymorphism, which will be discussed later, is fairly common in people of Western European descent but found only rarely in those of African or Asian descent.

Table 1. Conditions associated with thrombophilia

Acquired	Inherited
Surgery	Protein C deficiency
Trauma	Protein S deficiency
Malignancy	Antithrombin deficiency
Pregnancy	Dysfibrinogenemia
Oral contraceptives	Factor V Leiden
Hormone replacement therapy	Prothrombin G20210A
Immobilization	
Aging	
Antiphospholipid syndromes	
Hyperhomocysteinemia	
Liver disease	
Sepsis	

GENETIC VARIANTS IMPORTANT IN VTE:

An inherited predisposition can now be identified in approximately 60% to 70% of VTE patients.[5] Autosomal dominant inherited protein C, protein S, and antithrombin deficiencies and, to a much lesser extent, recessive deficiencies or abnormalities in proteins such as factor VII, fibrinogen, and thrombomodulin are thought to account for anywhere from 5% to 15% of venous thromboembolism.[6,7] Over 160 genetic variations have been identified in the protein C gene. In the absence of normal levels and/or decreased functionality of the above-mentioned proteins, the function of other coagulation factors may be impaired. For example, without activated protein C, the procoagulant factors Va and VIIIa are not completely inactivated. Similarly, a deficiency of protein S can impair the function of activated protein C.

Currently, laboratory diagnosis of these deficiencies involves functional or immunological testing, as clinical genetic testing presents somewhat of a challenge. The genetic changes underlying these protein deficiencies are due to many different mutations and types of mutations spread throughout the individual genes, thus making rapid detection difficult. Missense, nonsense, frameshift, deletion, and insertion mutations have all been found (Table 2). Mutation databases for the protein C, protein S, antithrombin, and fibrinogen

Table 2. Types of mutations

Type	Description
Missense	A base change that results in an amino acid substitution.
Nonsense	A base change that results in a stop codon, prematurely terminating the protein.
Insertion	The addition of 1 or more base pairs.
Deletion	The deletion of 1 or more base pairs.
Frameshift	The insertion or deletion that is not a multiple of three within the coding sequence that results in a subsequent change to all the following amino acids.

genes have reported a total of 161, 131, 127, and 282 unique genetic changes, respectively.[8-11] The detection of so many different DNA changes throughout the entire gene(s) is an expensive, time consuming, and labor intensive process. However, since only a single base pair substitution is involved, single nucleotide polymorphisms (SNPs) are ideally suited for molecular testing. The list of SNPs associated with VTE is continually growing (Table 3), but we will focus on three that are among the most commonly tested clinically in thrombophilia: factor V Leiden, prothrombin G20210A, and MTHFR C677T. Other tests clinically available include FXIII Val34Leu, Protein S Heerlen, PAI-1 4G/5G, MTHFR 1298, and platelet GP IIIa Leu33Pro.

FACTOR V LEIDEN

The discovery of activated protein C resistance (APCR) by Dahlbäck in 1993, and the subsequent discovery of the Factor V Leiden (FVL) mutation, which causes at least 90% of

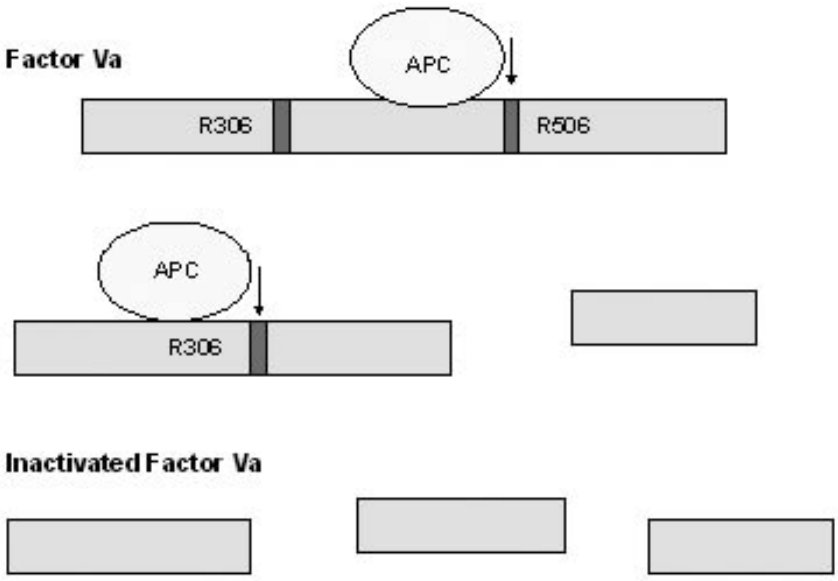
Table 3. Clinical associations of genetic polymorphisms (Modified from Hooper and De Staercke 2002)

Polymorphism	Phenotype	Association with VTE
Protein C anticoagulant pathway		
Factor V Leiden: G1691A (Arg506Gln)	APC resistance	Clear risk factor
Factor V Cambridge: Arg306Thr	APC resistance	Tentative
Factor V Hong Kong: Arg306Gly	APC resistance	Tentative
Factor V HR2 haplotype	Mild APC resistance	Tentative
Thrombomodulin C1481T (Ala455Val)	Unknown	Unlikely
Thrombomodulin-33 G to A	Unknown	Possible
EPCR 23bp insertion in exon 3	Unknown	Unlikely
Protein S Heerlen	Decreased free protein S	Unknown
Procoagulant Proteins		
Prothrombin G20210A	Increased FII	Clear risk factor
Fibrinogen Bcl-1 allele in β chain	Increased fibrinogen	Possible
Fibrinogen-148 CtoT in β promoter	No increased fibrinogen	Not known
Fibrinogen G448A in β chain	Increased fibrinogen	Not known
Fibrinogen Thr312Ala in α chain	Abnormal FXIII cross-linking?	Possible
Factor VII Arg353Gln	Low-normal FVII	Not known
Factor VII H7H7	Low-normal FVII	Not known
Factor VII G73A	Low-normal FVII	Not known
Factor XIII A subunit Val34Leu	Increased activity	Protective?
Homocysteine metabolism		
Cystathionine β-synthase T833C		Possible
5,10-methylenetetrahydrofolate reductase C677T		Unlikely
Platelet surface glycoproteins		
GP IIIa Leu33Pro (PL A2 or HPA-1b)	Increased sensitivity to platelet activation; altered sensitivity to aspirin	Unlikely
GP Iba VNTR	Unknown	Not known
GP Iba C3550T (Thr145Met)	Unknown	Not known
GP Ia/IIa, a2 A1648G	Altered surface expression of receptor	Not known
Thrombin receptor PAR-1-5061D	Unknown	Protective?

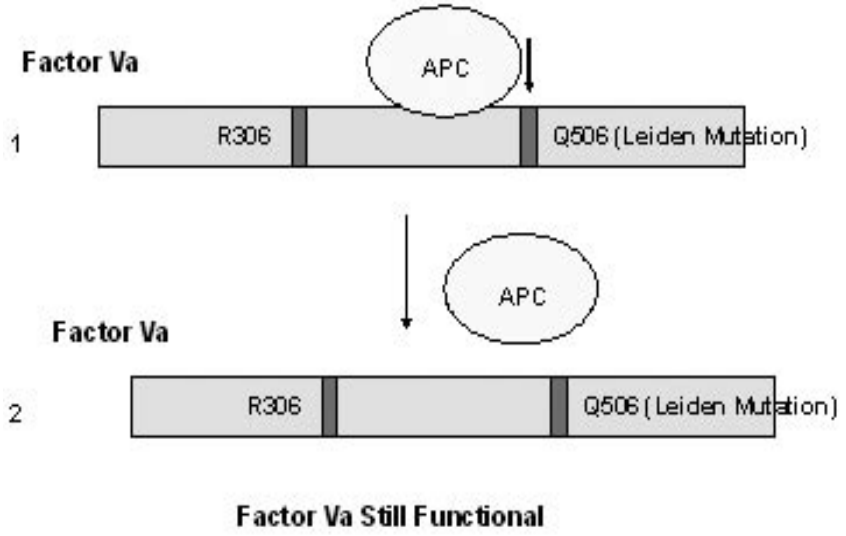
APCR, by several groups the following year, revolutionized the molecular diagnosis of thrombophilia.[12-16] The FVL mutation is a G to A transition at position 1691 in the factor V gene which changes arginine 506 to glutamine. Arginine 506 is a cleavage site for activated protein C (Figure 1). When glutamine is present, activated protein C cannot completely inactivate FVa, causing activated protein C resistance. Present in 15% to 20% of patients with VTE and in 2% to 5% of the general U.S. population, it is by far the most common genetic factor in inherited thrombophilia.[17] However, while FVL is common in Caucasian populations, especially those of Western European descent, it is not common in African American and Asian populations.[18,19] Heterozygosity increases the risk of VTE by three- to eight-fold and homozygosity by 50- to 100-fold.[20-22]

Figure 1. Inactivation of Factor Va by activated protein C (APC)

1A: 1) APC cleaves factor Va at Arginine 506; 2) APC cleaves factor Va at Arginine 306; 3) Factor Va is now inactivated.



1B: 1) APC cannot cleave factor Va at Glutamine 506; 2) Factor Va remains functional



PROTHROMBIN G20210A

A few years after FVL was reported in 1996, Poort described another single base change associated with VTE, this time in the 3' untranslated region of the prothrombin gene.[23] The G to A transition at position 20210 results in increased levels of circulating prothrombin and hence an increased potential for thrombin generation. As with FVL, PT G20210A is not common in African American or Asian populations, but is found in the Caucasian population at a frequency of 1% to 4%.[24] Among patients with VTE, the frequency rises to 6% to 18% and confers a two- to five-fold increased risk.[23,25-27] Diagnosis of PT G20210A can only be done using molecular genetic testing.

HYPERHOMOCYSTEINEMIA AND MTHFR C677T

In some individuals, plasma levels of the amino acid homocysteine are abnormally high. Hyperhomocysteinemia has been found to increase thrombotic risk by about two- to four-fold.[28] It is believed that atherogenic damage occurs when homocysteine binds to LDL and induces the expression of inflammatory chemokines, such as MCP-1 and IL-8. These chemokines

attract monocytes and neutrophils which attach to endothelial cells, become macrophages, and phagocytose the homocysteine-LDL aggregates. This process forms foam cells, a precursor to atherosclerotic lesion formation.[29] Because there is almost no free homocysteine in foods, circulating plasma homocysteine results from the metabolism of methionine. The enzymes cystathionine, β-synthase (CBS), methionine synthase (MTR), methionine synthase reductase (MTRR), 5,10-methylenetetrahydrofolate reductase (MTHFR), and thymidylate synthase (TS) and several cofactors, betaine, folate, and vitamins B2, B6, and B12, are key components of methionine metabolism. While there are some acquired causes of these elevated levels, such as vitamin B6 or B12 deficiency and renal failure, genetic factors have also been identified. More than 200 different genetic variants have been reported and linked to elevated homocysteine levels.[29] Most are rare and detrimental only when found in the homozygous state or have been found to have no association with VTE. One exception is MTHFR 677 C→T. The mutation results in the substitution of valine for alanine causing a reduction in enzyme activity and an increase in sensitivity to heat inactivation (thermolability).[30,31] A fairly common polymorphism in the general population, MTHFR 677 C→T has been associated with mild hyperhomocysteinemia and considered by some to be a weak VTE risk factor.[32]

GENE-GENE AND GENE-ENVIRONMENT INTERACTIONS

Due to the multigenic and multifactorial nature of VTE, it is possible that two or more genetic changes, or one or more genetic changes and an environmental risk factor, can be present in any individual at any one time. These interactions can have a dramatic effect on risk and consequently it is important to take these interactions into consideration. For instance, patients who are protein C, protein S, or antithrombin deficient and heterozygous for FVL have increased risk of VTE over that of the deficiency alone.[33-36] Significant increases in risk have also been found in patients who carry both the FVL and PT G20210A variants as compared to those with only a single variant.[37] Gene-environment interactions have also been noted between FVL and PT G20210A and oral contraceptives, hormone replacement therapy, pregnancy, and antiphospholipid syndrome.[38-42]

Gene-gene and gene-environment interactions are possible explanations for the conflicting data surrounding the relationship between the MTHFR C677T polymorphism and VTE. While Jacques found elevated plasma homocysteine levels only in individuals who had the T/T genotype and low folate, findings from other studies that looked at interactions were mixed.[43-45]

APPROACHES FOR MOLECULAR ANALYSIS

As mentioned previously, molecular testing for protein C, protein S, antithrombin, and fibrinogen deficiencies requires mutation detection methodology. Genotyping of SNPs such as FVL, PT G20210A, and MTHFR C677T, however, can be done using a wide variety of assays in single or multiplex formats using standard laboratory equipment or specialized instrumentation. Additionally, analyte specific reagents (ASRs) or FDA approved kits are available for some of the polymorphisms. Although they are generally more expensive, the validation and quality control they receive from the manufacturer may justify the extra expense in certain settings. Each assay's accuracy, costs, labor intensity, and multiplexing capabilities should be assessed before deciding on a method to use for a particular study or implement in a clinical laboratory. While it is beyond the scope of this paper to cover all possible methods, several will be discussed and compared.

The polymerase chain reaction (PCR) is the basis of the majority of genotyping methods. PCR allows short fragments of a relatively small amount of genomic DNA to be amplified into quantities suitable for many different types of analysis in only a few hours. Typically, the target DNA is amplified about a million-fold. DNA extracted from tissue, buccal cells, whole blood of certain patients with low white cell counts, or small volumes of blood can have lower DNA concentrations. PCR is especially useful when testing these types of samples. One common PCR-based assay is restriction fragment length polymorphism (RFLP). Although RFLP has not been around for a long time, it is a proven method. While it is quite labor intensive and perhaps not ideally suited for large sample sizes, it is fairly easy to optimize, reagent costs are low, and standard laboratory equipment is all that is necessary to perform the assay. By fluorescently labeling the fragments, throughput can be increased, multiplexing is made easier, and labor intensity decreased with only a minor raise in reagent costs.[46] The major drawback would be the need to purchase a capillary electrophoresis instrument, e.g., ABI Prism® 3100, or Beckman Coulter CEQ™ 8000. Another popular PCR-based method is allele specific amplification (ASA). In this method, each allele is amplified individually and separated by agarose gel or capillary electrophoresis. Although the optimization and design of ASA assays can be more difficult than RFLP, sample manipulation is decreased and therefore less labor is involved.[47,48]

Gene sequencing is generally cost-prohibitive for SNP genotyping. However, Pyrosequencing™ provides 50 to 100 base pairs of sequence data in a cost-effective platform with

assays for FVL and MTHFR C677T already available.[49,50] While there are benefits to having actual sequence data, such as detecting assay problems or nearby polymorphisms, this method requires specialized instrumentation and several post-PCR manipulations, making it fairly labor intensive.

Many new types of assays for SNP genotyping resulted from the advent of fluorescently labeled probe technologies. If carefully optimized, they afford great flexibility because they can be customized to any target sequence and with different fluorophores. Although each type of probe uses a slightly different mechanism to detect the genetic change, they share a common methodology of measuring fluorescent signals after laser excitation. Specific instrumentation is required to perform these assays, but there are many different instruments available in all price ranges, e.g., ABI Prism® 7900, Roche LightCycler®, Stratagene Mx4000®, and Rotor-Gene 2000™. Even though reagent and instrument costs are higher than some other assays, they hold one very distinct advantage: the PCR and genotyping are done simultaneously in the same tube. This significantly decreases manual manipulations, lowering the labor intensity, improving throughput, and decreasing the chance of sample mix-up. Two commonly used assays for genotyping thrombophilic mutations are the Roche Diagnostics LightCycler® Factor V Leiden Mutation and Prothrombin Mutation Detection Kits and Applied Biosystems' TaqMan Allelic Discrimination Assays.[51-55] The LightCycler® has a smaller capacity compared to ABI Prism® 7900 (32 versus 96 wells), but the run times are 45 minutes and 2 to 3 hours, respectively. One advantage to the Roche system is its ability to discriminate between the mutation of interest and other polymorphisms nearby that are not associated with VTE. As an example, there are three rare polymorphisms, two in the factor V gene and one in the prothrombin gene that can cause genotyping errors. The LightCycler® assays are able to discriminate between the different base changes.[56,57] Besides the LightCycler and TaqMan assays, genotyping can also be done using other types of fluorescent probes, such as molecular beacons and Scorpion probes.[58,59]

For high throughput and multiplexing capabilities, arrays such as Nanogen's NanoChip® Systems or GenomeLab's™ SNPstream® Genotyping System, may be a good solution.[60-63] Array technology is well-suited to large sample sizes and is very accurate and precise once optimized. Additionally, an analyte-specific reagent FVL and PT G20210A multiplex kit has been developed by Nanogen. Mass spectrometry is another robust method capable of high throughput and multiplexing when applied to genotyping. Masscode™ SNP Genotyping Technology from BioServe and MassARRAY from Sequenom are two available options.[64,65] Of course, purchasing the specialized instrumentation for these types of assays is a large investment and specialized training is required.

The Luminex® platform has also been utilized for SNP genotyping, taking advantage of its capacity for multiplexing and high throughput using fluorescently labeled microspheres and flow cytometry.[66,67] While assays can be custom-designed, kits are available from Tm Bioscience to test FVL alone, multiplexed with PT G20210A, or multiplexed with PT G20210A, MTHFR 677, and MTHFR 1298. Again, special instrumentation is necessary and there are several post-PCR manipulations, but it is a flexible platform, not only for genotyping, but for measurement of proteins as well.

Variations of single base extension (SBE) and oligonucleotide ligation assay (OLA) are additional PCR-based choices.[68-71] One non-PCR based option of note is the Invader® assay from Third Wave Technologies.[72,73] This assay requires only a plate reader and heat block, inexpensive items found in

Table 4. Methodology comparison

Method	Labor intensity	Throughput	Cost	Special instrument needed	ASR reagents
RFLP	Med-High	Low-Med	Low-Med	If fluorescently labeled	No
ASA	Med-High	Low-Med	Low-Med	If fluorescently labeled	No
Pyrosequencing	Med	Med	Med	Yes	No
Probe-based	Low	Med	Med	Yes	Yes
Arrays	Med	High	High	Yes	Yes
Mass spectrometry	Med	High	High	Yes	No
Luminex	Med	High	Med	Yes	Yes
Invader	Med	Med	Med	No	Yes

most laboratories, and with an assay set-up similar to that of PCR, technician time is minimal. Several additions and incubations are required before reading the plate. No assay development would be required for FVL, PT 20210, and MTHFR as ASR kits have already been developed.

Obviously, there are many choices for SNP genotyping, including some not mentioned here. As each method has its own advantages and disadvantages in regards to cost, accuracy, and labor intensity, careful evaluation and validation should be performed for each assay under consideration to find the best fit (Table 4). When testing in a clinical setting, using FDA approved or ASR reagents may be worth their additional expense. As always, a comparison of how several methods perform in your own hands is always the best way to make any decisions.

FUTURE DIRECTIONS

Currently, the value of genetic testing is somewhat limited, as the results do not usually change medical treatment and issues regarding privacy, genetic testing, and genetic counseling of unaffected carriers have yet to be resolved.[74] Even though the utility of molecular diagnosis in thrombophilia is somewhat limited at this time, it will surely play a larger role in the future. As gene sequencing continues to become less expensive and labor intensive, candidate gene sequencing of additional proteins involved in hemostasis will continue at a rapid pace, identifying more genetic polymorphisms correlated to an increased risk of VTE. For instance, it has been found that elevated levels of fibrinogen, factor VIII, factor IX, and factor XI are all risk factors for VTE.[75-80] Genetic components for these increases have not yet been identified, but may be in the future.

Another area in which a great deal of research is being done is the effects of inflammation and the immune response on hemostasis. As our comprehension of the roles that they play in thrombosis increases, the molecular diagnosis of thrombophilia will most likely expand to include genes in these pathways and provide new candidates for drug therapies. Genes involved in the metabolism of drugs, such as Cytochrome P450, may also play a central role in the treatment of thrombophilia. Identifying particular genotypes that require higher doses of anticoagulant drugs for appropriate therapeutic effects or genotypes that require lower doses to prevent bleeding complications will be an important step forward in using molecular diagnosis for patient care.[81] Additionally, as the ability to multiplex many SNPs in a cost-effective way using small amounts of sample continues to improve, the understanding of gene-gene interactions will leap forward. A patient's genetic information will be an important piece in solving the puzzle of thrombophilia.

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