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Venous thromboembolism laboratory testing (factor V Leiden and factor II c.*97G>A), 2018 update: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

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Factor V Leiden and factor II c.*97G>A (formerly referred to as prothrombin 20210G>A) are the two most common genetic variants associated with venous thromboembolism (VTE). Testing for these variants is one of the most common referrals in clinical genetics laboratories. While the methodologies for testing these two variants are relatively straightforward, the clinical implementation can be complicated with regard to test indications, risk assessment of occurrence and recurrence of VTE, and related genetic counseling. This document provides an overview of VTE, information about the variants and their influence on risk, considerations before initiating genetic testing, and the clinical and analytical sensitivity and specificity of the tests. Key information that should be included in the laboratory report is also provided. Disease-specific statements are intended to augment the general American College of Medical Genetics and Genomics (ACMG) technical standards for clinical genetics laboratories. Individual laboratories are responsible for meeting the Clinical Laboratory Improvement Amendments (CLIA)/College of American Pathologists (CAP) quality assurance standards with respect to appropriate sample documentation, assay validation, general proficiency testing, and quality control measures. This 2018 edition of the ACMG technical standard updates and supersedes the 2005 edition on this topic. It is designed to be a checklist for genetic testing professionals who are already familiar with the disease and the methods of analysis.

Genetics in Medicine (2018) 20:1489–1498; https://doi.org/10.1038/s41436-018-0322-z

Keywords: factor V; factor V Leiden; factor II; thrombophilia; venous thromboembolism

INTRODUCTION

Thrombosis is one of the most common causes of morbidity and mortality in the United States. The incidence of venous thromboembolism (VTE) is approximately 1~1.5 per 1000 person-years and an individual's absolute lifetime risk of VTE is approximately 11%[1–3]. The risk of VTE is age-related. Before age 40, the risk is approximately 1 in 10,000 persons per year and it increases to 1 in 100 persons per year after age 75[4]. Consequently, the economic burden is also significant; the clinical management of VTE costs the health-care system an estimated $1.5 billion/year in the United States.[5,6] The recurrence risk is estimated to be approximately 20% within

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The Board of Directors of the American College of Medical Genetics and Genomics approved this technical laboratory standard on 13 July 2018.

Submitted 17 September 2018; accepted: 17 September 2018
Published online: 5 October 2018

5 years and 30% within 10 years after the first incidence.[7,8] Although the most frequent VTE event is deep vein thrombosis (DVT) in the legs, thrombosis can also occur in the veins of other sites such as the upper extremities, pelvis, abdomen, cerebral venous sinuses, etc. Pulmonary embolism is the main life-threatening complication of DVT. It is estimated that one-third of VTE manifests as pulmonary embolism and two-thirds as other DVTs.[3] The etiology of VTE is multifactorial. Both environmental factors and genetic predispositions influence the hemostasis of the coagulation system. Environmental factors include smoking, male sex, older age, malignant neoplasm, prolonged immobilization, and surgery.[9] Additional risks for women include pregnancy, postpartum period, use of oral contraceptives, estrogen replacement therapy, tamoxifen, and raloxifene treatment.[10,11]

While factor V Leiden and factor II c.*97G>A are the most common genetic predisposition factors, genetic defects in antithrombin, protein C, protein S, or factor XIII also contribute to VTE.[12] In addition, other single-nucleotide polymorphisms (SNPs) associated with VTE have also been identified.[13] Known genetic factors are present in about 25% of unselected VTE cases and up to 63% of familial cases.[14] Genetic predisposition factors often interact with various environmental factors to provoke thrombosis. However, approximately 50% of first-time VTE cases are apparently unprovoked.[15] Genetic counseling about genetic and non-genetic aspects of the risk is important.[16] Due to the coexistence of multiple risk factors for each individual, it is often challenging to integrate these risk factors to make a definitive prediction of occurrence or recurrence.

Arterial thrombosis is mainly caused by atherosclerosis. Stroke and coronary heart disease are the main manifestations of arterial thrombosis. While arterial and venous thrombosis are traditionally viewed as distinct conditions with different pathophysiology and treatments, they share some common risk factors such as aging, immobility, and obesity.[17–19]

Due in part to the high incidence of VTE, genetic testing for hypercoagulability is one of the most common tests in clinical genetics laboratories. Factor V Leiden and factor II c.*97G>A are among the most commonly requested tests by clinicians. Testing for the other key inherited thrombophilias (antithrombin, protein C, and protein S deficiency) is usually functional, determining activity or in some circumstances, antigen level. Numerous pathogenic variants have been reported in the antithrombin (*SERPINC1*), protein C (*PROC*), protein S (*PROS1*) genes, and sequencing is available if desired in special circumstances (Genetic Testing Registry, http://www.ncbi.nlm.nih.gov/gtr, accessed May 2016). Overall, pathogenic variants in the protein C, protein S, or antithrombin genes account for approximately 5–10% of patients with thrombosis.[9,20–22]

In this document, we will focus on genetic testing for factor V Leiden and factor II c.*97G>A variants.

BACKGROUND

Genomic information

Factor V:

Gene name: Coagulation factor V (proaccelerin, labile factor)

Gene symbol: F5

Chromosomal location: 1q24.2

Genomic coordinates (hg38): chr.1:169,514,166–169,586,588, reverse strand

OMIM Entry: 612309

Nomenclature: c.1601G>A (p.Arg534Gln) (rs6025, g.169549811C>T, NC_000001.11, NM_000130.4, NP_000121.2). This variant was previously designated as G1691A or Arg506Gln and is referred to as factor V Leiden or FVL.

Clinical significance: pathogenic, risk factor

Factor II:

Gene name: Coagulation factor II (prothrombin)

Gene symbol: F2

Chromosomal location: 11p11.2

Genomic coordinates (hg38): chr.11:46,719,180–46,739,506, forward strand

OMIM Entry: 176930

Nomenclature: c.*97G>A (rs1799963, g.46761055G>A, NC_000011.9, NM_000506.3.) This variant was previously designated as G20210A or 20210G>A and is commonly referred to as factor II or prothrombin G20210A or 20210G>A.

Clinical significance: pathogenic, risk factor

The pathophysiology of factor V Leiden and factor II c.*97G>A

In the normal coagulation system, activated protein C (APC) functions as a natural anticoagulant by inactivating coagulant factor Va and factor VIIIa in the presence of protein S. The initial APC cleavage at position p.Arg534 of factor V is required for the optimal exposure of factor V to subsequent cleavage. Subsequently, a rapid inactivation of factor V occurs by the APC cleavage at positions p.Arg334 and p.Arg707 (previously referred to as positions p.Arg306 and p.Arg679 respectively).[23,24] The factor V Leiden eliminates the first APC cleavage site at p.Arg534. As a result, factor V is inactivated to a lesser extent than the normal protein and persists longer in the circulation, leading to more thrombin generation. Factor V Leiden is found in 90–95% of all patients with APC resistance.[25,26]

Factor II is a vitamin K–dependent protein. Prothrombin is converted to thrombin in the presence of factor Va, factor Xa, calcium ions, and phospholipids. Thrombin not only has the function of catalyzing the conversion of fibrinogen to fibrin, the building block of a hemostatic plug, but it also activates platelets, factor V, factor VIII, and factor XIII.[27] The c.*97G>A variant is located in the 3'UTR of the factor II gene. It is associated with an elevated prothrombin level of 30% above normal in heterozygous individuals and 70% above normal in homozygous individuals.[28,29] The elevated prothrombin level is believed to play a key role in the

pathogenesis of thrombosis.[28,30] Molecularly, the wild-type guanine at the cleavage site is the least efficient nucleotide to support 3' end processing.[31] Factor II c.*97G>A variant upregulates the 3' end processing efficiency of the precursor messenger RNA (pre-mRNA), resulting in an increased pre-mRNA accumulation and elevated protein synthesis.[31,32] This variant is, therefore, a gain-of-function mutation.

Mode of inheritance, population genetics, occurrence risk, and recurrent risk

Both the factor V Leiden and factor II c.*97G>A exhibit a semidominant trait in that both heterozygotes and homozygotes are at an increased risk of VTE, with a greater risk in homozygotes, especially for factor V Leiden.

Factor V Leiden

In the United States, factor V Leiden heterozygosity is present in 5.1%, 2.0%, and 1.2% of Caucasians, Hispanics, and African Americans respectively; the frequencies of homozygosity for the above populations are 65, 10, and 4 per 100,000 individuals correspondingly.[33–35] The population frequency of the factor V Leiden variant also varies among European countries. Greece and Sweden seem to have higher frequencies than Portugal and Italy (~7% vs.1.4%)[9]. The factor V Leiden variant almost does not exist among Sub-Saharan Africans, East Asians, and indigenous populations of America and Australia.[9,36]

Factor V Leiden is present in approximately 20% of individuals with an initial episode of isolated DVT (19% heterozygous and 1% homozygous), 8.3% with isolated pulmonary embolism (8% heterozygous and 0.3% homozygous), and 16% with both DVT and pulmonary embolism (15% heterozygous and 1% homozygous).[37,38] The relative risk for VTE is approximately six to eight-fold for heterozygotes and 80-fold for homozygotes.[39,40] For individuals with factor V Leiden, a positive family history increases the risk of VTE 2.9-fold (95% confidence interval [CI], 1.5–5.7), and if there is VTE in a relative before age 50, the risk increases up to five-fold (95% CI, 2.0–14.6). If there are multiple affected relatives, the risk could increase to 17-fold (95% CI, 2.2–143.1)[16,41].

Lifetime risk of VTE in heterozygotes is approximately 10% and close to 100% for factor V Leiden homozygotes (2.9 VTE events/1000 person/year for heterozygotes and 15 VTE events/1000 persons/year for homozygotes).[42,43] Lifetime risks of VTE are higher when environmental risk factors such as obesity and smoking are also present.[42–45]

Heterozygosity for factor V Leiden has at most a modest effect on recurrence risk after a first VTE, with conflicting results between studies.[46] Some studies have demonstrated no increased recurrence for factor V Leiden heterozygotes.[47] However, homozygous factor V Leiden leads to a significant increase in recurrence. A systematic review reported odds ratios of 1.56 and 2.65 for heterozygotes and homozygotes respectively.[48]

*Factor II c.*97G>A*

The heterozygous factor II c.*97G>A variant is found in approximately 1–3% of Caucasians, 1% of Hispanics, and 0.3% of African Americans in the United States.[16,33] The frequencies of homozygosity for factor II c.*97G>A are 12 per 100,000 and less than 1 per 100,000 individuals among Caucasians and Hispanics respectively.[33]

Among symptomatic individuals, this variant is present in 6% of individuals with an initial episode of VTE.[35,49] In the absence of other acquired risk factors, the relative risk for venous thrombosis associated with the factor II c.*97G>A ranges from 1.9- to 11.5-fold; the majority of studies have shown a risk of two to four-fold for heterozygotes.[49,50]

For individuals with the factor II c.*97G>A variant and a family history of VTE the risk of VTE increases three to four-fold.[38,41] The risk tends to be higher if the VTE occurred at a younger age or there are multiple affected family members.[38,41]

Homozygotes for the factor II c.*97G>A are rare. The prevalence among the general population is 0.001–0.012% and 0.2–4% among individuals with VTE.[16] The annual risk of VTE in homozygotes has been reported to be 1.1%/year.[16] From a literature review of 49 cases, homozygous individuals display a striking phenotypic heterogeneity, ranging from asymptomatic individuals who were identified through family studies to individuals suffering from a fatal event in the neonatal period.[51]

The recurrence risk for VTE due to factor II c.*97G>A heterozygosity is at most moderate, with conflicting data and many studies showing no increased recurrence.[35,38] The recurrence risk of VTE for factor II c.*97G>A homozygotes is presumed to be higher than for heterozygotes, but this is not well defined due to limited numbers of patients identified with this genotype.[38]

*Factor V Leiden and factor II c.*97G>A double heterozygotes*

Because both factor V Leiden and factor II c.*97G>A are relatively common among Caucasian populations, individuals may harbor both variants. The estimated prevalence of double heterozygotes is 22 per 100,000[33]. Six to twelve percent of individuals who are heterozygous for the factor V Leiden with a VTE event also harbor the factor II c.*97G>A.[52] In the same meta-analysis consisting of 2310 Caucasian cases and 3204 controls, the odds ratio for VTE of double heterozygotes was 20.0 (95% CI, 11.1–36.1)[52].

Reports have demonstrated that patients who have had VTE and are heterozygous for both factor V Leiden and factor II c.*97G>A have a three to nine-fold increased risk for recurrent VTE, though one family study did not find an increased risk.[38] A systematic review showed a five-fold increased risk.[48] A prospective study found an annual incidence of recurrent VTE of 12% per year in individuals heterozygous for both the factor V Leiden and factor II c.*97G>A versus 2.8% in those with neither variant.[53]

Pathogenic variant spectrum

Factor V Leiden accounts for at least 90–95% of cases with APC resistance.[54,55] Another variant in the factor V gene, called factor V R2 (rs1800595, c.3980A>G [p.His1327Arg] also known as His1299Arg), has also been widely studied. Unlike the factor V Leiden, which is almost exclusively found in Caucasian populations, the minor allele frequency (MAF) of the R2 variant ranges from 0% in Nigeria to about 10% in Bangladesh with an average global frequency of 5% (www.1000genomes.org, last accessed May 2016). It appears to confer a modest additional thrombotic risk when present in a compound heterozygous state with the factor V Leiden.[56] Compared with normal individuals, this variant has 73% of the APC cofactor activity.[57] In the homozygous state, factor V R2 allele appears to cause a mild APC resistance.[58] With rare exceptions, it is usually in *trans* with the factor V Leiden and rarely found in factor V Leiden homozygotes.[56,59] The R2 allele alone is not associated with an increased risk of VTE.[60,61] However, it has been speculated that homozygous R2 can contribute significantly to APC resistance in the Japanese population due to a relatively high prevalence of homozygosity (1 in 350) and an extremely low presence of the factor V Leiden variant.[62]

Other alleles in the factor V gene have also been described. Factor V Cambridge (rs118203906, c.1001G>C [p.Arg334Thr] also known as Arg306Thr) and factor V Hong Kong (rs118203905, c.1000A>G [p.Arg334Gly] also known as Arg306Gly) located in the APC cleavage site of the factor V gene were speculated to have functional implications.[63,64] By in vitro functional analysis, both factor V Cambridge and factor V Hong Kong variants showed a mild APC resistance with the APC response being in between that of the wild-type and factor V Leiden variant.[65] In the 1000 Genomes Project, factor V Hong Kong is reported in approximately 1% of Chinese and Vietnamese populations and in 0.2% of African Caribbeans. Factor V Cambridge was found in approximately 0.2% of African Caribbeans and 0.5% of Colombians (www.1000genomes.org, accessed May 2016). While anecdotal reports exist, studies do not support an association of these two variants with an increased risk of VTE at least in Chinese and Mexican populations.[66–68] Large-scale studies of these variants and their risks related to thrombosis are still lacking.

Other rare alleles such as factor V Liverpool (rs118203911, c.1160T>C [p.Ile387Thr], also known as Ile359Thr) and factor V Nara (c.5842T>C [p.Trp1948Arg], also known as Trp1920Arg) have also been described in patients with VTE.[69,70] More and more rare alleles are expected to be discovered in the future due to the frequent use of exome sequencing (ES) and genome sequencing (GS) in the clinical arena. However, it may not be necessary to test routinely for these rare alleles.

Regarding the factor II gene, the c.97*G>A variant accounts for the majority of reported alleles in patients with VTE. Other variants such as prothrombin Yukuhashi (c.1787G>T [p.Arg596Leu], rs387907201) have been described.[71] These alleles do not have a frequency high enough to warrant routine clinical testing.

TESTING INDICATIONS

General indications

Testing for factor V Leiden and factor II c.97*G>A is recommended (1) in patients with VTE when the results will influence treatment and clinical management decisions and (2) in patients and certain asymptomatic relatives to reduce the risk of provoked VTE through counseling about preventive measures in circumstances of elevated risk.[16,46,72] Factor V Leiden and factor II c.*97G>A genotyping provides information on the recurrence risk of VTE and can inform decisions relevant to avoidable circumstantial risks such as extended travel, contraceptive use, approach to long-term immobilization, etc.[16] Factor V Leiden and factor II c.97G>A homozygotes or double heterozygotes are defined as having high-risk thrombophilias, and the severity of the inherited thrombophilia (high or low risk) is a consideration for treatment decisions.[38,42,46,73–76] Testing is recommended for certain targeted populations/circumstances; it is not recommended indiscriminately for all patients with VTE or for the general population. Testing indications from different professional organizations vary. Some suggest there is limited clinical utility of testing for inherited thrombophilia in a majority of patients with VTE.[35,74,77–79] However, it is acknowledged that this approach could miss the identification of homozygotes, for whom knowledge of this genotype would influence treatment or prevention.[74] Others recommend targeted testing of patients and relatives with increased risk.[45,47] A review of testing indications from scientific societies and working groups is provided by de Stefano and Rossi.[46]

Testing for factor V Leiden and factor II c.*97G>A is recommended in the following circumstances:

1. A first unprovoked VTE, especially <50 years old
2. VTE at unusual sites (such as hepatic portal, mesenteric, and cerebral veins)
3. Recurrent VTE
4. Personal history of VTE with (a) two or more family members with a history of VTE or (b) one first-degree relative with VTE at a young age
5. Patients with low activated protein C (APC) resistance activity

Testing may be considered in the following circumstances:

1. Females under the age of 50 who smoke tobacco and have a history of acute myocardial infarction
2. Siblings of individuals known to be homozygous for factor V Leiden or factor II c.*97G>A, because they have a 1 in 4 chance of being a homozygote
3. Asymptomatic pregnant female or female contemplating pregnancy, with a first-degree relative with unprovoked VTE or VTE provoked by pregnancy or contraceptive use
4. Pregnant female or female contemplating pregnancy or

estrogen use who has a first-degree relative with a history of VTE and is a known carrier for factor V Leiden and/or factor II c.97*G>A variant
5. Pregnant female or female contemplating pregnancy with a previous non-estrogen-related VTE or VTE provoked by a minor risk factor, because knowledge of the factor V Leiden or factor II c.*97G>A status may alter pregnancy-related thrombophylaxis

Routine testing is not generally recommended for patients with a personal or family history of arterial thrombotic disorders (such as coronary artery disease or ischemic stroke) due to a lack of evidence of the association between thrombophilia and arterial ischemic events.

Several clinical scenarios requiring special considerations

Testing of symptomatic versus asymptomatic individuals

Current genetic technologies have high analytical sensitivity and specificity for the testing of factor V Leiden and factor II c.*97G>A. Currently, these tests are predominantly used for individuals with clinical symptoms of VTE. In a review of data from Europe, Australia, and United States,[46] VTE accounts for 42% of the clinical referrals for testing. Other indications include arterial thrombosis (15–23%), obstetric complication (13–17%), and asymptomatic relatives (12–16%). From a meta-analysis, factor V Leiden genotype was shown to be predictive of the recurrence of VTE for the proband and of the occurrence for family members especially when factor V Leiden homozygosity was detected.[48] For factor II c.*97G>A, the predictive value is not conclusive with most studies demonstrating no increased recurrence risk for heterozygotes and not enough data for homozygotes.[42,46,48,72,73] Knowing the factor V or factor II genotype will neither alter the clinical management nor affect the decision for prophylaxis for many patients.[35,48] However, under certain circumstances the knowledge of high-risk genotypes could influence the clinical management.[46,72] Further investigation is needed to demonstrate whether testing of asymptomatic relatives to promote awareness of their risk of VTE would decrease the incidence of VTE; knowledge of their genotype might facilitate counseling on avoidable circumstantial situations.[16]

Asymptomatic family members may sometimes request genetic testing before being exposed to certain risk factors. It is generally not recommended to test asymptomatic minors as VTE rarely occurs before young adulthood even in the homozygous state.[42]

Prenatal testing and population screening

Factor V Leiden and factor II c.*97G>A are relatively common among the general population and VTE can be fatal. However, prenatal testing and population screening are not indicated due to the low penetrance of these variants, later age of onset, and lack of genotype-directed prophylaxis.

Testing of pregnant women and patients with recurrent adverse pregnant outcomes

Pregnancy is associated with an increased clotting risk and decreased anticoagulant activity. Factor V Leiden and factor II c.*97G>A have been detected in approximately 40% and 17% of VTE cases respectively during pregnancy.[80–82] Risk stratification for pregnancy-associated VTE is reviewed by Rodger.[76] The risk of VTE in heterozygous women without a personal history or affected first-degree relative(s) is only minimally increased compared with the general population.[75,81,82] A comprehensive investigation of a patient's personal and family history of thrombosis and individualized risk assessment is recommended before the initiation of genetic testing.[16,75,83] However, it is often challenging to collect a thorough personal and family history in the current clinical settings.[83] It may be appropriate to test these variants in women with unexplained recurrent first-trimester pregnancy loss, an unexplained fetal loss after 10 weeks gestation, or still birth.[42] For individuals with a known genotype, some professional organizations recommend prophylactic treatment for homozygotes and double heterozygotes.[75]

Women who have experienced utero-placental thrombosis-related adverse pregnancies such as fetal loss, preeclampsia, fetal growth restriction, and placental abruption are often referred for genetic testing.[46] However, the relationship between inherited thrombophilia and utero-placental thrombosis or preeclampsia is unclear.[84,85] Routine genetic testing for these conditions is currently not recommended by the American College of Obstetricians and Gynecologists (ACOG).[84]

Women considering taking estrogen-containing oral contraceptives (OC) or hormone replacement therapy (HRT)

It is well known that oral contraceptives pose an additional risk of thrombosis among individuals harboring the factor V Leiden and/or factor II c.*97G>A.[86] In a meta-analysis, OC users showed an odds ratio of 1.8 (95% CI, 1.20–2.71) compared with nonusers among factor V Leiden carriers where the odds ratio is 1.63 (95% CI, 1.01–2.65) for factor II c.*97G>A carriers.[87] While ACOG recommends a consideration of alternative contraceptive options, screening all women for genetic thrombophilias before initiating contraception is not recommended.[79,88,89]

HRT is associated with a two to four-fold increased risk of VTE in users compared with nonusers.[42] Women on HRT with the factor V Leiden have an odds ratio of 13.16 (95% CI, 4.28–40.27) for VTE compared with women without this variant.[90] Retrospective studies suggest that transdermal HRT is not as prothrombotic as oral HRT.[91–93] Genetic screening of prospective HRT users has not proven to be beneficial.[92] A family and personal history of thrombosis should be carefully evaluated for all women before initiating HRT and a positive history may warrant thrombophilia screening.

For asymptomatic relatives of patients with factor V Leiden or factor II c*97G>A considering oral contraceptives or HRT, there is no published data regarding whether genetic testing would benefit or change the clinical management of oral contraceptive or HRT use.[35] Testing of these variants may be

useful when there is a strong family history of thrombotic disorders or a first-degree relative with both a history of VTE and known factor V Leiden or factor II c*97G>A.[16]

*Factor V Leiden and factor II c.*97G>A variants as secondary findings during ES or GS testing*

Exome sequencing (ES) or genome sequencing (GS) are now frequently used as a diagnostic tool for pediatric and adult patients. Due to the relatively high population frequency of the factor V Leiden and factor II c*97G>A, it is not surprising that these variants are often identified as secondary findings during ES or GS testing. Currently, there is not a general consensus regarding whether or not to report these variants. Individual laboratories may have different policies. Factor V and factor II are not among a list of genes proposed by ACMG in which secondary findings of pathogenic variants are recommended to be reported.[94,95] While these two variants increase the risk of VTE, which can have fatal outcomes such as pulmonary embolism, it is important to be aware that the penetrance of these variants is rather low. If a laboratory decides to report these variants as secondary findings, genetic counseling should be recommended. Additionally, the ES/GS consent should indicate specific genes that the laboratory includes in the secondary findings and it should be obtained from the patients or guardians before testing.

Informed consent

Obtaining informed consent is generally not mandatory for factor V Leiden and factor II c.*97G>A testing unless required by state-specific laws/regulations for genetic testing. However, individuals should be aware that any genetic test could possibly have implications for insurability or have other social and psychological implications. Other family members may be at an increased risk of VTE if the proband tests positive. Genetic counseling should be available when necessary. As for all other genetic tests, testing laboratories are encouraged to have mechanisms to collect pretest clinical information that includes the patient's date of birth, racial/ethnic background, indication for testing, and specific family history. When testing indications are found to be inappropriate by the clinical laboratory, testing laboratories are encouraged to communicate with the referring physician to recommend cancellation of the test.

CLINICAL VALIDITY, CLINICAL UTILITY, CLINICAL SENSITIVITY, AND CLINICAL SPECIFICITY

Clinical validity is defined as the test's ability to accurately and reliably identify or predict the disorder or phenotype of interest. Several meta-analyses support the clinical validity of factor V Leiden (either heterozygote or homozygote) to predict the recurrence of VTE in the proband and VTE occurrence in family members.[35,48] For factor II c.*97G>A, there is only limited evidence regarding the predictive value for the recurrence risk of VTE in probands and it is inconclusive whether this variant could predict VTE in family members.[48] Factor V Leiden and factor II c.*97G>A double heterozygotes seem to be predictive for the occurrence of VTE among family members, but there is insufficient information to draw a firm conclusion. However, the risk of VTE in family members with this genotype is likely to be at least as high as for factor V Leiden alone.[35,48]

Clinical utility is defined as whether the clinical test results could change the patient's clinical management. There is no consensus regarding the role of genotype for determining the treatment regimen for VTE. Current antithrombotic recommendations from professional organizations largely do not focus on genotype for most VTE patients.[81,96] For many patients, the clinical utility of genetic testing for VTE is not high.[48] However, for certain circumstances, such as pregnant women with previous VTE and positive family history, the clinical utility has been acknowledged.[46,72,74,81]

The clinical sensitivity of factor V Leiden or factor II c.*97G>A can be defined as the proportion of individuals who have had (or will have) VTE and are pathogenic variant positive. Overall, the clinical sensitivity of factor V Leiden for isolated VTE is between 20% and 50%[37,97]. It is 16% for individuals with both DVT and pulmonary embolism and for those with isolated pulmonary embolism.[37] Age is a strong risk factor for thrombosis. The risk for VTE in heterozygous carriers of factor V Leiden increased with age at a rate significantly greater than that in noncarriers.[98] The clinical sensitivity was found to be approximately 29.5% in a study of 380 individuals with at least one thromboembolic event.[99] Factor V Leiden has been found in 20–46% of women with VTE during pregnancy.[100,101] The clinical sensitivity of the factor II c.*97G>A variant for an initial episode of VTE is about 6%[49].

Clinical specificity can be defined as the proportion of individuals who do not have or will not develop VTE and do not have a pathogenic variant. The false positive rate is 1 minus the clinical specificity. The low penetrance of these two variants is the main reason for less than 100% clinical specificity. Analytical error is possible, but this is likely to be a much smaller factor in cases of clinical false positive test results. The clinical specificity for factor V Leiden has not been firmly established, but can be no lower than 95% (this assumes that all 5% of the population with a pathogenic variant are clinical false positives). Similarly, the clinical specificity for the factor II c.*97G>A test is likely to be no lower than 98% (if all 2% of pathogenic variant carriers are clinical false positives). Given the low penetrance of these variants (i.e., most individuals with a pathogenic variant will not develop VTE), the estimations of clinical specificity of these two variants are reasonably reliable.

The penetrance of the factor V Leiden variant is generally considered to be low for heterozygotes. The cumulative incidence of VTE at age 60 is about 6.5% for heterozygotes and the lifetime risk for heterozygotes is estimated to be approximately 10%[42,73]. Penetrance for homozygotes has been estimated at 15–20% from population screening studies.[43,73,102] Another study showed an incidence of 15 VTE/1000 person-

years for homozygotes.[43] The risk of VTE is expected to be higher in factor V Leiden positive asymptomatic individuals identified from thrombophilic families than those who were identified from population screening. It is difficult to estimate the absolute penetrance for these variants alone because it is very common that additional risk factors also co-exist.

TECHNICAL PERFORMANCE

Assay considerations

Because both factor V Leiden and factor II c.*97G>A variants are single-nucleotide substitutions, any assay that is amenable to detecting single-nucleotide changes can be used for clinical testing. Currently, laboratory developed tests (LDTs), research use only reagents (RUOs), and FDA-approved testing platforms are all being used by clinical laboratories. Individual laboratories may choose assays based on their sample volume, laboratory workflow, number of employees, etc. Assays can be designed based on polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), allele-specific PCR, flap endonuclease + FRET (Fluorescence Resonance Energy Transfer), melting curve analysis, Taqman real-time PCR, fluorescent probe-based allelic discrimination, etc. For details of the underlying chemistry, quality control and advantage/disadvantage of individual assays, please refer to the general technical standards published by American College of Medical Genetics and Genomics (www.acmg.net). Sanger sequencing is conventionally used as the "gold standard" for small nucleotide changes and it may be useful for clinical laboratories to use this technique to establish the controls during the validation stage of the assays. Routine use of Sanger sequencing is not necessary and not common due to cost, turnaround time, as well as limited capacity for multiplexing. Each laboratory is responsible for the in-house validation/verification required by regulatory agencies such as CLIA and CAP. Participation in proficiency testing or sample exchange with other clinical laboratories is recommended to ensure the assay quality.

Positive controls

Positive controls can be obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository (http://catalog.coriell.org) or other resources. Genomic DNA from patients identified as heterozygous or homozygous and confirmed by alternative methodologies, other laboratories, or Sanger sequencing can also be used as the assay control if consent is obtained from these patients.

Sample preparation

Most assays are amenable to the use of genomic DNA prepared from blood or other tissue sources using a variety of extraction protocols. Platforms integrating DNA extraction and PCR steps are also available.

Analytical sensitivity and specificity

The analytical sensitivity of an assay is defined as the proportion of biological samples with a known pathogenic variant that is correctly classified as having a positive test result. The analytical specificity is the proportion of biological samples without a specific pathogenic variant that is correctly classified as having a negative test result.

Segal et al. carried out a meta-analysis to investigate the analytical sensitivity and specificity of these two variants. This analysis included 43 individual studies with more than 11,000 subjects collectively for the genotyping of factor V Leiden and factor II c.*97G>A using different platforms.[48] The majority of the studies used PCR-RFLP as the reference standard. The concordance rate between various platforms and reference standard ranged between 98% and 100%, indicating that the analytical sensitivity and specificity are not lower than 98%. In the clinical setting, the analytical sensitivity and specificity of assays testing these two variants are also very high. A collection of data from ACMG/CAP external proficiency testing between 1999 and 2003 demonstrated a 99.1% and 99.7% analytical sensitivity and specificity for factor V Leiden (total of 7054 alleles tested) and 98.8% and 99.8% for factor II c.*97G>A (total of 6100 alleles tested) (http://www.cdc.gov/genomics/gtesting/ACCE/FBR/index.htm; accessed May 2016). Similar results were obtained in clinical laboratories from National External Quality Assessment Schemes (NEQAS) from the United Kingdom and Europe (http://www.cdc.gov/genomics/gtesting/ACCE/FBR/index.htm; accessed May 2016). Hertzberg et al. reported the result of a 5-year external quality assurance program in Australia.[103] Among 3799 responders, the rate of successfully identifying specific genetic alterations was 98.13% and 98.84% for factor V Leiden and factor II c.*97G>A, respectively.

We can conclude that the analytical sensitivity and specificity are excellent for both variants regardless of the testing platforms. Commercial kits and new methodologies for detecting the factor V Leiden and factor II c.*97G>A are being introduced into the market frequently. It is the responsibility of the laboratory director and/or medical director to evaluate and validate any new methodology before the implementation of clinical testing. If a proper clinical quality assurance protocol is instituted, the majority of the testing platforms will yield consistent genotyping results.

LABORATORY RESULT INTERPRETATIONS

Each laboratory may develop its own reporting format with content pertaining to the requirements of federal, state, and other regulatory agencies. Information regarding genotype, related risk for thrombosis, and potential clinical implications are integral components in a clinical genetic report. A recommendation for genetic counseling may also be included. Reports may be tailored for the specific clinical indications, if available, especially when the testing indication is not VTE (e.g., recurrent pregnancy losses, planning to use oral contraceptives, testing of asymptomatic individuals due to family history). Reports should clearly state that a positive result can only suggest an elevated risk, but cannot definitively predict the occurrence or recurrence of a VTE in a specific individual.

Normal results

Venous thrombosis is a relatively common disorder in the general population. Genetic causes can only be identified in about 25% of Caucasian patients without a family history.[14] The genetic causes in other ethnic groups are largely unknown. Health-care providers need to be aware that negative genetic testing results are unlikely to significantly reduce the recurrence risk derived from clinical and family history. Patients' clinical management and implementation of a healthier lifestyle toward preventing recurrent VTE should not be altered due to a negative genetic testing result.

Factor V Leiden heterozygote

Individuals heterozygous for factor V Leiden have an approximately four to seven or eight-fold increased risk of venous thrombosis compared with individuals without this variant.[86,104]

Factor V Leiden homozygote

Individuals homozygous for factor V Leiden have an approximately 80-fold increased risk of venous thrombosis compared with individuals without this variant.[40]

Factor II c.*97G>A heterozygote

Individuals heterozygous for factor II c.*97G>A have an approximately two to four-fold increased risk of venous thrombosis compared with individuals without this variant.[40]

Factor II c.*97G>A homozygote

The associated risk of the homozygous c.*97G>A genotype and VTE is not conclusive due to the relatively few number of individuals with this genotype.[105] However, it is presumed to be higher than the risk for the heterozygous c.97*G>A genotype.[38] The risk of VTE is estimated to be 1.1% per person per year for homozygotes.[16]

Factor V Leiden and factor II c.*97G>A double heterozygote

Between 1.4% and 10% of symptomatic carriers of factor V Leiden also harbor the factor II c.*97G>A.[10,33,52] Individuals harboring both factor V Leiden and factor II c.*97G>A have about 20-fold increased risk of VTE compared with individuals without either variant (about four-fold compared with individuals carrying factor V Leiden alone).[106] The pooled odds ratio for recurrence of VTE in a proband is 4.81 (95% CI, 0.50–46.3) compared with normal controls.[48]

ALTERNATIVE TESTING METHODS

Activated protein C (APC) resistance can be diagnosed by a functional coagulation assay that measures the ability of activated protein C to inactivate factor Va. A positive APC resistance may indicate factor V Leiden pathogenic variant, however, depending upon the assay used, it may be affected by pathogenic variant or conditions other than factor V Leiden. Testing of prothrombin levels is not a functional alternative assay for factor II c.*97G>A genetic testing.[38]

DISCLOSURE

S.Z. and A.K.T. are clinical laboratory directors at their respective institutions and perform the assays described herein as a clinical service. The other authors declare no conflicts of interest.

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