

CLINICAL STUDY

Laboratory testing of hereditary thrombophilia: Previous data in the face of verification

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Abstract: *Objective:* Mutations in hemocoagulation factors genes are nowadays routinely examined parameters, important in hereditary thrombophilia determination. We have complexly evaluated laboratory data from the 211 unselected patients with venous thrombosis, and compared with the former studies in the same Slovakian population.

Results: The Factor V Leiden mutation (FVL) was found in 43 of 211 patients (20.38 %), with the mutant allele frequency=10.2 %, while the prothrombin G20210A mutation was revealed in 11/205 individuals (5.37 %), with the mutant PT 20210A allele frequency=2.68 %. In both mutations, all carriers of mutant allele were heterozygotes. In 81 individuals was examined the PCAT-NR with the ProC[®] Global assay. Of them, 24 was heterozygotes for FVL mutation with considerably lower PCAT-NR levels (median=0.67; range: 0.53–0.83) compared with homozygotes without FVL mutation (n=57; median=0.84; range: 0.54–1.67; p<0.001). The sensitivity of the assay was 0.88 (95 % CI: 0.68–0.97) and the specificity =0.67 (95 % CI: 0.53–0.79).

Conclusions: Our examination revealed considerably lower frequency of the FVL mutation in the target population, by contrast to the neighbouring Caucasian populations. As for the PT 20210 mutation, the differences are milder. Even though the PCAT-NR was validly sensitive (sensitivity=0.88, 95 % CI: 0.68–0.97) to the FVL mutation, only DNA-testing is the definitive assay for FVL-carriership (Ref. 19). Full Text (Free, PDF) www.bmj.sk.
Key words: factor V Leiden, PT 20210 mutation, PCAT-NR, venous thrombosis.

During the past two decades, a considerable progression in understanding the thromboembolic disease occurrence and treatment has been achieved. Among all human single nucleotide DNA polymorphisms, only two were revealed to be most significantly associated with venous thrombosis:

Factor V Leiden mutation (FVL) is a single nucleotide G to A transition at position 1691, causing arginine by glutamine substitution at amino acid residue 506 in the proaccelerine (human coagulation factor V) leading to a loss of the activated protein C cleavage site on the proaccelerine (1). Prolongation of activated factor V activity subsequently increases the thrombin generation (2, 3). Relative risk of venous thrombosis was found to be increased 3–8 times in heterozygotes, as compared to individuals without the mutant allele. Homozygotes with both mutant alleles have even higher risk: 30–140 times (4).

Prothrombin (human coagulation factor II) mutation at nucleotide position 20210 is the second most common mutation associated with pathologic thrombosis and consists of G to A nucleotide transition, however without any amino acid residue

change, due to its localization within the 3'-untranslated region of the prothrombin gene (5). Eligible mechanism of the mutation effect is changing in mRNA structure and its post-translational processing, however exact knowledge is still missing (6, 7).

For more than a decade, ProC[®] Global assay has also been known to be a highly sensitive tool in the detection of FVL mutation, protein C and S deficiency (8). The test result is expressed in protein C activation time normalised ratio (PCAT-NR), which is a units-free parameter. The test has its limitations, namely in cases of oral anticoagulant treatment and liver failures it gives falsely reduced PCAT-NR values indicating that there is no abnormality present (9).

Nowadays there are available data regarding the prevalence of FVL and PT 20210 mutations not only in Europe, but also in worldwide studies. The primary goal of this study was to investigate the FVL mutation frequency in a specific Slovak sub-region (Lower Nitra region, population of ca 250.000) and to compare it with former studies performed in Slovakia (10, 11). However, in contrast to previous works we conceived our study as a complex evaluation of laboratory data including FVL along with FII 20210 mutations in all patients with venous thrombosis. Being a part of our consolidated laboratory, the Molecular Unit confronted its results with PCAT-NR from Hematology Division as an interesting methodological antinomy between PCAT-NR as a global assessment of the protein C pathway and molecular analysis of the particular mutation.

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Another important aspect of our work was the insistence on established quality assessment process involving all lab tests to ensure reliability of all obtained data.

Materials and methods

Patients: In our retrospective study we collected data of 211 patients diagnosed with venous thrombosis, 136 women and 75 men being tested at the laboratory primarily for FVL mutation. Patients with other diagnoses were excluded. No additional laboratory parameters except for those ordered by physicians were tested. Patients' data were de-identified and subsequently statistical analysis was performed.

Ethics: Faculty Hospital Nitra has ethical standards in accordance with the Declaration of Helsinki requirements, and all physicians are obliged to obtain patient's informed consent before treatment.

Genetical analysis: DNA was isolated from whole blood samples, collected into the S-Monovette® EDTA 2.7 ml tubes (EDTA=1.6 g/dm³ blood, Sarstedt Nümbrecht, Germany), with the use of the isolation kit NucleoSpin®Blood (Macherey-Nagel Düren, Germany). Both FVL and PT 20210 mutations were detected simultaneously by multiplex polymerase chain reaction (Taq polymerase from New England Biolabs, MA, USA) with the pairs of primers: 5'-TCA GGC AGG AAC AAC ACC-3' and 5'-GTT ACT TCA AGG ACA AAA TAC CTG TAA AGC T-3' for FVL, 5'-TCT AGA AAC AGT TGC CTG GC-3' and 5'-ATA GCA CTG GGA GCA TTG AAG C-3' for PT 20210 (5, 12). After initial denaturation (95 °C/5 min) followed 39 cycles (95 °C/1 min – 57 °C/1 min – 72 °C/1:40 min) and final extension (72 °C/10 min) on the thermal cycler MJ-Mini (Bio-Rad). In the next step restriction analysis of the both mutations with the use of *HindIII* (New England Biolabs, MA, USA) was performed, followed by fragment separation with electrophoresis on the polyacrylamide gel.

PCAT-NR analysis: Venous blood was collected into the Monovette® Citrate 3.0 ml tubes (Citrate=3.13 %, Sarstedt Nümbrecht, Germany). For protein C activation time normalised ratio (PCAT-NR) the ProC® Global assay (Dade-Behring, Marburg, Germany) was used with Sysmex CA-1500 analyser (Sysmex, Japan).

Quality control: For the ProC® Global assay two control plasmas were used: Control plasma N for the normal range (batch nr. 502799D) and ProC Control Plasma for the pathological range (batch nr. 524429B). External quality assessment was supplied by Equalis, Sweden (DNA analysis) and SEKK, Czech Republic (haematological tests).

Statistical analysis: Numerical results are expressed as median values with their ranges because of the lack of normality of data distributions. These data were evaluated with the non-parametrical tests: the Kruskal-Wallis test and the Mann-Whitney test. Categorical parameters were tested with the Chi-square test and the Fischer's exact test. *p* value below 0.05 was considered as significant test statistics.

Results

We investigated data from 211 patients, obtained in the period from 12/2006 till 06/2007, with age range=74 (from 9 to 83

years, median=48). Of them, 136 (64 %) were females and 75 (36 %) were males.

FVL mutation was found in 43 (28 women and 15 men) of 211 patients (20.38 %), all were heterozygote carriers of the factor V mutation. No homozygote with both mutant alleles was found. Allele frequencies amounts 10.2 % for the mutant A-allele and 89.8 % for the standard G allele respectively. Chi-square test revealed that there were no significant differences between estimated vs. observed frequencies of particular genotypes (*p*=0.3) according to the Hardy-Weinberg definition.

PT 20210 mutation was tested in 205 of 211 patients while only 11 of them (5.37 %) were prothrombin mutation carriers in heterozygous form. Allele frequency of the mutant A-allele takes 2.68 %; frequency of the standard G-allele reaches 97.32 %.

The comparison of age between particular genotypes of FVL and PT 20210 mutations detected no relevant differences (Mann-Whitney test, *p*=0.86 and *p*=0.73, respectively).

PCAT-NR was investigated in 81 patients (57 with standard homozygotes, 24 heterozygotes) with the assay cut-off level equal to 0.78. The difference in PCAT-NR between the both genotypes was very significant (*p*<0.001), whereby lower levels were found in heterozygotes (median=0.67; range: 0.53–0.83) and standard homozygotes had higher levels (median=0.84; range: 0.54–1.67). Of 57 patients without FVL mutation, 38 individuals were found to be above that value, in contrast to 24 heterozygotes with mutation, of whom only three were above the level (*p*<0.001). Test sensitivity was 0.88 (95 % CI: 0.68–0.97), test specificity=0.67 (95 % CI: 0.53–0.79) and likelihood ratio=2.63. The positive predictive value of high response of FVL mutation carriers to the ProC® Global assay was 0.53 (95 % CI: 0.36–0.69), negative predictive value=0.93 (95 % CI: 0.80–0.98).

The median of age of 81 patients examined for PCAT-NR was 52 (range: 16–83). Among the four groups considerable age differences were found (Kruskal-Wallis test, *p*=0.02): 33 females with PCAT-NR values ≥0.78 had median=53 (range: 16–78), 23 females with PCAT-NR values <0.78 showed median=36 (range: 18–83). In males we found 9 patients with PCAT-NR values 0.78 (median=28, range: 21–72) and 16 men with PCAT-NR below 0.78 (median=59, range: 28–81). In contrast to subgroups defined by gender and PCAT-NR levels, no significant age differences were revealed when comparing males and females (Mann-Whitney test, *p*=0.11), genotypes of FVL mutation (*p*=0.46) and PT 20210 mutation (*p*=0.91).

Discussion

FVL allele frequency found in our set of patients (10.2 %) is inferior to previous results – 19.71 % (Simkova et al, 2004); 16.19 % (Hudecek et al, 2003) obtained in the Slovak Caucasian population. The latter differences can be possibly explained by the fact that previous studies were carried out at centers with larger tributary areas while their records of patients' origin were not precise. *A contrario sensu*, we did not sort out individual patients clinically, only evaluated the existent data of individuals (diagnosed with venous thrombosis) previously treated at our

hospital. Among 43 heterozygotes, 28 females showed lower age (median=40, range: 18–83) than males (n=15, median=54, range: 7–78). Although the difference is not significant (p=0.21), yet it gives reasons for the effect of risk factors such as oral contraceptives and pregnancy, considered to be the most frequent risk factors in females with the FVL mutation (13).

Up to day, the data considering PT 20210 mutation frequencies in Slovakia are not known. In our study, heterozygosity for PT 20210A mutant allele was found in 11 of 205 patients (5.37 %). This is in accordance with the studies from countries neighbouring Slovakia: 4.8 % in Hungary (14) and 6.5 % in Poland (15). Allele frequency of PT 20210A in our study reached 2.68 %, which is slightly lower than in Austrian patients with venous thrombosis -4.5 %; (16) and French caucasian men with peripheral arterial disease -3.5 % (17).

The PCAT-NR was significantly lower in FVL mutation carriers in contrast to standard homozygotes. Unfortunately, no homozygotes with both FVL mutations were revealed, therefore their parameters remain unknown in the target population. Our test parameters (sensitivity=0.88; specificity=0.67) corresponded with the previous study containing more detailed patients' data (9): sensitivity=0.889, specificity=0.618 with the cut-of level of PCAT-NR=0.75. However, in our study a slightly higher cut-off level of PCAT-NR was used (0.78). Lower age in females with PCAT-NR below 0.78 beside those with the upper PCAT-NR values indicates that hormonal therapy is possible (18), but the data we had at our disposal were not appropriate for serious examination of our population. Other challenge to the future still includes other important interfering factors, such as anti-vitamin K therapy, low levels of protein S and/or protein C, liver disease, etc. This task requires however more patients in favour of sufficient counts in particular categories for correct and precise statistical analysis.

In the light of methodic suitability we revealed that the most appropriate method is the multiplex PCR creating a restriction site for the *Hind*III restriction endonuclease with electrophoretical separation and visualisation of resulting DNA fragments. It is not only a reliable and robust method (19), but also a highly economical technique for a medium-sized laboratory. We also appreciated the external quality assessment of DNA analysis based on both, whole blood and DNA solution samples. Such a combination encompasses a whole test procedure including DNA isolation as opposed to DNA-lyophilised samples often used in external quality controls.

In conclusion, a confirmative study can reveal differences in neighbouring populations. Our examination showed a considerably lower frequency of the FVL mutation in the Nitra population as opposed to neighbouring Caucasian populations. As for PT 20210 mutation, the differences are milder. Even though the PCAT-NR was validly sensitive (sensitivity=0.88, 95 % CI: 0.68–0.97) to the FVL mutation, only DNA-testing is the definitive assay for FVL-carriership.

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