ORIGINAL PAPERS

Adv Clin Exp Med 2010, **19**, 1, 51–55
ISSN 1230-025X
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The Genetic Background of Thrombosis – The Distributions of Factor V Leiden, Prothrombin G20210A, and MTHFR C677T Polymorphisms

Genetyczne podłoże zakrzepicy – występowanie polimorfizmów czynnika V Leiden, protrombiny G20210A oraz MTHFR C677T

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Abstract

Background. Polymorphisms of factor V (Leiden) (G1691A) and the gene for prothrombin (G20210A) are the main single-nucleotide polymorphisms (SNPs) connected with thrombotic disease. The polymorphism C677T in *MTHFR* gene may be implicated in the development of hyperhomocysteinemia, what is also a risk factor of thrombosis. The genetic diagnostics of these mutations is a valuable complement to the clinical diagnosis. It permits establishing the causes of disease and planning therapy.
Objectives. To evaluate associations of the polymorphisms with thrombosis, the distributions of the three major SNPs in factor V, prothrombin, and *MTHFR* genes in patients with diagnosed venous thromboembolic disease were investigated.
Material and Methods. DNA was isolated from the patients' whole blood. A very sensitive method, ASO-PCR, to detect point mutations in the selected genes was used. The PCR products were analyzed by agarose gel electrophoresis with ethidium bromide.
Results. Among 117 Polish patients, 99 mutation carriers were found: 26 of factor V Leiden, 13 of prothrombin G20210A, and 60 of *MTHFR* C677T. The frequencies of the obtained genotypes were compared with those of a healthy Caucasian population. Positive correlation of two SNPs (factor V Leiden and prothrombin G20210A) was found in study group; however, the *MTHFR* C677T polymorphism was not directly linked with thrombotic incidence.
Conclusions. These data indicate that only two of the SNPs, FVL and prothrombin G20210A, are related to disease phenotype (**Adv Clin Exp Med 2010, 19, 1, 51–55**).

Key words: factor V Leiden, MTHFR, prothrombin, single-nucleotide polymorphism (SNP), thrombosis.

Streszczenie

Wprowadzenie. Polimorfizm czynnika V krzepnięcia (Leiden) G1691A i genu protrombiny G20210A to główne polimorfizmy pojedynczego nukleotydu (SNP) związane z zakrzepicą. Polimorfizm C677T w genie *MTHFR* może być uwikłany w rozwój hiperhomocysteinemii, będącej również czynnikiem ryzyka zakrzepicy. Genetyczna diagnostyka tych mutacji jest doskonałym uzupełnieniem standardowych badań klinicznych. Pomaga ustalić przyczyny choroby oraz pozwala na planowanie terapii.
Cel pracy. Aby ocenić związek między polimorfizmami i zakrzepicą, zbadano 3 główne mutacje typu SNP w genach: czynnika V krzepnięcia, protrombiny i MTHFR u pacjentów ze zdiagnozowaną żylną chorobą zakrzepowo-zatorową.
Materiał i metody. DNA izolowano z krwi obwodowej pacjentów. Do detekcji mutacji punktowych w wybranych genach użyto czułej metody ASO-PCR. Produkty reakcji PCR rozdzielano elektroforetycznie w żelu agarozowym z dodatkiem bromku etydyny.
Wyniki. Wśród 117 pacjentów pochodzenia polskiego znaleziono 99 nosicieli mutacji; 26 nosicieli mutacji FVL, 13 nosicieli mutacji G20210 w genie protrombiny oraz 60 nosicieli mutacji C677T w genie *MTHFR*. Częstości genotypów w badanej grupie pacjentów porównano z częstościami w zdrowej populacji kaukaskiej. Wykazano

pozytywną korelację zakrzepicy z polimorfizmem czynnika V Leiden i protrombiny G20210A w badanej grupie pacjentów, nie zaobserwowano natomiast związku polimorfizmu C677T w genie *MTHFR* z chorobą.
Wnioski. Uzyskane dane pokazują, że dwa spośród trzech badanych polimorfizmów SNP predysponują do rozwoju choroby (**Adv Clin Exp Med 2010, 19, 1, 51–55**).

Słowa kluczowe: czynnik V Leiden, MTHFR, protrombina, polimorfizm pojedynczego nukleotydu (SNP), zakrzepica.

Blood clots are a very common incident in human pathology and their formation in blood vessels causes tissue damage. Their appearance is the basis of thrombotic disease, a frequent cause of death in developed countries. In fact, thrombosis is associated with vessel perturbation, changes in blood flow, and variations in the constitution of blood elements [1]. There are many factors that predispose to thrombotic disease, for example non-genetic factors such as atherosclerosis, diabetes, or cancer or perturbation of blood coagulation and fibrinolysis resulting from injury or a surgical operation. Many authors provide information about genetic predisposition to thrombosis [1–3]. The most common inherited risk factor is factor V Leiden (FVL) mutation [2, 4, 5]. This is a missense mutation of guanine (G) to adenine (A) at nucleotide 1691 in the gene sequence and it substitutes glutamine for arginine at position 506 in the protein [4]. The expressed protein is therefore much more resistant to proteolytic degradation by activated protein C [2, 4, 5]. New reports give the frequency of factor V Leiden mutation in the general European population as about 5% and as about 20% in venous thrombosis patients [4]. Data show that the estimated risk of developing deep venous thrombosis is 5- to 10-fold higher in heterozygous and 80- to 100-fold higher in homozygous carriers of the FVL polymorphism than in the general population. Another highly characterized variation is the transition of guanine to adenine at nucleotide 20210 in the prothrombin gene [2, 5]. It is also viewed as a probable causative factor for venous and arterial thrombosis [6]. This is a substitution in the 3'-untranslated region (3`-UTR) of the prothrombin gene [7]. This results in an increased synthesis of prothrombin and an elevated level of this protein. The frequency of the G20210A mutation in the prothrombin gene in the general population is about 1.2–2.0% [2, 8] and mutation carriers among patients with venous thrombosis are about 5.5 to 7.0% [2, 8]. It is associated with a threefold increase in the risk of disease [2].

Hyperhomocysteinemia is a strong and independent risk factor for thrombosis. A high concentration of total homocysteine can result from a cytosine to thymine transition at nucleotide 677 in the sequence of the methylenetetrahydrofolate reductase (*MTHFR*) gene [9]. This variation leads to a change of alanine to valine at amino-acid position 222 in the protein [10, 11]. Because MTHFR converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a carbon donor in the remethylation of homocysteine to methionine [12], the substitution makes it impossible to remove homocysteine by MTHFR [9]. The increased concentration of homocysteine in the blood can be accompanied by increased expression of tissue factor, over-activation of factor V, or perturbation of protein-C activation [13]. The polymorphism C677T in *MTHFR* is quite common in some populations. In general, in the Caucasian population the frequency of heterozygous carriers is 50% and of homozygous 10–15% [9].

Material and Methods

Specimens

Peripheral blood samples from 117 Polish patients with diagnosed venous thromboembolic disease (70 woman, 47 men) were received from the Department and Clinic of Angiology, Arterial Hypertension, and Diabetology, Wroclaw Medical University. Written informed consent was obtained from each patient.

DNA Extraction

DNA was isolated from whole blood using a QIAmp DNA Mini Kit (QIAGEN) according to the instructions.

PCR Assay

Detection of mutations was performed with a very sensitive method based on an allele-specific oligonucleotide (ASO-PCR). The initial PCR denaturation at 94°C for 2 min was followed by 35 cycles of 94°C for 30 s, 57–59°C for 20 s, and 72°C for 30 s, a final extension at 72°C for 5 min. The annealing temperatures for each set of primers differed: for factor V it was 58°C, for prothrombin 59°C, and for *MTHFR* 57°C. The ASO-primers were synthesized according to Hessner et al. [13] and the PCR products were analyzed by electrophoresis in 2% agarose stained with ethidium bro-

Table 1. Sequences of the primers used in this study

Tabela 1. Sekwencje starterów użytych w badaniach

Name (Nazwa)	Sequence (Sekwencja)	Product size (Wielkość produktu)
FII forward (consensus)	5'-tctagaaacagttgcctggcaga-3'	340 bp
FII reverse (normal G)	5'-cactgggagcattgaaggcac-3'	
FII reverse (mutant A)	5'-cactgggagcattgaaggcat-3'	
FV forward (consensus)	5'-gggggacaattttcaatatattttctttcaggcag-3'	270 bp
FV reverse (normal G)	5'-gggggttcaaggacaaaatacctgtattccac-3	
FV reverse (mutant A)	5'-gggggttcaaggacaaaatacctgtattccat-3	
MTHFR forward (normal C)	5'-gagaaggtgtctgcgggatc-3'	193 bp
MTHFR forward (mutant T)	5'-gagaaggtgtctgcgggatt-3'	
MTHFR reverse (consensus)	5'-aagacggtgcggtgagagtg-3'	

mide (0.5 μg/ml). The primer sequences and sizes of the PCR products are presented in Table 1.

Statistical Analysis

The results were compared with those of a healthy Polish population according to Lewandowski et al. (2005) [13]. The χ^2 test was used in the statistical analysis. Statistical significance was set at $p < 0.05$. The χ^2 test was also used to determine the fit to the Hardy-Weinberg distribution.

Results

It was found that the studied population was in genetic equilibrium as assessed by the Hardy-Weinberg distribution. The frequencies of the genotypes of factor V, prothrombin, and the *MTHFR* locus are presented in Table 2.

Homozygous carriers of FVL and prothrombin G20210A mutations were not present in the study group. The results were compared with a healthy Polish population according to Lewandowski et al. (2005) [13] (Table 3).

Statistically significant differences were found in the frequency of FVL in the study and control groups ($p < 0.05$). Four (15.4%) cases of compound heterozygotes for FVL and the prothrombin G20210A variation as well as 9 (34.6%) cases of FVL and *MTHFR* C677T mutation were also found. Linkage disequilibrium (D) for the two-compound heterozygotes FVL/prothrombin and FVL/*MTHFR* was 0.03 and 0.04, respectively, which indicates that the loci segregate independently. A statistically significant difference for carriers of the prothrombin gene mutation compared with the healthy population was also found ($p < 0.05$). Furthermore, it was established that in this group of carriers, 23.1% of patients had heterozygous and 7.7% of patients had homozygous *MTHFR* C677T mutations.

Table 2. Frequencies of factor V, prothrombin, and *MTHFR* locus genotypes

Tabela 2. Częstotliwości genotypów czynnika V, protrombiny i *MTHFR*

Genotype (Genotyp)	Frequency – n (Częstotliwość – n)
FVL (G1691A)	
GG	0.778 (91)
GA	0.222 (26)
AA	0.000 (0)
Prothrombin (G20210A)	
GG	0.889 (104)
GA	0.111 (13)
AA	0.000 (0)
MTHFR (C677T)	
CC	0.487 (57)
CT	0.453 (53)
TT	0.060 (7)

These results showed statistically significant differences in the frequencies of C677T homozygous mutations in the *MTHFR* gene between the study and control groups; however, there was no correlation regarding heterozygous carriers.

Discussion

The investigation of mutations which increase the tendency to particular diseases is an important step in establishing disease causes and planning

Table 3. Comparision of FVL, prothrombin G20210A, and MTHFR C677T mutation frequencies with these of a healthy Polish population according to Lewandowski et al. (2001), n – frequency

Tabela 3. Porównanie częstotliwości występowania mutacji FVL, G20210A w protrombinie i C677T w MTHFR z częstotliwością występowania w zdrowej polskiej populacji wg Lewandowskiego et al. (2005), n – częstotliwości

Genotype (Genotyp)	Study group (Badana grupa)		Control group (Grupa kontrolna)		*p*
	n	%	n	%	
GG (FVL)	91/117	77.8	116/121	95.9	0.3168
GA (FVL)	26/117	22.2	5/121	4.1	0.0006
AA (FVL)	0/117	–	0/121	–	–
GG (prothrombin)	104/117	88.9	120/121	99.2	0.6212
GA (prothrombin)	13/117	11.1	1/121	0.8	0.0036
AA (prothrombin)	0/117	0	0/121	–	–
CC (*MTHFR*)	57/117	48.7	78/172	45.3	0.8158
CT (*MTHFR*)	53/117	45.3	74/172	43.0	0.812
TT (*MTHFR*)	7/117	6.0	20/172	11.6	0.0011

therapy. Therefore genetic analysis is very important in clinical practice. Molecular biology offers many sensitive and fast methods which can detect changes in DNA. Genetic studies allow establishing mutations present in patients with suspicion of genetic disease and provide information about the type of mutation (heterozygous or homozygous) [15]. The frequencies of the FVL, prothrombin G20210A, and *MTHFR* C677T polymorphisms in the study group (22.2%, 11.1%, and 51.3%, respectively) were quite similar to those obtained by Lewandowski et al. (2005) in a group of patients with diagnosed vein thromboembolic disease (19.2%, 8.6%, and 44.8%, respectively). The present results were compared with a control group of Lewandowski et al. consisting of healthy Polish people [13].

The results of this study show that the presence of FVL, prothrombin G20210A, and *MTHFR* homozygous C677T mutations could be a basis for developing thrombosis in affected patients ($p < 0.05$). These genetic predispositions can be inherited by the next generations and can also be a cause the increasing tendency of thrombotic incidence. The study found that there was no association between heterozygous mutants of C677T in the *MTHFR* gene and disease ($p > 0.05$). It also established that there was no coexistence of *MTHFR* mutation with FVL or prothrombin mutation in the examined patients ($D << 1$).

Hessner et al. investigated the distribution of alleles of the FVL, prothrombin G20210A, and *MTHFR* C677T mutations in individuals of many populations. It was shown that the frequency of the A allele in FVL of the Caucasian population is the highest compared with African Americans, Asian Indians, Hispanics, Native Americans, and Koreans. Koreans presented a frequency of the T allele in *MTHFR* C677T of over 40% and Asian Indians 10.1%. Among the studied populations, the highest frequency of the mutant allele (A) of prothrombin G20210A was found in Hispanics [14]. These studies showed that geographic variability of the distribution of SNPs increased the predisposition to thrombosis.

It is known that other polymorphisms are connected with this disease. Inherited thrombotic incidence may be a result of deficiency of antithrombin III, protein C, or protein S [2, 8]. The latest sources report that elevated factor VIII level can increase the risk for venous and arterial thrombosis in hyperhomocysteinemic subjects [16]. Because of the many inherited causes of thrombosis, genetic analyses are a significant step in diagnosis. Genetic predisposition to thrombosis and new techniques for detecting mutations are still being investigated.

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Conflict of interest: None declared

Received: 21.09.2009
Revised: 6.11.2009
Accepted: 1.02.2010