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Abstract

Background/Aim. Ischemic stroke is a heterogeneous disorder caused by several genetic and environmental risk factors. It was suggested that coagulation disorders cause 1-4% of cases with ischemic stroke, especially in patients with early-onset of ischemic stroke. Case report. Here, we describe a case of patient who developed an unprovoked ischemic stroke in young adult. Biochemical, immunological and thrombophilia screening, as well DNA sequencing were performed in order to reveal molecular pathology underlying stroke of patient. Thrombophilia testing showed that patient was homozygous carrier for PAI-1 4G/5G and MTHFR C677T mutations. Additional genetic analysis revealed the presence of recently reported FII c.1824C>T gene variant, which is located in the last exon of prothrombin gene and previously shown to cause hyperprothrombinemia, hypofibrinolysis and altered fibrin clot phenotype. Conclusion. Our results suggest that newly reported FII c.1824C>T gene variant might have synergistic effect with PAI 4G/4G and MTHFR 677TT genotype in formation of altered fibrin clot phenotype characterized by thin, densely packed fibrin fibers, which makes clot less susceptible to fibrinolysis and greatly increases the risk for early ischemic stroke onset.

Key words: ischemic stroke, thrombophilia, FII c.1824C>T gene variant, fibrin clot, PAI 4G/4G, MTHFR 677TT

Apstrakt
**Uvod/Cilj.** Ishemijski moždani udar je heterogeni poremećaj koji može biti uzrokovan genetskim kao i sredinskim faktorima rizika. Poremećaji koagulacije mogu biti uzročnici 1-4% slučajeva ishemijskog moždanog udara, naročito kod bolesnika kod kojih se ishemijski moždani udar dogodi u mladoj životnoj dobi. **Prikaz bolesnika.** U ovom radu, prikazan je slučaj bolesnika koji je u mladoj životnoj dobi razvio ishemijski moždani udar nepoznatog uzroka. S ciljem da se utvrdi molekularna patologija koja može biti u osnovi moždanog udara kod ovog bolesnika, urađeni su biohemijski, imunološki i trombofilni testovi kao i sekvenciranje DNK. Testovima za trombofiliju je pokazano da je bolesnik homozigotni nosilac mutacija PAI-1 4G/5G i MTHFR C677T. Dodatnom genetičkom analizom detektovano je prisustvo nedavno opisane FII c.1824C>T varijante, koja se nalazi u poslednjem egzonu gena za protrombin i za koju se prethodno pokazano da izaziva hiperprotrombinemiju, hipofibrinolizu i izmenjeni fenotip fibrinskog ugruška. **Zaključak.** Naši rezultati ukazuju da bi nova FII c.1824C>T varijanta mogla imati sinergistički efekat sa PAI 4G/4G i MTHFR 677TT genotipom u nastanku fibrinskog ugruška sa izmenjenim fenotipom koji se odlikuje tankim, gusto upakovanim fibrinskim vlaknima, što čini ugrušak manje podložnim fibrinolizi i povećava rizik od nastanka ishemijskog moždanog udara u ranijoj živonoj dobi.

**Ključne reči:** ishemijski moždani udar, trombofilija, FII c.1824C>T genska varijanta, fibrinski ugrušak, PAI 4G/4G, MTHFR 677TT

**Introduction**

Ischemic stroke (IS) is a heterogeneous disorder that counts for 3/4 of all stroke cases and could be provoked by multiple risk factors, both genetic as well as environmental (1,
A great number of genetic mutations has been shown to have a role in the etiology of several subtypes of stroke (2), but it is still challenging to identify specific causative mutations (3). Studies performed on twins and siblings, shown that IS onset is greatly affected by inherited risk factors (4). Majority of genetic factors for IS have rather polygenic than monogenic influence. Additionally, IS could have a wide range of phenotypes, which could differ in their genetic background. Almost all human studies to date have employed a candidate gene approach (4).

Previous studies have suggested that coagulation disorders are the major cause of only a 1% to 4% of all IS, but may be of relevance for the pathogenesis of subgroups of stroke patients such as strokes in young ones (5, 6). The perturbation of the coagulation cascade, due to the gene variants found in several genes involved in hemostasis (fibrinogen, prothrombin, FV, FVII, FXIII, thrombomodulin, PAI-1, TAFI) are associated with increased coagulability and thrombotic risk (4). The most frequently studied genetic variants in the pathogenesis of IS are FV G1691A (FV Leiden), FII G20210A and the MTHFR C677T (7).

One of the potential candidate genes involved in the pathogenesis of IS is prothrombin (FII) gene. Its unusual non canonical architecture makes the 3` end of prothrombin gene sensitive to gain-of-function mutations, which are associated with increased prothrombin expression (8). Recent studies reported two novel gain-of-function variants in this region of prothrombin gene, FII c.1787G>A (prothrombin Belgrade) and FII c.1824C>T, which are recognized as significant risk factors for the IS occurrence (9, 10).

We present a case of the patient who suffered from early-onset unprovoked IS. Since the cause of this IS remained unknown after routine testing, we performed additional genetic analyses which revealed that patient is heterozygous carrier of the recently described FII c.1824C>T gene variant.

**Case report**

The patient, 42-year-old, right-handed male, nonsmoker, was initially presented to emergency department of the local hospital due to the sudden onset of left-sided weakness. Upon the arrival, he was alert and cooperative, while left facial palsy and severe left-sided hemi paresis were noted. Findings of the other neurological examination was normal. Computed Tomography (CT), performed on admission, revealed no evidence of acute
cerebral infarction or any other brain pathology (Figure 1. a). Brain magnetic resonance imaging (MRI) performed on day 4, showed a lesion in the region of the right thalamus with characteristics of possible ischemia and less likely large demyelinating plaque (Figure 1. b1). MRI of cervical spinal cord was normal (Figure 1. b2). Radiographic examination of the heart and lung and carotid ultrasonography were also normal. A cardiac evaluation, including echocardiography revealed no source of embolism. Cerebrospinal fluid (CSF) examination was performed and revealed normal cells counts, glucose levels and mild increase of protein levels (0.83). The levels of hematological, biochemical parameters and the levels of Vitamin B12 and ACE were regular. Antibodies to *Borelia burgdorferi*, HIV, HCV, HBS were negative. The origin of the brain lesions was unknown; the patient was treated conservatively with acetylsalicylic acid (100 mg/day) and simvastatin (10 mg/day). His condition gradually improved over the four weeks until he was able to walk independently. The patient was admitted to our hospital 90 days after the beginning of symptoms to define the nature and etiology of the thalamic lesions. Physical examination was normal; the left-sided weakness was stationary during a three-month follow-up. The estimated NIHSS (National Institute of Health Stroke Scale) score, on admission was 3, Multi-slice Computed Tomography (MSCT) with contrast showed hypodense lesions restricted to the right putamen and corona radiate (Figure 1. c1). The liquid density lesions represent the porencephaly cavity resulting in the area of the prior ischemia. MSCT-angiography was done, brains blood vessels were normal. Ophthalmic examination and Visual Evoked Potentials (VEP) showed no particularities.

A cardiac evaluation, including transesophageal echocardiography (mitral valve prolapse with minimal mitral regurgitation, aortic valve were intact, atrial septal defect was not seen) revealed no valid source of embolism. Biochemical and immunological results (Antinuclear antibodies, Antineutrophil cytoplasmic antibodies and Anticardiolipin antibodies) were within normal range. Thrombophilia screening showed normal results for PT, APTT, Lupus anticoagulant (LA), biological activity of Antithrombin, Protein C and protein S. Additionally, FII activity of 1.07 (reference range 0.80-1.40) and D-dimer level of 0.07 mg/L (reference range <0.25 mg/L) were obtained. Since the cause of the patient’s stroke was unknown we performed routine PCR tests which included genotyping for FV Leiden, FII G20210A, MTHFR C677T and PAI-1 4G/5G mutations. PCR tests showed that
patient is homozygous carrier of PAI-1 4G/5G and MTHFR C677T mutations, non-carrier for FV Leiden and FII G20210A mutations.

As it was previously shown that certain gain of function variants within 3’ end of the FII gene could be associated with early onset of IS (10), we performed additional analysis: DNA sequencing of this region in FII gene and screening for variants, as well as determination of patients plasma prothrombin level by Western blot analysis. DNA sequencing analysis, performed as described previously (11), revealed the presence of FII c.1824C>T gene variant, located in the last exon of the prothrombin gene in heterozygous state. In Western blot analysis, SDS-PAGE was used for separation of proteins in the patient’s and control’s plasma samples. Standard human plasma (Siemens, Germany) was used for the normalization, with the given referent value of 100 and prothrombin deficient plasma (HemosIL, USA) was used as a negative control. As additional control, plasma of symptomatic heterozygous carrier of FII G20210A mutation, suffered from deep venous thrombosis and plasma of healthy volunteer, who was non-carrier for all known mutations in the 3’ end of the prothrombin gene (confirmed by direct sequencing), was used. The plasma dilution, protein transfer, membrane blocking, antibodies used for protein detection and protein quantification were performed as described previously (10). Results of Western blot analysis showed that examined stroke patient had elevated prothrombin level (170.83±34.68) compared to standard plasma (referent value 100). Patients plasma prothrombin level was similar to prothrombin level of heterozygous carrier for FII G20210A mutation (163.64±21.74) and higher than prothrombin level in healthy volunteer (105.49±13.54, P<0.0001) (Figure 2).

Written approval from the Ethic committee of Institute of Molecular Genetics and Genetic Engineering, University of Belgrade (registration number: O-EO-004/2015, registration date: 29/07/2015), in accordance with Declaration of Helsinki for experiments involving humans, has been obtained. Patient signed the informed consent form.

Discussion

Here we present a patient with early-onset ischemic stroke of unknown etiology. Thrombophilia testing showed that patient was homozygous carrier for PAI-1 4G/5G and MTHFR C677T mutations. Additional analysis revealed the presence of recently reported
FII c.1824C>T gene variant in heterozygous state (10) and elevated plasma prothrombin level.

Impaired fibrinolysis and decreased fibrin network permeability are shown to represent substantial prothrombotic mechanisms contributing to the IS onset (11). The PAI-1 (plasminogen activator inhibitor 1) has a crucial role in the inhibition of two types of plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (u-PA), thus representing one of the most potent inhibitors of plasma fibrinolytic activity (12). The common PAI-1 4G/5G polymorphism within the promoter region of PAI-1 gene influences transcription of the gene and expression level of PAI-1 protein. The additional guanine in the DNA strand of the promoter region (5G allele) creates a repressor protein binding site that is absent in 4G allele. This reflects on PAI-1 expression and its plasma concentrations with 4G homozygotes having the highest and the 5G homozygotes the lowest PAI-1 concentrations (13). High levels of PAI-1 in 4G/4G carriers lead to suppressed fibrinolysis and consequent pathological fibrin deposition and tissue damage (14). The results on the association of PAI-1 4G/5G polymorphism with IS are conflicting, however the meta-analysis which included population-based association studies from 1966 up to 2006 showed that PAI-1 4G/4G genotype is likely to be associated with IS (15).

Elevated plasma homocysteine (hiperhomocysteinemia) and homozygosity for MTHFR C677T variant has been associated with the increased risk for IS, however the exact mechanism is not fully determined (16, 17). MTHFR (Methylenetetrahydrofolate Reductase) is a key enzyme in the folate pathway and homocysteine conversion to methionine. The presence of C677T variant leads to reduced enzyme activity and re-methylation of homocysteine to methionine, resulting in elevated homocysteine plasma levels (17). Homocysteine or its metabolites interact with plasma coagulation proteins and affect their function in vivo (16). It has been shown that elevated homocysteine level alters coagulation factor V in vitro and inhibits its cleavage by activated protein C (18). Hyperhomocysteinemia might also promote fibrinogen modification thereby impairing activity of fibrinolytic enzymes and fibrin polymerization. This results in altered fibrin clot structure, composed of thinner and tightly packed fibers resistant to fibrinolysis (16), which correlates with the fibrin clot phenotype observed in IS patients (17).
The third most common genetic risk factor associated with IS are gain of function variants in FII gene, which cause elevated prothrombin level, hypercoagulability and tendency towards hyper production of fibrin clot (19, 20). One of the most commonly tested prothrombotic variants in IS patients is FII G20210A mutation (19, 21). Taking into account that thrombophilia testing showed our patient is not a carrier of this variant, we decided to look into novel potential variants in FII gene associated with early IS onset and did sequencing of 715 bp within its 3’ end, where a recently described FII c.1824C>T gene variant was detected. The FII c.1824C>T represents a synonymous FII variant leading to the CGC to CGT codon replacement, on the Arg608 position in the protein (10). The study by Pruner et al. showed increased frequency of c.1824C>T gene variant in patients who suffered IS compared to healthy controls (4.8% vs. 0.9%), and 5.4-fold greater risk for IS occurrence in variant carriers (10), in vitro examination demonstrated that FII c.1824T allele is associated with elevated expression of prothrombin mRNA (10). In addition, ex vivo study indicated that FII c.1824C>T variant leads to hyperprothrombinemia, hypofibrinolysis and formation of denser and thinner fibrin fibers within the clot, which makes it resistant to fibrinolysis (10). The properties of fibrin clot are shown to have clinical relevance in patients with acute IS. Undas et al. enrolled 45 patients with acute IS and showed that the detected fibrin clot are less susceptible to fibrinolysis and that fibers form a less porous fibrin network (22). However, in comparison to the increased fiber thickness in acute IS patients (22), Pruner et al. showed that fibrin clot in FII c.1824C>T patients is structured from densely packed, but thinner fibers, which implicates that the fiber thickness could be FII c.1824C>T specific (10).

The result of elevated plasma level of prothrombin detected in our IS patient is in concordance with the study which examined the prothrombin level in carriers of FII c.1824C>T who suffered from IS or venous thromboembolism (VTE) (10). The increase in prothrombin level that we detected in the presence of FII c.1824C>T variant is similar to the one detected previously in IS and VTE patients. When compared to the effect of FIIG20210A, which increased prothrombin plasma level by approximately 60%, FII c.1824C>T variant could be a potentially new equally potent prothrombotic risk factor which leads to development of a prothrombotic phenotype (10). In this case study, we did not investigated the fibrin clot structure and thickness of fibrin fibers in our IS patient as did the Undas et al (22). However, electron microscopy scanning of fibrin clot structure from
FII c.1824C>T carriers, FII G20210A and healthy noncarriers by Pruner et al, revealed that fibrin fibers in plasma are denser and thinner in case of FII c.1824C>T compared to clots in FII G20210A and healthy noncarriers (10).

Fibrin clot phenotype is dictated by number of variables, involving pH, ionic strength, and concentrations of calcium, fibrinogen (23). However, thrombin concentration during clot formation has a crucial role in the density and stability of fibrin clot. Higher thrombin concentrations produce thin fibrin fibers that are densely packed, less susceptible to fibrinolysis and associated with thrombosis, whereas lower concentrations lead to production of thick, loosely-woven, permeable clots and bleeding disorders (23). Fibrin clot structure differs in terms of stability depending on the type of stroke, characterized as clots more prone to lysis in acute intracerebral hemorrhage, as opposed to more stable clots in the case of IS (24). Taking into account that our IS patient had elevated prothrombin level and previous results that FII c.1824C>T variant leads to the production of denser clots (10), we could hypothesize that this mechanism of clot formation is involved in the pathogenesis of early onset IS, but further studies concerning the association of fibrin clot phenotype and early onset IS are necessary.

Genetic predisposition for cerebral ischemia may result from an additive effect of several genes or from synergistic co-effects. Several studies have shown that PAI-1 4G/4G and MTHFR 677TT genotype could affect the fibrin clot microstructure. High levels of PAI-1 lead to suppressed fibrinolytic activity, while high homocysteine levels modify fibrinogen to be more resistant to cleavage by fibrinolytic enzymes (14, 16, 25, 26). Altogether, the conjunct effect of PAI-1 4G/4G and MTHFR 677TT genotypes observed in our patient might influence the susceptibility of fibrin clot to lysis and cause reduced clot permeability.

**Conclusion**

Based on our findings, we hypothesize that PAI-1 4G/5G and MTHFR C677T variants in synergy with hyperprothrombinemic and, hypofibrinolytic FII c.1824C>T variant, could lead to formation of altered fibrin clot phenotype characterized by densely packed, fibrinolysis resistant fibrin fibers, which could contribute to the early-onset ischemic stroke in our patient.
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Conflict of interest
All authors declare no conflict of interest.

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Figure captions

Figure 1. Brain scan and cervical spinal cord of stroke patient. a: Brain Computed Tomography performed on patient admission. b: Scans performed on day 4 after stroke onset; b1: Brain Magnetic Resonance Imaging (MRI); b2: MRI of cervical spinal cord. c: Scans performed 90 days after stroke; c1: Brain Multi-Slice Computed Tomography with contrast; c2: Brain Multi-Slice Computed Tomography angiography.
**Figure 2.** Relative quantification of plasma prothrombin level by densitometry of Western blot bands (Image Studio Lite, LI-COR). 1: Standard human plasma (assigned referent value of 100); 2: Plasma sample of healthy volunteer (non-carrier of mutations in 3’end of the prothrombin gene); 3: Plasma sample of heterozygous carrier of FII G20210A mutation; 4: Plasma sample of stroke patient with novel FII c.1824C>T gene variant. ***P<0.0001 compared to healthy volunteer.
