CASE REPORT

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Early-onset ischaemic stroke in a patient with the novel *F2 c.1824C>T* gene variant and *PAI-1 4G/4G, MTHFR 677TT* genotype

Ishemijski moždani udar u mlađem životnom dobu kod bolesnika sa novom *F2 c.1824C>T* genskom varijantom i *PAI-1 4G/4G, MTHFR 677TT* genotipom

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Abstract

Introduction. Ischemic stroke (IS) is a heterogeneous disorder caused by several genetic and environmental risk factors. It was suggested that coagulation disorders cause 1-4% of cases with IS, especially in patients with early onset of IS. **Case report.** We describe a case of a young adult male who developed an unprovoked IS. Biochemical, immunological, and thrombophilia screening, as well as DNA sequencing, were performed in order to reveal molecular pathology underlying the stroke of the patient. Thrombophilia testing showed that patient was a homozygous carrier for *PAI-1 4G/5G* and *MTHFR C677T* mutations. Additional genetic analysis revealed the presence of the recently reported *F2 c.1824C>T* gene variant, located in the last exon of the prothrombin gene and has previously been shown to cause hyperprothrombinemia, hypofibrinolysis, and altered fibrin clot phenotype. **Conclusion.** Our results suggest that the newly reported *F2 c.1824C>T* gene variant might have a synergistic effect with *PAI 4G/4G* and *MTHFR 677TT* genotype in the 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:
fibrin; genes; genetic variation; genotype; sequence analysis, dna; stroke; thrombophilia.

Apstrakt

Uvod. Ishemijski moždani udar (IMU) je heterogeni poremećaj koji može biti uzrokovan genetskim faktorima rizika i faktorima sredine. Poremećaji koagulacije mogu biti uzročnici u 1-4% slučajeva IMU, naročito kod bolesnika kod kojih se IMU dogodi u mlađem životnom dobu. **Prikaz bolesnika.** Prikazan je slučaj bolesnika koji je u mlađem životnom dobu razvio IMU nepoznatog uzroka. Urađeni su biohemijski, imunološki i testovi za trombofiliju kao i sekvenciranje DNK sa ciljem da se utvrdi molekularna patologija koja je mogla biti u osnovi moždanog udara kod tog bolesnika. Testovima za trombofiliju utvrđeno je da je bolesnik homozigotni nosilac mutacija *PAI-1 4G/5G* i *MTHFR C677T*. Dodatnom genetičkom analizom otkriveno je prisustvo nedavno opisane *F2 c.1824C>T* genske varijante, koja se nalazi u poslednjem egzonu gena za protrombin i za koju je prethodno pokazano da izaziva hiperprotrombinemiju, hipofibrinolizu i izmenjeni fenotip fibrinskog ugruška. **Zaključak.** Naši rezultati ukazuju na to da bi nova *F2 c.1824C>T* genska 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 IMU u ranijem životnom dobu.

Ključne reči:
fibrin; geni; geni, varijacije; genotip; dna, analiza sekvenci; moždani udar; trombofilija.

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 and environmental [1, 2]. A great number of genetic mutations have 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].

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Studies performed on twins and siblings have shown that IS onset is greatly affected by inherited risk factors [4]. The 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 1% to 4% of all IS, but may be relevant 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, plasminogen activator inhibitor-1 (PAI-1), thrombin activatable fibrinolysis inhibitor (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), *F2 G20210A*, and the *MTHFR C677T* [7].

One of the potential candidate genes involved in the pathogenesis of IS is the prothrombin *(F2*) gene. Its unusual non-canonical architecture makes the 3' end of the prothrombin gene sensitive to gain-of-function mutations associated with increased prothrombin expression [8]. Recent studies reported two novel gain-of-function variants in this region of the prothrombin gene, *F2 c.1787G>A* (prothrombin Belgrade) and *F2 c.1824C>T*, which are recognized as significant risk factors for the IS occurrence [9, 10].

We present a case of a 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 the patient is a heterozygous carrier of the recently described *F2 c.1824C>T* gene variant.

Case report

A 42-year-old, right-handed male, nonsmoker, was initially presented to the emergency department of the local hospital due to the sudden onset of left-sided weakness. Upon arrival, he was alert and cooperative, while left facial palsy and severe left-sided hemiparesis were noted. The findings of the other neurological examination were normal. Computed tomography (CT), performed on admission, revealed no evidence of acute cerebral infarction or any other brain pathology (Figure 1a). 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 1b1). MRI of the cervical spinal cord was normal (Figure 1b2). Radiographic examination of the heart and lung and carotid ultrasonography were also normal. A cardiac evaluation, including echocardiography, revealed no source of embolism. A cerebrospinal fluid (CSF) examination was performed and revealed normal cell counts, glucose levels, and a mild increase of protein levels – 0.83 mg/L (normal range is 0.15–0.6 g/L). The levels of hematological and biochemical parameters and the levels of vitamin B12 and angiotensin-converting enzyme (ACE) were regular. Antibodies to *Borrelia burgdorferi*, human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B surface (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 National Institute of Health Stroke Scale (NIHSS) score was 3 on admission, and multi-slice computed tomography (MSCT) with contrast showed hypodense lesions restricted to the right putamen and corona radiate (Figure 1c1). The liquid density lesions represent the porencephaly cavity resulting in the area of the prior ischemia. MSCT- angiography was done (Figure 1c2), and brain blood vessels were normal. Ophthalmic examination and visual evoked potentials (VEP) showed no particularities.

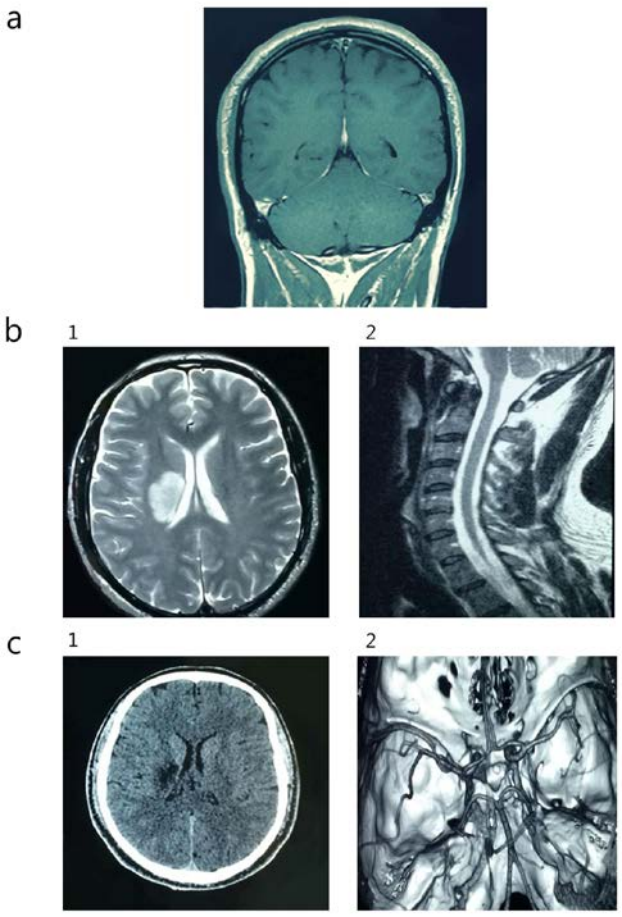

Fig. 1 – Brain scan and cervical spinal cord of stroke patient: a) brain computed tomography (CT) performed on patient admission; b) scans performed on day 4 after stroke onset [b1 − brain magnetic resonance imaging (MRI); b2 − MRI of the cervical spinal cord]; c) scans performed 90 days after stroke [c1 − brain multi-slice CT (MSCT) with contrast; c2 − MSCT angiography].

A cardiac evaluation, including transesophageal echocardiography (that demonstrated mitral valve prolapse with minimal mitral regurgitation, the aortic valve was intact, and the 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 prothrombin time (PT), activated partial thromboplastin time (APTT), lupus anticoagulant (LA), the biological activity of antithrombin, protein C, and protein S. Additionally, F2 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 polymerase chain reaction (PCR) tests that included genotyping for *FV* Leiden, *F2 G20210A*, *MTHFR C677T*, and *PAI-1 4G/5G* mutations. PCR tests showed that the patient is a homozygous carrier of *PAI-1 4G/5G* and *MTHFR C677T* mutations, non-carrier for FV Leiden and *F2 G20210A* mutations.

As previously shown that certain gain of function variants within the 3' end of the *F2* gene could be associated with the early onset of IS [10], we performed additional analysis: DNA sequencing of this region in the *F2* gene and screening for variants, as well as determination of patient's plasma prothrombin level by Western blot analysis. DNA sequencing analysis, performed as described previously [11], revealed the presence of the *F2 c.1824C>T* gene variant, located in the last exon of the prothrombin gene in the heterozygous state. In Western blot analysis, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for the 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 an additional control, the plasma of a symptomatic heterozygous carrier of *F2 G20210A* mutation suffered from deep venous thrombosis, and the plasma of a 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 patients had elevated prothrombin levels (170.83 ± 34.68%) compared to standard plasma (referent value 100%). The patients' plasma prothrombin levels were similar to the prothrombin level of the heterozygous carrier for *F2 G20210A* mutation (163.64 ± 21.74%) and higher than the prothrombin level in the healthy volunteer (105.49 ± 13.54%, $p < 0.0001$) (Figure 2).

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

Discussion

Here we present a patient with early-onset IS of unknown etiology. Thrombophilia testing showed that the patient was a homozygous carrier for *PAI-1 4G/5G* and *MTHFR C677T* mutations. Additional analysis revealed the presence of recently reported *F2 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].

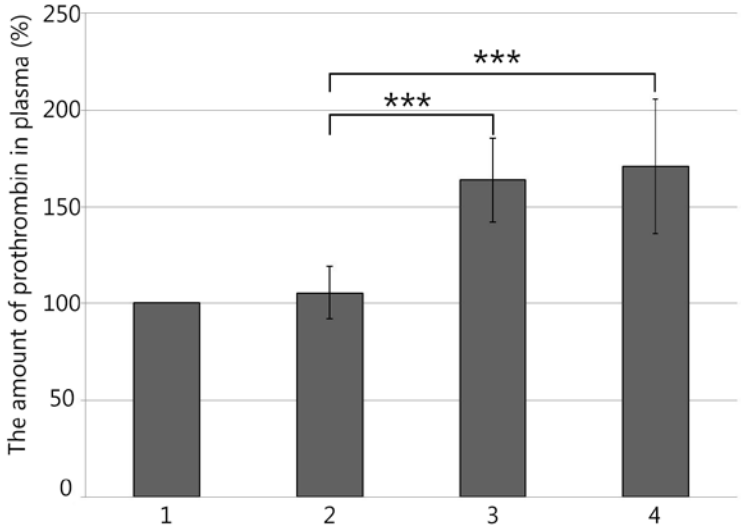

Fig. 2 – Relative quantification of plasma prothrombin level by densitometry of Western blot bands (Image Studio Lite™, LI-COR).
1: Standard human plasma (assigned referent value of 100%); 2: Plasma sample of a healthy volunteer (non-carrier of mutations in 3' end of the prothrombin gene);
3: Plasma sample of the heterozygous carrier of *F2 G20210A* mutation;
4: Plasma sample of stroke patient with novel *F2 c.1824C>T* gene variant.
***** $p <$ 0.0001 compared to healthy volunteer.**

PAI-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 the *PAI-1 gene* influences the 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 absent in the 4G allele. This reflects on PAI-1 expression and its plasma concentrations with 4G homozygotes having the highest and the 5G homozygotes having 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 to 2006, showed that *PAI-1 4G/4G* genotype is likely to be associated with IS [15].

Elevated plasma homocysteine (hyperhomocysteinemia) and homozygosity for the *MTHFR C677T* variant have been associated with the increased risk for IS; however, the exact mechanism is not fully determined [16, 17]. Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the folate pathway and homocysteine conversion to methionine. The presence of the *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 FV *in vitro* and inhibits its cleavage by activated protein C [18]. Hyperhomocysteinemia might also promote fibrinogen modification, thereby impairing the activity of fibrinolytic enzymes and fibrin polymerization. That 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 the *F2* gene, which cause elevated prothrombin level, hypercoagulability, and a tendency towards hyperproduction of fibrin clot [19, 20]. One of the most commonly tested prothrombotic variants in IS patients is *F2 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 the *F2* gene associated with early IS onset and did the sequencing of 715 bp within its 3' end, where a recently described *F2 c.1824C>T* gene variant was detected. The *F2 c.1824C>T* represents a synonymous *F2* variant leading to the CGC to CGT codon replacement on the Arg608 position in the protein [10]. The study by Pruner et al. [10] showed an 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, *in vitro* examination demonstrated that *F2 c.1824T* allele is associated with elevated expression of prothrombin mRNA [10]. In addition, an *ex vivo* study indicated that *F2 c.1824C>T* variant leads to hyperprothrombinemia, hypofibrinolysis, and the formation of denser and thinner fibrin fibers within the clot, which makes it resistant to fibrinolysis [10]. The properties of the fibrin clot are shown to have clinical relevance in patients with acute IS. Undas et al. [22] enrolled 45 patients with acute IS and showed that the detected fibrin clots are less susceptible to fibrinolysis and that fibers form a less porous fibrin network. However, in comparison to the increased fiber thickness in acute IS patients [22], Pruner et al. [10] showed that fibrin clot in *F2 c.1824C>T* patients is structured from densely packed, but thinner fibers, which implicates that the fiber thickness could be *F2 c.1824C>T* specific.

The result of the elevated plasma level of prothrombin detected in our IS patient is in concordance with the study which examined the prothrombin level in carriers of *F2 c.1824C>T* who suffered from IS or venous thromboembolism (VTE) [10]. The increase in prothrombin level that we detected in the presence of the *F2 c.1824C>T* variant is similar to the one detected previously in IS and VTE patients. When compared to the effect of *F2 G20210A*, which increased prothrombin plasma level by approximately 60%, *F2 c.1824C>T* variant could be a potentially new, equally potent prothrombotic risk factor that leads to the development of a prothrombotic phenotype [10]. In this case study, we did not investigate 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 *F2 c.1824C>T* carriers, *F2 G20210A* and healthy noncarriers by Pruner et al. [10] revealed that fibrin fibers in plasma are denser and thinner in case of *F2 c.1824C>T* compared to clots in *F2 G20210A* and healthy noncarriers.

Fibrin clot phenotype is dictated by the number of variables involving pH, ionic strength, and concentrations of calcium and fibrinogen [23]. However, thrombin concentration during clot formation has a crucial role in the density and stability of the 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 the 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 the *F2 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 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 [4, 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 the 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 *F2 c.1824C>T* variant, could lead to the formation of altered fibrin clot phenotype characterized by densely packed, fibrinolysis resistant fibrin fibers, which could contribute to the early-onset IS in our patient.

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Conflict of interest

All authors declare no conflict of interest.

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