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ORIGINAL ARTICLE

Clinical and biochemical characterization of the prothrombin Belgrade mutation in a large Serbian pedigree: new insights into the antithrombin resistance mechanism

P. MILJIC,*† M. GVOZDENOV,‡ Y. TAKAGI,§ A. TAKAGI,§ I. PRUNER,‡ M. DRAGOJEVIC,‡ B. TOMIC,‡ J. BODROZIC,† T. KOJIMA,§ D. RADOJKOVIC‡ and V. DJORDJEVIC‡

**Faculty of Medicine, University of Belgrade; †Clinic of Hematology, University Clinical Center; ‡Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia; and §Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya, Japan*

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Essentials

- Prothrombin Belgrade mutation leads to antithrombin resistance.
- Clinical and biochemical phenotypes in a large family with this mutation were investigated.
- In carriers, we detected decreased factor II activity and increased endogenous thrombin potential.
- Prothrombin Belgrade mutation represents a strong prothrombotic risk factor.

Summary. *Background:* The recently reported c.1787G>A mutation in the prothrombin gene leads to Arg596Gln replacement in the protein molecule (prothrombin Belgrade). This substitution impairs binding of antithrombin to thrombin and results in inherited thrombophilia, known as antithrombin resistance. *Objectives:* We aimed to elucidate the clinical and biochemical characteristics of thrombophilia associated with antithrombin resistance in a large Serbian family with the prothrombin Belgrade mutation. *Patients and methods:* Nineteen family members were investigated, among whom 10 were carriers of the c.1787G>A mutation. In all subjects the clinical phenotype was determined and laboratory investigations of hemostatic parameters were performed. *Results:* Six out of the 10 mutation carriers developed thromboembolic events, mainly deep venous and mesenteric vein thrombosis. The median age of the first thrombotic event was 26.5 (12–41) years, whereas the incidence rate of first thrombosis was 2.2% per year. In all mutation carriers prothrombin activity was significantly decreased in comparison with non-carriers, clearly distinguishing each group. However, the presence of the mutation did not affect the prothrombin antigen level in plasma. The endogenous thrombin potential was significantly increased in all carriers in comparison with non-carriers, indicating the presence of blood hypercoagulability. Interestingly, levels of D-dimer and the F1 + 2 fragment were similar in both groups. *Conclusions:* Although rare, the prothrombin Belgrade mutation represents strong thrombophilia with early onset of thrombosis in the investigated family. According to our results, decreased prothrombin activity may be a simple screening test for detection of this mutation in thrombotic patients.

Keywords: mesenteric venous thrombosis; mutation; prothrombin; thrombophilia; venous thromboembolism.

Correspondence: Valentina Djordjevic, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, 11010 Belgrade, Serbia.
Tel.: +381 11 3976658; fax: +381 11 3975808
E-mail: pg20210a@gmail.com

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Introduction

Several inherited abnormalities in the coagulation system are associated with increased risk of venous thromboembolism. Hereditary deficiencies of natural anticoagulants described more than three decades ago, represent strong but uncommon hypercoagulable conditions. On the other hand, *F5* Leiden and *F2* G20210A mutations, although more common, are weak thrombophilias that usually cause thrombosis in the presence of other acquired or inherited prothrombotic factors [1].

Recently, a novel mechanism of inherited thrombophilia associated with antithrombin resistance has been described. It involves a c.1787G>T mutation in the gene

encoding prothrombin, which results in substitution of arginine by leucine at residue 596 (prothrombin Yukuhashi). Arginine at residue 596 is positioned within one of the antithrombin-binding sites and its substitution leads to impaired inhibition of the mutant thrombin by antithrombin, resulting in thrombophilia due to antithrombin resistance [2]. Lately, we reported another prothrombin mutation, c.1787G>A, in two Serbian families with thrombosis. This mutation affects the same Arg596 position, but results in a different amino acid substitution, p.Arg596Gln (prothrombin Belgrade), and likewise leads to antithrombin resistance [3]. The prothrombin Belgrade mutation has been detected as well in one Indian and one Japanese thrombophilic family [4,5]. Most recently, prothrombin Padua 2 (*F2* c.1786C>T, p.Arg596Trp), also characterized by a mutation at the Arg596 position and the presence of antithrombin resistance, has been described [6].

However, despite these reports on prothrombin gene mutations involving Arg596 substitution, a wider picture of clinical expression of thrombophilia caused by inherited antithrombin resistance is missing. Data related to clinical penetrance, relative risk of thrombosis or common localization of thrombotic events in mutation carriers are scarce.

In addition to its influence on the thrombin/antithrombin interaction, Arg596 is positioned within the sodium-binding region, which is important for the procoagulant role of thrombin. Consequently, Arg596 substitutions can result in impaired procoagulant function of mutant thrombin [7,8]. This reasoning is supported by experiments with recombinant prothrombin Yukuhashi and prothrombin Padua 2, which demonstrated decreased clotting activity in comparison with native prothrombin [2,6]. However, it is still unclear whether decreased procoagulant activity of prothrombin variants with Arg596 substitution is associated with increased risk of bleeding, especially in high-risk conditions, such as delivery or surgery.

In an attempt to gain closer insight into the clinical and biochemical characteristics of thrombophilia caused by the prothrombin Belgrade mutation, we collected clinical data and conducted laboratory investigations in members of a large Serbian pedigree with this mutation.

Patients and methods

In an earlier paper we reported six symptomatic carriers of prothrombin Belgrade in two Serbian families [3]. The first one consisted of four members, and for the purpose of this study we further investigated members of the second, larger, family. The proposita, who suffered recurrent thrombosis, was monitored from 2006 and upon identification as a prothrombin Belgrade mutation carrier in 2012, an investigation of the complete family was undertaken. Written approval from the local ethical committee has been obtained and all participants signed informed consent form. The genealogical chart of the investigated family is shown in Fig. 1.

A detailed medical history with special attention to thromboembolic events and bleeding episodes was obtained for the proposita and 14 of her blood relatives. The proposita's husband and three wives of her blood relatives were also included in the investigation in order to explain the inheritance pattern of the prothrombin gene mutation. The participants were considered symptomatic if they experienced at least one episode of objectively documented venous or arterial thrombosis. Superficial thrombophlebitis without affecting the deep venous system was not encountered as a thrombotic event. Participating subjects were also interviewed regarding abnormal bleeding symptoms. Bleeding was considered as abnormal if denoted by the attending physician in the medical records as excessive or unusual. We also collected data, when available, for deceased family members regarding the age and cause of death and previous thromboembolic events.

Four family members were receiving chronic anticoagulant therapy with warfarin. In three of them warfarin was stopped 30 days before blood sampling and replaced with Dalteparin subcutaneously at 100 J kg^{-1} body weight once daily. The last dose was administered 48 h before blood sampling. In one family member warfarin therapy was not interrupted so coagulation assays were not performed in this individual.

For laboratory investigations blood samples were taken from all investigated subjects. Blood samples for hemostatic tests were immediately centrifuged at 1800 × *g* and plasma and cells separated. Plasma was aliquoted and frozen at −80 °C until required for analysis, and the layer of white blood cells was used for DNA extraction. Extracted DNA was stored at +4 °C until use.

In the context of laboratory investigation of hemostasis, the following tests were performed: prothrombin time (PT), activated partial thromboplastin time (APTT), activities of antithrombin, protein C and protein S, *F5* Leiden and *F2* G20210A mutations, lupus anticoagulant, anti-cardiolipin and anti-beta2-glycoprotein I antibodies.

Also, the clotting activity of factor (F) II, V, VII and X was measured in the PT-based assay, whereas activities of FVIII, FIX, FXI and FXII were determined in the APTT-based one-stage assay. Prothrombin fragment F1+2 was measured using ELISA kit-Enzygnost F1+2 micro (Dade Behring, Marburg, Germany) and D-dimer was determined by an immunoturbidometric method on the IL-7000 instrument (Instrumentation Laboratory, Milan, Italy).

DNA sequencing

Direct DNA sequencing of the 715 base-pairs region within the 3′end of the prothrombin gene including the last intron and exon was performed. This fragment was amplified by polymerase chain reaction and further sequenced with forward (5′-TCTAGAAACAGTTGCCTGGC-3′) and reverse

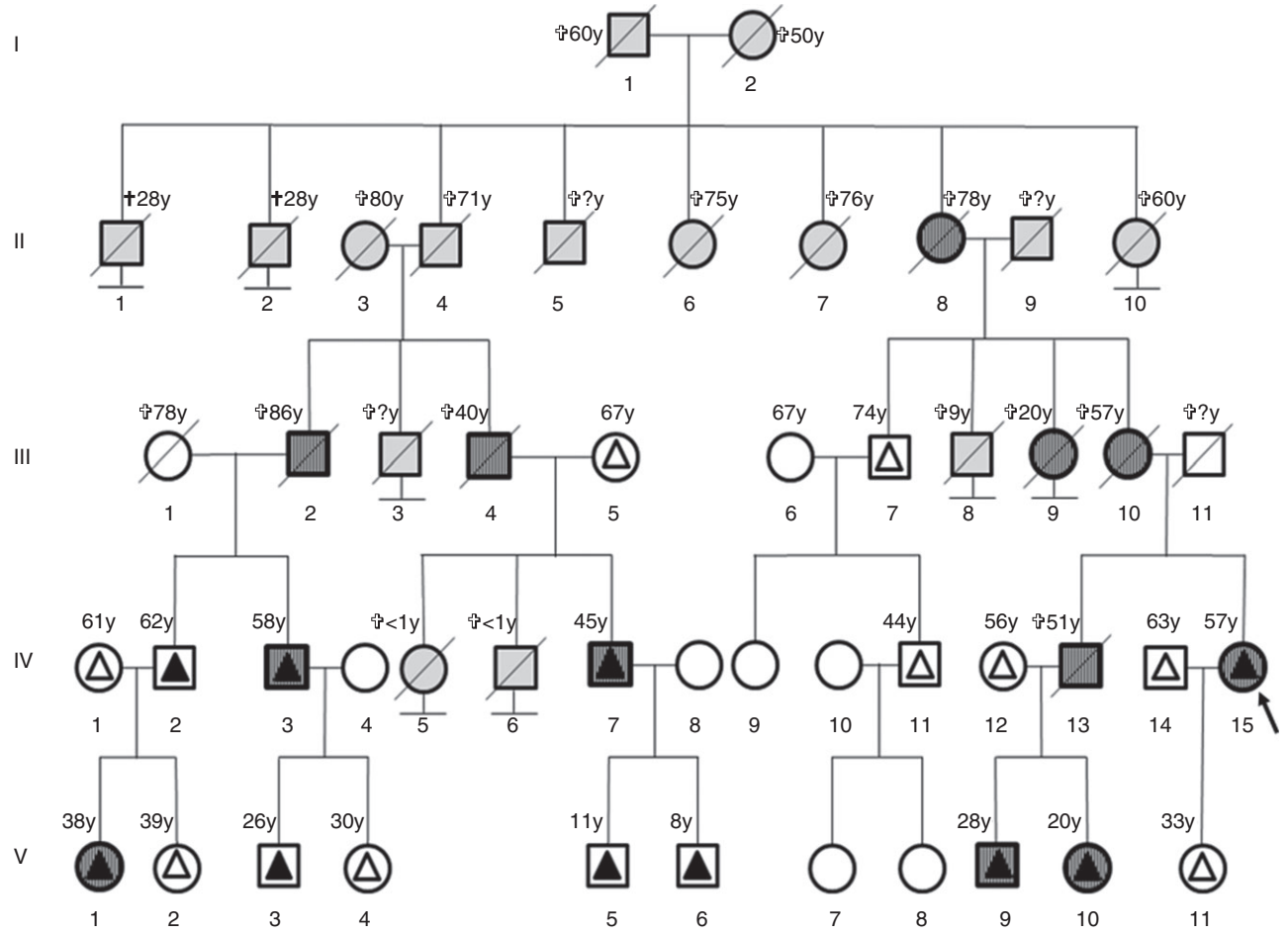


Fig. 1. Family pedigree. The proband (IV-15) is indicated by an arrow. Hatched objects represent affected family members with thromboembolic complications, empty objects are unaffected members and grey objects indicate those with no data. Triangles represent tested family members: solid triangles are heterozygous *F2* c.1787G>A mutation carriers; open triangles are non-carriers. Slashed objects represent deceased family members: †, killed in a war; ✞, natural death.

(5′-TCAATGCTCCCAGTGCTATTC-3′) primers using the BigDye™ Terminator Version 3.1 Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol, on a 3130 Genetic Analyzer (Applied Biosystems).

Antithrombin resistance detection assay

The ability of antithrombin to inactivate thrombin in plasma samples was evaluated using an antithrombin resistance assay [9]. In the first step, 500 μL of diluted plasma samples were treated for 2 min at 37 °C with 100 μL of *Oxyuranus scutellatus* venom (Sigma Aldrich, Taufkirchen, Germany) in the presence of phospholipid and $CaCl_2$. The second step involved thrombin inactivation by addition of human antithrombin (100 μL) (Mitsubishi Tanabe Pharma, Osaka, Japan). The incubation time ranged from 0 to 30 min. In the final step, relative residual thrombin activity (RRTA) was estimated with S-2238 (Chromogenix, Milan, Italy) as a synthetic chromogenic substrate for thrombin, by measuring the changes in absorbance per minute (ΔA/min) at 405 nm with TBA-180 (Toshiba Medical Systems Co, Tokyo, Japan).

Detection of prothrombin plasma level by western blot

The prothrombin plasma level (FII : Ag) was evaluated by relative quantification of western blot results. Diluted plasma samples (1 : 20 in sterilized water) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by transfer of the proteins to PVDF membrane (Millipore, Darmstadt, Germany). The membrane was incubated in blocking solution at 4 °C overnight. This was followed by incubation with Thrombin K-20 goat polyclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1 : 3000 dilution) and anti-goat IgG peroxidase conjugate secondary antibody (1 : 80 000 dilution) (Sigma, St Louis, MO, USA). Three independent experiments were performed, each carried out in triplicate. Immunoreactive bands were detected by the ECL kit (Millipore). The amount of prothrombin was determined by relative quantification of the

Table 1 Thromboembolic events and significant bleeding challenges in carriers of the prothrombin Belgrade mutation

Family member	Age (years)	Site of thrombosis (age at onset; provocative factors)	Significant bleeding challenges (excessive bleeding)
V-1	38	1. Proximal DVT (36; pregnancy)	Tonsillectomy (no) Two deliveries (no)
V-3	26	Asymptomatic	/
V-5	11	Asymptomatic	/
V-6	8	Asymptomatic	/
V-9	28	1. Proximal DVT (17; no) 2. AMT (24; no)	Large abdominal surgery (no)
V-10	24	1. DVT (12; surgery)	Kidney surgery (no)
IV-2	62	Asymptomatic	Minor surgery (no)
IV-3	58	1. DVT (41; surgery) 2. DVT + PE (48; no)	Orthopedic surgery (no)
IV-7	45	1. DVT (36; no) 2. DVT (38; no)	Tonsillectomy (no)
IV-15	57	1. DVT (16; no) 2. DVT (24; pregnancy) 3. AMT (32; no) 4. MI (41; no)	Tonsillectomy (no) Delivery (no) Abdominal surgery (no)

DVT, deep venous thrombosis; PE, pulmonary embolism, AMT, acute mesenteric vein thrombosis; MI, myocardial infarction.

signal using Image Studio Lite software Ver. 3.1 (LI-COR, Bad Homburg, Germany). The results obtained were normalized with standard human plasma (Siemens, Munich, Germany), reference value 100%.

Thrombin-generation assay

The thrombin-generation assay was performed by the calibrated automated thrombogram method (Thrombinoscope BV, Maastricht, the Netherlands) in accordance with the manufacturer's instructions, using tissue factor (TF) trigger at a final concentration of 5 pmol L^{-1}. We monitored the reactions for 2 h using a Fluoroscan Ascent FL (Thermo LabSystems, Beverly, MA, USA) set at an excitation wave length of 390 nm and an emission wave length of 460 nm with appropriate software (Thrombinoscope BV). Normal pooled plasma was used as a control.

Statistical analysis

Statistical analysis was performed by the Statistical Package for Social Sciences 13.0 for Windows (SPSS Inc., Chicago, IL, USA). The median values were calculated. Statistical analysis was conducted using the Mann–Whitney *U*-test. A P value less than 0.05 was considered as statistically significant.

Results

The c.1787G>A mutation with consequent Arg596Gln substitution had already been detected in the proposita and her two nephews [3]. Investigation of the rest of the family revealed this mutation in seven more members (Fig. 1). Altogether, the mutation was detected in seven male and three female subjects of median age 33 (8–62) years. All carriers inherited the mutation in the heterozygous form. The five family members were without the mutation. Also, the proposita's husband and three investigated wives of the proposita's blood relatives were found not to be a prothrombin Belgrade carrier (Fig. 1).

Clinical data

Among the 10 mutation carriers, six experienced at least one episode of thrombosis, whereas four remained asymptomatic. No subject without the mutation suffered a thrombotic episode. The median age of the first thrombosis in symptomatic carriers was 26.5 (12–41) years, whereas the median age of asymptomatic carriers was 18.5 (8–62) years. When calculated retrospectively from birth, the incidence rate of first thrombosis in carriers of prothrombin Belgrade mutation was 2.2% per year. Six symptomatic carriers had a total of 12 thrombotic episodes. Data regarding age at onset of thrombosis, its localization and the presence of provocative factors are shown in Table 1. All six symptomatic family members were treated with warfarin for different periods of time without recurrence of thrombosis during the treatment. Episodes of recurrent venous thrombosis occurred exclusively after interruption or cessation of anticoagulant therapy.

Seven carriers experienced significant challenges associated with risk of bleeding, but in none of them was excessive bleeding recorded (Table 1).

Laboratory investigations

Deficiency of natural anticoagulants and the presence of antiphospholipid antibodies and *F5* Leiden or *F2* G20210A mutation were excluded in all family members except the husband and daughter of the proposita, who were heterozygous carriers of the *F2* G20210A mutation.

Values of PT were at the upper limit of normal or slightly prolonged in the prothrombin Belgrade mutation carriers. Factor II activity was decreased in all carriers (range 46–70%), while in all family members without mutation were normal (Table 2, Fig. 2). In contrast to decreased FII activity, in all carriers the level of FII : Ag was normal and similar to levels in non-carriers of the prothrombin Belgrade mutation (Table 2, Fig. 3). Elevated plasma levels of FII : Ag were detected in carriers of the *F2* G20210A mutation (proposita's husband and daughter; Fig. 3). Coagulation FV, FVII and FX activities were within the normal range and similar in carriers and non-carriers (data not shown).

Markers of in vivo *activation of coagulation*

The levels of D-dimer were similar in carriers and non-carriers of the prothrombin Belgrade mutation (Table 2). Values

Table 2 Results of laboratory tests

Family member	Prothrombin Belgrade carrier status	PT (s)	APTT (s)	FII clotting activity (%)	FII : Ag (%)	D-dimer (mg L^{-1})	F1+2 fragment (pmol L^{-1})
V-1	Heterozygous	18	30.7	46	148.1	0.19	119
V-3	Heterozygous	15.1	29.2	70	87.1	0.2	87
V-5	Heterozygous	17.4	32	54	157.6	0.25	79
V-6	Heterozygous	17.1	33.7	54	118.9	0.31	79
V-9	Heterozygous	17.4	33.7	56	150.7	0.33	88
V-10	Heterozygous	17.4	34.2	60	117.4	0.21	76
IV-2	Heterozygous	15.9	32.2	63	147.9	0.38	153
IV-3*	Heterozygous	26	28.5	/	/	1.04	119
IV-7	Heterozygous	16.5	30	60	139.8	0.24	99
IV-15	Heterozygous	17.1	27	46	144.1	0.73	174
V-2	Non-carrier	14.7	29.2	97	130.1	0.27	160
V-4	Non-carrier	14.8	25.5	94	141.1	0.23	97
V-11†	Non-carrier	14.9	26.7	105	166.5	0.28	186
IV-1	Non-carrier	15.0	32.2	87	125.1	0.27	137
IV-11	Non-carrier	13.8	28	101	148.2	0.2	119
IV-12	Non-carrier	14.4	28.5	97	126.3	0.32	235
IV-14†	Non-carrier	13.6	31.7	101	154.9	0.21	176
III-5	Non-carrier	14.7	28.7	97	108.3	0.47	166
III-7	Non-carrier	14.8	23.5	84	120.2	1.46	219

Normal ranges: for PT, 12.4–15.2 s; for APTT, 27–35 s; for FII clotting activity, 75–150%; for D-dimer, < 0.5 mg L−1; for F1+2 fragment, 69–229 pmol L−1. *Warfarin therapy; †*F2* G20210A heterozygous carrier. PT, prothrombin time; APTT, activated partial thromboplastin time; FII : Ag is expressed relative to standard plasma (100%).

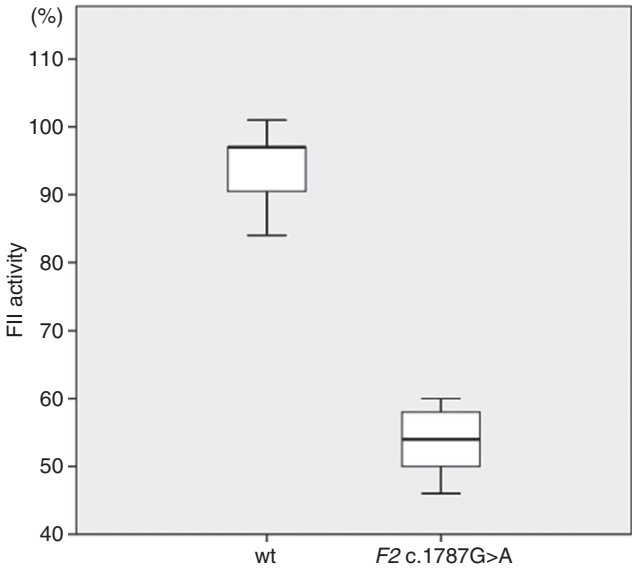


Fig. 2. Factor II clotting activity in non-carriers (wt) and heterozygote carriers of the mutation (*F*2 c.1787G > A).

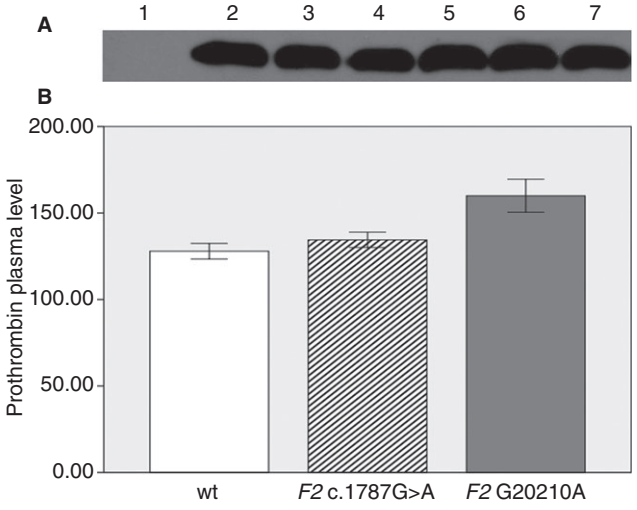


Fig. 3. Western blot analysis and relative quantification of prothrombin plasma level. A) Western blot analysis: prothrombin deficient plasma 1; standard plasma 2, 3, 4; heterozygous *F*2 c.1787G > A mutation carrier plasma 5, 6, 7; B) Relative quantification of non-carriers (wt), heterozygous *F2* c.1787G > A mutation carriers (*F*2 c.1787G > A) and heterozygous *F*2 G20210A mutation carriers (*F*2 G20210A) plasma prothrombin level.

of the F1+2 fragment were in the normal range in all carriers of the mutation. Moreover, no differences in levels of the F1+2 fragment between symptomatic and asymptomatic carriers were found, whereas a slightly elevated D-dimer level was observed in two symptomatic carriers (Table 2).

Antithrombin resistance analysis

The test for assessment of thrombin inactivation by antithrombin was performed on plasma samples from 10 carriers and nine non-carriers of prothrombin Belgrade. At all time-points during measurement there were clear distinctions in RRTA between carriers and non-carriers. At 30 min from starting the test the RRTA values in carriers were 50.9–54.7%, whereas in non-carriers and control plasma these values were below 10%. RRTA was similar in symptomatic and

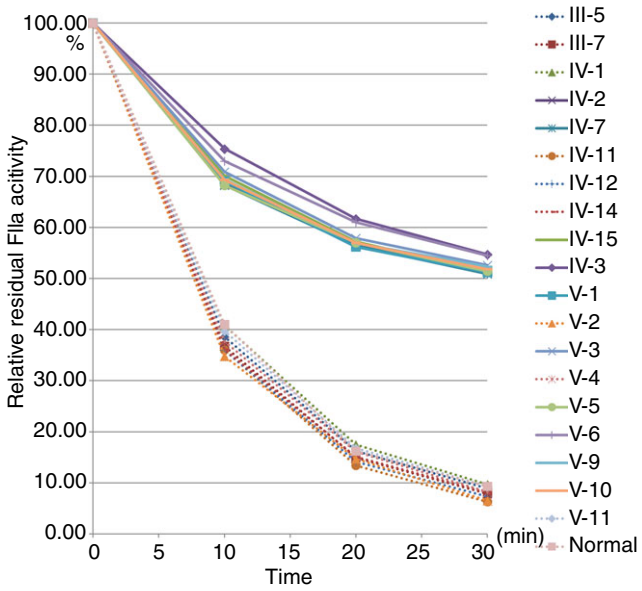


Fig. 4. Antithrombin resistance analysis in plasma samples. Sample numbers correspond to the Family pedigree (Fig. 1). Full lines represent *F*2 c.1787G > A mutation carriers, interrupted lines represent non-carriers and the control plasma sample (Normal). [Color figure can be viewed at wileyonlinelibrary.com].

asymptomatic carriers of the prothrombin Belgrade mutation (Fig. 4).

Thrombin-generation assay

The detected values of endogenous thrombin potential (ETP) were significantly higher in mutation carriers than in non-carriers, and there was a clear distinction between these two groups with no overlap. The increased ETP in carriers was a result of prolonged decay time, whereas values of lag time and peak levels were comparable between carriers and non-carriers (Fig. 5). The results presented in Fig. 5 are calculated without taking into account the values for subject IV-3, who was on warfarin therapy.

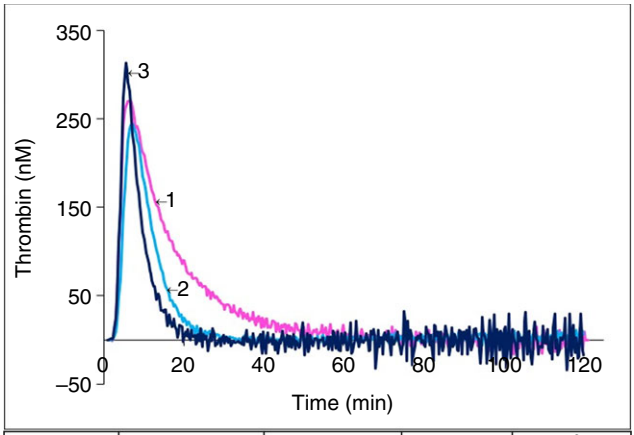
Discussion

In this study we collected clinical data and performed laboratory investigations in a large family with prothrombin mutation p.Arg596Gln conveying antithrombin resistance (prothrombin Belgrade). Six out of 10 identified mutation carriers had experienced one or more thrombotic episodes, usually at a young age, whereas all nine non-carriers remained asymptomatic, demonstrating that prothrombin Belgrade mutation is associated with a strong thrombotic tendency in this family.

It seems that clinical penetrance of the prothrombin Belgrade mutation in the investigated family could be at least at the same level as that for antithrombin deficiency. Earlier it was established that about 50% of heterozygous carriers of antithrombin deficiency developed venous thrombosis during their lifetime [10,11]. Similarly, 60% of heterozygous carriers of the prothrombin Belgrade mutation in this family experienced thrombosis, with the earliest occurrence at the age of 12 years. Moreover, it should be noted that penetrance of prothrombin mutation in this family may be even higher because four asymptomatic carriers were significantly younger (median age 18.5 years) than symptomatic carriers at the time of their first thrombosis (median age 26.5 years). In a retrospective study, Tormene *et al.* reported an annual incidence rate of thrombosis of 0.55% in persons with a deficiency of natural anticoagulants, whereas in a prospective investigation it was 0.92% [12]. In our retrospective study, the annual incidence rate of the first thrombosis from birth was 2.2% in carriers of the prothrombin Belgrade mutation. All these data lead to the conclusion that in this family, the prothrombin Belgrade mutation represents a serious thrombophilic condition, at least comparable with thrombophilia associated with deficiency of natural anticoagulants.

The most common sites of thrombosis in carriers of natural anticoagulant deficiency, *F5* Leiden or *F2* G20210A mutation are in deep leg veins, iliac veins and pulmonary arteries [13]. In the investigated family with prothrombin Belgrade mutation, deep venous thrombosis (DVT) was the most common manifestation, but unusual clustering of acute mesenteric vein thrombosis (AMT) has also been recorded (in investigated subjects V-9 and IV-15, and documented as cause of death in subjects III-9 and IV-13; Fig. 1). This unusual clinical expression of the prothrombin Belgrade mutation may be explained hypothetically by a different mechanism of thrombosis in carriers of this mutation compared with other thrombophilias that are mainly associated with increased risk of DVT.

The arginine at residue 596 in the prothrombin molecule is not only important for thrombin/antithrombin interactions, but it is also located in the sodium-binding region of thrombin [7,8]. The compromised sodium binding due to mutation of Arg596 was reported by Dang and coworkers in 1997 [8]. Sodium-bound thrombin (known as the fast form) is optimized for procoagulant activity as a result of increased specificity for fibrinogen. On the other hand, sodium-free thrombin (slow form) predominantly demonstrates anticoagulant activity because of increased affinity for thrombomodulin and specificity for cleaving protein C [14]. Therefore, it could be expected that substitution at residue 596 may impair formation of the fast form of thrombin and negatively affect its procoagulant function. Recombinant prothrombin Yukuhashi showed decreased procoagulant function in a clotting assay and, interestingly, also manifested thrombomodulin resistance in terms of its fibrinogen clotting inhibition activity [2,15]. Also, Simioni and coauthors



	Carriers[1] median (range)	Non-carriers median (range)	Control plasma	P[2]
Lag time (min)	3.1 (2.6–3.6)	3.2 (2.7–4.2)	2.5	0.93
ETP (nM min)	3645 (3105–4233)	1653 (1482–2025)	1408	<0.001
Peak (nM)	301 (273–373)	283 (246–310)	309	0.05
Start tail (min)	60.0 (51.0–78.7)	24.0 (22.0–25.7)	21	<0.001

Fig. 5. Thrombin-Generation Assays Panel shows the thrombin-generation assay results for plasma of *F*2 c.1787G > A mutation carriers (pink line-1), non-carriers (light blue line-2) and normal plasma (dark blue line-3) as the mean curve of each genotype. The table shows the median and range values for: the Lag time; the total amount of thrombin activity, assessed as the area under the curve for the endogenous thrombin potential (ETP); the maximum concentration of thrombin (Peak), and the total duration of thrombin-generation activity (Start tail). [1]subject IV-3 was excluded; [2]carriers vs. non-carriers by Mann-Whitney U test. [Color figure can be viewed at wileyonlinelibrary.com].

reported diminished FII activity in plasma of prothrombin Padua 2 mutation carriers [6]. In agreement with these findings, we observed decreased FII clotting activity in all carriers of the prothrombin Belgrade mutation, without overlap with non-carriers who had normal FII activities (Fig. 2). On the other hand, the prothrombin antigen level was normal in all carriers, indicating that partial deficiency of prothrombin function is caused by a dysfunctional molecule. Interestingly, the clotting activity of prothrombin in some carriers of the prothrombin Padua 2 mutation was at the lower limit of referent values [6], indicating that the procoagulant function of prothrombin Belgrade may be more affected than that of prothrombin Padua 2. According to our results, the finding of decreased FII clotting activity (with reference values for activity of other factors) in a patient with thrombosis may become a simple screening test for the prothrombin Belgrade mutation.

However, despite the decreased level of FII activity (range 46–70% of normal), bleeding diathesis was not recorded in any of the prothrombin Belgrade mutation carriers, even in situations of increased risk of hemorrhage, such as childbirth or exposure to major surgery or intensive anticoagulant therapy. This paradox of thrombosis in individuals with significantly decreased levels of FII activity could be at least partially explained by overcompensation for prothrombin procoagulant dysfunction by delayed thrombin inactivation. In all carriers of prothrombin Belgrade we observed increased ETP in comparison with non-carriers, mainly on account of delayed decay of thrombin (Fig. 5). However, in contrast to blood hypercoagulability assessed by the ETP assay, the levels of D-dimer and F1+2 fragment did not differ between carriers and non-carriers. The results of these tests, which are sensitive markers of thrombin formation *in vivo*, argue against increased activation of the coagulation system in basal conditions in mutation carriers. The absence of increased levels of molecular markers of blood coagulation activation, even in symptomatic family members, may implicate participation of platelets in the pathogenesis of thrombosis in carriers of the prothrombin Belgrade mutation. On the basis of our findings, we hypothesize that in the basal condition activation of the coagulation system to a level sufficient to produce a burst of thrombin and generate increased concentrations of F1+2 fragment and D-dimer is prevented by impaired mutant prothrombin activity. However, it could be speculated that even traces of mutant thrombin, which is poorly inactivated as a result of antithrombin resistance, may be able to activate platelets for a prolonged period of time. Namely, a significantly lower concentration of thrombin (0.5–1 nmol L^{-1}) is required for full platelet activation in comparison with the amount that is generated during blood coagulation (10–100 nmol L^{-1}) [16]. On the other hand, it is possible that stronger stimuli, such as pregnancy, may generate a sufficient amount of mutant thrombin for conversion of fibrinogen to fibrin, leading to overt thrombosis.

Despite supposed platelet activation as a participating factor in a thrombotic tendency, it appears that use of warfarin is an efficient way to prevent recurrence of thrombosis in carriers of the prothrombin Belgrade mutation in this family. Six symptomatic carriers have received warfarin for 49 years in total, and no recurrence has been observed during that period.

In conclusion, prothrombin Belgrade seems to be a rare prothrombotic condition, but it has been found so far in Serbian, Indian and Japanese populations [3–5]. It is well known that the risk of thrombosis in families with inherited thrombophilia depends highly on the clinical phenotype of the proband [17]. For that reason, it is not clear whether our findings could be generalized to other families with the prothrombin Belgrade mutation or other prothrombin variants involving substitution at the Arg596 residue. However, our results indicate that prothrombin Belgrade behaves in a similar way to prothrombin Yukuhashi and prothrombin Padua 2 in terms of increased antithrombin resistance, and we believe that prothrombin variants with Arg596 substitutions should be considered as severe thrombophilic conditions, in the same way as deficiencies of natural anticoagulants.

Addendum

P. Miljic and V. Djordjevic were responsible for data management and study design; J. Bodrozic and P. Miljic were responsible for the thrombophilia testing; B. Tomic and I. Pruner were responsible for the genetic analyses; M. Gvozdenov and M. Dragojevic were responsible for the prothrombin western blot analyses; Y. Takagi, A. Takagi and T. Kojima were responsible for the functional assay of plasma to detect antithrombin-resistance and thrombin generation assay; P. Miljic, T. Kojima and V. Djordjevic were responsible for writing the manuscript; and D. Radojkovic and V. Djordjevic were responsible for supervision of the manuscript.

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Disclosure of Conflict of Interests

T. Kojima reports personal fees from Pfizer, Baxter, Boehringer Ingelheim, Nihon Pharma, Sysmex, and LSI Medience; and grants and personal fees from Bristol-Myers Squibb, Bayer, and Daiichi-Sankyo, outside the submitted work. A. Takagi reports grants from Baxalta, outside the submitted work.

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