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THE PROTHROMBIN GENE G20210A VARIANT IN AN UNSELECTED THROMBOEMBOLIC POPULATION. A belgian prospective clinical study.

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SUMMARY

The presence of the 20210A allele of the prothrombin gene has recently been shown to be a risk factor of venous thromboembolism, probably mediated through increased prothrombin levels. The aim of the study was to determine the frequency of the prothrombin 20210A allele in 193 consecutive unselected patients with venous thromboembolism and 100 healthy controls and to analyze the clinical profile associated with this new inherited thrombophilic factor. In agreement with previous reports, we found a frequency of 7.3% of heterozygous carriers of the 20210A allele among patients and 1% among controls. We confirm that plasma prothrombin levels are more elevated in the individuals bearing the prothrombin 20210A allele compared with those who do not. We did not find any relationship between the presence of the prothrombin 20210A allele and either a family history of thromboembolism, the rate of recurrences or the age at disease onset. However, the co-inheritance in the same individual of both prothrombin 20210A allele and factor V Leiden was associated with a significantly lower age at disease onset suggesting a synergistic contribution of both abnormalities.

INTRODUCTION

Venous thromboembolism is a frequent disease with an annual incidence in the general population of approximately 1 per 1000. Risk factors involve both hereditary and acquired conditions. Mutations in genes coding for coagulation proteins play an increasingly significant role. Until a few years ago, inherited deficiency of antithrombin III, protein C or protein S were the only single gene disorders related to the development of venous thrombosis but their prevalence remained low. Recently, the discovery of a single mutation of factor V responsible for activated protein C resistance, also called factor V Leiden, emphasised the pivotal role of genetic analysis in understanding thrombophilic mechanisms (1,2). Factor V Leiden is encountered in more than 20% of thromboembolic patients, hence representing the most frequent thrombophilia inherited factor (3,4). Despite this recent advance, many thrombotic events remained unexplained. At the end of 1996, another point mutation located at position 20210 in the 3' untranslated region of the prothrombin gene and consisting in a G to A nucleotide transition was identified and associated with a 2.8 fold increased risk for venous thrombosis (5). It was reported in 6.2 % of unselected patients with a first episode of deep venous thrombosis and in 18% of patients with a familial history of venous thrombosis. The presence of the 20210A allele was also correlated with elevated plasma prothrombin levels permitting the hypothesis that the hypercoagulability conferred by the 20210A allele may be due to hyperactivity of the common coagulation pathway (5). This observation prompted us to analyse a large cohort of patients presenting with well-documented initial or recurring venous thromboembolism.

PATIENTS AND METHODS

Patients with thrombosis and healthy control subjects

The study population involved 193 unrelated patients treated in a single academic hospital between June 1993 and September 1997. One hundred volunteers (62 women and 38 men) with neither a personal nor a family history of thrombosis, recruited among the hospital employees and students were included as control subjects. The mean age of the control subjects was 28 years (range: 20 to 64). Patients and controls were of West European descent.

Assays

a) Blood sampling:

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Blood was collected by clean venipuncture into tubes containing 0,106 M trisodium citrate for coagulation assays and kalium-EDTA (1,6 mg EDTA/ml blood) for DNA processing. At blood sampling, none of the patients were receiving any medication that would interfere with the coagulation study.

b) Coagulation assays.

Platelet free plasma was obtained by centrifugation at 5000 g for 20 minutes at 4°C and stored at — 20°C until required. Protein C and antithrombin levels were determined with functional methods using chromogenic substrates from Chromogenix® on a centrifugal auto-analyser ACL200 (Instrumentation Laboratory, Milan, Italy). Protein S was analysed by ELISA testing (free protein S). Lupus anticoagulant was evaluated by 3 screening tests (activated recalcification time, activated partial thromboplastin time (AM), tissue thromboplastin inhibition test (TTI) (using Innovin Dade thromboplastin) and 2 confirming methods involving plasmatic mixture and platelet neutralisation. Factor II (prothrombin) assays were performed by a thromboplastin-based assay using factor II deficient plasma on a centrifugal autoanalyser ACL200.

c) DNA amplification and sequencing:

DNA was extracted from peripheral blood leukocytes using a QI Aamp Blood kit (Qiagen, Germany). For the screening of the Leiden mutation, the Arg 506-Gln mutation of the factor V gene resulting from G-A 1691 substitution was detected using digestion by the restriction enzyme Mnl-I of the products amplified by polymerase chain reaction, as described by Bertina (2). We adapted the detection of the factor II mutation from a previous report (5). A 204-bp fragment from exon 14 and the 3'-untranslated region was amplified by PCR using two, rather than one, mutagenic primers as follows:

Sense 5'-CGGGATGGGAAATAAAGCTTCTA-CA-3' 20030-20056

Antisense 5'-ATAGCACTGGGAGCATTGAAGC-3' 20233-20212

The antisense mutagenic primer was previously described by Poort *et al* (5). The design of the sense primer was based on the published human prothrombin nucleotide sequence (6). The nucleotides underlined are not present in the normal sequence. The mutagenic primers introduced two Hind III restriction sites in the mutated PCR products and only one in the wild type (WT) amplicons. After enzymatic digestion, three fragments (15, 23 and 166-bp) were yielded in mutated amplicons versus two (23 and 181-bp) in WT. This modification of the original method was in order to control the quality of the enzymatic digestion and to avoid false-negative results in the case of unsuspected digestion failure.

Diagnosis of thromboembolism

Deep venous thrombosis was confirmed by duplex Doppler ultrasonography or venography. Pulmonary embolism was diagnosed by high probability lung scintigraphy according to Pioped criteria, angiography or spiral computed tomography.

Statistical Analysis

Dichotomic values were compared using Fischer's exact test. Continuous values were analysed using the Wilcoxon test (p are two-sided).

RESULTS

Prothrombin 20210A allele prevalence

Fourteen out of 193 patients (7.3%) were heterozygote carriers of the prothrombin gene 20210A allele (mutant allele frequency 0.036) whereas this variant was detected in only 1 out of 100 healthy controls (1%) ($p < 0.05$)(table 1). The 20210A allele was

Table 1. Clinical profile of patients with thromboembolic disease: comparison of overall population, patients with 20210A allele and patients with the wild allele of the prothrombin gene.

	OVERALL	factor II 20210A allele	factor II wild allele	statistical significance
HEALTHY CONTROLS				
number	100	1 (1%)	99	
male/female	38/62	0/1	38/61	
THROMBOEMBOLIC PATIENTS				
number	193	14 (7.3%)	179	
male/female patients	75/118	4/10	71/108	NS
mean age (years) at 1st thrombotic event	46.3	39.9	46.8	NS
presenting thrombotic event				
proximal DVT	108	8 (57%)	100 (56%)	
distal DVT	57	3 (21%)	54 (30%)	
lung embolism	19	3 (21%)	16 (9%)	
varia	9		9 (5%)	
number of patients with positive family history of thromboembolic disease (%)	46/163 (28%)	5/13 (38%)	41/150 (27%)	NS
number of patients with recurrence of thromboembolic disease (%)	65/181 (36%)	5/14 (36%)	60/167 (36%)	NS
n of patients with predisposing risk factors	109/193 (56%)	8/14 (57%)	101/179 (56%)	NS
frequency of lung embolism	71/161 (44%)	6/14 (43%)	65/147 (44%)	NS

not identified in the homozygous state.

Plasma prothrombin levels

The relationship between plasma prothrombin levels and the prothrombin genotype was analyzed in the first 10 patients presenting the prothrombin 20210A allele and in the first 40 patients with the wild type allele. The median prothrombin level was significantly increased in the first group: 1.16 U/ml compared to 1.04 U/ml in the latter ($p \leq 0.02$, Wilcoxon test).

Clinical profile

No correlation was found between heterozygous carriage of the 20210A allele and several parameters determining the clinical profile of thromboembolic disease (table 1): the distribution of thrombosis location (proximal versus distal venous thrombosis), the frequency of associated pulmonary embolism, the proportion of patients with a family history of thromboembolism, the percentage of patients suffering from recurrences and the age at disease onset (table 2) were not affected by the presence of the prothrombin gene variant. Similarly, the risk factors commonly involved in venous thromboembolism, i.e. postoperative state, immobilisation, associated clotting disorders, were found with a similar frequency in both the normal and the variant prothrombin gene population.

Association with other thrombophilia factors

According to our previous study (4), factor V Leiden mutation was found in 42 out of 193 patients (21.8 %). Among the 14 patients with prothrombin 20210A allele, the association with protein S deficiency was encountered in 2 cases and with protein C deficiency in 1 case. Coexistence of both factor V Leiden and prothrombin 20210A allele was identified in 3 additional patients (3/193; 1.6%). Interestingly, although the median age at first thromboembolism event was similar for patients with either factor V Leiden or factor II mutation alone compared to those with wild type alleles (between 43 and 47 years), the figure was markedly lower (25 years) for the individuals bearing both abnormalities. This difference was statistically significant (table 2).

Table 2. combined thrombophilic risk factors: comparison of patients with either no mutation, factor V Leiden mutation or factor II 20210A allele and individuals bearing both defects. The association of both defects results in a significantly lower age of disease onset compared to factor V Leiden alone or no mutation.

	patients number	median age (years) at 1st thrombotic event
total	193	
no mutation	140	47[a]
factor V Leiden (alone)	39	46 [b]
factor II 20210A allele (alone)	11	43 [c]
fact.V Leiden and fact.II 20210A allele (combined)	3	25[a][b][c]

[a] $p < 0.025$; [b] $p < 0.025$; [c] NS

DISCUSSION

The frequency of 7.3 % for heterozygous carriers of the prothrombin gene 20210A allele is in accordance with the initially reported prevalence of 6.3 % among consecutive unselected thromboembolic patients (5). Similar observations were made by Cumming *et al.* (7) reporting a prevalence of 5.5 % among patients with venous thrombosis and 1.2 % among healthy controls. Likewise, Hillarp *et al.* found a prevalence of 7.1 % among 99 Swedish unselected outpatients with deep venous thrombosis and 1.8 % in the healthy control group (8). Moreover, we were able to confirm the relationship between prothrombin 20210 AG genotype and significantly elevated plasma prothrombin levels. However, no cut-off point was identified in order to discriminate reliably between the prothrombin 20210A variant and the wild allele on the basis of a plasma prothrombin level.

Furthermore, the exact mechanism linking elevated plasma prothrombin levels to the risk of venous thromboembolism remains speculative.

A more in-depth analysis allowed assessment of the clinical profile of patients bearing the 20210A prothrombin variant. Firstly, the frequency of prothrombin 20210A variant among our patients with and without a familial history of thromboembolism was 10.8 % (5/46 patients) and 6.8% (8/117 patients), respectively, this difference was not significant. The frequency of prothrombin variant carriers was reported by Poort *et al.* to be as high as 18% among patients with a personal and a family history of thromboembolism but the figure may have been influenced by selection bias, as suggested by the authors. Secondly, the frequency of factor V Leiden was similar among the patients bearing the 20210 AG genotype (3/14 patients, 21%) and the patients bearing the wild prothrombin allele (36/179 patients, 20%). The rate of 40% found by Poort *et al.* can be explained by the smaller size of their cohort and the selection of patients with a familial clustering of venous thromboembolism. Our data are in agreement with the prevalence reported elsewhere (9). Thirdly, we found that the frequency of associated risk factors, i.e. postoperative state, immobilisation or other clotting defects, was similar among the carriers of 20210 AG variant and the patients with the wild type genotype. The same applied to the location of the thrombotic process (dis-

tal versus proximal) and the frequency of associated pulmonary embolism that were identical, whether the prothrombin mutation was present or not. Contrary to observations regarding factor V Leiden (4,10), the relapse rate was not higher among carriers of the prothrombin 20210A allele. Moreover, the mean age at disease onset was identical in normal and prothrombin gene heterozygous patients. This observation contrasts with the analysis of Hillarp *et al.* who did not find any carriers of the 20210A allele among patients less than 62 years (8). This discrepancy has already been underlined by Rosendaal *et al.* (11).

Overall, the data suggest that factor II mutation is probably a moderate thrombophilic factor. The strikingly lower median age at the first thrombotic event in patients with co-inheritance of both prothrombin 20210A allele and factor V Leiden suggests a synergistic contribution of both factors. Although this latter finding relied on a limited number of patients and required further confirmation, it was in agreement with the very recent observations of Makris *et al.* who demonstrated that among patients with either factor V Leiden, protein S deficiency, protein C deficiency or antithrombin deficiency, the simultaneous carriage of prothrombin 20210A allele was responsible for an increased number of venous thromboembolic episodes in comparison with the wild prothrombin allele carriers (12). This synergistic action could favour the hypothesis that a higher concentration of prothrombin linked to 20210A allele results in an increased thrombin generation which appears to be also the result of either APC resistance or deficiencies of protein C and S (13).

While the association of prothrombin 20210A variant with venous thromboembolism has been established beyond doubt, its potential contribution to arterial disease was suggested by several studies (14,15). These preliminary findings, however, need to be confirmed before assessing the role of prothombin variant in arterial disease.

In conclusion, the prothrombin 20210 A allele appears to be a moderate but relatively common thrombophilic factor. In our series of patients, the frequency of thrombosis recurrence, age at onset and the prevalence of a family history were not influenced by the carriage of prothrombin 20210 A allele. Interestingly, the co-inheritance of prothrombin 20210 AG variant and factor V Leiden in the same individual, although rare, may act synergistically as suggested by a very low age at disease onset.

RESUME

L'existence de l'allèle 20210A du gène de la prothrombine a été récemment identifié comme un facteur de risque de la maladie thromboembolique veineuse; son action est probablement médiée par l'augmentation du taux plasmatique de prothrombine. Notre but a été d'une part, de déterminer la fréquence de l'allèle 20210A de la prothrombine parmi 193 patients consécutifs traités pour une maladie thromboembolique et parmi 100 volontaires sains et d'autre part d'analyser le profil clinique associé à ce nouveau facteur de thrombophilie. En concordance avec d'autres observations, nous mettons en évidence une fréquence de 7,3 % de porteurs hétérozygotes de l'allèle 20210A parmi les patients et de 1% parmi les contrôles. Nous confirmons que les taux plasmatiques de prothrombine sont statistiquement plus élevés chez les individus porteurs de l'allèle muté par rapport aux autres.

Nous ne mettons pas de relation en évidence entre la présence de l'allèle 20210A et une histoire familiale de maladie thromboembolique, la fréquence des récidives ou l'âge de début de la maladie thromboembolique. Cependant, la présence chez le même individu de la mutation de Leiden (facteur V) et de l'allèle 20210A de la prothrombine est associée à un début significativement plus précoce de l'affection suggérant l'intervention synergique de ces deux facteurs de thrombophilie.

SAMENVATTING

Het bestaan van het allel 20210A van het protrombine gen werd recent geïdentificeerd als een risikofaktor van veneuze tromboembolie. Zijn werking is waarschijnlijk gemedieerd door een verhoging van het protrombine plasmagehalte. Ons doel was enerzijds de frekwentie van het protrombine allel 20210A te bepalen bij 193 opeenvolgende patiënten behandeld voor veneuze tromboembolie en bij 100 gezonde vrijwilligers, en anderzijds het bijkomend klinisch profiel te analyseren in verband met deze nieuwe trombose bevorderende faktor. In akkoord met vroegere bevindingen, vonden wij een frekwentie van 7,3% heterozygote dragers van het allel 20210A bij de patiënten, en 1% bij de controles. Wij bevestigen dat het plasmagehalte aan protrombine statistisch hoger is bij dragers van het gemuteerde allel dan bij controles. Wij kunnen geen verband aantonen tussen de aanwezigheid van het allel 20210A en een familiale voorgeschiedenis van tromboembolische ziekte, de frekwentie van recidieven of de leeftijd bij het begin van de aandoening. Nochtans, de aanwezigheid bij ëenzelfde persoon van de mutatie van Leiden (faktor V) en het allel 20210A van protrombine is geassocïeerd aan een duidelijk vroegtijdiger begin van de aandoening, veronderstellend een synergische tussenkomst van deze twee trombofilie risikofaktoren.

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REFERENCES

1. Dahlbäck B, CarIsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognised mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Nail Acad Sci.* 1993; 90: 1004-8.
2. Bertina RM, Koeleman BP, Koster T *et al.* Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature.* 1994; 369: 64-7.
3. Koster T, Rosendaal FR, de Ronde H, Briët E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. *Lancet.* 1993; 342: 1503-6.
4. Hainaut P, Azerad MA, Lehmann F *et al.* Prevalence of activated protein C resistance and analysis of clinical profile in thromboembolic patients. A Belgian prospective study. *J Intern Med.* 1997; 241: 427-33.
5. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is asssociated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood.* 1996; 88: 3698-3703.
6. Friezner Degen SJ, Davie EW. Nucleotide sequence of the gene for human prothrombin. *Biochemistry.* 1987; 26: 6165-77.
7. Cumming AM, Keeney S, Salden A, Bhavnani M, Shwe KH, Hay CRM. The prothrombin gene G20210A variant: prevalence in a U.K. anticoagulant clinic population. *Br Haematol.* 1997; 98: 353-5.
8. Hillarp A, Zöller B, Svensson PJ, Dahlbäck. The 20210 A allele of the prothrombin gene is a common risk factor among Swedish outpatients with verified deep venous thrombosis. *Thromb Haemost.* 1997; 78: 990-2.
9. Alhenc-Gelas M, Le Cam-Duchez V, Emmerich J *et al.* The A20210 allele of the prothrombin gene is not frequently associated with the factor V Arg 506 to Gln mutation in thrombophilic families. *Blood.* 1997; 90: 1711.
10. Simioni P, Prandoni P, Lensing A *et al.* The risk of recurrent venous thromboembolism in patients with an Arg506-to-Gln mutation in the gene for Factor V (Factor V Leiden). *N Engl J Med.* 1997; 336: 399-403.
11. Rosendaal FR, Vos HL, Poort SL, Bertina RM. Prothrombin 20210A variant and age at thrombosis. *Thromb Haemost.* 1998; 79: 444.
12. Makris M, Preston FE, Beauchamp NJ *et al.* Co-inheritance of the 20210A allele of the prothrombin gene increases the risk of thrombosis in subjects with familial thrombophilia. *Thromb Haemost.* 1997; 78: 1426-9.
13. Zöller B, Holm J, Svensson PJ, Dahlbäck B. Elevated levels of prothrombin activation fragment 1+2 in plasma from patients with inherited APC-resistance and/or protein S deficiency. *Thromb Haemost.* 1996; 75: 270-4.
14. Arruda VR, Annichino-Bizzacchi JM, Gonçalves MS, Costa FF. Prevalence of the prothrombin gene variant (nt20210A) in venous thrombosis and arterial disease. *Thromb Haemost.* 1997; 78: 1430-3.
15. Rosendaal FR, Siscovick DS, Schwartz SM, Psaty BM, Raghunathan TE, Vos HL. A common prothrombin variant (20210 G to A) increases the risk of myocardial infarction in young women. *Blood.* 1997;90: 1747-50.