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Rapid Communications

The Prothrombin 20210A Allele Is the Most Prevalent Genetic Risk Factor for Venous Thromboembolism in the Spanish Population

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Summary

We investigated the prevalence of the new recently reported mutation in the prothrombin gene (20210 A) in a sample of 116 unrelated patients with venous thromboembolism. We found 20 heterozygous carriers (17.2%, CI 95% 10.4-21.1). In comparison, we observed 13 carriers among 201 healthy unmatched controls (6.5%, CI 3.5-10.8). The 20210 A mutation seems to increase the risk of venous thrombosis 3-fold (odds ratio 3.1, 95% CI 1.4-6.6). Considering only patients with a first event (n = 62) the OR was 2.0 (p = 0.18, NS) while those with recurrent events (n = 54) showed an OR of 5.9 (95% CI 2.5-14.4). A majority of heterozygous patients (55%) presented a second thrombophilic factor and 60% of affected females had their first event before 30 years of age, while on oral contraceptive treatment. The prevalence found in this study for healthy people is the highest reported to date. The 20210 A variant appears to be the most prevalent genetic risk factor among patients with thrombosis in our geographical area.

Introduction

Inherited thrombophilia is a clinical entity characterised by an increased risk to develop venous thromboembolism (VTE) (1). To date, only a few genetic abnormalities have been considered as independent risk factors for VTE. In other words, these deficiencies on their own would be responsible for the thrombotic tendency (i.e. mutations in genes encoding antithrombin, protein C, protein S and fibrinogen). From an epidemiological point of view, this has been demonstrated for protein C in a population study (2), whereas for antithrombin, protein S and fibrinogen the increase in the thrombotic risk has been deduced from family studies (3, 4). These protein deficiencies have been shown to be relatively strong risk factors, although their prevalence is low (1). In 1993 the phenomenon known as activated protein C resistance appeared as a very frequent cause of inheritable thrombophilia (5) determined in the great majority of cases by the factor V Leiden mutation (6). Apart from these hereditary disorders, another abnormality, moderate hyperhomocysteinemia is associated with an increase in the thrombotic risk, although in some patients it could have an acquired origin (7).

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In a large multicentric Spanish study we described the prevalence of the above mentioned genetic defects among patients with VTE in our country. Considering all these hereditary abnormalities we can explain only 25% of the cases of VTE (8). Furthermore, hyperhomocysteinemia could account for an additional 17% of our cases (unpublished data).

In 1996 Poort et al. published a new genetic risk factor for VTE. It is a G to A transition at position 20210 in the 3'untranslated region of the prothrombin gene (9). These authors found a prevalence of 18% in selected VTE patients and 6.2% in patients with a first episode of VTE, whereas the prevalence in healthy controls was 2.3%. According to these figures, the risk of VTE in heterozygous carriers of the 20210 A allele was estimated to be 2.8 times higher than in non carriers.

A few studies appeared during 1997 and more recently reporting the prevalence of this variant in different countries, with a range between 1% to 19.3% in patients with VTE and 0% to 4.3% in controls (10-18). An increase of thrombotic risk when this variant is associated with other hereditary thrombophilic defects has also been reported (19).

The aim of the present study was to investigate the prevalence of the prothrombin gene 20210 A allele in a Spanish population of not related patients suffering from confirmed VTE. The clinical characteristics, association with other risk factors and influence of sex and age on these VTE episodes are also described.

Materials and Methods

Patients and controls. The Hemostasis and Thrombosis Unit at the Hospital de la Santa Creu i Sant Pau, Barcelona, is a reference Centre for several hospitals in our region. All such patients who had been referred to or had primarily visited our Hospital for thrombophilia screening between October 1996 and November 1997 were included. This patient group had 116 not related individuals studied after a first (62 patients) or recurrent (54 patients) episode of objectively confirmed venous thrombosis or pulmonary embolism. There were 51 males and 65 females, with an age mean of 47.8 years (range 16-83). The control group consisted of 201 unmatched individuals (103 healthy blood donors and 98 volunteers without any known disease, recruited from the laboratory staff and from the general population, when undergoing routinary health screening). There were 113 males and 88 females with an age mean of 40 years (range 18-74), all unrelated and living in or around Barcelona City.

Blood collection. Blood was collected from the antecubital vein. Samples for hemostatic tests were immediately anticoagulated with 1/10 volume of 0.129 M sodium citrate. Platelet poor plasma was obtained by centrifugation at 2000 g for 20 min. Plasma samples were stored at –80° until use. Samples for homocysteine determination were collected in disodium EDTA and kept on ice until plasma was harvested after centrifugation.

Laboratory determinations. Fibrinogen and APC resistance were assayed in the STA automated coagulometer (Boehringer Mannheim, Mannheim).

Fibrinogen was measured by the von Clauss method (20) with thrombin from BioMerieux (Marcy-l'Etoile. APC resistance was measured with the kit Coatest APC Resistance from Chromogenix, (Mölndal). Antithrombin (AT), protein C and heparin cofactor II (HCII) were measured in a biochemical analyser (CPA Coulter, Coulter Corporation, Miami FL) using chromogenic methods from: Chromogenix for AT and protein C and from Diagnostica Stago for HCII. Total and free protein S were assayed using ELISA methods from Diagnostica Stago. Homocysteine was separated by HPLC and determined by a fluorimetric method (21). Lupus anticoagulant was investigated using Exner's method (22) and antiphospholipid antibodies (APA) were screened by means of an ELISA that uses cardiolipin and phosphatidilserine as antigens (23).

The criteria for the diagnosis of protein deficiency were plasma levels below the lower limit of normal, as used in our laboratory. These lower limits are 80% for antithrombin; 70% for protein C; 73% and 72% for total and free protein S respectively, in men and women older than 44 years old; 63% and 54% for total and free protein S in women younger than 45; 65% for heparin cofactor II; a ratio below 2.3 for APC resistance. Hyperhomocysteinemia was defined as fasting plasma levels of homocysteine higher than 11.3 mmol/l and/or a postmethionine load increase in plasma levels higher than 31mmol/l.

Genetic analyses. Genomic DNA was isolated from peripheral blood leukocytes according to standard protocols (24).

Factor V-Leiden detection. Factor V Leiden genotype was screened using the two primers described previously (25), with minor modification in the reaction conditions. Briefly, 50 ml of mixture containing 20 mM TRIS HCl pH 8.2, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, 250 ng of DNA and 0.5 U Taq polymerase (Ecogen, Barcelona) were subjected to 30 cycles of 40 sec at 95° C, 40 sec at 55° C and 2 min at 72° C. The 220 bp PCR product was digested with *MnlI* (New England Biolabs, Inc. Beverly, MA) and analysed by ethidium bromide UV-fluorescence after electrophoresis in a 3% Nusieve GTG agarose gel (FMC Bioproducts, Rockland ME).

Detection of the prothrombin gene 20210 variant. The 3´-UTR region of the prothrombin gene was obtained by PCR as previously described (9), with minor modifications in the reaction conditions. The 345-bp fragment was digested with *Hind III* endonuclease (Life Technologies Inc. Gaithersburg, MD) according with recommendation from the suppliers. Digestion products were analysed by ethidium bromide UV-fluorescence after electrophoresis in 3% Nusieve GTG agarose gel (FMC Bioproducts, Rockland ME). Another fragment, spanning position 19889 to 20307, from exon 14 and the 3´-UTR region of the prothrombin gene was amplified with the sense primer 5´-TCTAGAAACAGTTGCCTGGC-3´ (19889 to 19908) and the antisense primer 5´-AGGTGGTGGATTCTTAAGTC-3´(20307 to 20288). PCR was performed for 30 cycles of 30 sec at 94° C, 30 sec at 55° C and 2 min at 72° C in a 50 μl reaction mixture that contained 0.5 mg of genomic DNA, 50 pmol of each primer, 0.3 mM of each dNTP and 2U of Eco-taq polymerase (Ecogen, Barcelona) in 10mM TRIS HCL pH8.3, 50mM kCl and 1.5 mM MgCl. This 418-bp fragment was sequenced directly in an Applied Biosystem 310 DNA sequencer following the instructions from the supplier. The primers used to sequence were the same as those used for the PCR amplification.

Statistical analysis. We performed the Chi-square test for group comparison of frequencies. Kaplan-Meier analysis was performed to evaluate age differences at first thrombotic event. A logistic regression method was used to calculate the odds ratio (OR) associated to VTE. Adjustments for sex and age of first thrombotic event were made. P values of <0.05 were considered significant and 95% confidence intervals were established for all the estimated parameters.

Results

We found 13 heterozygous carriers of the 20210 A variant among control subjects. The observed genotype prevalence of 6.5% (95% CI 3.5-10.8) corresponds to an allele frequency of 0.0325. Among the patient group we found 20 heterozygous carriers. Prevalence was therefore 17.2% (95% CI 10.4-24.1) with an allele frequency of 0.086. No homozygous 20210 A individuals were detected either in the control group or among the patients.

In relation to the 96 remaining patients not carrying the 20210A allele, we found a completely normal biologic study in 71, factor V Leiden in 2, the presence of APA in 9, moderate hyperhomocysteinemia in 10 and protein S deficiency in 4. There was no age difference in the first thrombotic event between 20210 A carriers (mean 39 years, range15-68) and non-carriers (mean 42 years, range 16-77).

Taking in consideration the whole group of patients, the crude odds ratio (OR) of VTE associated to carriership of the prothrombin gene variant was 3.0 (95% CI 1.4-6.3). When adjusted for sex and age, it was very similar: 3.1 (95% CI 1.4-6.6). In other words, sex and age did not seem to be confounding variables for the risk of thrombosis related with 20210 A allele. However, considering separately those patients after their first event from those with recurrent thromboembolic events, we observed relevant differences: While the adjusted OR of VTE associated with carriership of the mutated allele in the patients with only one episode of thrombosis was 2.0 ($p = 0.18$, NS), the adjusted OR in the subgroup of recurrent patients was 5.9 (95% CI 2.5-14.4). In addition, the risk of VTE associated with the female sex in the subgroup of first events was significantly higher in comparison with the male sex (OR 2.1, 95% CI 1.1-3.9). This difference was not observed in the subgroup of patients with recurrent events (OR 1.3, $p = 0.48$, NS). The age at first event was 38.9 (CI 95% 35.3-42.5) for the patients without recurrences and 44.9 (CI 95% 40.8-49) for those with recurrent events. This result was not statistically significant.

Among the 20 patients carrying the prothrombin variant, we observed the following clinical characteristics: 10 were women; 13 (65%) had recurrent VTE before entering the study; 11 (55%) had other associated biological thrombophilic defects: 4 (20%) had factor V Leiden, 5 (25%) presented APA, 1 had heparin cofactor II congenital deficiency and 1 had moderate hyperhomocysteinemia. One patient with APA, also suffered breast cancer and developed VTE during chemotherapy. From these 11 individuals combining two prothrombotic abnormalities, 7 showed recurrent VTE. The mean age at first VTE event was 47.1 years-old (range 20-68) for the carriers of the isolate defect, while it was 32.6 year-old (range 15-64) for those carriers who associated another combined defect. Six women were under 30 years of age at the time of first VTE event, and all were associated with the intake of oral contraceptives. A common feature of this association was the short period of time between the start of this treatment and the VTE event: average 3.8 months (range 1-10). Furthermore, two of the six suffered recurrent VTE during pregnancies.

Discussion

We detected 20 individuals carrying the prothrombin gene 20210 A variant in a sample of 116 patients with VTE. In contrast, in the control group only 13 of 201 were carriers. This difference is statistically significant and confirms that this variant is probably a risk factor for VTE, with the adjusted OR for sex and age of first event being slightly higher than 3. This result is in agreement with most previous reports, which have found OR between 2 and 4.7 (9-12). However, our data failed to demonstrate that the 20210 A variant is a risk factor among patients with only one thrombotic episode. In this subgroup, the prevalence of carriers was 11.3% (7/62), which means a non-significant OR of 2.0, compared to controls. This can be considered a trend and this result in fact closely resembles data reported by Poort et al. in their series of unselected patients after a first episode of VTE (9). On the other hand, the prevalence of carriers among patients with recurrent

Thromb Haemost 1998; 80: 366–9

episodes of VTE was 24.1% (13/54), leading to a significant OR of 5.9. The interpretation of this OR as a relative risk must be done cautiously, because it can be an overestimation due to bias (a first event affects the risk of recurrence).

The prevalence of the prothrombin gene 20210 A variant in our control group (6.5%) is the highest reported to date in individuals without VTE. According to this figure and after considering the results in other series, this variant in the prothrombin gene should not be considered as a rare mutation. The prevalence we found in patients would place this variant as the most frequent genetic abnormality related with thrombosis in our population, in comparison with the prevalences reported for other genetic causes of VTE in a previous study which used the same inclusion criteria (8). These data contrast surprisingly with another Spanish study, which reported frequencies of 7.3% and 1.4% in patients (after a first episode of VTE) and normal controls respectively. This study was performed in a different region where there is perhaps a different subpopulation with another allele frequency (13). Nevertheless, the comparison between our subgroup of nonrecurrent VTE patients and that study does not reveal significant differences: frequency of 20210 A carriership of 11.3% *versus* 7.3% (6/82). As previously mentioned, we can not exclude the possibility that our results were biased because our sample included some selected patients from referral Hospitals. Nevertheless, this would not account for findings in the control group. On the other hand, this control group was recruited in the region of Barcelona, a big area which received an important migratory flow between 1950-1975 from the rest of Spain. Taking into account this heterogeneous genetic background, the results in Barcelona population would be extrapolable to the general Spanish population.

A cooperative study including more than 5500 healthy subjects from nine European and American countries, has estimated the overall prevalence of heterozygous carriers of 2% (being the confidence interval of 95% 1.4-2.6) (26). The same study found a difference between southern European (3.0%) and northern European countries (1.7%) (26). Our results seem to confirm this regional difference.

Interestingly, a high number (55%) of patients with the 20210 A variant present other thrombophilic abnormalities, mainly factor V Leiden and APA. In comparison, only 26% of the patients without 20210 A allele exhibited another known biological cause of thrombosis. It is difficult to interpret this higher frequency of associated defects in the 20210 A carriers, because we did not perform the complete biological study of thrombosis risk factors in the control group. Perhaps the 20210 A variant is a moderate risk factor for VTE which often needs the presence of additional circumstances or other biologic defects in order to develop the disease. Other studies seem to corroborate this idea. The first report on this subject by Poort et al. found the coexistence with factor V Leiden in 40% of carrriers of factor II 20210 A allele in their patient group (9). Makris et al. found that 7% of probands with heritable thrombophilia due to antithrombin, protein C or protein S deficiencies or carriers of factor V Leiden, showed coinheritance with factor II 20210 A variant (19). Ferraresi et al. reported that 10 of 17 carriers of 20210 A variant had a second prothrombotic defect again, mainly factor V Leiden (15). In contrast, other study found no carriers of 20210 A allele in 288 members of families with factor V Leiden (27).

One of the most striking results in this sample is the very frequent association of this new mutation with oral contraceptives in young women suffering VTE. Sixty per cent of female carriers and 100% of carrier women under 30 years old showed this association in our study. A similar strong association has been clearly demonstrated between oral contraceptives and factor V Leiden mutation (28). Considering the very high prevalence among the normal population of both genetic risk factors and the wide use of oral contraceptives in western countries, further scientific and epidemiological efforts are required to explain these pathologic relationships.

In conclusion, the prothrombin gene 20210 A allele is the most prevalent genetic risk factor for VTE in Spanish patients and its frequency in normal Spanish controls (at least in the area of Barcelona) is the highest reported to date. In the future, screening of patients with VTE must include this new factor. Patients with VTE carrying this allele, frequently present another prothrombotic abnormality. Young women taking oral contraceptives and carrying this allele seem to be at a very high risk for disease. Further studies are needed to clarify this interaction and to elucidate its pathogenic mechanisms.

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