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Homozygotes for prothrombin gene 20210 A allele in a thrombophilic family without clinical manifestations of venous thromboembolism

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ABSTRACT

Background and Objective. **A new genetic risk factor for venous thromboembolism has recently been described which involves a G to A transition at position 20210 in the 3' untranslated region of the prothrombin gene. To date, only a few homozygotes for this mutation have been reported and in most of cases, they suffered from thrombotic disease. Here, we describe a pedigree including both heterozygous and homozygous subjects for prothrombin (PT) 20210 A.**

Design and Methods. **This family was recruited in 1996 as part of our GAIT (*Genetic Analysis of Idiopathic Thrombophilia*) project. To qualify for the GAIT study, a pedigree was required to have at least 10 living individuals in three or more generations (i.e. extended pedigree). The pedigrees were selected through probands with idiopathic thrombophilia. A complete set of plasma and DNA determinations related to hemostasis was performed on this family.**

Results. **The plasma studies yielded normal results in all of the individuals. The family members who had a history of thromboembolism were heterozygous carriers of the PT 20210 A variant. In addition, 4 relatives who were heterozygous, and two who were homozygous for this A allele, failed to show clinical manifestations. These two homozygotes were 51 and 19 years old.**

Interpretation and Conclusions. **This case exemplifies the complexity of thrombotic disease since individuals homozygous for a mutant gene do not exhibit symptoms while heterozygous individuals often do exhibit the disease. This case suggests that the new genetic risk factor for thrombosis (i.e. PT 20210 A) may not be as strong as most of the previously described genetic risk factors.**

Key words: thrombophilia, prothrombin gene, factor II, prothrombin gene 20210 A mutation

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Thrombophilia is a common disease clinically defined by early age of onset, repeated episodes of venous thromboembolism (VTE) and frequent co-existence in related individuals.[1] This last suggests that heredity plays a role in susceptibility to thrombophilia. Only a few inherited deficiencies are considered as independent risk factors for VTE. Among these are mutations in structural genes encoding for antithrombin, protein C, protein S, and fibrinogen.[1] Some individuals with VTE are heterozygous carriers of one of these mutant genes. In contrast, the rare homozygous individuals exhibit very severe thrombotic symptoms.[2] In 1993, activated protein C resistance was identified as a very frequent cause of inheritable thrombophilia determined in the great majority of cases by the factor V Leiden mutation.[3] In 1996, Poort *et al.* described a G to A transition at position 20210 in the 3' untranslated region (UT) of the prothrombin gene, which was also a new genetic risk factor for VTE. An important finding from this seminal work was the significant increase of plasma levels of prothrombin in the carriers of the A allele. Unfortunately, we do not know the pathogenic mechanisms associated with this genetic variant. The risk of VTE in heterozygous carriers of the 20210 A allele was estimated to be 2.8 times higher than in non-carriers.[4]

More than 40 epidemiological studies appeared during 1997 and 1998 that reported the prevalence of this variant in different countries or ethnic groups, ranging from 0% to 18% in patients with VTE or arterial disease and ranging from 0% to 8.1% in control individuals.[5-7] The biggest study published up to now estimated a prevalence in the normal population between 1.4% and 2.6%.[8] Recently, our study in Spain found one of the highest prevalences reported to date in healthy people: 6.5% (confidence interval 95% 3.5-10.8). Furthermore, the 20210 A variant appears to be the most prevalent genetic risk factor for thrombosis in our geographical area, accounting for the condition in 17.2% of the patients.[9]

It is important to note that the great majority of individuals described in these studies were heterozy-

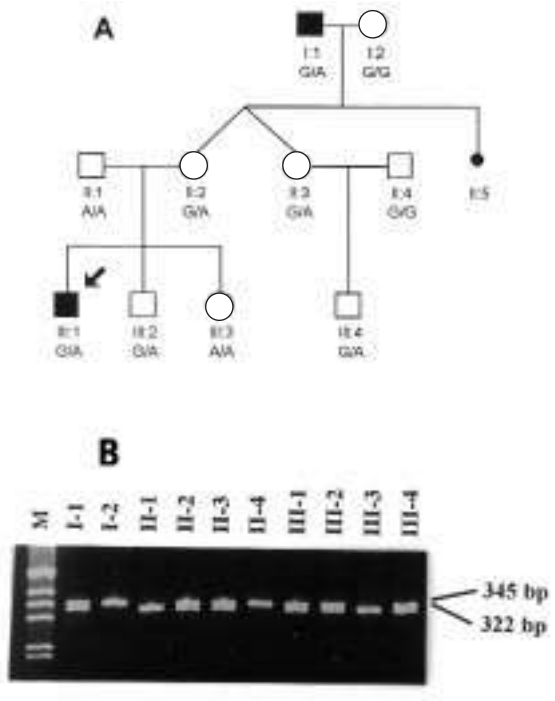

Figure 1. A: Pedigree of the reported family: The proband is indicated by an arrow. The 20210 variant genotype present in each member is also shown under his / her symbol. Filled-black symbols indicate thrombotic disease. Symbol II-5 refers to spontaneous abortion. B: Familial segregation of the 20210 variant. A new *HindIII* site is introduced in the amplified fragment when the 20210 A allele is present, yielding two fragments (322 bp and 23 bp in length) after digestion. The 20210 G allele lacks the restriction site and therefore generates only a 345 bp fragment by PCR-*HindIII* digestion. M is the Φ174 DNA / *HaeIII* marker. Individual numbers along the top refer to the same numbers as the pedigree.

gous. To our knowledge, only 34 cases of homozygous individuals for the 20210 A allele have been reported.[4,7,10-22] Of these, 9 were in 3 families.[16,18,22] The remaining 25 individuals are unrelated. A total of 17 cases had thrombosis, including 14 individuals with venous thrombosis. Nine cases remain asymptomatic; four of them belonging to the same pedigree.[18] There is no clinical information about the 8 cases mentioned by Zivelin *et al*.[17] Here, we present two new cases of homozygous individuals for this mutation. Remarkably, neither of these homozygotes has experienced thrombosis in spite of the fact that they belong to a family in which hereditary thrombophilia is clearly evident.

Design and Methods

Case Report

The family was recruited in 1996 as part of our GAIT (Genetic Analysis of Idiopathic Thrombophilia) project.[23] To qualify for the GAIT study, a pedigree was required to have at least 10 living individuals in three or more generations (i.e. extended pedigree). The pedigrees were selected through probands with idiopathic thrombophilia. The proband's thrombophilia was considered idiopathic because all known (during the recruitment period of 1995-1997) biological causes of thrombophilia were excluded (i.e., antithrombin deficiency, protein C and S deficiencies, activated protein C resistance and factor V Leiden, plasminogen deficiency, heparin cofactor II deficiency, dysfibrinogenemia, lupus anticoagulant and antiphospholipid antibodies).

The proband of this family (individual III-1, see Figure 1), is a 25 year-old male who suffered from spontaneous deep venous thromboses at the age of 19 in the vein cava and right iliac vein. There were diagnosed by means of venography and abdominal CT-scan. Initially, he received heparin treatment followed by a six-month treatment with acenocoumarol. As a sequel, a minor post-thrombotic syndrome remained in his right leg. At the age of 22, he developed a new episode of spontaneous left iliofemoral vein thrombosis. An objective diagnosis was made by ultrasonography. Since then, he has been under oral anticoagulant therapy as prophylaxis against the disease. His maternal grandfather (individual I-1, Figure 1) suffered from deep venous thrombosis in his right leg after a surgical repair of a groin hernia at the age of 62. He had no other predisposing conditions to thrombosis through his life.

None of the remaining pedigree members has had thromboembolic disease, although some of them have been exposed to some risk factors for thrombosis such as pregnancy and puerperium (individuals I-2, II-2 and II-3), surgical procedures (II-3) and oral contraceptives (II-2 and II-3). Specifically, II-1 and III-3 have not been exposed to acquired risk factors. The individual I-2 had a spontaneous abortion during the second trimester of her second pregnancy, presented as II-5 in the family tree.

After we had finished the required analyses of all of the members of this family for our GAIT project, we stored samples of plasma and DNA in the event that further investigations were warranted. When Poort *et al.* reported the discovery of the prothrombin gene 20210 A allele as a risk factor for thrombosis,[4] we retrospectively tested the probands in our GAIT families. We found that the proband of this family was a carrier of the A allele at position 20210 of the prothrombin gene. Consequently, we investigated all of his relatives.

Methods

Plasma study. APTT, PT, fibrinogen, coagulation factors IIc, Vc,VIIc,VIIIc, IXc, Xc, XIc and XIIc (coagulative methods); von Willebrand factor (antigen) ; total, free and functional protein S; APC resistance, antithrombin (functional), protein C (functional),

heparin cofactor II (functional), plasminogen (functional), t-PA (antigen), PAI-1 (functional), HRG (antigen), TFPI (functional), tissue factor (antigen) and homocysteine were determined by means of standard methods.

Factor V-Leiden detection. Factor V Leiden genotype was screened using the two primers described previously,[24] with minor modifications in the reaction conditions.

Detection of the prothrombin gene 20210 variant. The 3'-UT region of the prothrombin gene was obtained by PCR as previously described,[4] with minor modifications in the reaction conditions. The 345-bp fragment was digested with *Hind*III endonuclease (Life Technologies Inc. Gaithersburg, MD, USA) according to the recommendation from the supplier. Digestion products were analyzed by ethidium bromide UV-fluorescence after electrophoresis in 3% Nusieve GTG agarose gel (FMC Bioproducts, Rockland, ME, USA). To confirm the results, another 418-bp fragment, spanning position 19889 to 20307, from exon 14 and the 3'-UT region of the prothrombin gene was amplified and sequenced directly in an Applied Biosystem 310 DNA sequencer following the instructions from the supplier.

Results

Initially, all of the classical risk factors for thrombophilia tested normal in the family members. The 20210 prothrombin variant analysis using PCR-*Hind*III digestion (Figure 1) demonstrated that the proband (III-1) was heterozygous for the 20210 A allele as was his maternal grandfather (I-1). These were the only members of the family with a history of thromboembolic disease. We completed the analysis in the remaining family members and found 4 additional heterozygous (II-2, II-3, III-2 and III-4) and two homozygotes: the father (II-1) and the sister (III-3) of the proband. Figure 2 shows the genomic sequence of the propositus DNA (using the reverse primer), the DNA of his father and a control DNA known to be homozygous G/G for the 20210 variant (C/C in the antisense strand). Table 1 shows the clinical data, current ages, prothrombin levels and 20210 genotypes for all of the individuals in this study. The paternal grandparents of the proband (not tested) were not known to be related. The maternal grandparents were not likely to be related to the paternal ones, since the maternal branch came from Barcelona (Catalunya, in East Spain) and the paternal came from the region of Castilla-León, in Central Spain.

Discussion

To our knowledge, this is the second report of homozygous individuals for the prothrombin gene 20210 variant, belonging to the same thrombophilic pedigree, in whom there is no evidence of any thromboembolic events, despite the fact that one of the patients is 51 years old. A considerable number of

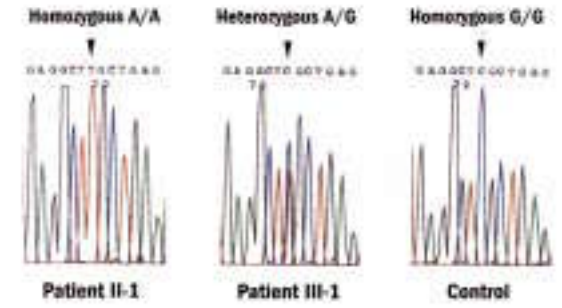

Figure 2. Partial sequences of genomic DNA of the 3'-UTR region of the prothrombin gene from the proband (III-1), his father (II-1) and a control. The antisense strand sequence is shown with the 20210 G→A variant (C to T in the antisense strand) indicated by an arrow.

Table 1. Clinical and laboratory features of the family.

Family member	*Current age (yr)*	*History of VTE*	*20210 genotype*	*Factor IIc levels**
I-1	78	Yes	G/A	149
I-2	77	No	G/G	152
II-1	51	No	A/A	168
II-2	49	No	G/A	156
II-3	49	No	G/A	133
II-4	51	No	G/G	147
III-1 (proband)	25	Yes	G/A	140°
III-2	21	No	G/A	142
III-3	19	No	A/A	183
III-4	17	No	G/A	145

**Values for Factor IIc plasma levels are given in %. Normal values in our laboratory are 70-125%. °The proband stopped oral anticoagulation 1 month before testing factor II levels.*

cases of homozygous individuals for the G20210A variant in the prothrombin gene, have been clinically reported. Table 2 summarizes the clinical information available from all of these reported PT 20210 AA individuals. In some of them, the associations with factor V Leiden or hyperhomocysteinemia make it difficult to interpret the role played by PT 20210 A allele in the thrombotic events, although a synergy could be suspected. Among the published series there are 17 cases of thromboembolic disease; at least 4 cases were spontaneous and in another 4 there were related triggering factors. At least 6 patients have had recurrent events.

Apart from these sporadic cases, there are no data about the specific risk associated with the homozygous state of the 20210 A allele. The fact that even homozygotes and heterozygotes may not show any symptoms makes the prognosis of the thrombotic risk extremely tenuous. More studies are needed to resolve this dilemma. But, from the observed clinical

Table 2. Reported cases of homozygous individuals PT 20210 AA.

Case	Ref.	Sex	Current age	Thrombosis (age of first)	Location	Triggering factors	Recurrence	Associated risk factors	Factor IIc levels (%)
1	4	F	#	Y (#)	#	#	#	F.VLeiden	#
2	7	M	Elderly	N	–	–	–	#	#
3	10	M	24	Y (24)	DVT/PE	MI	N	F.V Leiden	146
4	11	F	18	Y (18)	DVT	Pregnancy	N	N	136
5	12	M	>70	Y (66)	retina	#	Y	Hyper Hcy	#
6	13	M	26	Y (24)	stroke	#	Y	N	#
7	13	M	26	Y (26)	stroke	#	N	Foramen ovale	#
8	14	#	#	N	–	–	–	#	#
9–13	15	#	#	Y (#)	DVT	#	#	#	#
14	16	M	56	Y (40)	DVT	N	Y	N	154
15	16	F	52	Y (26)	STP	Pregnancy	Y	N	170
16–23	17	#	#	#	#	#	#	#	#
24	18	M	44	Y (#)	DVT/PE	N	N	N	132
25–28	18	F	33–74	N	–	–	–	N	113–129
29	19	M	65	Y (65)	DVT	Surgery	Y	N	142
30–31	20	#	#	N	–	–	–	#	96/137*
32	21	M	72	N	–	–	–	N	#
33	22	M	48	Y (40)	DVT/PE	N	Y	N	148*
34	22	F	>48	Y (30)	PE	N	N	N	205*
35	Ours	M	51	N	–	–	–	N	168
36	Ours	F	19	N	–	–	–	N	183

*F: female, M: male, #: not reported, –: not applicable, Y: yes, N: no; DVT: deep venous thrombosis, PE: pulmonary embolism, STP: superficial thrombophlebitis; MI: myocardial infarction, hyperHcy: moderate hyperhomocysteinemia; *antigen levels of factor II.*

data we can make some comparisons with other genetic thrombophilic defects.

Individuals homozygous for the 20210 A allele seem to be much less affected than individuals homozygous for protein C, protein S or antithrombin deficiency.[2] This can be reasonably concluded because none of the previously reported patients suffered from thrombosis in their childhood. Further, one of our cases was an asymptomatic homozygote even at the age of 51. In the report from Morange *et al.* the four asymptomatic individuals are a mother aged 74 and 3 sisters, all older than 33 years. Each of these women had several pregnancies without thrombotic complications.[18] Furthermore, the individual mentioned by Akar *et al.* is an asymptomatic grandfather.[7] The case reported by Alatri *et al.* is an asymptomatic man aged 72 who has had several risk situations for thrombosis during his life.[21] In this sense, the PT 20210 A allele would be more similar to factor V Leiden, since there are several cases of homozygous individuals for this mutation without thrombotic disease.[25, 26]

There are two family cases in which two homozygous siblings have suffered from recurrent venous thrombosis.[16,22] In addition, there are at least two other families including six homozygotes without thromboembolic disease (Morange *et al.* and the present study). Theoretically, it would be expected that individuals homozygous for a thrombophilia risk factor would have a higher probability of developing thrombotic disease than individuals who are heterozygous. In fact, our cases argue against this expectation, since thrombosis has appeared only in heterozygotes, and not in homozygotes. One explanation might be that thrombotic risk is in fact higher in homozygous than in heterozygous, but that there is an epistatic locus inhibiting the risk. Alternatively, there may be an unknown risk factor (genetic or not) associated with the heterozygote, leading perhaps to gene conversion and subsequent clinical manifestations. Moreover, we must emphasize that a complete set of hemostatic parameters was normal in all of the members of our family.

An interesting question arises concerning the higher prothrombin plasma levels in 20210 AA homozygotes than in the heterozygotes or normal relatives. As with any complex phenotype, plasma prothrombin levels are determined by the interaction of genetic and environmental factors. It is also likely that the prothrombin levels are controlled, in part, by multiple genes (mainly regulatory). For this reason, it is necessary to compare relatives (who share genetic and environmental backgrounds) because such family studies would avoid interfamilial heterogeneity.[27] As a general trend, in the four pedigrees mentioned here, the prothrombin levels are higher in homozygotes than in heterozygotes, and also higher in heterozygotes than in non-carriers. Nevertheless, in all of these homozygous individuals the plasma levels of prothrombin are far from those expected if the genet-

ic effect of this variant were additive. Another remarkable point is that some individuals with normal alleles have plasma levels above the upper limit of the normal range (Table 2, individuals I-2 and II-4). This must be due to the above-mentioned specific genetic and environmental factors. It has been demonstrated recently that prothrombin levels have a wide range of values both in carriers of PT 20210 A and in normal controls.[20] In relation to the prothrombotic state, it is perhaps more important to investigate the ability of the affected individuals to generate active thrombin, rather than their levels of circulating plasma prothrombin. Interestingly, the cases reported by Kyrle *et al.* had normal levels of prothrombin fragment F1+2, indicating the absence of ongoing hemostatic system activation but, simultaneously, they showed a clear increase in their endogenous thrombin potential.[16] Because physiopathological mechanisms responsible for thrombosis underlying this variant are unknown, further investigations, both epidemiological and biochemical are needed to answer the intriguing questions arising from this new thrombosis-related genetic abnormality, among which, why some homozygotes are asymptomatic.

Contributions and Acknowledgments

JCS and JM were responsible for the recruitment of the family, data analysis and writing the manuscript. JMS was responsible for the genetic analysis, wrote part of the manuscript and supplied the figures. DL and IC developed and carried out the molecular biology assays. MB was in charge of all the plasma studies and analyzed their results. JF was responsible for the conception of the study and its interpretation. We thank Elisabeth del Río, from the Servei de Genètica, Hospital de la Santa Creu i Sant Pau, Barcelona, for technical assistance with DNA sequencing and Professor William H. Stone, from the Department of Biology, Trinity University, San Antonio, TX, USA, for critically reviewing the manuscript.

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Disclosures

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Manuscript processing

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