LETTER TO JMG

Gain-of-function gene mutations and venous thromboembolism: distinct roles in different clinical settings

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Objective: To calculate the prevalence of common gain of function gene mutations in patients with different clinical manifestations of venous thromboembolism.
Design and setting: Case–control study in two hospitals in Italy.
Participants: 387 patients with venous thromboembolism and 286 controls.
Main measures: Factor V (FV) Leiden, factor II (FII) A20210 and JAK2 V617F mutations.
Results: Among patients with deep vein thrombosis in one leg, 23 (20.9%) carried FV Leiden and FII A20210 mutations. Similar figures were observed in patients with cerebral vein thrombosis (CVT; n = 9; 20.0%) and in patients presenting with splanchnic vein thrombosis (SVT; n = 26; 18.7%). A lower prevalence was obtained in patients with retinal vein thrombosis (n = 11; 11.8%). The JAK2 F617 mutant allele was found in 27 (21.1%) patients with SVT, but in none of the patients presenting with a thrombotic event from different districts. 13 of the 27 JAK2 V617F-positive subjects with SVT were previously known to have a myeloproliferative disease (MPD). Three other patients had a diagnosis of MPD after the occurrence of the thrombotic event.
Conclusion: Carriership of FV Leiden or FII A20210 mutations identifies an at-risk condition for venous thrombosis in the lower extremities, SVT or CVT. In patients with SVT, screening for the JAK2 V617F mutation may be useful in recognising patients who should be carefully observed for the subsequent development of overt MPD. Thus, genetic tests may play a different role, various clinical manifestations of venous thromboembolism being associated with distinct risk profiles.

Venous thrombosis is the third most common cardiovascular affliction after ischaemic heart disease and stroke.[1] It is common in Caucasians, affecting 1 in 1000 individuals every year, and is strongly associated with life-threatening pulmonary embolism. The pathogenesis of venous thrombosis is multifactorial, involving acquired and genetic factors. In addition to circumstantial predisposing factors (eg, surgery, pregnancy, immobilisation and malignancy), genetic predisposition due to molecular abnormalities of components of the coagulation pathway have been found in subjects who had had thromboembolic disease.[2] Abnormalities within the gene loci encoding for natural anticoagulants (antithrombin, protein C and protein S) and for fibrinogen have been shown to be rather uncommon risk factors for venous thrombosis.[3] In patients of European ancestry, common gain-of-function mutations within the gene of the coagulation factor V (FV Leiden mutation) and the factor II (FII) gene (a G→A transition at nucleotide position 20210) have been shown to account for a large number of cases of thromboembolism.[2 3] Recently, the JAK2 V617F mutation, an acquired somatic event occurring in most patients with polycythaemia vera (PV) and in about half of the patients with essential thrombocythaemia (ET) or myelofibrosis (MF),[4–8] has been found in a high proportion of patients with Budd–Chiari syndrome[9] and in patients without cirrhosis with portal and mesenteric venous thrombosis, a heterogeneous group of disorders.[10]

Thus, the high frequency of common mutations in patients presenting with venous thromboembolism raised the hypothesis that it can be considered as a feasible and practical approach for the identification of predisposing genetic risk factors. Genetic test results can better inform individuals about their risk of recurrence and appropriated tailored strategies for the prevention of venous thrombosis.

However, depending on the different clinical manifestations of venous thromboembolism, the prevalence of inherited coagulation abnormalities varies, suggesting pathogenic differences.[11–13] These data support the hypothesis that mechanistic differences are involved in the pathogenesis of thrombosis in different clinical settings. Thus, before considering whether it is advisable to investigate a subject for inherited risk factors, we need to know the prevalence of these risk factors in different clinical settings.

We, therefore, calculated the prevalence of inherited thrombophilic risk factors in a large cohort of patients referred for a thrombophilic investigation with different clinical manifestations of venous thromboembolism.

PATIENTS AND METHODS

Patients and controls

Between January 1997 and October 2006, we investigated 139 patients who were not anticoagulated (median (range) age 47 (10–86) years), 56 men and 83 women, with a splanchnic vein thrombosis (SVT); 93 patients (median (range) age 51 (20–81) years), 51 men and 42 women, with retinal vein thrombosis (RVT); and 45 patients (median (range) age 40 (13–55) years), 14 men and 31 women, with cerebral vein thrombosis (CVT). In parallel, 110 patients (median (range) age 49 (12–81) years), 59 men and 51 women, who enrolled between January 2005 and October 2006 with deep vein thrombosis (DVT) in one leg, served as the reference group.

Patients with DVT in one leg were consecutively referred to the Thrombosis Centre of the IRCCS ''Casa Sollievo della Sofferenza'', San Giovanni Rotondo (FG), Italy. Patients with RVT or CVT were referred for a thrombophilic investigation, to the Thrombosis Centre of the IRCCS Casa Sollievo della Sofferenza or to the Thrombosis Centre of the ''A Cardarelli'' Hospital, Naples, Italy. Patients with SVT consecutively diagnosed and followed up at the Gastroenterology Unit of

Abbreviations: CVT, cerebral vein thrombosis; DVT, deep vein thrombosis; ET, essential thrombocythaemia; FII, factor II; FV, factor V; MF, myelofibrosis; MPD, myeloproliferative disease; PV, polycythaemia vera; RVT, retinal vein thrombosis; SVT, splanchnic vein thrombosis

Table 1 Prevalence of factor V Leiden and factor II A20210 mutations among different groups investigated

	n (m/w)	FV Leiden, n (%)	FII A20210, n (%)	Both, n (%)	JAK2 617F, n (%)
Controls	286 (119/163)	10 (3.5)	9 (3.1)	19 (6.6)	None
OR (95% CI)		Reference	Reference	Reference	
DVT	110 (59/51)	14 (12.7)	9 (8.2)	23 (20.9)	None
OR (95% CI)		4.0 (1.7 to 9.4)	2.7 (1.4 to 7.1)	3.7 (1.9 to 7.2)	
SVT	139 (56/83)	14 (10.1)	13 (9.4)	26* (18.7%)	27 (21.1)
OR (95% CI)		3.1 (1.3 to 7.2)	3.2 (1.3 to 7.6)	3.2 (1.7 to 6.1)	
CVT	45 (14/31)	1 (2.2)	8 (17.8)	9 (20.0)	None
OR (95% CI)		0.6 (0.1 to 5.0)	6.7 (2.4 to 18.3)	3.5 (1.5 to 8.4)	
RVT	93 (41/52)	6 (6.5)	5 (5.4)	11 (11.8)	None
OR (95% CI)		1.9 (0.7 to5.4)	1.8 (0.6 to 5.4)	1.9 (0.9 to 4.1)	

CVT, cerebral venous thrombosis; DVT, deep venous thrombosis in a leg; FII, factor II; FV, factor V; m, men; SVT, splanchnic venous thrombosis; RVT, retinal venous thrombosis; w, women.
*A patient with SVT carried both mutations.

the A Cardarelli Hospital were referred to the Thrombosis Centre of the same hospital. All subjects with liver cirrhosis or hepatocellular carcinoma were excluded from the study. All were investigated at least 3 months after the thrombotic episode.

All patients with venous thrombosis were diagnosed by Doppler ultrasonography, spiral CT or MRI as required during the routine diagnostic investigation. A complete clinical summary with emphasis on personal and family history for thromboembolic disease, and circumstantial vascular risk factors (surgery, immobilisation, pregnancy, postpartum, trauma, oral contraception, varicose veins and malignancy) was obtained from all subjects by a specially trained staff. A myeloproliferative disease (MPD) was diagnosed according to established criteria.[14]

The protocol for the investigation was similar in the two thrombosis centres and included testing for deficiencies of antithrombin, protein C and protein S, either before the start or after the termination of the anticoagulation, and the presence of antiphospholipid antibodies. In addition, all patients were tested for the presence of FV Leiden, FII A20210, and JAK2 V617F mutations at the Thrombosis Centre of the IRCCS Casa Sollievo della Sofferenza.

A total of 286 apparently healthy subjects (119 men and 163 women; median (range) age 44 (21–73) years) randomly selected from a Southern Italian general population of employees of the IRCCS Casa Sollievo della Sofferenza, without a history of venous thromboembolism, served as controls. All subjects who reported a personal history of clinical venous thrombosis were excluded from the study. Both cases and controls were Caucasian and were from the same region. The two groups were comparable for sex, social status and age.

After approval of the local ethics committees, the study was carried out according to the principles of the Declaration of Helsinki; informed consent was obtained from all the subjects.

Blood collection and coagulation tests

Blood samples were collected into vacuum plastic tubes containing 3.8% trisodium citrate and centrifuged at 2000 *g* for 15 min to obtain platelet-poor plasma. The platelet-poor plasma was frozen and stored in small aliquots at −70°C until tested. Antiphospholipid antibodies—lupus anticoagulant and IgG anticardiolipin antibodies (ELISA, Byk Gulden, Italy)—antithrombin, protein C, amidolytic and immunological (Behring, Marburg, Germany) and total and free protein S antigen (ELISA, Diagnostica Stago, Asnières, France) were determined in all patients, as reported elsewhere.[15] Clotting assays were performed on a KC4 Amelung coagulometer (Amelung, Germany). Interassay and intra-assay coefficients of all the variables did not exceed 8.0% and 5.0%, respectively.

DNA extraction and analysis

DNA was extracted from peripheral blood leucocytes according to standard protocols.[16] A 220 bp DNA fragment of the FV gene that includes the nucleotide 1691, was amplified and digested with *Mnl*I, as described previously.[17] To identify the G→A mutation of the FII gene a 345 bp fragment was obtained and then digested using the *Hin*dIII endonuclease, as reported previously.[18]

Amplifications of regions of JAK2 gene containing the V617F mutation were performed as described previously.[10] Genomic DNA was amplified by PCR, and amplification products were subjected to restriction enzyme analysis using 2I U of the *Bsa*XI endonuclease (New England Biolabs, Beverly, Massachusetts, USA). The G→T transversion in exon 12 leads to the missing of a site of digestion of *Bsa*XI, giving rise to an uncut product instead of the expected two restriction fragments (96 and 56 bp). Then, amplified DNA fragments showing an abnormal pattern of digestion were cleaned with QIAquick PCR purification Kit (Qiagen, Valencia, California, USA, and subjected to direct cycle sequence analysis using the Taq dye-deoxy terminator method, using the same primers used for amplification, and an ABI PRISM 310 Genetic Analyzer Sequencer (PE Biosystems, Foster City, California, USA). Because the JAK2 V617F mutation is an acquired somatic event, we assessed the sensitivity of methods used to detect it using scalar dilutions of the amplification product from a patient homozygotic for the FV Q506 allele and that from a subject homozygotic for the FV R506 allele. Using restriction enzyme analysis, we were able to detect dilutions as low as 5%, whereas the lower limit of the direct cycle sequence analysis was 20%.

Statistical analysis

All analyses were performed according to the SPSS software V.11.0 for Windows. The significance of any difference in means was evaluated by the non-parametric test, whereas the significance of any difference in proportions was tested by χ^2 statistics. The allele frequencies were estimated by gene counting, and genotypes were scored. The observed numbers of each gene mutation (FV Leiden, FII A20210 and JAK2 V617F) were compared with those expected for a population in Hardy–Weinberg equilibrium using a χ^2 test. The significance of the difference of observed alleles and genotypes between the groups were tested using the χ^2 analysis after grouping

Table 2 Clinical characteristics of patients carrying the JAK2 V617F mutation

Patient	Sex	Age at diagnosis (years)	Year	MPD	Risk factors	Vein thrombosis
1	Female	40	1997	PV known	None	Budd–Chiari syndrome
2	Female	49	1997	PV during the work-up	None	Portal, mesenteric
3	Female	61	1997	CML known	Hyperhomocysteinaemia	Spleen, portal, mesenteric
4	Female	30	1997	No	None	Budd–Chiari syndrome
5	Female	52	1998	ET known	None	Portal, mesenteric
6	Female	31	1998	PV known	None	Budd–Chiari syndrome
7	Male	42	1999	MF known	None	Budd–Chiari syndrome
8	Female	44	2000	MF during follow-up	Abdominal surgery	Portal, mesenteric
9	Female	46	2000	No	None	Portal
10	Male	25	2000	No	None	Budd–Chiari syndrome
11	Female	35	2000	MF known	None	Portal, mesenteric
12	Female	80	2000	MF known	None	Portal
13	Female	52	2001	No	None	Portal, mesenteric
14	Female	66	2001	MF known	None	Budd–Chiari syndrome
15	Female	49	2001	MF known	FV Leiden	Budd–Chiari syndrome
16	Male	50	2001	MF during follow-up	None	Portal, mesenteric
17	Female	43	2001	No	FV Leiden	Budd–Chiari syndrome
18	Male	42	2002	ET known	None	Portal
19	Male	85	2003	No	Hyperhomocysteinaemia	Portal
20	Male	42	2003	PV known	None	Portal, mesenteric
21	Female	35	2003	No	None	Budd–Chiari syndrome
22	Female	28	2003	No	None	Spleen, portal
23	Female	34	2004	No	Oral contraceptives	Spleen, portal, mesenteric
24	Female	41	2004	MF known	None	Budd–Chiari syndrome
25	Male	80	2006	No	None	Spleen, portal, mesenteric
26	Female	36	2006	MF known	None	Portal, mesenteric
27	Female	38	2006	No	None	Portal

CML, chronic myeloid leukaemia; ET, essential thrombocythaemia; FV, factor V; MF, idiopathic myelofibrosis; MPD, myeloproliferative disease; PV, polycythemia vera.

homozygotic and heterozygotic carriers of the FV Leiden mutation. Odds ratio (OR) and 95% CIs were calculated using the modified Wald or exact method as required. Significance was considered as $p<0.05$.

RESULTS

Prevalence of FV Leiden and FII A20210 mutations

Table 1 shows the distributions of FV Leiden and FII A20210 mutations according to the type of clinical manifestations of venous thromboembolism. Overall, one 60-year-old man who had a DVT in one leg and a SVT, was homozygotic for the FV Leiden mutation. With regard to the FII A20210 gene variant, none of the patients were homozygotic.

Among patients with DVT in one leg, 23 (20.9%) carried at least one of these gene variants. Similar figures were observed in the group of patients with CVT (n = 9; 20.0%) and in patients presenting with SVT (n = 26; 18.7%), whereas a lower prevalence was obtained in the group of patients with RVT (n = 11; 11.8%). Likewise, carriers of the FV Leiden or the FII A20210 mutation presented less frequently with RVT (table 1).

Prevalence of the JAK2 V617F mutation

The JAK2 F617 mutant allele was not found in controls and in any of the patients presenting with a thrombotic event that occurred in cerebral or retinal vein of the leg. Among patients with SVT, a total of 27 (21.1%; 95% CI 14.4 to 29.2), 7 men and 20 women, carried the JAK2 V617F mutation (table 2); 26 were heterozygotic and 1 was homozygotic. No-one in other patient groups or in the control group was found to carry the mutant allele. Samples from all subjects with SVT and those carrying the JAK2 V617F mutation were sequenced. In 26 of 27 patients, sequencing analysis showed concordance with results obtained with the restriction fragment length analysis. Allele quantitation, estimated taking into account the relative area of both alleles, gave comparable results for the wild (G) and the mutant (T) alleles, the ratio never exceeding 60%, in all subjects but one who was homozygotic for the mutant (T) allele. In the remaining patient, a 28-year-old woman, the mutant allele was detected only by restriction enzyme analysis. Two of the patients presenting with the JAK2 V617F mutation carried the FV Leiden mutation, whereas none of them had the FII A^{20210} allele.

Circumstantial vascular risk factors

Circumstantial vascular risk factors were registered in 59 (53.6%) patients with DVT in one leg, 58 (41.7%) with SVT, 8 (8.6%) with RVT and 27 (60.0%) with CVT. Frequencies of common inherited mutations did not differ among patients presenting with or without circumstantial vascular risk factors.

The JAK2 V617F mutation and MPDs

The knowledge of an MPD at the occurrence of the venous thrombotic event was recorded in 13 (48.2%; 95% CI 32.0 to 71.3) patients with and 8 (7.9%; 95% CI 3.5 to 15.0) patients without the JAK2 V617F mutation (table 2). Overall, the JAK2 V617F mutation was detected in 13 of 22 patients (59.1%; 95% CI 40.7 to 82.8) presenting with a known MPD. In all, 7 of 12 patients with MF, 2 of 3 with ET, 3 of 6 with PV and a woman with chronic myeloid leukaemia showed the JAK2 V617F mutation.

All patients presenting with SVT without a known diagnosis of MPD had serial evaluations of haematocrit and erythrocyte, leucocyte and platelet counts. In addition, a karyotype analysis and the search for the *bcr-abl* rearrangement were performed in all subjects. During the follow-up (median (range) 41 (3–114) months), 5 of 104 patients (4.8%; 95% CI 0.2 to 10.9) went on to develop MPD: 2 with MF (1 man and 1 woman), 1 with PV (woman) and 2 with ET (2 women). Among the 14 patients (4 men and 10 women) carrying the JAK2 V617F mutation and presenting with SVT without a diagnosis of MPD, 3 (1 man and 2 women) were then diagnosed as having MF (n = 2) or PV (n = 1). Thus, in 11 patients (3 men and 8 women) an MPD was not detected.

DISCUSSION

Venous thrombosis is increasingly recognised as a multifactorial disorder, although the specific combination of genetic and acquired risk factors or local precipitating events may differ between patients. The knowledge of the significance of inherited risk factors stems essentially from settings of patients with thrombosis of the legs, which is by far the most frequent localisation of venous thromboembolism. Thus, despite inherited thrombophilic risk factors being strongly associated with venous thrombosis, decisions on whom to screen for these risk factors are complex and vary in different clinical settings. We investigated whether the prevalence of the common gain-of-function gene mutations, FV Leiden, FII A20210 and JAK2 V617F, vary significantly in different clinical settings of patients with venous thrombosis.

Patients with DVT in one leg, CVT or SVT had, as would be expected,[19 20] a significantly higher prevalence of FV Leiden and FII A20210 mutations than did apparently healthy individuals from a general population. The prevalence was also significantly higher than that recorded in patients with RVT. However, owing to the sample size this subgroup analysis may present some limitations and has to be considered with caution.

We have found a high prevalence of the JAK2 V617F mutation in patients with SVT, but in none of the patients presenting with venous thrombosis from other districts. The prevalence of inherited coagulation abnormalities, anticoagulant factors, FV Leiden and FII A20210 mutations have been reported to be significantly different depending on the site where venous thrombosis occured.[11–13] In some,[21 22] but not all studies,[23] the prevalence of inherited thrombophilic risk factors among patients with RVT was comparable to that among controls. The present report extends these data to the JAK2 V617F mutation, and further suggests mechanistic differences in the pathogenesis of venous thrombotic events in different districts.

Results from the present study suggest that screening for inherited thrombophilic risk factors can identify high-risk individuals among patients who have had a venous thrombosis of the leg, SVT or CVT. By contrast, our data further confirm that genetic screening programmes have less relevance among patients with RVT.

Chronic MPDs, such as PV and ET, follow a chronic clinical course with increased risk of thrombosis and of evolution to MF with myeloid metaplasia or acute leukaemia.[24 25] Thrombosis remains the most frequent complication during follow-up and is the main predictor of death in both PV and ET. Notably, only 13 of the 27 JAK2 V617F-positive subjects with SVT were previously known to have MPD. Three other patients had a diagnosis of MPD after the occurrence of the thrombotic event, therefore representing a group with a latent form of MPD. The remaining cases failed to fulfil the accepted diagnostic criteria for MPD, and we suggest that they be carefully observed for the subsequent development of overt MPD. These findings further suggest that the JAK2 V617F mutation is often found in patients with SVT, but some of them did not have an overt MPD.

The present study has been performed in patients referred to specialised thrombosis centres for a thrombophilic investigation and, therefore, constitutes a highly selected group. Thus, results may not apply to less highly selected patient populations.

Understanding of the genetic factors predisposing to venous thrombosis is important in identifying subgroups of patients at risk. From a clinical prospective, whether carriers of inherited thrombophilic risk factors are more likely to have specific clinical manifestations of venous thromboembolism is an important issue that needs to be addressed. Overall, the available information indicates that carriership of common inherited thrombophilic defects identifies an at-risk condition for venous thrombosis in the lower extremities that eventually will be complicated by pulmonary embolism, SVT or CVT. In addition, present findings suggest that, in patients presenting with splanchnic venous thrombosis, screening for the JAK2 V617F mutation may be useful in recognising patients who should be carefully observed for the subsequent development of overt MPD. Therefore, genetic tests may play a different role, with various clinical manifestations of venous thromboembolism being associated with distinct risk profiles.

Key points

- Common gain-of-function mutations within coagulation factor genes predispose to venous thromboembolism.
- The JAK2 V617F mutation is an acquired somatic event, which occurs in several patients with splanchnic vein thrombosis.
- Screening for common gain-of-function mutations plays an important role, various clinical manifestations of venous thromboembolism being associated with distinct risk profiles.

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