The association of the β1-tubulin Q43P polymorphism with intracerebral hemorrhage in men

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ABSTRACT

Background and Objectives

Platelets play a fundamental role in hemostasis and alterations of their function can be determinant in the onset of stroke. A polymorphism in β1-tubulin (TUBB1 Q43P), a protein specifically expressed in the megakaryocytic line, has been described as a protective factor in cardiovascular disease. The potential effect of this variant in the pathogenesis of hemorrhagic stroke has not yet been investigated.

Design and Methods

We evaluated the role of the TUBB1 Q43P polymorphism and its synergism with other polymorphisms in the risk of developing subarachnoid (SAH) and intracerebral hemorrhage (ICH). We performed the study in 109 patients with SAH, 259 patients with ICH, and 449 subjects from the general population from southern Spain.

Results

No relationship was found between the TUBB1 Q43P polymorphism and SAH. In contrast, this polymorphism significantly increased the risk of ICH in men (OR, 2.78; 95% CI, 1.16-6.63; p=0.021) and was associated with an earlier age of occurrence of an ICH event (p=0.011). Carriers of the TUBB1 Q43P polymorphism displayed lower platelet reactivity towards collagen. A potent synergistic effect was observed in ICH patients carrying the TUBB1 Q43P polymorphism combined with either FVII -323 Del/Ins of a decanucleotide (OR 20.76; 95% CI, 3.57-120.71; p<0.001) or FXIII V34L (OR 7.19; 95% CI, 1.99-25.95; p=0.003).

Interpretation and Conclusions

This is the first evidence linking the TUBB1 Q43P platelet polymorphism with hemorrhagic stroke in humans. The TUBB1 Q43P polymorphism, by causing a lower reactivity in platelets carrying the variant form of β1-tubulin, protects against thrombotic disorders but increases the risk of ICH in men.

Key words: platelets, β1-tubulin, intracerebral hemorrhage, polymorphisms, synergism.

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Platelets play a critical role in hemostasis through the formation of the hemostatic plug at sites of vessel injury. In addition, platelets with a more reactive state play an important role in stroke events.[1-3] In particular, patients with atherosclerotic plaque rupture show an enhanced platelet activation and have increased amounts of platelet-leukocyte aggregates.[3] In the last years, studies have investigated the role that genetic alterations which increase platelet reactivity play in the risk of thrombosis, while the role of genetic changes leading to platelet hyporeactivity in the development of hemorrhagic disorders has been rarely surveyed. Recently, a polymorphism affecting platelet function, TUBB1 Q43P, was characterized in β1-tubulin.[4] This isoform of tubulin is specifically expressed in platelets and megakaryocytes and, together with α-tubulin, forms heterodimers which assemble into high-ordered microtubule polymers.[5,6] Microtubules play an important role in megakaryocyte physiology, being an essential element during thrombocytopoiesis, involved in proplatelet elongation, granule trafficking, and platelet separation.[7] In platelets, microtubules form a subcortical ring, called the marginal band, which maintains the discoid form of platelets. However, little is known about the role of β1-tubulin in platelet activation and aggregation. It has been shown that β1-tubulin-deficient mice develop thrombocytopenia with spherical platelets that are less responsive to thrombin activation.[6] Interestingly, platelets carrying the TUBB1 Q43P polymorphism have a phenotype similar to that of the platelets from β1-tubulin-deficient mice.[4] This polymorphism affects a highly conserved residue within a region implicated in the binding of β1-tubulin with other isoforms of tubulin. The TUBB1 Q43P polymorphism leads to a malformation of microtubule polymers and, consequently, to the generation of an abnormal platelet marginal band. This polymorphism seems to have a protective effect on arterial thrombosis since the prevalence of the TUBB1 Q43P polymorphism was higher in a healthy Belgian population than in patients with cardiovascular disease.[4]

To date, very few studies have associated polymorphisms of elements of the hemostatic system with intracranial hemorrhage. Given the severity of intracranial hemorrhage and the high mortality and morbidity associated with such bleeding, it is very relevant to identify new risk factors that will help not only to prevent the disease but also to define new therapeutic approaches. The aim of this study was to evaluate the role of the TUBB1 Q43P polymorphism in the development of hemorrhagic strokes.

Design and Methods

Study population

Our study involved 259 patients suffering from non-traumatic intracerebral hemorrhage (ICH) and 109 subjects with subarachnoid hemorrhage (SAH). Of these patients, 164 with ICH (108 men and 56 women) and 102 with SAH (40 men and 62 women) were from Arrixaca Hospital and University General Hospital (Murcia, Spain) and were collected during a 3-year period from 1997 to 2000,[8,9] whereas 95 with ICH (all women) and seven with SAH (three men and four women) were from Vall d'Hebron Hospital and were also collected during a 3-year period from 2003 to 2006.

Patients were diagnosed with non-traumatic intracranial hemorrhage by computed tomography scan within 24 hours of admission to hospital. Patients with hemorrhagic transformation of a previous infarct, with acquired or congenital bleeding disorders, or with primary or metastatic brain tumors, and patients receiving anticoagulant or antiaggregant treatment were not included in the study.[8] The frequency of TUBB1 Q43P polymorphism in the general population was studied in 449 healthy unrelated controls, recruited from among blood donors (215 controls), and traumatology and ophthalmology patients undergoing minor outpatient surgery (234 controls). The latter set of controls had no documented history of vascular disease, hemorrhagic episodes, or personal history of thromboembolic disease. All the participants were Caucasians and gave informed consent before enrollment. The clinical features and the prevalence of selected risk factors in the patients and in the general population are summarized in Table 1. The study was approved by the local Ethics Committee and carried out in accordance with the Declaration of Helsinki.

Genetic analysis

Genomic DNA was isolated from whole blood samples according to standard procedures. β1-tubulin DNA was amplified by polymerase chain reaction (PCR) using primers described previously.[4] Genotyping was performed by SSCP on 12% polyacrylamide and sequencing of all identified patterns. Additionally, the TUBB1 Q43P polymorphism was further genotyped by PCR-ASRA using *PvuII* (Fermentas, Vilnius, Lithuania). The -323 decanucleotide Del/Ins polymorphism of the FVII gene, FXIII V34L, and HPA-1b (GPIIIa L33P) were genotyped by PCR as described elsewhere.[9,10]

Platelet aggregation

The effect of the TUBB1 Q43P polymorphism on platelet function was investigated by performing aggregation assays in 11 healthy volunteers of known genotype [six Q/Q (three men/three women) and five Q/P (two men/three women)]. Platelet aggregation was measured in citrated platelet-rich plasma (300×10^9 platelets/L) stimulated with different doses of thrombin receptor agonist (6.25 and 12.5 μmol/L) (TRAP from Calbiochem, San Diego, CA, USA), collagen mixture (0.5 and 1 μg/mL) (Menarini Diagnostics, Florence, Italy), or ADP (0.625, 1.25, and 2.5 μmol/L) (Menarini Diagnostics, Florence, Italy). Time course changes in light transmission of platelet-rich plasma over baseline (platelet-poor plasma) were recorded for 5 minutes using an Aggrecorder II aggregometer (Menarini Diagnostics), and maximal aggregation was determined to examine differences between groups.

Table 1. Clinical features and prevalence of selected risk factors in patients and in the general population.

	Controls (n=449)	*ICH (n=259)*	*p**	*SAH (n=109)*	*p**
Age (y)					
Range	29-90	25-99		19-90	
Mean±SD	56.7±16.5	71.9±12.1	<0.001	59.4±13.6	0.26
Male sex (%)	63.2	41.7	<0.001	39.4	<0.001
Risk factors (%)					
Current/former smoker	32.3	18.5	<0.001	34.9	0.61
Hypertension	28.5	67.6	<0.001	45.0	<0.001

*Values are mean±SD or percentage of individuals. Current/former smoker: subjects consumed >10 cigarettes per day. Hypertension was a systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg on repeated observations over 3 months or, if no blood pressure values were available, when the subject was under treatment with chronic antihypertensive therapy. *Statistical analysis was performed vs. controls, a t-test or χ^2 test was used to evaluate statistical differences between groups. Two-sided p values less than 0.05 were considered statistically significant. ICH: intracerebral hemorrhage; SAH: subarachnoid hemorrhage.*

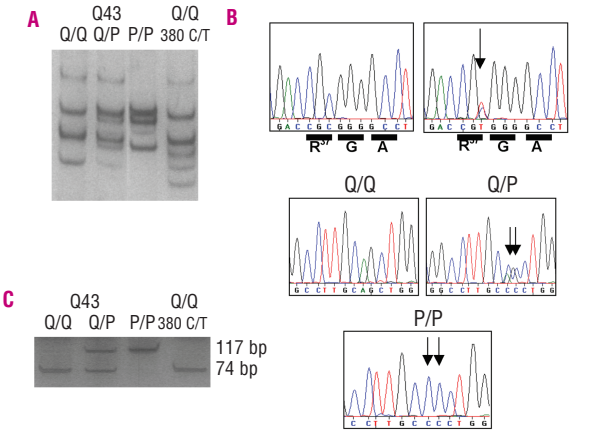

Figure 1. Genetic determination of the TUBB1 polymorphisms. SSCP patterns of all possible genotypes identified in this study. (B) Sequencing was carried out for subjects with a newly identified SSCP pattern, revealing a new C380T silent polymorphism (nucleotide and amino acid numbering as per mRNA GenBank entry NM_030773). Sequence analyses of the three genotypes determined by the TUBB1 Q43P polymorphism are also shown. (C) All possible genotypes were confirmed by PCR-ASRA. Arrows indicate nucleotide changes.

Statistical analyses

All results are expressed as mean ± SD for continuous variables and as percentages for categorical variables. Multivariate analysis was performed through multiple logistic regression using the SPSS statistical package (SPSS Inc., Chicago, IL, USA) adjusting for sex, age, and cerebrovascular risk factors (smoking habit and hypertension). Differences between groups with two-tailed *p*-values <0.05 were considered statistically significant. The strength of association between the TUBB1 Q43P polymorphism and haemorrhage was estimated by calculating the odds ratio (OR) with 95% confidence intervals (CI).

Results

Prevalence of the TUBB1 Q43P polymorphism in the study

The general characteristics of patients and controls are shown in Table 1. Polymorphism genotyping performed by SSCP analysis showed four different patterns (Figure 1A). Sequence analysis revealed all three possible genotypes of the TUBB1 Q43P polymorphism; however, we also identified a new transition C380T affecting Arg37 without an amino-acid change (nucleotide and amino-acid numbering as per mRNA GenBank entry *NM_030773*) (Figure 1B). This new variant was found in a heterozygous state in one control person from the general population and in one patient with ICH. Both individuals had a normal 43 Q/Q genotype for TUBB1. The TUBB1 Q43P polymorphism was additionally determined by PCR-ASRA (Figure 1C).

The genotype and allelic frequencies of TUBB1 Q43P polymorphism among the cases and among the controls are summarized in Table 2. In our control group (a Caucasian population from the south of Spain) the prevalence of the TUBB1 Q43P polymorphism was 5.8% (26 cases out of 449). This value was lower than that previously reported in the Flemish population (10.4%).[4] Therefore, the frequencies of the CC (Q43) and AG (P43) alleles were 0.971 and 0.029, respectively. The distribution of genotypes was not significantly different from that predicted by the Hardy-Weinberg equilibrium (*data not shown*). We next calculated the prevalence of this polymorphism in patients who had had non-traumatic hemorrhagic strokes. We subdivided the group into those who had had intracerebral and subarachnoid hemorrhages. As shown in Table 2, the prevalence of TUBB1 Q43P polymorphism was different in the two subgroups of patients. The prevalence was 7.3% in patients with SAH, and 12.0% in those with ICH. The only subject homozygous for TUBB1 Q43P polymorphism in our study had had a SAH. Multivariate analysis indicated that TUBB1 Q43P polymorphism was not a risk factor for SAH (p=0.433; OR, 1.40; 95% CI, 0.61-3.22). In contrast, the crude analysis suggested that this polymorphism increased the risk of ICH (p=0.004; OR, 2.21; 95% CI, 1.28-3.82). After adjusting for sex, age, and risk factors, logistic regression analysis showed that TUBB1 Q43P polymorphism still increased the risk of developing ICH by 2.36-fold (p=0.008) (Table 2). In a previous study, Freson *et al.* showed that the TUBB1 Q43P polymorphism was a protective factor in cardiovascular disease only in men.[4] Therefore, we used logistic regression analysis to evaluate the role of this polymorphism in relation to gender. Interestingly, the presence of the TUBB1 Q43P polymorphism had no effect on ICH in women (p=0.148; OR, 1.97; 95% CI, 0.79-4.95). However, male carriers had a significantly increased risk of ICH (p=0.021; OR, 2.79; 95% CI, 1.16-6.63). We also analyzed the association of the studied polymorphism with different clinical features of ICH patients (Table 3). Remarkably, we found that the presence

Table 2. Impact of the TUBB1 Q43P polymorphism on risk of intracerebral and subarachnoid hemorrhage.

Group	Number of subjects	P43 carriers (%) Q/P + P/P	Adjusted OR; 95% CI	Adjusted p value
Controls	449	26 (5.8)	NA	NA
Men	284	15 (5.3)	NA	NA
Women	165	11 (6.7)	NA	NA
Total ICH	259	31 (12.0)	2.36; 1.25-4.45	0.008*
Men	108	13 (12.0)	2.79; 1.16-6.63	0.021*
Women	151	18 (11.9)	1.97; 0.79-4.95	0.148
Total SAH	109	7+1 (7.3)	1.40; 0.61-3.22	0.433
Men	43	3 (7.0)	1.34; 0.37-4.92	0.658
Women	66	4+1 (7.6)	1.77; 0.56-5.60	0.331

*OR: odds ratio obtained on multivariate logistic regression after controlling for age, sex, smoking, and hypertension (adjusted OR). CI: 95% confidence interval. ICH: intracerebral hemorrhage. SAH: subarachnoid hemorrhage; NA: not applicable. *Adjusted p values less than 0.05 were considered statistically significant.*

of the polymorphic allele (P43) was associated with an earlier age of onset of ICH. Indeed, within the ICH group, 12.9% of TUBB1 Q43P polymorphism carriers were less than 45 years old compared with 3.1% in non-carriers (p=0.011) (Table 3). Our results also highlighted a lower prevalence of hypertension in those ICH patients with the polymorphic allele P43.

Finally, we analyzed the potential synergistic effect between pro-hemorrhagic (FXIII V34L and FVII -323 Ins/Del) and pro-thrombotic (HPA-1b) risk factors and the TUBB1 Q43P polymorphism (Table 4). Our results showed that the co-inheritance of TUBB1 P43 and FXIII L34 alleles increased the risk of developing ICH by more than 7-fold (OR, 7.19; 95% CI, 1.99-25.95; p=0.003). However, a dramatic effect was observed when the TUBB1 Q43P polymorphism was associated with FVII-323 Del/Ins (OR, 20.76; 95% CI, 3.57-120.7; p<0.001). In contrast, the association with the HPA-1b pro-thrombotic polymorphism neutralized the pro-hemorrhagic effect of the TUBB1 Q43P polymorphism observed in ICH.

Platelet function in subjects carrying the TUBB1 Q43P polymorphism

We tested the influence of the TUBB1 Q43P polymorphism on the function of platelets from 11 healthy subjects divided into two groups (heterozygous carriers and non-carriers) by assessing the aggregation of platelets induced by different doses of TRAP, collagen, and ADP (Figure 2A). We found no significant difference in platelet aggregation between the two groups upon stimulation with TRAP (6.25 and 12.5 μmol/L). When using ADP, we observed no significant impairment in platelet aggregation although there was a trend toward a lower platelet response at low concentrations of agonist (0.625 μmol/L) in TUBB1 Q43P carriers. Additionally, platelets carrying the TUBB1 Q43P polymorphism were significantly less sensitive to collagen (0.5-1 μg/mL) than were normal platelets (Figure 2B). We observed no difference in platelet aggregation to collagen or other agonists according to gender (*data not shown*). Since several studies have shown that β1-tubulin intervenes in thrombocytopoiesis and that β1-tubulin-deficient mice are macrothrombocytopenic, we investigated platelet characteristics (platelet count and mean platelet volume) in the healthy volunteers described above. As shown in Table 5, platelets from carriers of TUBB1 Q43P polymorphism did not differ significantly from those of healthy non-carriers.

Table 3. Selected hemorrhagic risk factors in patients with non-traumatic intracerebral hemorrhage according to β1-tubulin genotype.

	Q43 (Q/Q) (n=228)	P43 (Q/P) (n=31)	p
Age (y): mean ± SD	72.3±11.6	68.9±15.4	0.142
Age < 45 years N (%)	7 (3.1)	4 (12.9)	0.011*
Male sex N (%)	95 (41.7)	13 (41.9)	0.977
Current/former smoker N (%)	41 (18)	7 (22.6)	0.536
Alcohol consumption N (%)	18 (7.9)	2 (6.5)	0.778
Hypertension N (%)	158 (69.3)	14 (45.2)	0.007*
30-day survival N (%)	178 (78.0)	22 (70.1)	0.376

*Values are n (%) or mean±SD . Current/former smoker: see table 2. Alcohol consumption: subject consumed >300 g alcohol/week. Hypertension definition: see Table 2. A t-test or χ^2 test was used to evaluate statistical differences between groups. *Two-sided p values less than 0.05 were considered statistically significant.*

Table 4. Combined effect of the TUBB1 Q43P polymorphism with other classical polymorphisms.

Genotype	ICH (N=259)	Controls (N=449)	Adjusted OR; 95% CI	Adjusted p value
FVII -323Ins	84 (32.4)	123 (27.4)	1.76; 1.18-2.64	0.002*
FXIII L34	99 (38.2)	172 (38.3)	1.06; 0.73-1.53	0.778
HPA-1b	67 (25.9)	126 (28.1)	1.18; 0.79-1.78	0.422
TUBB1 P43 and FVII -323Ins	12 (4.6)	2 (0.4)	20.76; 3.57-120.71	<0.001*
TUBB1 P43 and FXIII L34	11 (4.2)	6 (1.3)	7.19; 1.99-25.95	0.003*
TUBB1 P43 and HPA-1b	5 (1.9)	5 (1.1)	1.13; 0.26-4.92	0.873

*Values are n (%). OR: odds ratio obtained on multivariate logistic regression after controlling for age, sex, smoking, and hypertension (adjusted OR). CI: 95% confidence interval. ICH: intracerebral hemorrhage. *Adjusted p values less than 0.05 were considered statistically significant.*

Discussion

Intracerebral hemorrhage is a complex disease in which different factors, such as hypertension, male sex, or prior ischemic stroke, influence the risk of developing spontaneous bleeding. Genetic risk factors might also play a role in this disease. To date, few polymorphisms potentially implicated in the development of ICH have been described,[11,12] the more relevant being FVII Del/Ins -323 polymorphism, e2 and e4 alleles of the apolipoprotein E in lobar ICH, and FXIII Val34Leu, which has a more controversial role.[8,10,13-17] However, platelets play a major role in hemostasis and atherothrombosis and genetic alterations in platelet receptor proteins have been proposed as potential risk factors for thrombotic or hemorrhagic events.[18-23]

To our knowledge, few studies have addressed the importance of genetic changes in cytosolic platelet proteins in either thrombotic or hemorrhagic risk. Among these proteins, β1-tubulin is essential for platelet formation and

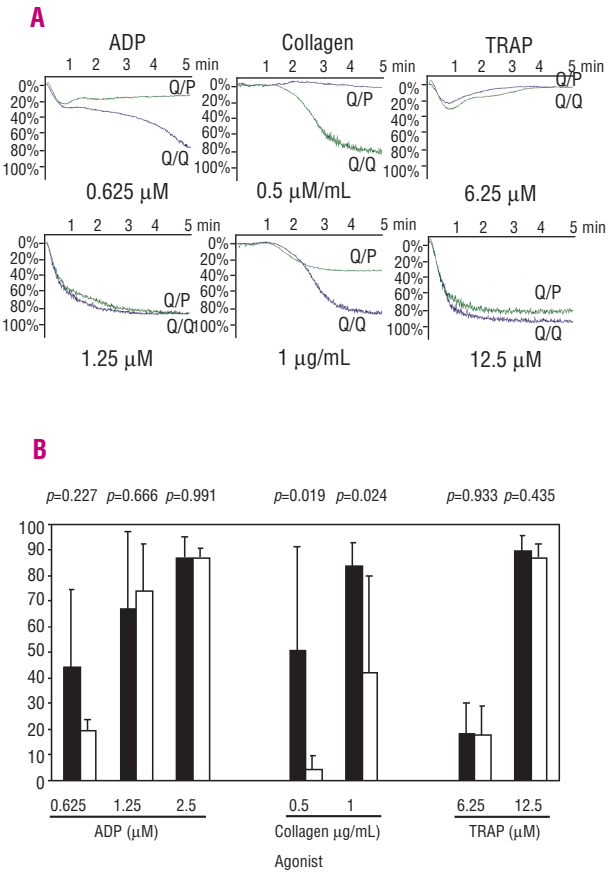

Figure 2. Aggregation of platelets in healthy carriers and non-carriers of the TUBB1 Q43P polymorphism. Representative aggregation tracings are shown (A). Changes in light transmission of platelet-rich plasma (0-100%) over baseline (platelet-poor plasma=0%) were recorded for 5 minutes: mean ± SD (six non-carriers of the TUBB1 Q43P polymorphism [▯] and five carriers of the polymorphism [▯]) (B). The *t*-test was used to evaluate statistical differences between groups. Two-sided *p* values less than 0.05 were considered statistically significant.

its loss or down-regulation has important effects on platelet function.[6] In particular, it has been recently shown that β1-tubulin-deficient platelets, i.e. platelets expressing the TUBB1 P43 allele, are less responsive to some agonists.[4] In this study, we describe that platelet aggregation in response to collagen, and to ADP to a lesser extent, is significantly reduced in healthy carriers of the TUBB1 Q43P polymorphism. Nevertheless, we were unable to demonstrate impaired platelet aggregation in response to TRAP in healthy heterozygous carriers of the TUBB1 Q43P. The exact mechanism by which tubulin participates in platelet aggregation remains undefined and further studies are needed to better understand the role of the marginal band in platelet function. Certainly, the study of platelet responses in subjects homozygous for the TUBB1 Q43P polymorphism (P/P) would be of help. Platelet hyporeactivity may also be relevant when using antiplatelet therapy. Aspirin is a drug of choice to lower the risk of thrombotic cerebro- and cardiovascular events, but some patients receiving aspirin therapy suffer from intracerebral hemorrhage.[24,25] In our study, we show that the TUBB1 Q43P polymorphism increases the risk of ICH. Thus, an interesting issue would

Table 5. Characteristics of platelets from healthy TUBB1 Q43P polymorphism carriers and non-carriers.

	Platelet count (x10⁹/L)	*Mean volume (fL)*
Non-carriers		
1	239	7.0
2	224	7.3
3	210	8.8
4	248	7.3
5	272	6.9
6	332	9.6
Mean values	*254.1±43.6*	*7.8±1.1*
Carriers		
1	262	8.1
2	170	8.6
3	245	7.9
4	379	6.4
5	278	7.5
Mean values	*266.8±75.1*	*7.6±0.8*
p	0.735	0.77

A t-test or χ^2 test was used to evaluate statistical differences between groups. Two-sided p values less than 0.05 were considered statistically significant.

be to test the prevalence of this polymorphism in patients taking aspirin who had hemorrhagic strokes to evaluate whether the TUBB1 Q43P polymorphism should be taken into account when prescribing aspirin prophylaxis.[26] In this study, we show that the TUBB1 Q43P polymorphism is present in 5.8% of the general population from the south of Spain. This prevalence is markedly lower than that (10.4%) observed in a healthy Belgian population.[4] The scarcity of population genetic data concerning this new polymorphism makes it impossible to define the existence of a European south-north gradient in prevalence as demonstrated for other polymorphisms.[27] However, we can wonder whether the higher prevalence of the TUBB1 Q43P polymorphism in the Flemish population could, in some way, be a protective alteration against cardiovascular disease, which has a higher incidence in northern European countries.

Our current data establish that the TUBB1 Q43P polymorphism is a risk factor for hemorrhagic stroke. When we subdivided the hemorrhagic strokes into ICH and SAH, we found that this new polymorphism only played a relevant role in the development of ICH. The lack of association of the TUBB1 Q43P polymorphism with SAH is intriguing, given the association found with ICH, and can probably be explained by the complexity, the nature, and the existing differences in the pathogenesis of the two diseases. On the other hand, as mentioned above, the TUBB1 Q43P polymorphism is an independent risk factor for ICH in the Mediterranean population with a calculated adjusted OR of 2.36, and its presence is associated with an earlier age of onset of ICH. In addition, we found that this genetic alteration increases the risk of ICH in men. In women, on the other hand, the statistical analysis showed no statistically significant different risk between patients and controls, in accordance with the finding of Freson *et al.*[4] Synergism analyses showed that when combined with other pro-hemorrhagic risk factors, the effect of the TUBB1 Q43P

polymorphism on the onset of ICH is greatly enhanced. Indeed, when co-inherited, TUBB1 Q43P and FVII -323 Del/Ins polymorphisms increase the risk of suffering from an ICH event by about 20-fold suggesting a dramatic effect due to the combination of low FVII plasma levels associated with reduced platelet activation probably accompanied by reduced procoagulant platelet surface exposure. Although the FXIII L34 allele was not a risk factor for ICH in our population, when associated with TUBB1 Q43P polymorphism, it clearly increased the occurrence of ICH episodes, showing how the combination of a platelet aggregating defect together with a polymorphism affecting clot stability strongly affects stroke events. On the other hand, HPA-1b counterbalanced the pro-hemorrhagic effect of the TUBB1 Q43P polymorphism in individuals expressing both polymorphic alleles. Thus, our results further support the suggestion that ICH is a multifactorial disease in which genetic alterations can play a fundamental role. Indeed, like other pathologies, ICH is probably due to the synergistic effect of different gene alterations that individually only have a slight effect, but when combined can greatly exacerbate the occurrence of pro-hemorrhagic events.

The TUBB1 Q43P polymorphism has a dual effect on hemorrhage or thrombosis, further supporting the concept that the same genetic alteration may have opposite effects, being protective against or a risk factor for thrombotic and hemorrhagic events, probably depending on the specific conditions and associations with other factors.[8]

In conclusion, our data add the TUBB1 Q43P polymorphism to the small list of genetic risk factors implicated in the development of ICH. Given the scarcity of data concerning the development of hemorrhagic strokes and, in particular, ICH, this new finding could help the understanding of the etiology of this disease and thus facilitate the diagnosis and therapeutic management of intracerebral hemorrhage.

Authors' Contributions

LN-N, MLL, JR, CM designed and performed research, analyzed data, and wrote the paper. JC, RG-C, JAI collected samples and acquired clinical data; VR did statistical analyses, JC, RG-C, and VV analyzed data and critically revised the paper.

Conflict of Interest

The authors reported no potential conflicts of interest.

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