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Original Article

Exome Sequencing and Clot Lysis Experiments Demonstrate the R458C Mutation of the Alpha Chain of Fibrinogen to be Associated with Impaired Fibrinolysis in a Family with Thrombophilia

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Aim: **We report the study of a familial rare disease with recurrent venous thromboembolic events that remained undiagnosed for many years using standard coagulation and hemostasis techniques.**
Methods: **Exome sequencing was performed in three familial cases with venous thromboembolic disease and one familial control using NimbleGen exome array. Clot lysis experiments were performed to analyze the reasons of the altered fibrinolytic activity caused by the mutation found.**
Results: **We found a mutation that consists of a R458C substitution on the fibrinogen alpha chain (*FGA*) gene confirmed in 13 new familial subjects that causes a rare subtype of dysfibrinogenemia characterized by venous thromboembolic events. The mutation was already reported to be associated with a fibrinogen variant called fibrinogen Bordeaux. Clot-lysis experiments showed a decreased and slower fibrinolytic activity in carriers of this mutation as compared to normal subjects, thus demonstrating an impaired fibrinolysis of fibrinogen Bordeaux.**
Conclusions: **The exome sequencing and clot-lysis experiments might be powerful tools to diagnose idiopathic thrombophilias after an unsuccessful set of biochemical laboratory tests. Fibrinogen Bordeaux is associated with impaired fibrinolysis in this family with idiopathic thrombophilia.**

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Key words: Coagulation, Dysfibrinogenemia, Fibrinogen, Thrombosis, Genetics

Introduction

Exome sequencing is an efficient strategy to search for genetic variants underlying rare Mendelian disorders. Here we report a family with an autosomal dominant heritable disease characterized by venous thromboembolic events that has been studied since 9 years. After multiple coagulative and biochemical tests performed in a university hospital, only a high concentration of tissue plasminogen activator (t-PA) was detected and the diagnosis remained elusive. Exome sequencing techniques in 2014 have finally permitted the identification of idiopathic thrombophilia, and proper clinical advice has been given to relatives affected by the disease.

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Aim

Our aim was to diagnose a family with a mendelian genetic disease using all the biochemical and genetic strategies available and to check the fibrinoly-

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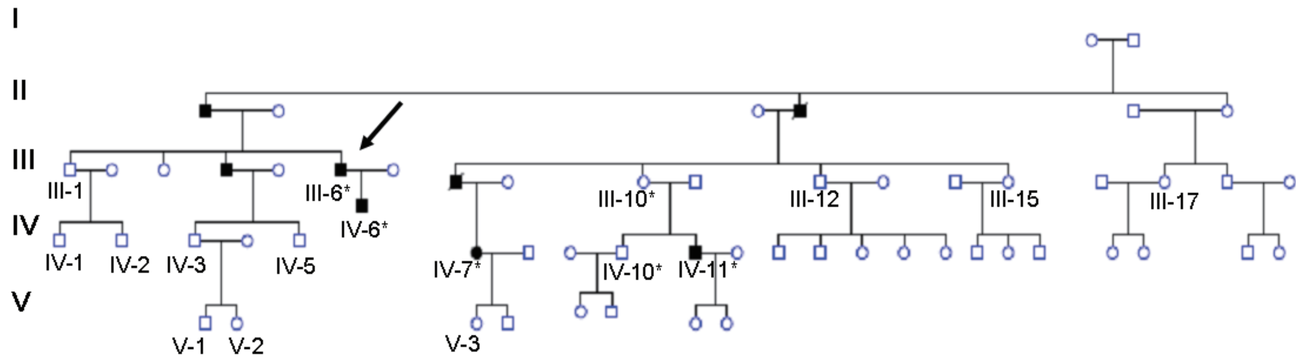

Fig. 1. Pedigree of the family

In black, subjects with documented venous thrombotic events, including pulmonary venous thrombosis. *indicates that the patient harbored the R458C mutation. The arrow indicates the proband.

sis activity of fibrinogen Bordeaux found in that family.

Materials and Methods

Study Population

Several members of a family had episodes of venous thromboembolism (VTE), mainly deep vein thrombosis of the lower extremities or pulmonary embolism as seen in **Fig. 1**. The proband (Ⅲ-6) suffered acute dyspnea when he was 36. A ventilation-perfusion scan showed multiple bilateral perfusion defects, and an ultrasonography revealed a bilateral deep vein thrombosis of the lower limbs. Placement of a Greenfield cava filter was required. He also reported to have abundant epistaxis as the only accompanying symptom. The most aggressive cases were those of a male (Ⅲ-8) and his daughter. He suffered right leg deep vein thrombosis and pulmonary embolism in 1991, received anticoagulation therapy for 6 months, and was then shifted to aspirin; 10 years later, he suffered a lethal massive pulmonary embolism. His daughter (Ⅳ-7) suffered a pulmonary embolism in the early postnatal period of her second child. Patients signed an informed consent, and this study was approved by our Hospital Ethics committee.

Biochemical Laboratory Tests

Routine biochemical and coagulation tests were performed according to standard procedures. Biochemical studies consisted the evaluation of thrombin time; reptilase time; levels of fibrinogen, PAI-1, alpha-2-antiplasmin, protein C, protein S, antithrombin, plasminogen, thrombin-antithrombin complex, t-PA, D-dimer, and beta-tromboglobulin; Fearnley test, coagulation factors activity, and t-PA zymography[1-5].

Tests for Coagulation System

Coagulation factors activity was determined by measuring activated partial tromboplastin time (APTT) in citrate plasma samples using the semi-automated coagulometer ST4 (Diagnostica Stago-Roche, Asnières, France) following manufacturer's users guide. Factors diluent, APTT reagents, deficient serums, normal control and calibration plasma were from IZASA (Werfen Group, Barcelona, Spain). Activities are indicated as a percentage of normal control plasma.

Thrombin clotting times of plasma were done in a mechanical test system containing 75 μl of plasma and 75 μl of thrombin. For reptilase times, 50 μl of plasma and 100 μl of enzyme were used. The reptilase clotting time was measured at 37℃ during one minute and the thrombin time at 37℃ during two minutes.

Tests for Coagulation System Regulation

Thrombin-antithrombin complex: Citrate tubes were used to obtain plasma samples and commercial ELISA kit was used to determine TAT complex following manufacturer user guide (Enzygnost TAT micro, Dade Behring Marburg GmbH, Marburg, German).

Protein C activity: Protein C activity was assayed by ProtClot Kit, following manufacturer's users guide (IZASA, Spain).

Protein S activity: Protein S activity was assayed by ProS Kit, following manufacturer's users guide (IZASA, Spain).

Antithrombin Ⅲ activity was assayed by a commercial kit, following manufacturer's users guide (IZASA, Spain).

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Tests for Fibrinolytic System

The presence and identity of t-PA and its inhibitors in circulating blood were analyzed using the euglobulin fraction of plasma by direct and reverse fibrin autography (zymography) following SDS-PAGE performed as described previously[1)] (expanded methods of tPA zymography are in the supplemental material).

Fearnley test: Tests were carried out in duplicate. In each case blood dilutions were made with phosphate buffer pH 7.4 and clots were made with bovine or human thrombin at 10 units/ml. The tubes were kept in ice-cold water until firm clots appeared and then transferred to a water-bath at 37℃. When the clots were set the tubes were rolled between the palms of hands in order to loosen the clots. The tubes were left in a water-bath at 37℃ and the clots were observed for lysis at regular intervals.

The clot lysis assay was performed following previous methods[6)]. The slopes were calculated as follow. We selected three points in the graph. Point 1 (start of clot formation), Point 2 (OD maximum) and Point 3 (end of clot lysis). Each of these points is indicated by two coordinates (OD and time in seconds). The slope of the clot formation is represented by the formula (OD2-OD1) / (t2-t1). The slope of the fibrinolysis is represented by the formula (OD3-OD2) / (t3-t2).

PAI-1, t-PA measurements: PAI-1 and t-PA were assayed by ELISA methods using kits from Biopool-Menarini, PAI-1 and t-PA antigens were from Biopool-Menarini as well.

Alpha-2-antiplasmin and Plasminogen activity: Alpha-2-antiplasmin and Plasminogen were assayed by commercial kits, following manufacturer's users guide (IZASA, Spain).

Exome Sequencing

Exome sequencing was performed in three familial cases (Ⅳ-6, Ⅳ-7, and Ⅳ-11) and one familial control (Ⅲ-12) (**Fig. 1**). The results were validated in the same samples by Sanger sequencing (**Supplemental Fig. 1**). In the second validation phase, 13 new familial cases and controls were analyzed.

Exome sequencing was performed in the Centro Nacional de Analisis Genómicos (CNAG, Barcelona, Spain). The Illumina TruSeq DNA Sample Prep kit and the NimbleGen SeqCap EZ Exome were used for library preparation and exome enrichment, respectively.

PCR Primers and Conditions

For the validation study the Exon 5 of the FGA gene, where the R458C mutation was located, amplification was performed by PCR. PCR was performed in a mixture containing 100-200 ng DNA, 5 μl (5 μM) forward primer 5′-ATGTAAGTCCAGGGACAAGG-3′, 5 μl (5 μM) reverse primer 5′-GGTGAGAAGAA ACCTGGGAA-3′, 5 μl 10× PCR buffer, 4 μl dNTP buffer, 0.5 μl (5 U μl^{-1}) Taq polymerase (TaKaRaBio Inc.), and up to 50 μl water. The samples were subjected to denaturation at 95℃ for 5 min, followed by amplification consisting of 30 cycles of denaturation at 94℃ for 1 min, annealing at 55℃ for 30s, and extension at 72℃ for 1 min, with a final extension at 72℃ for 5 min, in a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA). Amplicons were purified with the QIAquick PCR purification kit and subjected to cycle sequencing on a GeneAmp PCR System 2700 with BigDye-labeled terminators (Applied Biosystems) and analyzed in an ABI Prism_310 DNA sequencer.

Results

Biochemical laboratory tests detected normal levels or activity for fibrinogen, PAI-1, alpha-2-antiplasmin, protein C, protein S, antithrombin, plasminogen, thrombin-antithrombin complex, D-dimer, beta-tromboglobulin, and coagulation factors activity.

Thrombin time (21.5 s; normal range: 18-25 s) and reptilase time (14.2 s; normal range <22 s) were also normal for the proband and Ⅳ-6 patient (**Fig. 1**). Fearnley test results were abnormally prolonged, indicating an impaired lysis; however, t-PA antigen levels measured by ELISA were high in cases, including the proband (23.5 ng/ml), compared with the normal range (0.15-13.4 ng/ml). The majority of t-PA appeared to be in complex with PAI-1 as detected by zymography (**Supplemental Fig. 2**). However, after an extensive study, the cause of the disease remained undiagnosed.

After quality control analysis of exome sequencing results, 21.787 new mutations not previously described as polymorphisms in dbSNP or 1.000 Genomes project were identified. Sixty nine of these mutations were found in heterozygous in three cases and were not present in the control subject (**Table 1**) following an autosomal dominant heritability. In 6 out of 69 mutations an amino acid change was identified with a moderate or high impact on the protein function coded by the gene, based on Polyphen results (http://genetics.bwh.harvard.edu/pph2/) (**Table 1**). The six mutations and genes were: R458C of *FGA gene*, Splice-site of *ARHGEF37* gene, V67G of *TPRG1L*, T612P of *RARA*, V265G of *ATL3* and V1G of *CELF2*.

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Table 1. Mutations obtained after exome sequencing analysis

The table shows the mutations that have not been described previously in dbSNP or 1000 genomes project and were present in the three cases and absent in the familial control.

CHR	POS	REF	ALT	Effefct_Impact	Functional_Class	AA_change	Gene_Name
chr10	11299536	T	G	MODERATE	MISSENSE	V1G	*CELF2*
chr10	38466875	A	T	MODIFIER	TRANSCRIPT	NO	*RP11-508N22.9*
chr10	39108582	A	T	MODIFIER	INTERGENIC	NO	na
chr10	42355885	C	T	MODIFIER	INTERGENIC	NO	na
chr10	42369451	C	G	MODIFIER	INTERGENIC	NO	na
chr10	42369460	C	G	MODIFIER	INTERGENIC	NO	na
chr10	42380101	T	A	MODIFIER	INTERGENIC	NO	na
chr10	42380949	C	G	MODIFIER	INTERGENIC	NO	na
chr10	42398896	G	T	MODIFIER	INTERGENIC	NO	na
chr10	42398919	A	G	MODIFIER	INTERGENIC	NO	na
chr10	42532591	A	C	MODIFIER	INTERGENIC	NO	na
chr10	42947013	A	G	MODIFIER	INTRON	NO	*CCNYL2*
chr10	46187861	G	C	MODIFIER	TRANSCRIPT	NO	*CTGLF10P*
chr11	63410966	A	C	MODERATE	MISSENSE	V265G	*ATL3*
chr16	33497584	T	C	MODIFIER	INTERGENIC	NO	na
chr16	33866511	C	G	MODIFIER	INTERGENIC	NO	na
chr17	25264916	T	A	MODIFIER	INTERGENIC	NO	na
chr17	25267857	T	A	MODIFIER	INTERGENIC	NO	na
chr17	38512948	A	C	MODERATE	MISSENSE	T612P	*RARA*
chr17	77770440	A	C	MODIFIER	INTRON	NO	*CBX8*
chr18	14187255	A	T	MODIFIER	UPSTREAM	NO	*U6*
chr18	14187255	A	T	MODIFIER	DOWNSTREAM	NO	*ANKRD20A5P*
chr18	18512924	G	C	MODIFIER	INTERGENIC	NO	na
chr18	18515816	C	T	MODIFIER	INTERGENIC	NO	na
chr19	52568032	A	C	MODIFIER	UPSTREAM	NO	*AC011468.1*
chr19	52568032	A	C	MODIFIER	DOWNSTREAM	NO	*ZNF841*
chr1	3542312	T	G	MODERATE	MISSENSE	V67G	*TPRG1L*
chr1	121355224	A	G	MODIFIER	INTERGENIC	NO	na
chr1	142538644	T	C	MODIFIER	INTERGENIC	NO	na
chr1	167063884	T	G	MODIFIER	INTRON	NO	*DUSP27*
chr1	167063884	T	G	MODIFIER	UPSTREAM	NO	*DUSP27;GPA33*
chr20	21213251	G	T	MODIFIER	TRANSCRIPT	NO	*PLK1S1*
chr22	22707151	C	G	MODIFIER	UPSTREAM	NO	*IGLV1-47;IGLV5-48*
chr22	22730530	C	T	MODIFIER	TRANSCRIPT	NO	*IGLV5-45*
chr22	22730530	C	T	MODIFIER	UPSTREAM	NO	*IGLV1-44*
chr2	89853127	C	G	MODIFIER	INTERGENIC	NO	na
chr2	89869921	C	T	MODIFIER	INTERGENIC	NO	na
chr2	89875260	T	A	MODIFIER	INTERGENIC	NO	na
chr2	89875528	A	G	MODIFIER	INTERGENIC	NO	na
chr2	89875649	A	G	MODIFIER	INTERGENIC	NO	na
chr2	89877166	A	T	MODIFIER	INTERGENIC	NO	na
chr2	89879255	A	G	MODIFIER	INTERGENIC	NO	na
chr2	89879384	T	C	MODIFIER	INTERGENIC	NO	na
chr2	89879824	G	C	MODIFIER	INTERGENIC	NO	na
chr2	133019835	A	T	MODIFIER	UPSTREAM	NO	*ANKRD30BL;CDC27P1*
chr2	133020147	C	T	MODIFIER	TRANSCRIPT	NO	*CDC27P1*
chr2	133020147	C	T	MODIFIER	UPSTREAM	NO	*ANKRD30BL*
chr4	49100611	G	A	MODIFIER	INTERGENIC	NO	na
chr4	49100621	T	A	MODIFIER	INTERGENIC	NO	na
chr4	49121358	C	T	MODIFIER	INTERGENIC	NO	na
chr4	88537306	T	C	LOW	SILENT	D1164	*DSPP*
chr4	88537384	T	C	LOW	SILENT	S1190	*DSPP*
chr4	155507209	G	A	MODERATE	MISSENSE	R458C	*FGA*
chr4	190202975	G	A	MODIFIER	INTERGENIC	NO	na
chr5	6447959	C	G	MODIFIER	UPSTREAM	NO	*UBE2QL1*
chr5	148989259	G	A	HIGH	SPLICE_SITE_DONOR	NO	*ARHGEF37*
chr7	4947290	A	C	MODIFIER	INTRON	NO	*MMD2*
chr7	65306563	C	T	MODIFIER	DOWNSTREAM	NO	*RP13-254B10.1*
chr7	100550515	T	A	MODIFIER	UPSTREAM	NO	*MUC3A*
chr7	100550515	T	A	MODIFIER	DOWNSTREAM	NO	*AC118759.1;MUC3A*
chr7	142027951	C	G	MODIFIER	UPSTREAM	NO	*TRBV6-1;TRBV7-1*
chr8	43093147	A	T	MODIFIER	INTERGENIC	NO	na
chr9	33797630	G	A	MODIFIER	INTRON	NO	*PRSS3*
chr9	33797630	G	A	MODIFIER	TRANSCRIPT	NO	*PRSS3;RP11-133O22.6*
chr9	68419021	T	C	MODIFIER	UPSTREAM	NO	*RP11-764K9.2*
chr9	68419021	T	C	MODIFIER	DOWNSTREAM	NO	*CR786580.1*
chr9	68429913	G	A	MODIFIER	TRANSCRIPT	NO	*RP11-764K9.4*
chr9	72653362	T	G	MODIFIER	INTERGENIC	NO	na
chr9	131726634	A	C	MODIFIER	INTRON	NO	*NUP188*

CHR: Chromosome number, Pos: Nucleotide position on the chromosome, Ref: Reference sequence at the position, Alt: Alternative sequence at this position, Effect_impact: Effects are categorized by 'impact': High, Moderate, Low and Modifier depending on results of Polyphen web tool. Functional_Class: the functional class of the variant, AA_change: the change in amino acid produced by a variant in a coding region, given in the following format using one letter amino acid codes: Amino acid in the reference-amino acid position in the protein-amino acid in the variant. Gene_Name: the name of the gene overlapping this position in HGNC gene symbol format, na: non applicable. The same variant position may appear in more than one row, since the same variant can affect different transcripts and could be located in different genes.

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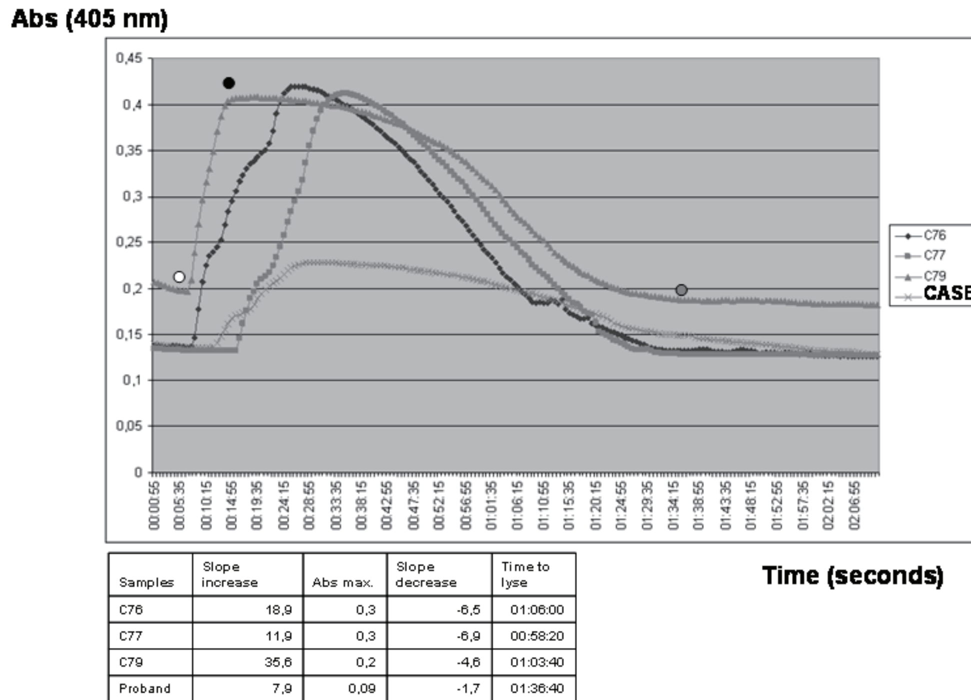

Samples	Slope increase	Abs max.	Slope decrease	Time to lyse
C76	18,9	0,3	-6,5	01:06:00
C77	11,9	0,3	-6,9	00:58:20
C79	35,6	0,2	-4,6	01:03:40
Proband	7,9	0,09	-1,7	01:36:40

Fig. 2. Clot lysis experiment of the proband

C76, C77, and C79 were healthy controls. The graph shows the lower slope decrease of the case compared with three controls, indicating difficulty to lyse the clot. The slopes were calculated selecting three points in the graph. Point 1 (when the clot begins to form, white dot), Point 2 [point of maximum Absorbance (Abs.), black dot], and Point 3 (When just lyses, gray dot). Each of these points are indicated by two coordinates (OD and time in seconds). The slope of the clot formation is represented by the formula (Abs2-Abs1)/(t2-t1). The slope of the fibrinolysis is represented by the formula (Abs3-Abs2)/(t3-t2).
In addition, the time for fibrinolysis was higher in cases compared with controls. Time was measured in hours: minutes: seconds. Abs: Absorbance at 450 nm. Slope was measured in absorbance units/time in seconds.

One of these six mutations, R458C mutation in *FGA*, was previously described[7]. *FGA* codifies for the alpha chain subunit of fibrinogen, and mutations located in this gene have been associated with dysfibrinogenemia, a disease with VTE episodes in some rare cases. Validation tests in three cases and control subjects and the analysis of thirteen new family individuals confirmed the link between the R458C mutation and the disease (**Supplemental Table 1**). In addition, an asymptomatic subject (IV-10) (**Fig. 1**) harboring a R458C mutation was detected; the patient had no history of venous thromboembolic events at the time of evaluation.

The proband and IV-6 patient showed an impaired fibrin polymerization and an impaired fibrinolysis based on clot lysis experiments (**Fig. 2** and **Supplemental Table 2**). The slope of the turbidity increase diminished approximately 2-3 times, and the final turbidity was 67% less than that of the mean result of controls, indicating an altered clot formation process.

The internal fibrin lysis time process after t-PA administration was delayed in both abnormal fibrinogens from proband and IV-6 patient vs. three healthy controls (95.8 ± 0.28 min vs. 62.3 ± 4.04 min, respectively).

The lysis rate was markedly reduced. The slope of the turbidity decrease diminished approximately 3-4 times in proband and IV-6 compared with controls (patients: 1.65 ± 0.07 OD/sec vs. controls: 6 ± 1.22 OD/sec). The diminished slopes and delayed fibrin lysis are associated with clot lysis difficulty, probably due to the difficult binding of t-PA to fibrin.

Discussion

Familial dysfibrinogenemia is a coagulation disorder with a bleeding tendency due to a functional

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alteration of the circulating fibrinogen. In several dysfibrinogenemias, an altered fibrinogen generates aberrant thrombus that in some rare cases increases the risk of venous thrombosis.

Dysfibrinogenemia may be due to mutations in *FGA*, *FGB*, or *FGG* that code for the alpha, beta, and gamma chains of fibrinogen, respectively. More than 580 cases of abnormal fibrinogens have been reported[8]. In our study, the origin of idiopathic thrombophilia was impossible to elucidate using the routine biochemical tests. Only the exome sequencing analysis was useful to find the causes of the familial thrombophilia disease. We studied three affected cousins and a control subject of the family without thromboembolic events. We found a heterozygous R458C mutation in *FGA*, and we considered this as a pathological mutation on the following considerations. First, the mutation was previously described in a family with dysfibrinogenemia and venous thromboembolic disease[7], and it is included in the Human Genome Variation database. Second, the mutation was present in five cases and was absent in 11 healthy family controls. Third, the mutation changes a conservative arginine to cysteine, probably affecting the secondary structure of the protein, as has been suggested previously[9, 10].

The biochemical tests to determine the origin of venous thromboembolic events were performed according to the conventional protocol of our hospital. Only the t-PA antigen and zymography assays detected higher t-PA and tPA-PAI-1 complexes in cases compared with controls. In addition, the Fearnley test detected an impaired fibrinolysis. Other clinical biochemistry results obtained, including fibrinogen levels and reptilase and thrombin times, were within the control range.

Thrombin and reptilase times are the gold standard clinical tests to identify dysfibrinogenemias. Fibrinogen levels could be normal in dysfibrogenemias but thrombin and reptilase times are prolonged. However, this is not the case in some rare cases of dysfibrinogenemia. This problem was documented by Hanss and colleagues[7] who reported a patient with the R458C mutation and normal thrombin and reptilase times, as we observed in our study.

This type of dysfibrinogenemias is very difficult to diagnose due to the normal results of fibrinogen levels and normal thrombin and reptilase times. In cases with idiopathic genetic thrombophilias, when all biochemical tests have been inconclusive and familial heritability has been observed, the use of exome sequencing could be an important clinical tool to perform the diagnosis of the disease, as suggested by our study.

The R458C mutation causes a fibrinogen variant previously identified as fibrinogen Bordeaux[7]. The base substitution found in fibrinogen Bordeaux implies the presence of an unpaired Cys residue. We observed in patients with the R458C mutation a delayed fibrin lysis process (the slope of the turbidity decrease was diminished in the clot-lysis experiments and the time that t-PA needs to do the complete fibrinolysis was prolonged), suggesting an impaired tPA-induced fibrinolysis. This impaired fibrinolysis was also observed with Fearnley tests. This effect has been previously observed in dysfibrinogenemia patients caused by a less permeable fibrin that is more difficult to lyse compared with normal fibrin[11]. Hanss and collegues hypothesized that the R458C mutation produces an aberrant thrombus that blocked the correct binding of t-PA to fibrin, inhibiting fibrinolysis and causing thrombosis. We speculate that our clot lysis experiment suggests the same hypothesis of Hanss and colleagues. The region of the alpha chain of fibrinogen molecule from amino acid 392 to 610, where our mutation is located, participates in fibrinolysis regulation, providing sites for plasminogen and t-PA binding, as it has been previously described[9, 10]. Consequently, the R458C could modify plasminogen–tPA binding to fibrin delaying normal fibrin lysis, as we observed in our clot lysis experiment. We hypothesize that the increased t-PA levels in plasma (measured by ELISA and zymography) are due to an attempt to lyse the altered thrombus. This is the first time that has been described the biological mechanism of the impaired fibrinolysis in fibrinogen Bordeaux, however further studies are needed to confirm these hypotheses. In addition clot-lysis experiments could be a useful strategy to diagnose idiopathic thrombophilias. In our case the fibrinogen Bordeaux was associated with an impaired fibrinolysis detectable by clot-lysis experiments. However, experiments focused on the diagnostic usefulness of clot-lysis experiments in clinical practise await further demonstration.

In summary, we could use exome sequencing to diagnose a family with dysfibrinogenemia that remained undiagnosed for 9 years. We suggest that exome sequencing could be a quick and economically affordable technique to diagnose monogenic idiopathic thrombophilias when current clinical biochemistry tests fail to find the cause. and importantly, we have observed an impaired fibrinolysis associated with fibrinogen Bordeaux, which could be the mechanism associated with the thrombotic accidents.

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Acknowledgments and Notice

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Conflicts of Interest

The authors declare nothing to disclose.

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Supplemental Material

Expanded Methods

Fibrin zymography. The presence and identity of t-PA and its inhibitors in circulating blood were analyzed using the euglobulin fraction of plasma by direct and reverse fibrin autography following SDS-PAGE performed as described previously (Supplemental reference 1). Briefly, MPs were lysed in 100 mM Tris-HCl buffer, pH 8.1, containing 1% Triton X-100. MPs lysates (10 μL from 2.10^5 MPs) and reference proteins (10 μL of t-PA 5 nM and uPA) were electrophoresed in a 7.5% polyacrylamide gel under non-reducing conditions. SDS was then exchanged with 2.5% Triton X-100. After washing-off excess Triton X-100 with distilled water, the gel was carefully overlaid on 1% agarose gel containing 1 mg/mL of bovine fibrinogen, 100 nM plasminogen, and 0.2 NIH U/mL of bovine thrombin. For reverse fibrin zymography, the fibrin gel was supplemented with 0.05 IU/mL of urokinase. Zymograms were allowed to develop at 37℃ for 24 h and photographed at regular intervals using dark-ground illumination. Active proteins in the samples were identified by reference to the migration of known markers (u-PA and t-PA). When required, the fibrin-agarose indicator gel was supplemented with antibodies (10 μg/mL) directed against specific plasminogen activators.

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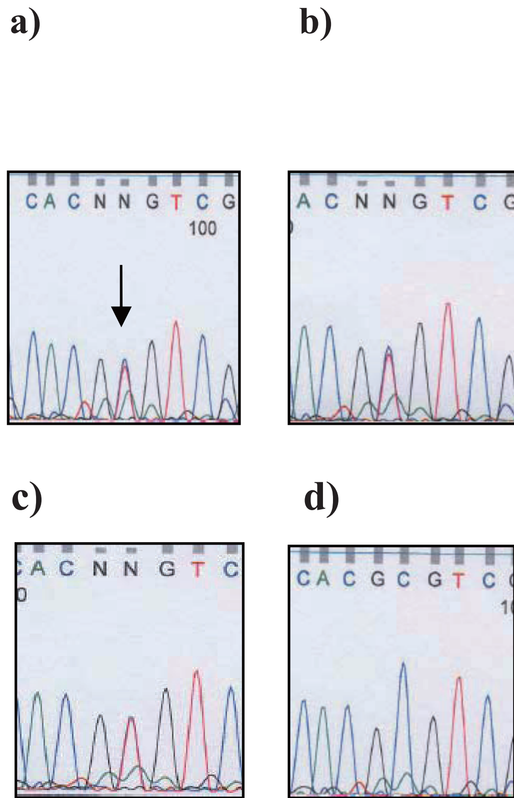
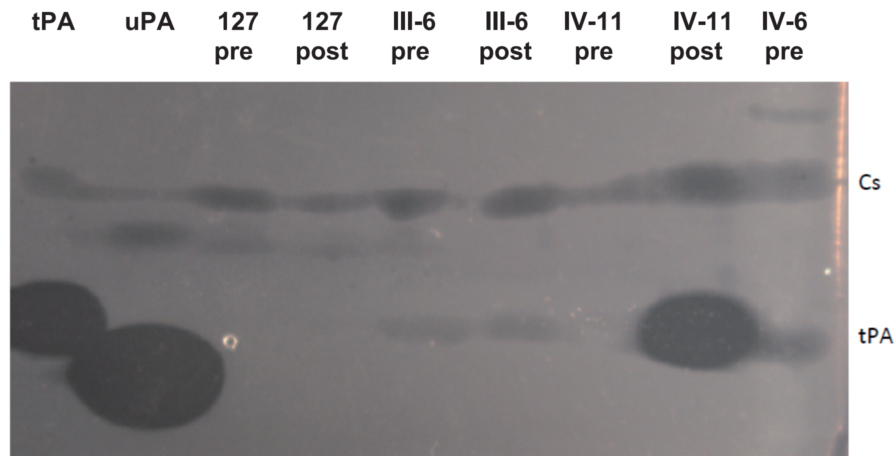


Supplemental Fig. 1. Validation electropherograms of the three cases (a, b and c) and one control (d) analyzed with exome sequencing

The electropherograms confirmed the C to T change, substituting an arginine for a cysteine at 458 position of FGA protein. The arrow indicates the substitution.



Supplemental Fig. 2. Zymography gel showing the activity of t-PA alone (t-PA) or forming complexes (Cs) with inhibitors

From left to right t-PA: marker of t-PA, u-PA: marker of u-PA, 127: healthy control, Ⅲ-6 (proband), Ⅳ-11, and Ⅳ-6 patients. The proband and the cases showed 3x higher t-PA activity (complexes and free t-PA). Pre: sample obtained previous Fearnley test, before physical occlusion of the arm. Post: sample obtained post physical occlusion of the arm. Fearnley test measures the fibrinolytic capacity of the patient.

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Supplemental Table 1. Genetic results of the exome study and validation analysis

Subjects	R458C Mutation	Study
Ⅲ-12	WT	Exome study
Ⅳ-6	MUT	Exome study
Ⅳ-7	MUT	Exome study
Ⅳ-11	MUT	Exome study
Ⅲ-1	WT	Validation study
Ⅲ-6	MUT	Validation study
Ⅲ-10	MUT	Validation study
Ⅲ-17	WT	Validation study
Ⅳ-1	WT	Validation study
Ⅳ-2	WT	Validation study
Ⅳ-3	WT	Validation study
Ⅳ-5	WT	Validation study
Ⅳ-10	MUT	Validation study
V-1	WT	Validation study
V-2	WT	Validation study
V-3	WT	Validation study
Ⅲ-15	WT	Validation study

MUT: mutated, WT: Wild-Type

Supplemental Table 2. Clot-lysis data of the two cases and the healthy controls

Samples	Slope increase (OD/time)	Slope decrease (OD/time)	Time to lyse (seconds)
Control-76	18.9	−6.5	01:06:00
Control-77	11.9	−6.9	00:58:20
Control-79	35.6	−4.6	01:03:40
Proband	7.9	−1.7	01:36:40
Ⅳ-6 patient	7.8	−1.6	01:35:20