ACTN1-related thrombocytopenia: identification of novel families for phenotypic characterization

Short title: Characterization of *ACTN1*-realated thrombocytopenia

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Text: 1199 words

Abstract: 150 words

Figure: 1

Table: 1

Reference: 12

Scientific category: Brief Report

Supplemental data: Table 1S. Table 2S, and Fig. 1S

Key Points

1. *ACTN1* mutations were identified in 10 of 239 families with inherited thrombocytopenia of unknown origin.
2. *ACTN1*-related thrombocytopenia is characterized by mild thrombocytopenia with platelet macrocytosis and low risk of bleeding.

Abstract

Inherited thrombocytopenias (ITs) are a heterogeneous group of syndromic and non syndromic diseases caused by mutations affecting different genes. Alterations of *ACTN1*, the gene encoding for α-actinin 1, have recently been identified in a few families as responsible for a mild form of IT (*ACTN1*-related thrombocytopenia; *ACTN1*-RT). To better characterize this disease, we screened *ACTN1* in 128 probands and found ten (eight novel) missense heterozygous variants in 11 families. Combining bioinformatics, segregation, and functional studies we demonstrated that all but one amino acid substitution had deleterious effects. The clinical and laboratory findings of 31 affected individuals confirmed that *ACTN1*-RT is a mild macrothrombocytopenia with low risk of bleeding. Low reticulated platelet counts and only slightly increased serum thrombopoietin levels indicated that the latest phases of megakaryopoiesis were affected. Given its relatively high frequency in our cohort (4.2%), *ACTN1*-RT has to be taken into consideration in the differential diagnosis of ITs.

Introduction

Inherited thrombocytopenias (IT) are a highly heterogeneous group of diseases characterized by different degrees of severity and complexity.[1] The variable clinical phenotype derives from a wide genetic heterogeneity, at least 22 genes having been identified so far.[2-4] Moreover, mutations in known genes account for only 50% of patients, indicating that not all the existing forms have yet been identified. One of the most recently recognized disorders is *ACTN1*-related thrombocytopenia (*ACTN1*-RT), an autosomal dominant form caused by mutations in the gene (*ACTN1*) encoding for one of the two non-muscle isoforms of α-actinin 1.[5] Mainly expressed in megakaryocytes and platelets,[2] *ACTN1* has a binding domain for cross-linking the actin filaments into bundles. Based on the seven families described in the literature, *ACTN1*-RT is characterized by mild macrothrombocytopenia and bleeding tendency and no additional defects associated with low platelet count.[5,6] To better characterize *ACTN1*-RT, we studied ten families identified after the screening of *ACTN1* in 128 probands with an IT of unknown origin.

Research design

Of 239 consecutive probands with IT examined at the IRCCS Policlinico San Matteo Foundation, (Pavia, Italy), we enrolled all the 128 propositi without a certain diagnosis after the IT diagnostic work-up.[7,8] Mutational screening of *ACTN1* was carried out by whole exome sequencing (WES) in seven individuals and Sanger sequencing in the remaining 121 probands, as indicated in Table 1S. Pathogenetic effects of variants were investigated by segregation analysis, bioinformatic tools, and immunofluorescence studies (Tables 1S; Figures 1 and 1S). Medical history (family history included), bleeding tendency, and outcome of possible surgeries and pregnancies were ascertained from medical records or patient interviews.

Results and Discussion

Characterization of *ACTN1* missense variants

Mutational screening of *ACTN1* identified ten different missense variants in 11 out of 128 probands (Table 1S). Whereas two (p.Arg738Trp and p.Arg752Gln) were known mutations,[5] the others were novel variants not enlisted in dbSNP databases. Interestingly, c.136C>T (p.Arg46Trp) was found in two families and affected the same residue hit by the known c.137G>A (p.Arg46Gln) mutation.[5,6] The eight novel variants affected highly conserved amino acid residues (data not shown) and different pathogenicity prediction tools indicated deleterious effects. All but one (p.Asp666Val) of the variants segregated with thrombocytopenia within pedigrees when family members were available (Figure 1S).

In order to determine the pathogenetic role of the novel missense variants, we performed immunofluorescence analysis in human fibroblasts (Figure 1A). Transfecting wild type constructs, we observed that the cytoskeleton was organized in filaments, with ACTN1 colocalizing with actin. In cells expressing all but one (p.Asp666Val) of the mutations, ACTN1 was instead distributed uniformly within the cytoplasm and actin was no longer organized in filaments. Consistent with segregation analysis, the correct organization of the cytoskeleton in cells expressing p.Asp666Val excluded this variant as a disease-causing mutation. It is worth noting that p.Asp666Val is outside the actin binding (ABD) and calmodulin-like (CaM) domains (Figure 1B), suggesting that only alterations of these functional regions are compatible with the disease.

ACTN1-RT as a mild form of thrombocytopenia with slight platelet macrocytosis

Of the ten families with pathogenetic variants, nine were from Italy and one from United Kingdom. Of the 31 affected family members, 22 were females and nine males, indicating a potential diagnostic bias due to menorrhagia. At the time of recognition of thrombocytopenia, the patients' mean age was 34.5 years (range, 3-82 years). Mean platelet count was slightly reduced ($103 \pm 26 \times 10^9$/L) with only one subject having a platelet count lower than the cut off value (50×10^9/L) for safe platelet count (Table 1).[9] Of note, two young girls (families 5 and 8) had a platelet count slightly above 150×10^9/L, but they were considered as thrombocytopenic according to the age- and gender-specific reference intervals in the Italian population.[10] Both mean platelet volume (12.6 ± 1.7 fL; 95% CI 12.0-13.2) and diameter (3.2 ± 0.5 μm; 95% CI 3.0-3.4) were significantly higher in *ACTN1*-RT than in 50 controls, where values were 10.1 ± 1.4 fL (95% CI 9.7-10.4) and 2.4 ± 0.3 μm (95% CI 2.3-2.5), respectively.

The mean reticulated platelet count evaluated in nine patients using a hematology analyzer was lower

($1.16\pm0.64\text{x}10^9$/L; 95% CI 0.48-2.07) than in 15 controls ($4.3\pm0.96\text{x}10^9$/L; 95% CI 3.55-5.24), indicating that thrombocytopenia derived from reduced platelet formation. To further investigate this aspect, we measured the serum thrombopoietin (TPO) concentration, which is inversely related to the total megakaryocyte and platelet mass,[11] with a commercially available ELISA kit (Quantikine Human TPO Immunoassay, R&D). The mean TPO level was slightly higher (22.3 ± 6.5 pg/mL, 95% CI 13.1-31.5) in the 14 examined patients than in controls (n=40; 14.6 ± 10.8 pg/mL; 95% CI 11.1-18.0), suggesting normal megakaryopoiesis, as also revealed by bone marrow examination of the only patient who received this test.[6] Both young platelet count and TPO levels indicate that thrombocytopenia derives from defects of the latest phases of megakaryocyte maturation. Consistent with this conclusion, investigations in mouse megakaryocytes showed that they had defective proplatelet formation and platelet release when expressing *ACTN1* mutants.[5]

In vitro platelet aggregation in response to ADP, collagen, and ristocetin was normal and the expression level of glycoproteins (GP) GPIbα, GPIX, GPIIb, and GPIIIa on the platelet surface was increased, as expected in individuals with platelet macrocytosis (Table 2S). Consistent with mild thrombocytopenia, as well as with platelet aggregation and expression of major platelet glycoproteins in the normal range, spontaneous bleeding tendency was absent or mild, with a few episodes of epistaxis, gum bleeding, easy bruising and menorrhagia (Table 1). Only three females received iron therapy for menorrhagia. Four of the 18 patients undergoing surgery had excessive bleeding, but only one needed platelet transfusions. Of the 30 deliveries, only one had increased blood loss. Although limited, these severe hemorrhagic episodes suggest caution when hemostatic challenges occurs.

Finally, we did not observe additional phenotypic defects consistently associated with *ACTN1* mutations. However, it is worth mentioning that three individuals from family 2, whose genotypes and platelet counts were not available, developed leukemia (both age of onset and type of leukemia were not available) (Figure 1S). Moreover, individuals from families 1 and 5 had mitral valve prolapse (MVP) and/or atrial sept defect (ASD) (Figure 1S). Since one individual with ASD (family 5) did not carry the mutation (p.Gly251Arg) segregating with thrombocytopenia in his family, a gene other than *ACTN1* is likely to be responsible at least for this heart malformation. Since the search for cardiac anomalies was not systematically performed in our case series, we cannot exclude that other subjects with *ACTN1*-RT had similar defects. Hence, echocardiography may be appropriate in this form of IT.

ACTN1-RT is relatively common among the different forms of IT at least in Italy and Japan. [5] Indeed, we identified *ACTN1* mutations in 10 of 239 (4.2%) consecutive IT families, a frequency similar to that reported in the Japanese population (3.7%).[5] Considering only the ITs with large platelets, the frequency is 6.6% in our population. However, we cannot exclude that *ACTN1*-RT is more frequent than expected, because affected subjects have no pathognomonic features and may be easily misdiagnosed as having immune thrombocytopenia.[12] Four of our patients had this diagnosis and three were treated with steroids without any effect on platelet count. Therefore, molecular genetic testing, combined with segregation analysis and functional assays carried out to discriminate between pathogenic and silent variants, is the only diagnostic approach to identify patients with *ACTN1*-RT.

Acknowledgements

This work was supported by grants GGP13082 from Telethon Foundation (to MS, PN, and AS) and grants 15/12 and 16/12 of IRCCS Burlo Garofolo (to AS). We thank Prof. Kerry J. Rhoden for his critical review of the manuscript.

Authorship

Contributions: R.B. and C.M. carried out mutational screening and analyzed data; M.F. and G.B. performed immunofluorescence analysis and analyzed data; S.K. generated vectors; A.P., C.C., U.R., S.P., L.N., C.B., and P.N. enrolled patients, provided biological samples and analyzed clinical data; C.L.B., M.S., A.S., and P.N. designed research, interpreted data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Figure Legend

Figure 1. **Functional studies of novel *ACTN1* variants.** (A) Immunofluorescence analysis in PD220 fibroblast cell line transiently transfected according to standard procedures. Both wild type (top panel) or mutant (lower panels) *ACTN1* cDNAs were cloned into the pcDNA3.1-Myc tagged expression vector.[5] The subcellular localization of exogenous α-actinin1 (green) was examined using c-myc antibodies (9E10; Santa Cruz Biotechnology, Dallas, Texas, U.S.A.) while the actin filaments were stained with AlexaFluor594 (red) conjugated phalloidin (Invitrogen, Marseille, France). Images were obtained with a Nikon C1si confocal microscope using a 60X Plan Apo objectives. Images were processed for z-projection (maximum intensity), brightness and contrast regulation using ImageJ 1.45 (NIH, Bethesda, USA). The cells shown are representative of three independent experiments. Scale bar =50 µm. (B) Domain structure of α–actinin and localization of ACTN1 mutations identified in Japanese families (arrowheads)[5] and in this paper (arrows). The p.Arg46Gln mutation was also identified in a French family.[6] α-actinin is organized in an actin binding domain (ABD) at the N-terminus, four spectrin repeats and a calmodulin-like domain (CaM) at the C-terminus. Antiparallel molecules dimerize in rod-like structures with the ABD at each extremity for cross-linking the actin filaments into bundles.

Table 1. Features of families with *ACTN1* mutations

Family (N. of individuals)	Mean age at diagnosis Years [range]	WHO bleeding score[a]	Mean platelet count[b,c] $x10^9$/L [range]	Mean platelet volume[b] fL [range]	Mean platelet diameter[d] μm [range]	ACTN1 mutation
F1 (4)	43 [22-55]	0 (1), 1 (3)	107 [89-134]	11.1 [10.1-12.0]	2.8 [2.7-3.0]	p.Asp22Asn
F2 (4)	46 [26-64]	0 (1), 1 (2), 2 (1)	103 [81-118]	12.5 [10.6-14.7]	3.3 [3.0-3.7]	p.Arg46Trp
F3 (4)	42 [14-72]	0 (1), 1 (1), 2 (2)	95 [66-124]	14.8 [14.0-15.6]	3.8 [3.5-4.1]	p.Arg46Trp
F4 (2)	30 [12-49]	0 (2)	103 [97-110]	11.8 [11.3-12.4]	3 [2.8-3.1]	p.Glu225Lys
F5 (6)	23 [7-44]	0 (1), 1 (3), 2 (3)	103 [78-**154**]	12.3 [10.4-14.0]	3.3 [2.6-4.3]	p.Gly251Arg
F6 (2)	58 [34-82]	2 (2)	58 [55-62]	10.5 [10.4-10.6]	3.2 [2.9-3.5]	p.Thr737Asn
F7 (1)	nd	0 (1)	110	12.1	2.5	p.Arg738Trp
F8 (3)	25 [3-44]	0 (2), 1 (1)	112 [65-**166**]	12.5 [11.3-14.3]	2.8 [2.6-3.0]	p.Arg752Gln
F9 (1)	48	0 (1)	117	14.4	3.3	p.Gly764Ser
F10 (4)	38 [3-66]	0 (1), 1 (2), 2 (1)	86 [46-120]	14-3 [12.6-15]	3.5 [2.9-3.9]	p.Glu769Lys

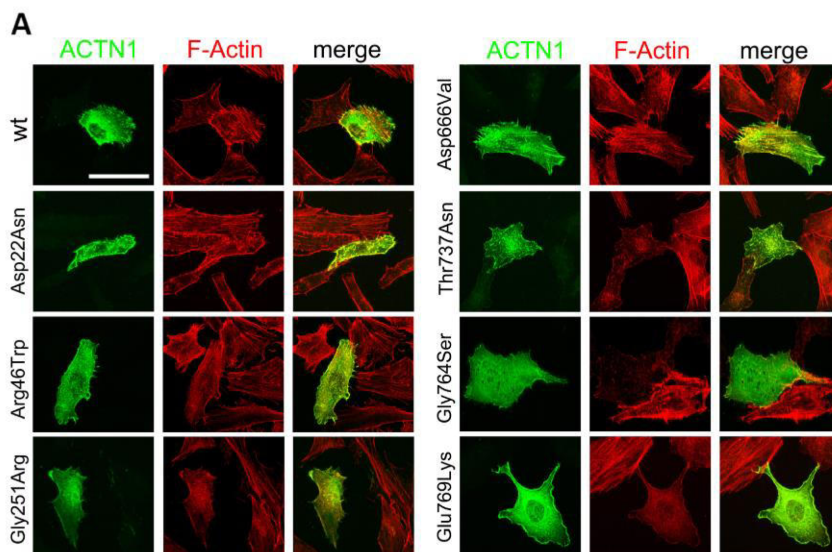
[a]WHO (World Health Organization) bleeding score: grade 0, no bleeding; grade 1, only cutaneous bleeding; grade 2, mild blood loss; grade 3, gross blood loss, requiring transfusion; grade 4, debilitating blood loss, retinal or cerebral associated with fatality.

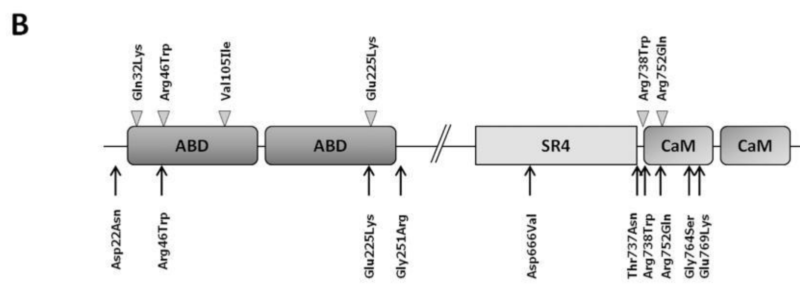
[b]Platelet count and mean platelet volume were measured with a Cell-Dyn Sapphire hematology analyzer (Abbott, Lake Forest, IL, USA) in EDTA-anticoagulated blood samples within one hour from venipuncture.

[c]One individual from family 5 and 8 with more than 150 x 10^9 platelets/L (bold) were classified as thrombocytopenic according to the recently proposed age- and gender specific reference intervals.[19]

[d]Mean platelet diameter was measured on peripheral blood films by optical microscopy and software-assisted image analysis (Axio-vision 4.5; Carl Zeiss, Göttingen, Germany) as previously reported.[17] In brief, blood smears prepared with non-anticoagulated blood from fingerstick were stained with May-Grünwald-Giemsa staining and the mean platelet diameter was calculated from 200 different cell.

Figure 1





SUPPLEMENTAL DATA

ACTN1-related thrombocytopenia: identification of novel families for phenotypic characterization

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Table 1S. Variants identified in the *ACTN1* gene.

cDNA[a,b]	Exon	Protein	Bioinformatic analysis[c]			Segregation (Family)[d]	Cytoskeleton organization[e]	References
			PlyPhen-2	SIFT	Mutation Taster			
c.64G>A	1	p.Asp22Asn	Possibly damaging	Not tolerated	Disease causing	Yes (F1)	Altered	This study
c.136C>T	2	p.Arg46Trp	Probably damaging	Not tolerated	Disease causing	Yes (F2, F3)	Altered	This study
c.673G>A	7	p.Glu225Lys	Possibly damaging	Not tolerated	Disease causing	Yes (F4)	Altered	This study
c.751G>A	8	p.Gly251Arg	Possibly damaging	Not tolerated	Disease causing	Yes (F5)	Altered	This study
c.1997A>T	17	**p.Asp666Val**	Probably damaging	Not tolerated	Disease causing	**No**	**Normal**	This study
c.2210C>A	18	p.Thr737Asn	Possibly damaging	Not tolerated	Disease causing	Yes (F6)	Altered	This study
c.2212C>T	18	p.Arg738Trp	Probably damaging	Not tolerated	Disease causing	nd (F7)	nd	Kunishima et al. 2013[1]
c.2255G>A	18	p.Arg752Gln	Probably damaging	Not tolerated	Disease causing	Yes (F8)	nd	Kunishima et al, 2013[1]
c.2290G>A	19	p.Gly764Ser	Possibly damaging	Tolerated	Disease causing	nd (F9)	Altered	This study
c.2305G>A (WES)[f]	19	p.Glu769Lys	Benign	Not tolerated	Disease causing	Yes (F10)	Altered	This study

[a]Nucleotide A of the ATG translation initiation start site of the *ACTN1* cDNA in GenBank sequence NM_001130004.1 is indicated as nucleotide +1. The only not pathogenetic variant is indicated in bold.

[b]All but one (c.2305G>A) the mutations were identified by Sanger sequencing of the ACTN1 gene. The analysis was restricted to exons encoding for the N-terminus actin binding domain (ABD; exons 1-8) and for the C-terminus calmodulin-like domain (CaM; exons 17-21), where mutations were previously identified.[1,2] PCR products was obtained using standard protocols according to standard protocols and sequenced using the 3130xl Genetic Sequencer (Applied Biosystems, Foster City, CA).

[c]Effect of variations was evaluated using three pathogenicity prediction programs: PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http:// http://sift.jcvi.org/), and Mutation Taster (http://www.mutationtaster.org/).

[d]For segregation analysis in families see Figure 1S.

[e]For deleterious effects of mutations on cytoskeleton organization see Figure 1.

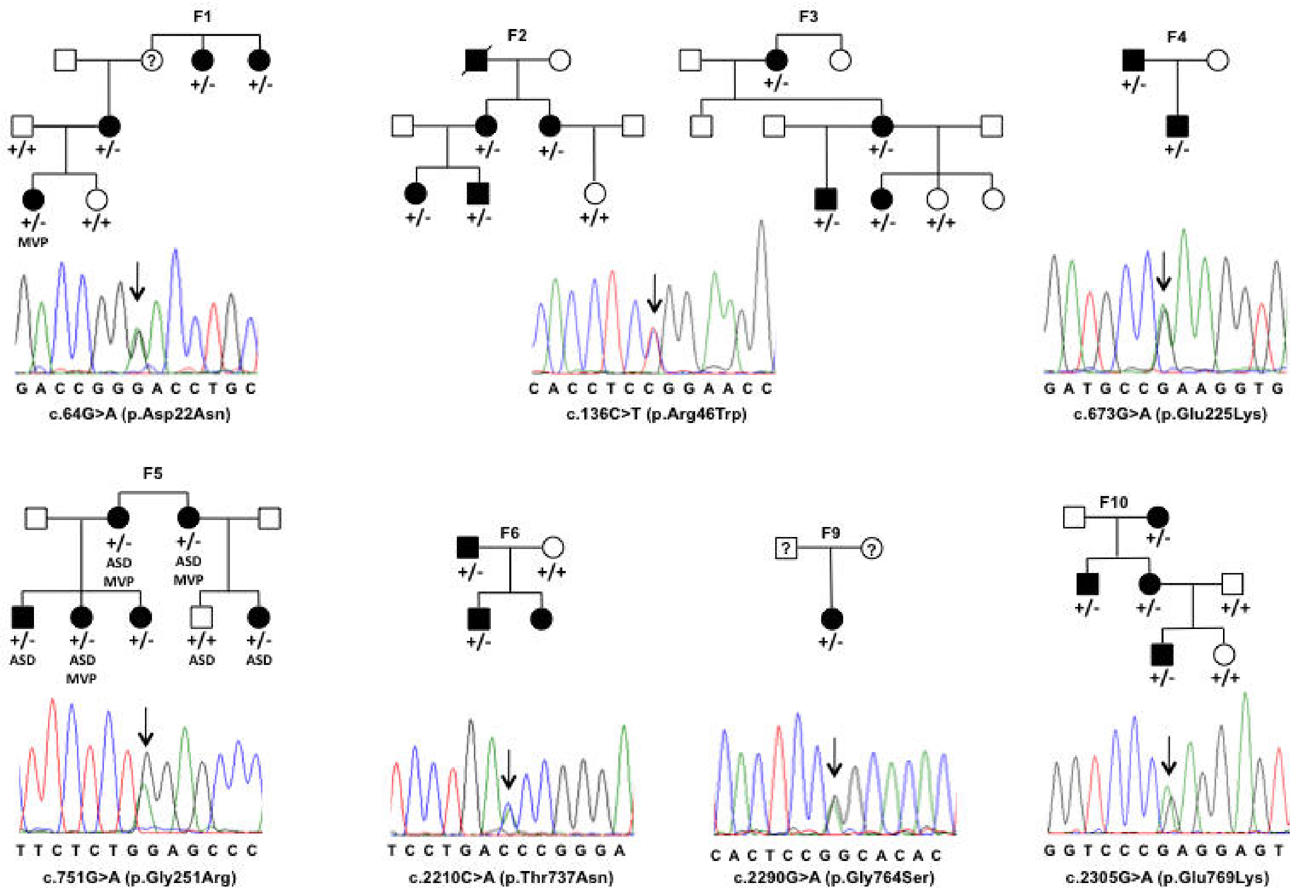
[f]Whole exome sequencing (WES) was performed using the solid-phase NimbleGen SeqCap EZ Exome 44Mb array (Nimblegen Inc., Madison, WI, USA) and sequenced as 91/100 bp paired-end reads on Illumina HiSeq2000 platform (Illumina Inc., San Diego, CA, USA). Variants passing quality filters were annotated using ANNOVAR[15] against NCBI RefGene (http://www.ncbi.nlm.nih.gov). Variants of the *ACTN1* gene were confirmed by Sanger sequencing as above. Variants were confirmed by Sanger sequencing.

Table 2S. In vitro platelet aggregation and flow cytometry of platelet glycoproteins.

Family	In vitro platelet aggregation[a] (N. of patients)			Flow cytometry of platelet glycoproteins (GP)[b] (N. of patients)			
	ADP	Collagen	Ristocetin	GPIba	GPIX	GPIIIa	GPIIb
F1	nd			98 ± 21 (4)	124 ± 8 (4)	94 ± 7 (4)	115 ± 17 (4)
F2	92 ± 10 (4)	90 ± 8 (4)	96 ± 5 (4)	121 ± 32 (4)	142 ± 37 (4)	108 ± 25 (4)	136 ± 21 (4)
F3	68 ± 11 (4)	83 ± 13 (4)	90 ± 10 (4)	154 ± 6 (3)	136 ± 29 (3)	114 ± 6 (3)	120 ± 24 (3)
F4	75 ± 7 (2)	77 ± 4 (2)	88 ± 3 (2)	182 (1)	174 (1)	138 (1)	134 (1)
F5	90 ± 2 (2)	94 ± 4 (2)	91 ± 8 (2)	138 ± 14 (5)	134 ± 12 (5)	126 ± 26 (5)	116 ± 20 (5)
F6	71 (1)	72 (1)	83 (1)	nd			
F7	nd						
F8	nd			134 ± 24 (2)	120 ± 14 (2)	115 ± 9 (2)	112 ± 10 (2)
F9	89 (1)	96 (1)	100 (1)	106 (1)	115 (1)	142 (1)	121 (1)
F10	88 ± 9 (4)	94 ± 5 (4)	89 ± 11 (4)	124 ± 15 (4)	143 ± 25 (4)	127 ± 21 (4)	130 ± 12 (4)

[a]Expressed as mean ± SD percentage of platelet aggregation after 5 min from the addition of ADP (5 μM), ristocetin (1.5 mg/mL) (both from Sigma Chemical Co, St. Louis, MO, USA) and collagen (4 μg/mL; Mascia Brunelli, Milan, Italy). Blood was collected in 3.8% (w/v) sodium citrate (blood:anticoagulant ratio 9:1). To minimize the loss of denser platelets, platelet-rich plasma (PRP) was obtained by sedimentation of the blood at 1 g for 20 to 30 min. Platelet aggregation was measured in PRP by the densitometric method of Born.[3]
[b]Expressed as mean ± SD fluorescence intensity compared to control. Flow cytometric analysis of surface platelet GP expression was performed in PRP obtained from EDTA-anticoagulated as previously reported.[4] The platelets were incubated with the following monoclonal antibodies: SZ2 (Immunotech, Marseille, France) or MB45 (CLB, Amsterdam, the Netherlands) for GPIbα (CD42b) detection; SZ1 (Immunotech) for GPIX (CD42a); SZ21 (Immunotech) for GPIIIa (CD61); P2 (Immunotech) for detection of GPIIb (αIIb) when correctly complexed with GPIIIa. The negative control MO2 and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (GAM-FITC) were purchased from Coulter Corporation, Miami, USA. Fluorescent antibody staining of the platelets was analyzed with the FC500 flow cytometer (Beckman Coulter, Brea, California, U.S.). Ten thousand platelet events were collected and the value of mean fluorescence, expressed in arbitrary units, was recorded. Results were expressed as % of the values obtained in control samples analyzed in parallel. The same control was used for all patients.

FIGURE 1S



Identification of novel *ACTN1* mutations. Pedigrees of eight families with heterozygous novel missense mutations of *ACTN1*. Symbols "+" and "-" indicate wild type and mutant alleles, respectively. In family members with question marks, both phenotype and genotype are unknown. Individuals with only thrombocytopenia are in black symbols; in those with mitral valve prolapse (MVP) and/or atrial sept defect (ASD), the cardiac defect is indicated below the symbol (Families 1 and 5). In Family 2, three relatives (two nieces and one grandniece) of the first-generation affected male developed leukemia (not shown). Chromatogram of each mutation is shown under the corresponding pedigree. Families 2 and 3 share the same c.136C>T mutation.

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