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FACOLTA' DI MEDICINA E CHIRURGIA

XXX Ciclo di Dottorato in Medicina Sperimentale

COMBINING NEXT GENERATION SEQUENCING WITH CLINICAL STUDIES TO UNRAVEL NOVEL INHERITED THROMBOCYTOPENIAS AFFECTING HALF OF THE PATIENTS

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SUMMARY

INTRODUCTION

Since the beginning of the century, our knowledge of inherited thrombocytopenias (ITs) greatly advanced, and we presently know about 30 forms with well-defined genetic defects. This great advancement changed our view of these disorders in that we realized that most patients have only mild thrombocytopenia with inconspicuous bleeding tendency or no bleeding tendency at all. However, better knowledge of ITs also revealed that some of the most prevalent forms expose to the risk of acquiring during infancy or adulthood additional disorders that, much more than hemorrhages, endanger the life of patients. Thus, ITs are complex disorders with quite different clinical features and prognosis (Melazzini *et al*, 2017) especially when referring to those forms we have addressed as "predisposing syndromes" (Table 1). Identification of more than 30 genes whose mutations result in low platelet count greatly advanced also our knowledge on the mechanisms of megakaryopoiesis and proved beyond any doubt that the defective proteins play an essential role in platelet biogenesis. Based on the study of ITs, we better understood the sequence of molecular events regulating megakaryocyte (Mk) differentiation and maturation, and platelet release.

Getting to a diagnosis of certainty is important not only for defining prognosis and personalizing follow-up, but also for using specific treatments that can increase platelet counts in some forms, prevent kidney failure in others, and manage the acquired and often life-threatening comorbidities (Balduini CL *et al,* 2013). Unfortunately, the differential diagnosis with the most common form of thrombocytopenia, the immune thrombocytopenia (ITP), requires a series of complex tests that are available only in a few centers (Balduini CL *et al,* 2003). In addition, nearly a half of patients with ITs do not meet the diagnostic criteria to fit into any known category (new ITs) (Figure 1) (Balduini CL *et al,* 2016). In these cases, prognostic definition is not possible, prenatal or neonatal diagnosis is difficult and genetic counseling is inconclusive. In addition, patients with new ITs cannot benefit from treatments available for the known forms. The simplification of the diagnostic method by developing a targeted sequencing platform and the increase in the number of known ITs by using whole exome sequencing (WES) into a large sample of subjects without a definite diagnosis would thus represent a major step forward in the field of knowledge and management of ITs.

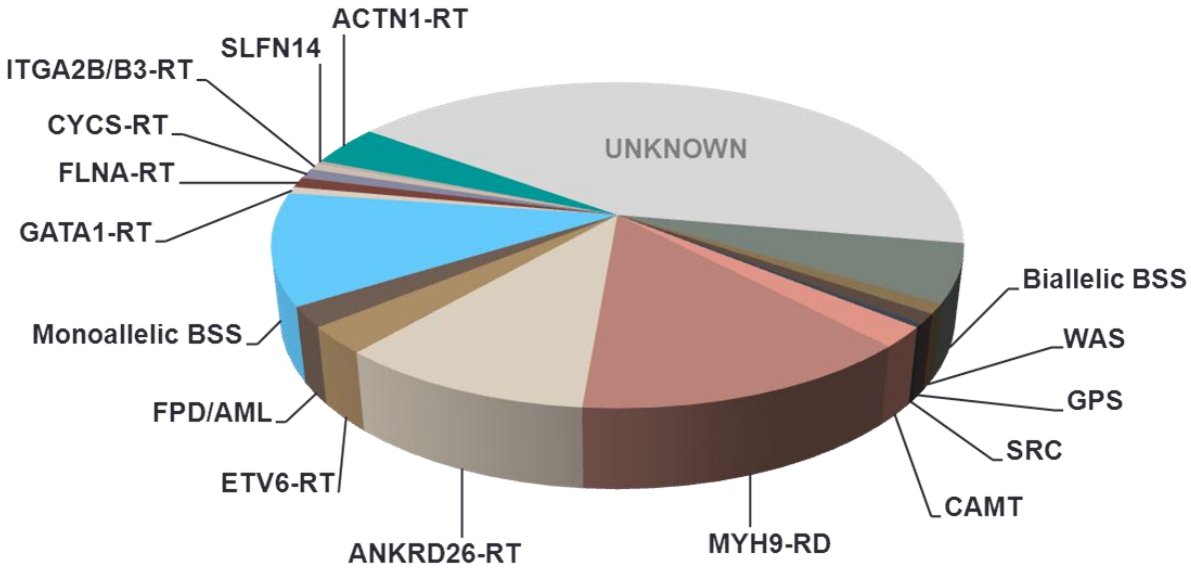

Figure 1. The spectrum of inherited thrombocytopenias nowadays. The reported figure describes IRCCS Policlinico San Matteo Foundation database comprising 303 consecutive families. In this cohort of patients not all known disorders are included, since many forms are extremely rare; 47% of patients belong to the category "IT of unknown origin"

Table 1. Main features of inherited thrombocytopenias predisposing to additional disorders.

Disease (abbreviation, OMIM entry)	Inheritance	Gene (locus)	Thrombocytopenia[1]	Platelet size	Additional features
FORMS PREDISPOSING TO EXTRA-HAEMATOLOGICAL DEFECTS					
MYH9-related disease (*MYH9*-RD, na)	AD	*MYH9* (22q12)	+/+++	Large/Giant	Risk of sensorineural deafness, nephropathy, cataract (genotype-phenotype correlations identified). Elevated liver enzymes in some cases. Döhle-like inclusions in granulocytes.
DIAPH1-related thrombocytopenia (DIAPH1-RT, na)	AD	*DIAPH1* (5q31.3)	+/+++	Large	Risk of sensorineural deafness during infancy. Possible mild transient leucopenia.
SRC- related thrombocytopenia (SRC-RT, na)	AD	*SRC* (20q11.23)	++/+++	Large	Congenital facial dysmorphism, juvenile myelofibrosis and splenomegaly, severe osteoporosis, premature edentulism. Platelets hypogranular or agranular. Abundant vacuoles.
FORMS PREDISPOSING TO HAEMATOLOGICAL MALIGNANCIES					
Familial platelet disorder with propensity to acute myelogenous leukemia (FPD-AML, 601399)	AD	*RUNX1* (21q22)	++	Normal-Slightly increased	Over 40% of patients acquire acute myelogenous leukemia or myelodysplastic syndromes. Increased risk of T acute lymphoblastic leukemia.
ANKRD26-related thrombocytopenia (*ANKRD26*-RT or THC2, 188000)	AD	*ANKRD26* (10p12)	++/+++	Normal-Slightly increased	About 8% of patients acquire myeloid malignancies. Some patients have increased levels of hemoglobin and/or leukocytes.
ETV6-related thrombocytopenia (*ETV6*-RT, na)	AD	*ETV6* (12p13)	+/++	Normal-Slightly increased	Increased risk of acute lymphoblastic leukemia (20%) mainly in childhood and other hematological malignancies.
MPIG6B- congenital Myelofibrosis *(MPIG6B*-cMF, na*)*	AR	*MPIG6B* (c.147insT) (c.6161+ 1dup)	+/+++	Large	Mild anemia, mild leukocytosis and bone marrow reticulin fibrosis centered around clusters of atypical megakaryocytes
FORMS PREDISPOSING TO BONE MARROW APLASIA					
Congenital amegakaryocytic thrombocytopenia (CAMT, 604498)	AR	*MPL* (1p34.2)	+++	Normal-Slightly reduced	Reduced/absent BM megakaryocytes. Evolution to severe bone marrow aplasia in infancy in nearly all patients.
THPO-related disease (*THPO*-RD, na)	AR	*THPO* (3q27.1)	++/ +++	Normal-Slightly reduced	Bone marrow hypoplasia-aplasia. Possible mild thrombocytopenia in heterozygous subjects.
Radioulnar synostosis with amegakaryocytic thrombocytopenia (RUSAT, 605432)	AD/AR	*HOXA11* (7p15) or *MECOM* (3q26.2)	+++	Normal-Slightly increased	Bilateral radio-ulnar synostosis +/- other malformations. Reduced/absent BM megakaryocytes. Possible evolution to bone marrow aplasia. The hematologic phenotype is more severe in the AR form due to *MECOM* mutations.
Thrombocytopenia-absent radius syndrome (TAR, 274000)	AR	*RBM8A* (1q21)	+++	Normal-Slightly increased	Bilateral radial aplasia +/- other upper and lower limb abnormalities. Reduced megakaryocytes in BM. Possible kidney, cardiac, and/or CNS malformations. Possible intolerance to cow's milk. Platelet count may raise over time.

[1] Degree of thrombocytopenia: +, > 100 x 10^9 platelets/L; ++, 50-100 x 10^9 platelets/L; +++, < 50 x 10^9 platelets/L

Abbreviations: Inh. = Inheritance. AD = autosomal dominant. AR = autosomal recessive. XL = X-linked. na= not available. CNS = central nervous system. BM = bone marrow.

BACKGROUND

ITs are a heterogeneous group of diseases characterized by a reduced number of platelets and bleeding tendency of various degree. They can be associated to other congenital or acquired defects/pathologies.

Managing ITs is still challenging because of several problems: half of the patients remains without a definite diagnosis because affected by unknown forms; diagnosing the known forms of ITs is still difficult because it is based on a diagnostic algorithm that requires a complex set of laboratory tests (Figure 2,); effective treatments are only available for a limited number of ITs.

This PhD project wants to address these shortcomings through three integrated approaches:

1. identification of genes involved in new forms of ITs through WES in a large sample of patients without a diagnosis despite extensive investigations;
2. development of a new generation sequencing platform aimed at recognizing all the genes involved in the known forms of ITs;
3. identification of possible and useful therapies that could lead to an increase in platelet count for the new forms of ITs.

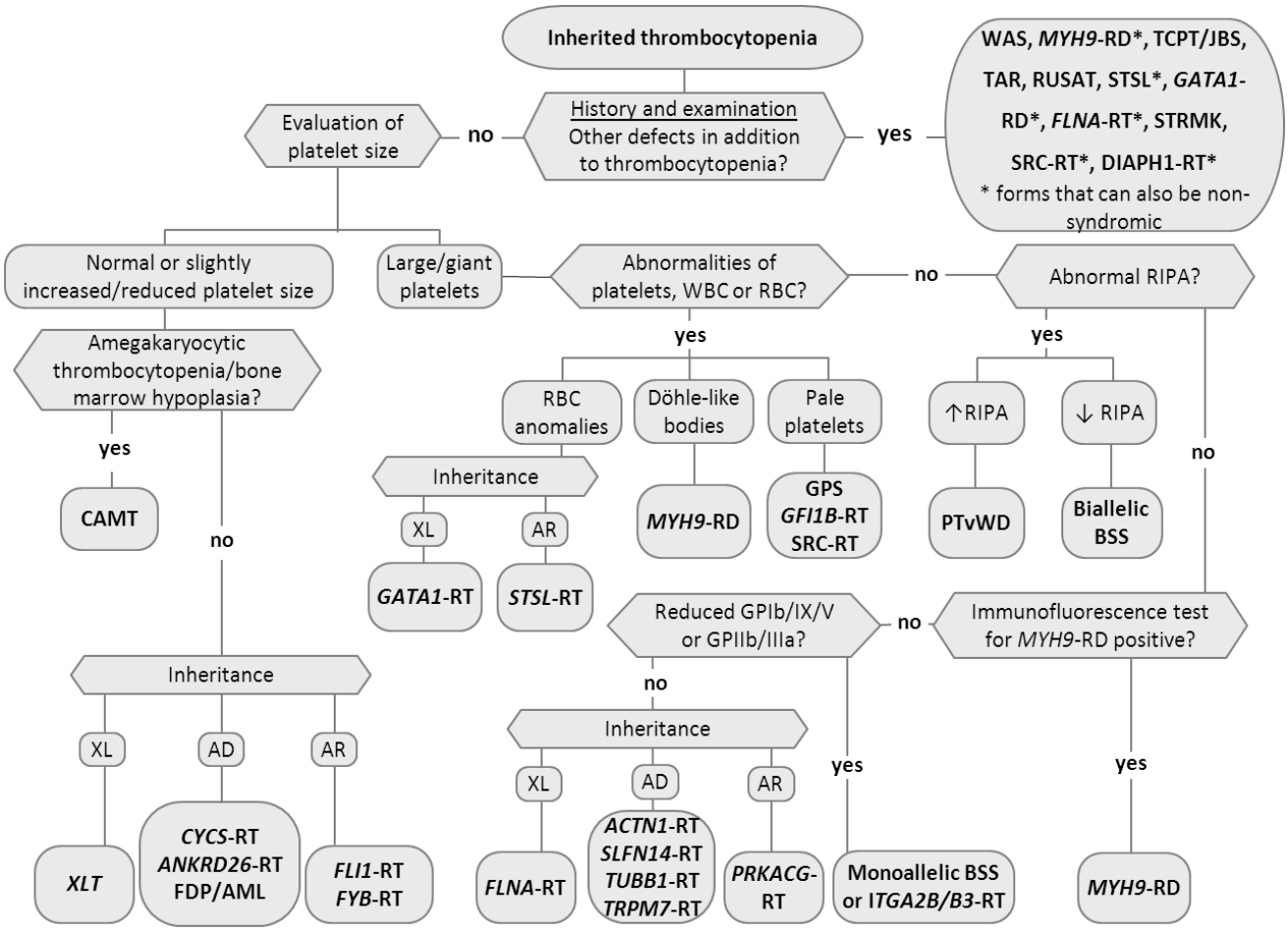

Figure 2. Diagnostic algorithm for inherited thrombocytopenias used for classification and enrollment of patients to the project. Algorithm proposed by the Italian Platelet Study Group in 2003 and updated at the time of the beginning of the study (modified from Balduini CL *et al*, 2003).

AIMS OF THE PROJECT

This project started in 2014. At that time 22 were the mutations of the genes responsible for the 19 forms of ITs identified so far, and our group, in collaboration with the Universities of Trieste and Bologna, hypothesized that such genetic heterogeneity could also re-emerge in patients affected by new forms of ITs. We expected that the next generation sequencing (NGS) approach, together with the phenotypical characterization in specialized centers, would allow us to identify new pathologies, thus making the diagnostic path even more complex. However, we believed that the targeted sequencing by Ion Torrent of all the old and new genes still represents an important step in terms of effectiveness, cost and speed in diagnosing these disorders, allowing differential diagnosis with ITP.

Specifics Aims

Aim 1: *characterization of new ITs*. Once the mutations in the new genes of ITs was confirmed, phenotypic characterization (as clinic and laboratory) of patients (and their affected family members) with mutations in the same gene (new ITs) has been performed. Through the realization of *in vitro* models of megakaryopoiesis, it would be possible to study the pathogenetic mechanisms underlying the new forms of thrombocytopenia and the efficacy of some promising drugs in stimulating platelet formation.

Aim 2: *recognition of new forms of ITs through the identification of causative mutations in new genes by using WES.* For this purpose, we studied a large case series of patients left without a definitive diagnosis, despite the careful application of the conventional diagnostic algorithm (table 2). In larger families, linkage studies were conducted to narrow the regions where the probability of finding candidate genes could be greater, thus facilitating the detection of causative mutations.

Aim 3: *development of a sequencing tool for the targeted analysis of genes involved in ITs* (those already known and those identified during this study), Ion Torrent PGM TM * (Life Technologies). This technique has been validated by applying it to a wide range of patients with known mutations.

Experimental Design-Aim 1: CHARACTERIZATION OF NEW ITs

All enrolled patients underwent to clinical and basic laboratory characterization according to the aforementioned diagnostic algorithm (Balduini CL *et al*, 2013; Balduini CL *et al*, 2003) so that the essential features of new ITs were available as soon as mutations in new genes were identified.

We also wanted to study the pathogenetic mechanisms of the thrombocytopenia and to identify possible treatments to increase platelet count. To achieve these goals, we developed, in collaboration with the Department of Molecular Biology of this University, an *in vitro* experimental model that reproduces human megakaryopoiesis from peripheral blood of patients. For these studies, hematopoietic progenitors has been separated from a few mL of patient blood and induced differentiation of Mks *in vitro* in the presence of thrombopoietin (THPO), IL-6, IL-11 and various components of the extracellular matrix (Balduini A *et al*, 2009; Bluteau D *et al,* 2014*).* After differentiation of Mks, proliferation, maturation, formation and release of pro-platelets and platelet formation have been evaluated. Finally, the effects of various molecules potentially stimulating platelet have been tested.

Experimental Design-Aim 2: RECOGNITION OF NEW FORMS OF ITs THROUGH THE IDENTIFICATION OF CAUSATIVE MUTATIONS IN NEW GENES BY USING WES

(in collaboration with the University of Bologna)

The searching for new genes responsible for ITs was performed by applying WES to 130 probands, which remained without a clinical/molecular definitive diagnosis after the conventional diagnostic approach. WES was also applied to patients enrolled prospectively where Ion PGM had not detected mutations in known genes.

In the larger families, we combined WES with linkage studies, which is in fact effective in identifying rare causative variants in Mendelian genetics. For this purpose, genotyping of these families was used to generate data sets for linkage analysis that were performed for each family separately and for all families exhaustively, in order to identify a chromosomal region shared by two or more

families. All regions approaching the maximum predicted LOD score had been considered candidate intervals and used as a filter to limit the number of candidate variants in WES data analysis.

For families where linkage analysis revealed candidate regions, only the variants included in these ranges were considered as candidates. In addition, all genes found mutated in at least two unrelated patients were considered candidates. The strongest candidates were validated through Sanger sequencing. Subsequently, the segregation of such genes within families were confirmed or not.

Experimental Design-Aim 3: DEVELOPMENT OF A SEQUENCING TOOL FOR THE TARGETED ANALYSIS OF GENES INVOLVED IN ITs

(in collaboration with the University of Trieste)

We are developing an Ion PGM sequencing tool (Figure 3) for screening all genes whose mutations are responsible for ITs. For its validation, Ion PGM has been applied to patients with known forms of ITs, whose mutations have been previously recognized/confirmed by Sanger sequencing. To estimate the quality of the process, we evaluated the correspondence rate of each amplicon (currently 650) and identified those that were not sufficiently concordant (<30X). By comparing the sequencing data of the same genomic regions obtained by Sanger and Ion Torrent methods, we calculated the number of false positive and false negative variants that together with the true positive and negative variants determined the specificity and sensitivity of the Ion Torrent platform.

Once validated, the Ion PGM platform has been applied to prospectively enumerated purposes. The study was performed blindly, without taking into account the results of the clinical and laboratory characterization of patients lost to follow-up. Thus the data obtained were able to define the diagnostic accuracy of the new platform compared to the traditional diagnostic algorithm (Balduini CL *et al*, 2013; Balduini CL *et al*, 2003).

Figure 3. Ion Torrent PGM TM* (Life technologies) sequencing tool for targeted analysis of IT genes.

Thanks to this multidisciplinary approach we expected to increase the number of known ITs, simplify the diagnostic approach to ITs, identify new pathogenic mechanism underlying ITs and find drugs that can be useful in increasing platelet count in these patients.

This approach allowed us to reach almost all the goals we prefigured to date. The following chapters will report the publications that this research brought.

MATERIALS & METHODS

The institutional review board of the San Matteo Foundation approved the study and all subjects or their legal guardians signed written informed consent in accordance with the Declaration of Helsinki.

CHARACTERIZATION OF NEW ITs

In addition to basic characterization (personal history, clinical evaluation, blood cell count), patients with new ITs received the following studies:

- ✓ measurement of bleeding tendency by using the World Health Organization (WHO) bleeding scale
- ✓ platelet morphology by optical microscopy (OM);
- ✓ platelet ultrastructure by electronic microscopy (EM);
- ✓ platelet membrane glycoproteins by flow cytometry (FC);
- ✓ *in vitro* platelet aggregation and release reaction by optical density and lumiaggregometry, respectively;
- ✓ *in vitro* platelet adhesion and spreading on various physiological substrates by immunofluorescence and OM.

WHO bleeding scale

The degree of bleeding tendency of patients enrolled in the study was evaluated by the bleeding scale proposed by the WHO (Miller AB *et al*, 1981), which contemplates the following levels of spontaneous bleeding:

- ✓ Grade 0: no bleeding
- ✓ Grade 1: cutaneous bleeding (i.e. petechiae)
- ✓ Grade 2: mild bleeding (i.e. gum bleeding, occasional epistaxis)
- ✓ Grade 3: moderate to severe bleeding (i.e. menorrhagia conditioning anemia)
- ✓ Grade 4: severe and debilitating bleeding requiring transfusions

Manual Platelet Count

Five µl of capillary blood were dilute with 105 µl of 1M ammonium chloride solution in order to induce red blood cell lysis; the platelet count was performed using the Bürker chamber by optical microscope using a 40x lens.

Evaluation of platelet count and mean platelet volume by automated counter

Automatic evaluation of platelet count and of their mean volumes were performed by analyzing K3-EDTA anticoagulated venous blood samples by ADVIA 120® automatic counter (Bayer, Tarrytown, NY, USA).

In optical counters the mean platelet volume (MPV) is obtained measuring the light scattered at the lower angle of laser diffraction; a platelet histogram is produced from measurements corresponding to the size and number of platelets and then the MPV is calculated from the geometrical mode of such histogram (Briggs C, 2009). Based on the specific technology, both platelet counting and sizing obtained by optical counters are expected to be more reliable than the impedance ones: in particular, large platelets are correctly identified based on their peculiar density and then correctly counted and sized.

The reference range of MPV has been investigated in different populations because of the increasing interest about its role in several disorders. Since population studies have been achieved with different cell counters and without standardization of reading times, MPVs of healthy peoples resulted very different, ranging from 6.0 to 13.2 fL in the different studies (Demirin H *et al*, 2011; Hoffmann JJ, 2012; Lippi G *et al*, 2012).

Peripheral and bone marrow smears examination and mean platelet diameters evaluation

Blood films were set up using capillary or bone marrow blood, May–Grünwald–Giemsa (MGG) stained, and examined by optical microscope for the assessments required by the aforementioned diagnostic algorithm for IT as well as for basic morphological evaluations. Platelet diameters were measured by optical microscopy with 100x magnification on peripheral

blood smears using Axiovision 4.5® software (Zeiss, Oberkochen, Germany) (Figure 4), calculating the average platelet diameter of 200 elements.

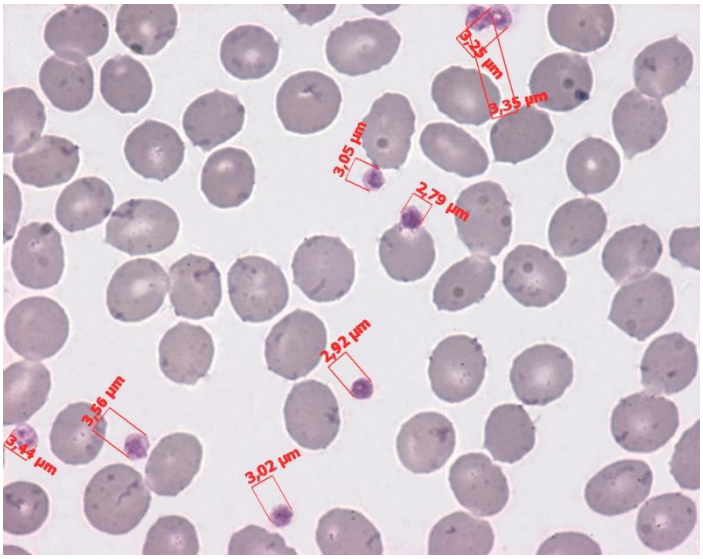

Figure 4. Platelets diameter evaluation, measured using AxioVision 4.5® software on peripheral blood films stained with May–Grünwald–Giemsa .

"In vitro" platelet aggregation

In vitro platelet aggregation was performed according to the turbidometric method developed by Born in 1962 (Born, 1962) using a Chrono-Log Lumi-Aggregometer (Chrono-Log Corporation, Hawerton, St. Louis, MO, USA). Platelet function was evaluated on Platelet Rich Plasma (PRP) obtained by centrifugation of sodium citrate anticoagulated blood for 10 minutes at 210xg; platelets were stimulated by collagen 4 µg/ml (Mascia Brunelli, Milan, Italy), adenosine diphosphate (ADP) 5 µM, and ristocetin 1.5 and 3.0 mg/ml (both obtained from Sigma-Aldrich, St. Louis, MO, USA). Platelet aggregation was evaluated after 5 minutes of platelet stimulation with single agonists as a percentage variation of optical density versus baseline value.

Surface platelet glycoprotein expression

Platelet surface glycoproteins (GP) expression was investigated by flow cytometry. A volume of 30 µl K_3-EDTA anticoagulated venous blood samples were incubated for 30 minutes in the dark

at room temperature with 10 µl of the solutions of the different fluorescein-labeled monoclonal antibodies. At the end of the incubation, the cells were suspended in 2.5 ml of PBS and analyzed by Cytomics FC 500 cytometer (Beckman Coulter, Brea, California, USA). Platelets have been identified based on their size and density (forward-scatter and side-scatter respectively); an electronic window on platelets allowed exclusion of white blood cells and red blood cells. For each single analysis, the mean fluorescence intensity value was recorded, expressed in arbitrary units. The values were compared to those obtained in a healthy subject, the same for all patients. The monoclonal antibodies used were as follows:

- P2 (Beckman Coulter) against the GP IIb-IIIa complex (CD41);
- SZ21 (Beckman Coulter) against GP IIIa (CD61);
- SZ2 (Beckman Coulter) against a GPIbα epitope, localized at the N-terminal region of the glycoprotein, the one involved in the vWF binding (CD42b);
- MB45 (Sanquin, Amsterdam, The Netherlands) against a GPIbα epitope (CD 42b);
- SZ1 (Beckman Coulter) against GPIX, belonging to GPIb-IX complex (CD42a);
- Gi9 (Beckman Coulter) against GPIa-IIa complex (CD49b);
- 679.1Mc7 (Beckman Coulter) fitched anti-IgG murine immunoglobulin (IgG1-FITC).

Platelet ultrastructure

Peripheral blood samples obtained by healthy volunteers and patients were collected in 3.8% of sodium citrate tubes. After 60 minutes of sedimentation, the supernatant was transferred to a test tube containing K_3-EDTA to avoid platelet activation in subsequent stages of sample setup. Cells were recovered by centrifugation at 730xg for 15 minutes and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 4% Cocodylated buffer for 4 hours; the cells were finally fixed in tetrahedron at room temperature for 60 minutes and included in eponaldehyde.

Ultra-thin sections (70 nm) were cut from resin blocks and images were captured with a Jeol JEM-1200 EX II (Tokyo, Japan) transmission microscope equipped with Mega View III Olympus CCD

Camera (Olympus, Tokyo, Japan) to a magnification of 25000x.

Platelet adhesion & spreading

Glass coverslips were coated with type I collagen, von Willebrand Factor, or fibrinogen, and blocked with bovine serum albumin (BSA) (Pecci A *et al*, 2011). Washed platelets were prepared (Canobbio I *et al*, 2013) and aliquots of 1 x 10^8 platelets in 2.5 mL HEPES with 1 mg/mL BSA, 5.5 mM glucose and 2 mM MgCl2 were incubated on coated coverslips for 45 minutes at 37°C. After washing, specimens were fixed and stained with Alexa Fluor 488-conjugated phalloidin (Life Technologies, Carlsbad, CA) (Pecci A *et al*, 2011). Samples of patients and controls were processed simultaneously. At least 10 random microscopic fields at 63x magnification were acquired per each specimen for image analysis, which was performed by the Axiovision 4.6 software (Zeiss). The following parameters were assessed: number of adherent platelets, percentage of spread platelets and average platelet area.

WHOLE EXOME SEQUENCING

WES results were analyzed as follows:

- ✓ Aligning sequences with the reference genome using BWA software (Li H *et al,* 2009) and verifying alignment parameters with SAMtools (Li H, Handsaker B *et al,* 2009);
- ✓ Recalibrating quality scores and realigning through the GATK package (DePristo MA *et al,* 2011);
- ✓ Removing duplicate PCRs with the Picardtools MarkDuplicates utility (picard.sourceforge.net);
- ✓ Calculating Coverage Extent, Variation Position, and Data Selection with GATK Package
- ✓ Annotating variants with "gene-based" methodology in RefSeqGene (ncbi.nlm.nih.gov) and selecting the same on the basis of their novelty by searching in dbSNP and EVS.

Once all the variants had been identified, genetists prioritized: variants in conserved residues among species (evaluated through PhyloP, PhastCons and GERP scores); variants with deleterious

effect on protein (evaluated via SIFT, Polyphen2, Mutation Taster); variants related to ITs-related genes (evaluated through ToppGene, Endeavor, Exomiser, Extasy); variants of expressed genes and/or specific genes for the megakaryocytic line (evaluated through Human Protein Atlas, Haematlas, PlateletWeb and an internal database of Mk expression profiles).

Details will be specifically described below, for each work reported.

ION TORRENT PGMI SEQUENCING

Recommended protocols from Life Technologies were used. The primers were designed with the Designer Ion Ampliseq software (https://www.ampliseq.com/browse.action) and clustered in the amplification libraries. PCR products were lined up with Ion Xpress Barcode adapters to read multiple samples on the same chip. The sequencing data were analyzed with the Suite Ion Torrent software. Using the Coverage Analysis plug-in (TSCA, v3.6), we evaluated the data quality. The Variant Caller plug-in (TSVC, v.3.6) aligned sequencing data with the human genome sequence hg19 to locate variants, whose functional annotation had been performed by ANNOVAR software.

For enrolled patients, Sanger sequencing was performed in exons where Ion Torrent PGMI detected variants with MAF <0.01, the effect of which was determined by pathogenicity prediction programs, such as PolyPhen-2 MutationAssessor, MutationTaster, NNSplice, and Human Splicing Finder.

Details will be specifically described below, for each work reported.

RESULTS

ARTICLE

Clinical and pathogenic features of *ETV6*-related thrombocytopenia with predisposition to acute lymphoblastic leukemia

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ABSTRACT

ETV6-related thrombocytopenia is an autosomal dominant thrombocytopenia that has been recently identified in a few families and has been suspected to predispose to hematologic malignancies. To gain further information on this disorder, we searched for *ETV6* mutations in the 130 families with inherited thrombocytopenia of unknown origin from our cohort of 274 consecutive pedigrees with familial thrombocytopenia. We identified 20 patients with *ETV6*-related thrombocytopenia from seven pedigrees. They have five different ETV6 variants, including three novel mutations affecting the highly conserved E26 transformation-specific domain. The relative frequency of *ETV6*-related thrombocytopenia was 2.6% in the whole case series and 4.6% among the families with known forms of inherited thrombocytopenia. The degree of thrombocytopenia and bleeding tendency of the patients with *ETV6*-related thrombocytopenia were mild, but four subjects developed B-cell acute lymphoblastic leukemia during childhood, resulting in a significantly higher incidence of this condition compared to that in the general population. Clinical and laboratory findings did not identify any particular defects that could lead to the suspicion of this disorder from the routine diagnostic workup. However, at variance with most inherited thrombocytopenias, platelets were not enlarged. *In vitro* studies revealed that the maturation of the patients' megakaryocytes was defective and that the patients have impaired proplatelet formation. Moreover, platelets from patients with *ETV6*-related thrombocytopenia have reduced ability to spread on fibrinogen. Since the dominant thrombocytopenias due to mutations in *RUNX1* and *ANKRD26* are also characterized by normal platelet size and predispose to hematologic malignancies, we suggest that screening for *ETV6*, *RUNX1* and *ANKRD26* mutations should be performed in all subjects with autosomal dominant thrombocytopenia and normal platelet size.

Introduction

Until the end of the last century, only a few forms of inherited thrombocytopenia were known, all of which were extremely rare and characterized by a severe bleeding tendency. Since then, knowledge of these thrombocytopenias has improved

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CLINICAL AND PATHOGENETIC FEATURES OF *ETV6*-RELATED THROMBOCYTOPENIA WITH PREDISPOSITION TO ACUTE LYMPHOBLASTIC LEUKEMIA

Melazzini F, Palombo F, Balduini A, De Rocco D, Marconi C, Noris P, Gnan C, Pippucci T, Bozzi V, Faleschini M, Barozzi S, Doubek M, Di Buduo C, Kozubik K, Radova L, Loffredo G, Pospisilova S, Alfano C, Seri M, Balduini CL, Pecci A and Savoia A. Clinical and pathogenetic features of ETV6-related thrombocytopenia with predisposition to acute lymphoblastic leukemia. Haematologica. 2016 Nov;101(11):1333-1342.

INTRODUCTION

In 2015, the IRCC Policlinico San Matteo Foundation and other three independent studies showed that mutations in the gene *ETV6* (Figure 5) are responsible for a new form of IT and suggested that *ETV6*-related thrombocytopenia (*ETV6*-RT) predisposes to acute lymphoblastic leukemia (ALL) (Moriyama T *et al*, 2015; Noetzli L *et al*, 2015; Topka S *et al*, 2015; Zhang MY *et al*, 2015). However, only a few families have been reported so far and the clinical and laboratory features of *ETV6*-RT remain poorly defined.

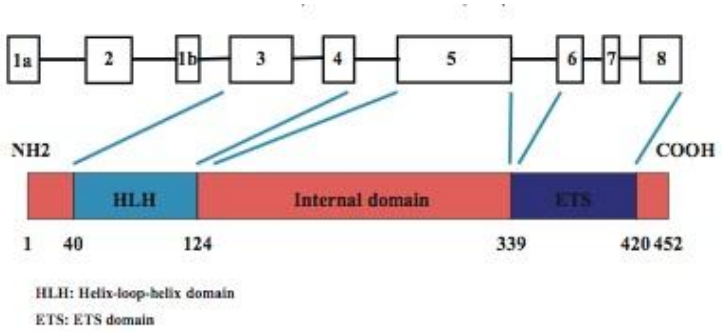

Figure5. *ETV6* and its protein. Diagram above: *ETV6* gene. White rectangles represent the exons with their numbering. Scheme at the bottom: ETV6 protein. The numbers refer to amino acid residues.

In order to gain further information on this disorder, we screened for *ETV6* mutations 130 consecutive unrelated propositi with IT of unknown origin and identified 7 affected families. Below we describe the genetic, clinical and laboratory features of 20 affected subjects and discuss the pathogenesis of *ETV6*-RT based on the results of *in vitro* study of patients' Mks and platelets.

PATIENTS & METHODS

Patients

From 2003 to 2014, 274 consecutive unrelated probands with familial thrombocytopenia were analyzed at the IRCCS Policlinico San Matteo Foundation of Pavia. By the application of a well-defined diagnostic algorithm for ITs (Balduini CL *et al*, 2003), we made a molecular diagnosis in 144 of these families, whereas 130 probands remained without a definite diagnosis as they did not fit the criteria for any known IT. These 130 consecutive propositi with IT of unknown origin have been screened for mutations in *ETV6*. Whenever *ETV6* mutations were identified, the available relatives of probands have been investigated.

Mutation screening and RT-PCR

Genomic DNA and RNA were extracted from peripheral blood. The *ETV6* gene was analyzed using Sanger and WES.

For Sanger sequencing PCR was carried out in 35 µl of total reaction volume with 25 ng of genomic DNA, 10 µM of each primer, and Kapa 2G Fast Hot Start ReadyMix 2X (KapaBiosystems, Cape Town, South Africa). PCR products were sequenced using the ABI PRISM BigDye v3.1 Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3130xl sequencer (Applied Biosystems, Foster City, CA). Whole exome sequencing (WES) was performed on DNA samples using the solid-phase NimbleGen SeqCap EZ Exome 44Mb array (Nimblegen Inc., Madison, WI) and sequenced as 91/100 bp paired-end reads on Illumina HiSeq2000 platform (Illumina Inc., San Diego, CA). Variants passing quality filters were annotated using ANNOVAR1 against NCBI RefGene (http://www.ncbi.nlm.nih.gov). cDNA was synthesized with the Go Script Reverse Transcription System kit (Promega, Madison, WI, USA). Amplification reactions were performed using the following pairs of primers: F (5'-ACCAGGAGAACAACCACCAG-3') in exon 5 and R (5'-AAGTGTCCCTGCCATTTCTG-3') in exon 8 using DNA polymerase KAPA2G Fast HS Ready Mix (Kapa Biosystems, Wilmington, MA, USA). PCR products were sequenced as indicated above. Bioinformatic tools and analysis of the structures Missense variants were evaluated using prediction programs, such as PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), Mutation Taster (http://www.mutationtaster.org/) Mutation Assessor (http://mutationassessor.org/), and SIFT (http://sift.jcvi.org). The only available structure of an ETV6 construct containing residues C338 to L442 (numbered in the file C8 and L112 using the construct numbering) was obtained from the PDB

database. The structure was displayed by the graphic program MOLMOL and analyzed by the DSSP program to assess water exposure.

Basic blood cell studies

Blood cell counts were evaluated by electronic counters for their count, and volume measured as MPV. Parameters relative to platelet diameter were measured by software-assisted image analysis on peripheral blood smears (Noris P *et al,* 2014). The following previously defined parameters were computed: mean platelet diameter (MPD), platelet diameter distribution width (PDDW), platelet diameter large cell ratio (PDLCR), and platelet diameter small cell ratio (PDSCR) (Noris P *et al,* 2014). Surface expression of platelet glycoproteins (GPs) was investigated by flow cytometry. Platelet aggregation was evaluated by the densitometric method of Born (Noris P *et al,* 2009).

Platelet activation

Platelet activation in response to ADP or TRAP was investigated by flow cytometry (Psaila B *et al*, 2012). Samples of patients and controls (unaffected relatives of *ETV6*-RT patients and age-matched healthy volunteers) were processed in parallel. Aliquots of whole blood were incubated with moAbs and either TRAP 25 µM, ADP 1 µM, or vehicle HEPES buffer for 10 minutes at 37°C and fixed with paraformaldehyde. The following moAbs were used: PAC1, which specifically binds to the activated conformation of GPIIb-IIIa (Becton Dickinson, San Josè, CA); CLB-Thromb/6 against P-selectin (Immunotech); SZ2 against GPIbα; P2 against GPIIb-IIIa. Platelets were gated by GPIIb-IIIa expression. Platelet activation was expressed as the ratio between mean fluorescence intensity (MFI) measured after stimulation with each agonist and MFI measured after incubation with the buffer alone.

Platelet adhesion and spreading

See “Materials & Methods”, page 14.

Investigation of megakaryocytes

Mks were differentiated in vitro from peripheral blood $CD45^{+}$ cells (Pecci A *et al*, 2009; Bluteau D *et al*, 2014). Morphological analysis of Mks was performed by phase-contrast and fluorescence microscopy, while the percentage of fully differentiated Mks and Mk ploidy at the end of the culture were investigated by flow cytometry (Bluteau D *et al*, 2014; Balduini A *et al*, 2012). Proplatelet

yields were evaluated both in suspension and in adhesion on fibrinogen at the end of the culture, as previously described (Pecci A *et al*, 2009; Di Buduo CA *et al*, 2014).

Statistical analysis

Data are presented as mean and SD or range. Statistical comparisons were performed by two-tailed Student t test. Incidences of hematologic malignancies (per 100 000 person years) together with their exact 95% confidence intervals (95%CI) were computed.

RESULTS

Mutation screening

The analysis of the *ETV6* gene allowed us to identify 5 different heterozygous variants. Two mutations (c.641C>T/p.P214L and c.1252A>G/p.R418G) were previously reported in two *ETV6*-RT families (Figure 5-A, families B and G). The remaining three are two missense variants and one deletion.

The two missense variants, c.1105C>T (p.R369W) and c.1138T>A (p.W380R), are likely to be pathogenic since they segregate in the affected family members and are not present in healthy relatives (Figure 6-A). They are absent in public genomic databases, such as dbSNP (www.ncbi.nlm.nih.gov/SNP), 1000 genomes (www.ww.1000genomes.org), and Exome Aggregation Consortium (www.exac.broadinstitute.org). The multiple-sequence alignment indicated that they affect highly conserved amino acid residues (data not shown). Moreover, they are deleterious for protein structure and function, as predicted by different tools, such as Polyphen, Mutation Assessor, and Mutation Taster. In the coordinate analysis, where the ETS domain (2DAO) spans residues 338 to 442 (numbered in pdb C8 and L112), R369 and W380 correspond to R39 and W50. Of these residues, W380 is well buried in the hydrophobic core and surrounded by a number of hydrophobic residues, such as L341 and M394 (L11 and M64 in the structure) (Figure 6-E). It is also close to the side chains of H383 and K384 (H53 and K54). Its substitution to an arginine will greatly destabilize the structure both by creating a hole in the hydrophobic core and an electrostatic repulsion with nearby positively charged residues. Residue R369, which is affected by a different substitution (p.R369Q) in a previously reported family with *ETV6*-RT (Zhang MY *et al,* 2015), is well

exposed on the surface of the protein. It is predicted to form an electrostatic interaction with the spatially nearby E361 (E31). Its substitution with a tryptophan could destabilize the fold by abolishing this interaction. Alternatively, the role of this residue could be that of being implicated in protein-protein interactions. In this case, its substitution with a much bulkier and uncharged residue could also be deleterious.

The c.1153-1_1165del deletion variant removes the last "G" nucleotide of intron 6 and the first 13 nucleotides of exon 7. In order to provide the effect of this deletion, we carried out RT-PCR on the three affected individuals of family F. Sequencing analysis of the altered 721 bp product showed skipping of exon 7 (r.1153_1253del/p.N385Vfs*7; Figure 6-B) resulting in truncation of the ETS domain. Since the 721 bp band is fainter than the wild type product (822 bp), we cannot exclude that the alternatively spliced mRNA is partially degraded. Inspection of the intron 6/exon 7 genomic boundary reveals repeats that are likely to be involved in non-allelic homologous recombination leading to micro deletions/duplications (Figure 6-C).

The five families carrying the p.P214L (family A), p.R369W (families C and D), p.W380R (family E), and p.N385Vfs*7 (family F) mutations, together with families B and G, which were previously reported (Noetzli *et al,* 2015), represent our cohort of 20 affected individuals who have been studied for the clinical and phenotypic characterization of *ETV6*-RT.

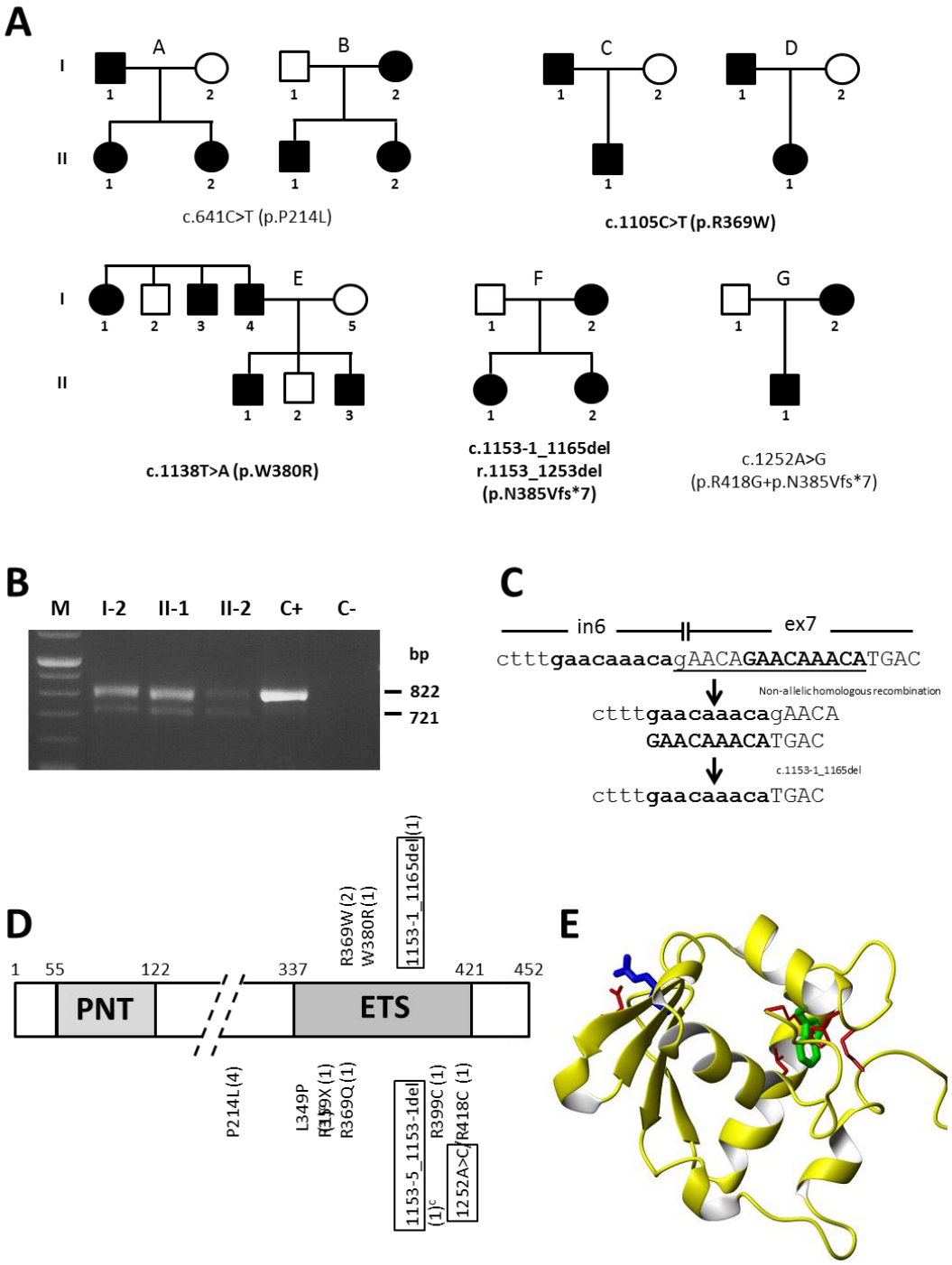

Figure 6. Mutations identified in the ETV6 gene. (A) Pedigrees of families enrolled in this study carrying different mutations as indicated (in bold novel mutations). Nucleotide numbering reflects the ETV6 cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (RefSeq NM_001987.4). Therefore, the initiation codon is residue 1 in the amino acid sequence. Families B and G where previously. (B) RT-PCR in affected members (I-2, II-1, and II-2) of family F to determine the consequence of the c.1153-1_1165del mutation on splicing. C+, wild type control; C-, negative control. The analysis shows two fragments, the wild type (822 bp) and the exon 7 skipping (721 bp) products. (C) The deletion of the 14 bp (gAACAGAACAAACA) of c.1153-1_1165del is likely due to non-allelic homologous recombination between the two GAACAAACA repeats located at the intron 6 and exon 7 boundary. (D) Domain structure of ETV6 (XP_011518909.1) based on Pfam annotation at http://www.ncbi.nlm.nih.gov/gene/2120 (PNT, N-terminal pointed domain; ETS, C-terminal DNA binding domain), with mutations identified in ETV6, already reported or identified in this study (top). The number of families carrying each mutation are in brackets. Mutations leading to skipping of exon 7 are boxed. (E) Structural modeling of the ETS domain with residues R369 (blue) and W380 (green) affected by the p.R369W and p.W380R mutations.

Clinical picture

As shown in Table 2, a mild bleeding tendency (WHO grade 1 or 2) was present in 11 patients, whereas 9 subjects presented no spontaneous bleeding. More common bleeding symptoms were petechiae, ecchymoses, gum bleeding, epistaxis, and menorrhagia. Thrombocytopenia was discovered in the adulthood in 5 patients. One of them was initially misdiagnosed with immune thrombocytopenia and had received steroids and splenectomy without any benefit. Ten patients underwent 17 surgeries and 6 had tooth extractions without excessive bleeding. Four women gave birth 6 children, three vaginally and three by cesarean section. Prophylactic platelet transfusion was deemed necessary to cover one vaginal delivery; excessive bleeding (800 mL) was reported in another woman who had given birth vaginally.

Unilateral polydactyly was observed in one patient, mitral valve prolapse in two subjects, and renal ectopia in one. So, no recurrent extra-hematological abnormalities have been identified.

Four patients from 4 families developed B-cell ALL (common ALL in three cases, not better defined in one) during childhood. The incidence of ALL in our case series was 731.3 per 100000 (95%CI, 274.5-1948.4), while it is 1.4 per 100000 in the general population according to the National Cancer Institute (*National Cancer Institute*, 2015). Three patients obtained remission after conventional chemotherapy, and one after hematopoietic stem cell transplantation (HSCT) from unrelated donor. One patient was diagnosed with *JAK2*-positive polycythemia vera at age 37 years.

A history of non-hematological neoplasms was present in three patients. The patient B/II-1 had breast fibroadenoma at age 35 and meningioma at age 42. The patient G/I-2 had breast carcinoma at age 49, while the patient F/II-2 had breast fibroadenoma at age 14.

Table 2. Main characteristics of investigated patients.

Family/ Individual	*ETV6* mutation[2]	Age[3], y/ Gender	Age at diagnosis[4], y	WHO bleeding score[5]	Platelets, x10^9/L	MPV, fL[6]	MPD, µm[7]	Hgb, g/dL	MCV, fL[8]	WBC, x10^9/L	Neutrophils x10^9/L	Hematological malignancies
A/I-1	c.641C>T p.Pro214Leu	57/M	31	1	115	8.8	2.44	14.6	99	7.13	4.9	
A/II-1		20/F	birth	2	59	8.6	2.24	10.4	68	4.98	2.3	
A/II-2		27/F	birth	2	82	8.2	2.23	13.6	98	5.5	3.39	Common ALL at age 7
B/I-2[1]		43/F	14	0	115	10	2.82	11.1	88	5.02	1.75	B-cell ALL at age 15
B/II-1[1]		15/M	birth	2	66	10.4	2.89	14.0	91	5.36	1.18	
B/II-2[1]		18/F	2	0	44	10.1	3.26	13.1	97	4.04	1.42	
C/I-1	**c.1105C>T p.Arg369Trp**	48/M	38	1	112	na	2.73	15.4	103	6.3	4	
C/II-1		13/M	3	0	87	na	2.53	14.1	86	3.84	1.81	
D/I-1		53/M	47	0	110	8.4	2.42	13.7	97	5.4	2.84	
D/II-1		7/F	1	0	109	9.2	2.28	12.6	79	6.82	1.87	
E/I-1	**c.1138T>A p.Trp380Arg**	37/F	8	0	105	8.1	na	14.2	97	7.50	5.2	
E/I-3		42/M	5	2	765[9]	8.91[9]	na	14.2[9]	93[9]	11.1[9]	6.29[9]	JAK2+ PV at age 37
E/I-4		45/M	20	0	93	7.9	na	16.9	101	8.30	4.24	
E/II-1		20/M	4	1	171[10]	na	2.73[11]	16.2[10]	94[10]	8.49[10]	5.37[10]	Common ALL at age 7
E/II-3		13/M	birth	2	99	7.430	na	14.0	90	6.15	2.45	
F/I-2	**c.1153-1_1165del r.1153_1253del p.Asn385Valfs*7**	49/F	7	0	105	8.9	2.55	13.4	107	7.11	4.4	
F/II-1		12/F	birth	1	57	8.6	2.40	14.2	97	6.59	4	
F/II-2		17/F	birth	1	70	8.7	2.36	14.4	97	8.24	5.3	Common ALL at age 3
G/I-2[1]	c.1252A>G p.Arg418Gly	51/F	20	0	101	7.6	3.17	13.6	97	4.71	2.02	
G/II-1[1]		28/M	3	1	101	7.8	2.99	15.9	97	5.3	2.39	

Notes: [1]Previously reported patients. [2]Nucleotide A of the ATG translation initiation start site of the ETV6 cDNA in GenBank sequence NM_001987.4 is indicated as nucleotide +1. Novel germline mutations are in bold. [3] Age at the last evaluation: the blood parameters and bleeding score reported here have been measured at the last evaluation, unless otherwise specified. [4]Age at diagnosis of thrombocytopenia. [5]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. [6]Normal range: 8-13.4 fL. [7]Normal range: 1.9-3.4 m. [8]Normal range: 82-98 fL. [9]Values obtained after the patient developed polycythemia vera. [10]Values obtained after the patient received bone marrow transplantation. Platelet count before transplantation was 60 x10^9/L. [11] Measured on a blood slide that had been collected before transplantation.

Abbreviations. na: not available; ALL: acute lymphoblastic leukemia; PV: polycythemia vera.

Blood cell counts and peripheral blood film examination

Eighteen of 20 patients presented thrombocytopenia at the last examination, while two patients previously recognized as being thrombocytopenic had more than 150 x 10^9 platelets/L: one had received HSCT for ALL, and one had developed thrombocytosis in the context of polycythemia vera. Nine patients had less than 100 x 10^9 platelets/L and only one less than 50 x 10^9/L.

MPV was slightly reduced in 4 cases and normal in the other 13 evaluable patients (Table 2). Peripheral blood film examination in 16 patients showed that MPD was similar to that of healthy subjects, confirming that average platelet size is consistently normal in *ETV6*-RT patients (Table 3). We found very mild but significant increases of PDDW and PDLCR, which indicate that a mild platelet anisocytosis and a slightly increased proportion of large platelets were frequent features of the investigated patients.

Mild anemia has been observed in one patient with iron deficiency (A/II-1) and in the subject who had received HSCT (B/I-2). Mean corpuscular volume (MCV) was reduced in the subject with iron deficiency, increased without any apparent cause in three subjects, and within the normal range in the remaining patients. White blood cell count was normal in all the cases.

Table 3. Parameters of platelet diameters measured on peripheral blood films in investigated patients.

	No.[1]	**MPD, µm** mean (SD)	**PDDW, µm** mean (SD)	**PDLCR, %** mean (SD)	**PDSCR, %** mean (SD)
Family A	3	2.30 (0.12)	2.57 (0.06)	8.27 (1.77)	5.87 (3.19)
Family B	3	2.99 (0.24)	2.97 (0.21)	12.3 (4.75)	1 (0.86)
Family C	2	2.63 (0.31)	2.75 (0.49)	7 (4.24)	1.25 (1.06)
Family D	2	2.35 (0.10)	2.10 (0.14)	4.5 (4.95)	5.5 (3.53)
Family E	1	2.73	2.9	8.5	1
Family F	3	2.44 (0.10)	2.57 (0.35)	8.83 (4.07)	3.16 (1.25)
Family G	2	3.08 (0.13)	3.15 (0.49)	13 (7.07)	0.5 (0.71)
Total *ETV6*-RT patients	16	2.63 (0.17)	2.70 (0.29)*	9.23 (4.47)*	2.85 (1.77)
Healthy subjects[2]	55	2.49 (0.32)	2.18 (0.58)	3.64 (4.93)	4.35 (5.9)

Notes: [1] Number of investigated subjects. [2] Values of healthy subjects previously measured in a cohort of 55 healthy volunteers (reference 8). *p <0.01 with respect to healthy subjects.
Abbreviations: MPD, mean platelet diameter. PDDW, platelet diameter distribution width = difference from the 2.5th to the 97.5th percentile of platelet diameter distribution. PDLCR, platelet diameter large cell ratio = proportion of platelets larger than the 97.5th percentile of MPD of healthy subjects (3.9 µm). PDSCR, platelet diameter small cell ratio = proportion of platelets smaller than the 2.5th percentile of the MPD of healthy subjects (1.6 µm) (reference 8).

In vitro platelet studies

Platelet aggregation. Among the 11 investigated patients, the three patients from family F had mildly reduced platelet aggregation after stimulation with collagen 4 mg/mL and ADP 5 µM, while individual C/II-1 showed a slightly reduced response to ristocetin 1.5 mg/mL (Table 4). However, all patients had completely normal responses to higher concentrations of these agonists (collagen 20 µg/mL, ADP 20 µM, ristocetin 3 mg/mL, data not shown), indicating that, if present, the aggregation defects were mild.

Table 4. In vitro platelet aggregation and surface expression of major platelet glycoproteins in investigated patients.

	Platelet aggregation, %[1] – mean (range)				Surface expression of platelet glycoproteins, % of controls- mean (range)			
Family	**No. of investigated subjects**	**Collagen, 4 mcg/mL**	**ADP, 5 µM**	**Ristocetin, 1.5 mg/mL**	**No. of investigated subjects**	**GPIbα (SZ2)**	**GPIX (SZ21)**	**GPIIb (P2)**
A	2	80 (71-89)	75 (66-84)	88 (76-100)	2	131 (130-132)	130 (116-144)	91 (85-97)
B	2	80 (74-87)	81 (77-85)	80 (68-93)	3	98.7 (98-99)	99 (98-100)	98.8 (97-100)
C	2	71 (69-73)	57 (44-70)	67 (57-77)	2	147.5 (143-152)	126 (122-130)	108 (94-122)
F	3	54 (50-56)	37 (35-39)	100 (100-100)	2	117.5 (110-125)	125 (121-129)	127.5 (118-137)
G	2	78 (67-90)	82 (78-87)	88 (77-100)	2	159.5 (136-165)	101 (89-113)	85 (78-92)

Note: [1] Normal ranges: collagen 66-88%; ADP 43-76%; ristocetin 67-90%.

Platelet flow cytometry. As shown in Table 4, flow cytometry performed in 11 patients did not identify any consistent defect of the major GPs of the platelet surface.

Platelet activation. Overall, the surface expression of activated GPIIb-IIIa and P-selectin and the reduction of GPIbα upon stimulation of platelets with ADP or TRAP, were not significantly different in 11 *ETV6*-RT patients with respect to controls (Figure 7). A mild reduction of activated GPIIb-IIIa expression (52 to 65% of controls) after stimulation with TRAP was observed in three patients.

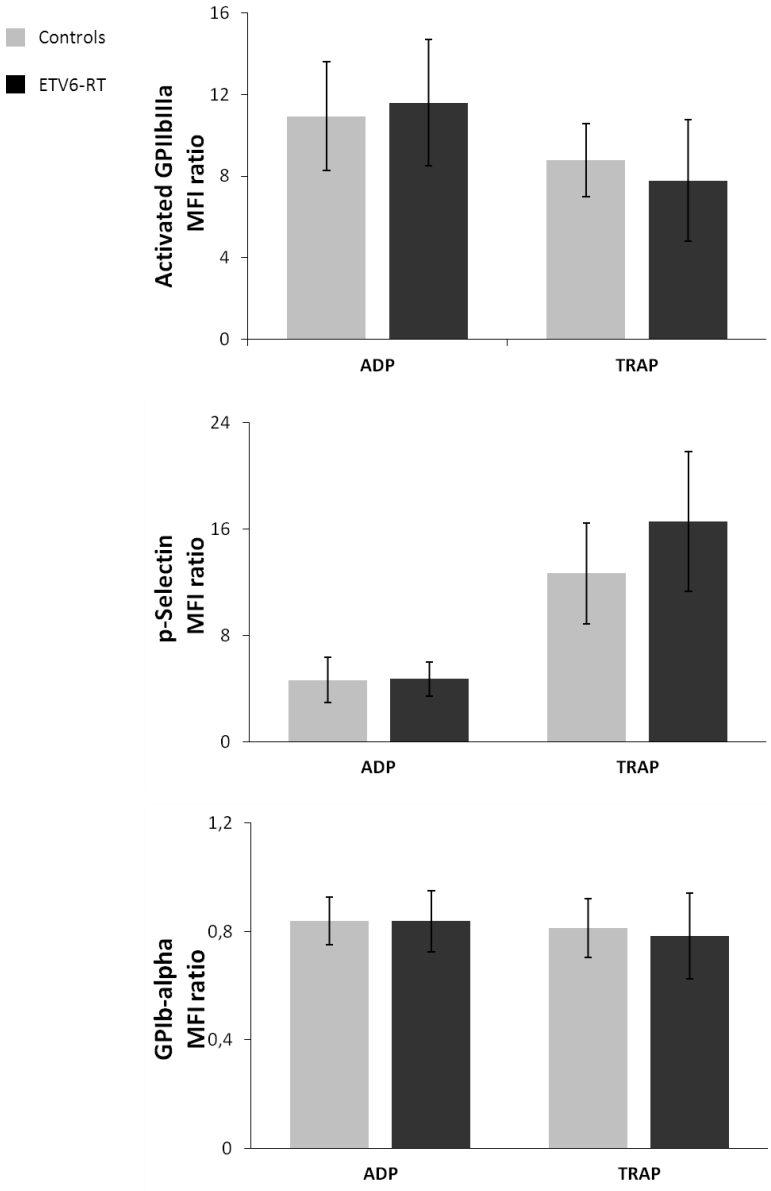

Figure 7. Flow cytometry analysis in response to ADP or TRAP in *ETV6*-patients. The expression of activated GPIIb-IIIa (GpIIb-IIIa, PAC1 antibody binding), P selectin and GPIbα was measured after the addition of TRAP 25µM, ADP 1µM or the vehicle buffer alone. Data are expressed as the ratio between mean fluorescence intensity (MFI) measured after stimulation with each agonist and MFI measured after incubation with buffer alone. No significant differences were observed between 11 *ETV6*-patients and 20 healthy subjects analyzed in parallel (two tailed Student's t test).

Platelet adhesion and spreading. *In vitro* adhesion of platelets from 7 patients to subendothelium components of the extracellular matrix was not different from that of controls. However, the ability of *ETV6*-RT platelets to spread on fibrinogen was consistently and significantly reduced, while spreading on collagen and von Willebrand Factor was normal (Table 5).

Table 5. In vitro platelet interaction with subendothelium molecules in 7 *ETV6*-RT patients.

	Platelet adhesion and spreading , % of controls mean (SD)		
	No. of adhering platelets	% of spread platelets	Surface area covered by platelets
Fibrinogen	102.9 (27.6)	51.5 (33.5)*	61 (28.5)*
Collagen	107.2 (45.1)	103.6 (41.7)	122.4 (29.3)
von Willebrand Factor	80.4 (36.5)	103.8 (41.5)	120.7 (22.7)

Note: * $p < 0.01$ with respect to controls

***In vitro* culture of megakaryocytes and assessment of proplatelet formation**

In vitro culture of Mks was performed in 8 patients and 8 healthy subjects. After 14 days of culture, expression levels of the major Mk differentiation surface markers (GPIIIa, GPIIb and GPIbα) were similar to those of healthy controls (Figures 8-A and 8-B). Conversely, Mk ploidy was significantly lower in patients than controls (Figure 8-C), and this was paralleled by differences in Mk diameters (Figure 8-D). The analysis of proplatelets formation revealed that Mks from patients elongated proplatelet shafts of shorter length and with decreased number of branches compared to controls. Further, the percentage of proplatelet-forming Mks was significantly reduced. Similar results were obtained with Mks in suspension (Figures 9-A and 9-B) and in adhesion on fibrinogen (Figures 9-C and 9-D).

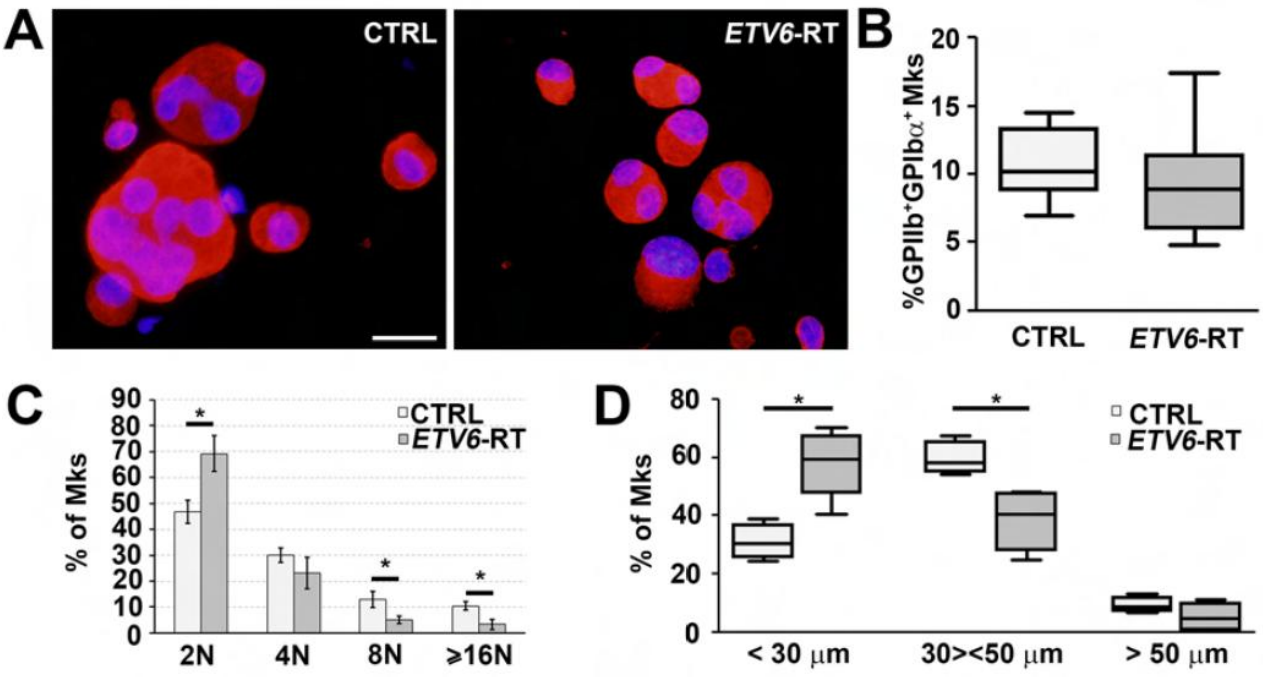

Figure 8. Normal differentiation but decreased ploidy by *ETV6*-RT megakaryocytes. Hematopoietic progenitors from peripheral blood samples of healthy controls (CTRL) and patients (*ETV6*-RT) were differentiated in vitro into megakaryocytes in presence of TPO, IL6 and IL11. **(A)** Representative immunofluorescence staining of plasma membrane GPIIIa in CTRL and *ETV6*-RT megakaryocytes (red=GPIIIa; blue=nuclei; scale bar=20 μm). **(B)** Flow cytometry analysis of GPIIb and GPIbα expression revealed comparable percentage of double stained population in CTRL and *ETV6*-RT at the end of the culture. **(C)** Ploidy of megakaryocytes at the end of the culture was significantly reduced in cells of *ETV6*-RT patients (*p<0.05). **(D)** Diameters of megakaryocytes were also significantly lower in *ETV6*-RT patients (total number of analyzed cells: 1,100, *p<0.01).

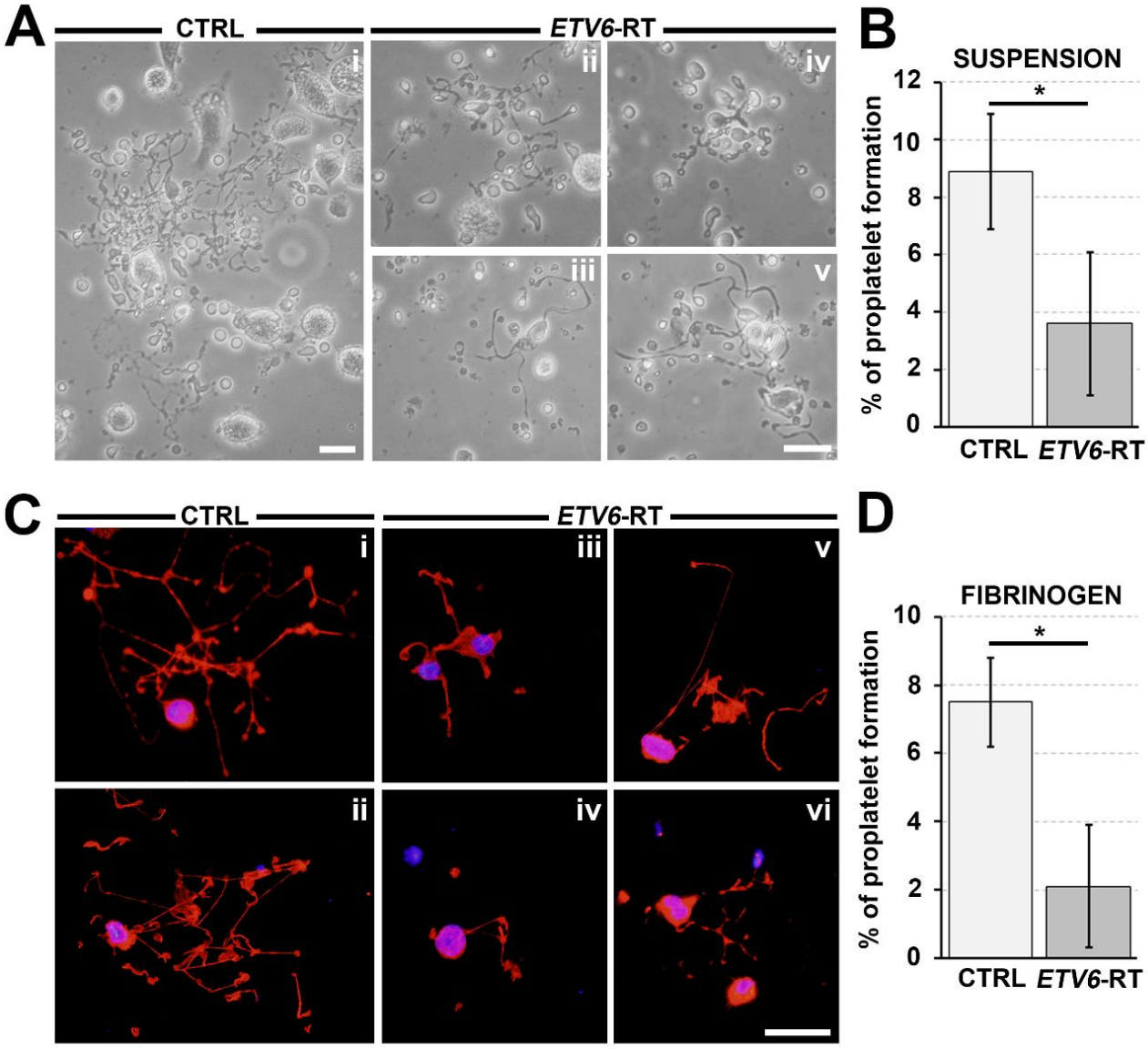

Figure 9. Aberrant proplatelet formation by *ETV6*-RT megakaryocytes (A) Representative light microscopy analysis of proplatelet formation and structure from controls (CTRL, i) and patients (*ETV6*-RT, ii-v) megakaryocytes cultured for 16 hours in suspension (scale bar=50 µm). **(B)** The percentage of proplatelet forming megakaryocytes was calculated as the number of megakaryocytes displaying at least one filamentous pseudopod with respect to total number of round megakaryocytes per analyzed field (*p<0.01). **(C)** Representative fluorescence microscopy analysis of proplatelet formation and structure from CTRL (i-ii) and *ETV6*-RT (iii-vi) megakaryocytes cultured for 16 hours in adhesion on fibrinogen. Pictures clearly show defective proplatelet elongation in *ETV6*-RT (red=β1-tubulin; blue=nuclei; scale bar=30 µm). **(D)** The percentage of proplatelet forming megakaryocytes was calculated as the number of β1-tubulin+ cells displaying at least one pseudopod with respect to total number of round megakaryocytes per analyzed field (*p<0.01)

DISCUSSION

ETV6, also known as *TEL* oncogene, is a transcriptional repressor involved in the embryonic development and hematopoietic regulation (Hock H, 2004.). In particular, animal studies suggested that *ETV6* has two independent roles in mouse hematopoiesis: on the one hand it is required for survival of hematopoietic stem cells, on the other it promotes the late phases of megakaryopoiesis. Interest in *ETV6* greatly increased at the end of the last century after demonstration that its deregulation due to rearrangement, fusion or deletion is involved in hematologic malignancies (Bohlander SK, 2005; De Braekeleer E, 2012). Moreover, somatic mutations in *ETV6* were recently found in a variety of hematologic malignancies, including AML, T and B cell ALL, mixed-phenotype acute leukemia, MDS, chronic lymphocytic leukemia and chronic myelogenous leukemia (Wang Q *et al*, 2014). Even more recently, targeted sequencing of *ETV6* in 4405 childhood ALL cases identified 31 germline exonic variants potentially related to leukemia in 35 cases (Moriyama T *et al*, 2015). Based on this evidence, it is not surprising that the 4 studies that identified *ETV6*-RT in 41 subjects from 9 families found that 16 patients (39%) had hematological malignancies, with 12 patients (29%) developing ALL (Moriyama T *et al*, 2015; Noetzli L *et al,* 2015*;* Topka S *et al*, 2015; Zhang MY *et al*, 2015). Of note, 11 of 12 subjects with ALL were children. Other observed blood neoplasms were mixed-phenotype acute leukemia, multiple myeloma, Myelodysplastic syndromes (MDS) and chronic myelomonocytic leukemia.

By the investigation of a series of consecutive subjects with *ETV6*-RT, we found that 4 of 20 patients (20%) developed ALL during childhood, thus confirming that early leukemic transformation is a major risk of these patients. Moreover, we found that one patient developed *JAK2* positive polycythemia vera at age 37, suggesting that this disease should be added to the list of malignancies to which the *ETV6*-RT predisposes. The frequency of hematologic malignancies is lower in our study than in the previous ones (25% versus 39%). This is explained by the fact that, in the previous investigations (Moriyama T *et al*, 2015; Noetzli L *et al,* 2015*;* Topka S *et al*, 2015; Zhang MY *et al*, 2015), the occurrence of hematologic malignancies was one of the criteria for the recruitment of patients, while we examined a series of consecutive, unselected patients with IT of unknown origin. This approach appears more suitable to provide a reliable estimation of the incidence of hematologic neoplasms among *ETV6*-RT patients. Of course, the analysis of a larger series of patients is needed to confirm this figure.

Similarly to this study, we previously searched for *ANKRD26* mutations a large series of unselected patients and revealed that 10 of 118 (8%) subjects with *ANKRD26*-RT had developed myeloid malignancies (Noris P *et al*, 2013). Thus, hematological malignancies seem much more frequent in *ETV6*-RT than *ANKRD26*-RT. The risk of malignancies appears even higher in FPD/AML, since over 40% of patients had myeloid neoplasms (Liew & Owen, 2011). However, as discussed for *ETV6*-RT, also *RUNX1* mutational screening was generally performed in pedigrees with hematological malignancies (Owen CJ *et al,* 2008), and it is therefore likely that the incidence of transformation has been overestimated. However, each patient with an IT caused by mutations in *ETV6*, *RUNX1* or *ANKRD26* has a relevant risk of hematological malignancies, and recognizing these patients is required not only to provide effective genetic counseling and appropriate follow-up, but also to give an appropriate treatment to patients who developed blood neoplasms and need HSCT. In fact, as shown in different disorders predisposing to myeloid malignancies (Churpek JE, 2016), the use of an affected family member as the donor would entail the risk of developing malignancies once again.

ETV6-RT is a relatively frequent form of IT. In fact, in our series of 274 consecutive propositi, *ETV6*-RT was identified in 7 families and had, therefore, a relative prevalence of 2.6% in the whole case series, and of 4.6% in the series of probands with known ITs (7/151). In our cohort, the frequency of *ETV6*-RT was lower only to that of monoallelic Bernard-Soulier syndrome (BSS, 12.2% in the whole series), *MYH9*-related disease (11.4%), *ANKRD26*-RT (9.4%), and biallelic BSS (5.7%). Since most of our patients with monoallelic BSS had the Ala156Val mutation of GPIbα ("Bolzano" mutation), which is exclusive of the Italian population (Noris P *et al*, 2012), it is expected that the relative frequency of *ETV6*-RT is even higher in other countries.

Our study did not identify any peculiar feature that can be used to raise the suspicion of *ETV6*-RT by routine diagnostic workup and the diagnosis remains therefore difficult. We could not confirm the previous observation that red cell macrocytosis is typical of this disorder (Noetzli L *et al,* 2015), since only a minority of our patients had increased MCV. Moreover, we did not identify any distinguishing defect of major platelet GPs or *in vitro* platelet aggregation. Also evaluation of peripheral blood films did not reveal any morphological abnormality, except for mild platelet anisocytosis. However, at variance with most ITs, MPD and MPV were consistently normal in *ETV6*-RT, and it is just the normal size of platelets that should raise suspicion of this condition in subjects

with an autosomal dominant thrombocytopenia. In fact, the other dominant ITs with this feature are FDP/AML, *ANKRD26*-RT, and *CYCS*-RT. Of note, *CYCS*-RT is a very rare condition described so far in only two pedigrees, whereas the other two disorders are more frequent and, as *ETV6*-RT, predispose to hematologic malignancies. Thus, we suggest that all subjects with a dominant IT and normal platelet size should be tested for mutations in *ETV6*, *RUNX1*, and *ANKRD26*, in order to recognize one of these predisposition syndromes.

ETV6 is a small gene that can easily be screened, together with *RUNX1* and the 5'UTR of *ANKRD26*, even by standard sequencing. Interestingly, of the ten different mutations identified in *ETV6*-RT families, all but one (p.P214L) affect the ETS domain (Figure 6-D), a conserved region that directly interacts with DNA consensus sequences. The alterations of the ETS domain are amino acid substitutions or lead to truncating proteins. Among the latter, three different mutations are associated with skipping of exon 7 (p.N385Vfs*7). Of these, c.1135-5_1153-1del and c.1153-1_1165del in the acceptor splice site of intron 6 are likely to be due to non-allelic homologous recombination between repeated units AACAG and GACAA, respectively. Instead, p.P214L alters a less conserved central domain that interacts with several transcription repressors further controlling expression of the target genes. Contrary to the other mutations, which are mainly private, this substitution was responsible for *ETV6*-RT in 4 of the 14 *ETV6*-RT unrelated families characterized so far, indicating that, together with the ETS domain, it represents a potential mutational hot spot.

Our study investigated for the first time *in vitro* megakaryopoiesis of *ETV6*-RT patients. We showed that *ETV6* pathogenetic variants impair Mk maturation, as demonstrated by the production of smaller Mks with decreased ploidy. These immature Mks showed an impaired ability to extend fully developed proplatelets, providing an explanation for thrombocytopenia. These findings seem consistent with the results of the studies in mice, which suggested a role for *ETV6* in terminal Mk maturation (Hock *et al*, 2004), and with the findings obtained with Mk differentiated from human $CD34^+$ cells transduced with some *ETV6* variants (Noetzli L *et al*, 2015). We also had the possibility to study in detail the function of platelets in a substantial number of patients. Although we did not identify any consistent defect of *in vitro* platelet aggregation, activation and adhesion, we found that the ability of platelets to spread on fibrinogen was reduced in all the investigated patients. As the platelet expression of GPIIb-IIIa was normal, this finding suggests that

mutations in the ETV6 transcription factor alter the expression of one or more proteins involved in the GPIIb-IIIa-mediated platelet outside-in signalling after interaction with fibrinogen. Moreover, this defect could contribute to the bleeding diathesis observed in some *ETV6*-RT individuals. In fact, although the degree of bleeding was always mild, the proportion of patients with spontaneous bleeding (55%) appeared globally high with respect to the very mild degree of thrombocytopenia.

In conclusion, our study showed that monoallelic *ETV6* mutations cause a relatively frequent form of IT and confirmed that affected subjects have mild bleeding tendency but high propensity to hematological malignancies, in particular ALL. Since *ETV6*-RT is one of the few autosomal dominant forms of IT without platelet macrocytosis, the screening for *ETV6* mutations is recommended in all patients with these characteristics.

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CORRESPONDENCE

WILEY AJH

Mutations of *RUNX1* in families with inherited thrombocytopenia

To the Editor:

Familial platelet disorder with propensity to myeloid malignancy (FPD/AML) is a rare autosomal dominant disease characterized by thrombocytopenia and platelet functional defects.[1] Although bleeding tendency is usually mild to moderate, an important hallmark of FPD/AML is the increased risk of myeloid neoplasms, such as myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).

FPD/AML is caused by different, usually private, mutations of *RUNX1*, a gene encoding the DNA binding subunit (also known as CBF-alpha) of the core binding factor (CBF) transcription complex.[2] The N-terminal domain of RUNX1 (runt-homologous domain) mediates DNA binding and heterodimerization to CBF-beta, the other subunit of the CBF complex. At the C-terminus, RUNX1 is constituted of domains involved in transcription activation and repression.

We report three families with pathogenic variants of *RUNX1* identified in our cohort of 274 consecutive unrelated probands with inherited thrombocytopenia (IT) (Supporting Information Materials and Methods). They carry three different heterozygous mutations, all segregating with thrombocytopenia in the respective families: one missense (c.578T > A/p.Ile193Asn) variant affecting a well conserved residue of the runt-homologous domain, and two nucleotide substitutions of the canonical "gt" dinucleotide in the donor splice sites of intron 4 (c.351 + 1G > A) and intron 8 (c.967 + 2_5del) (Supporting Information Figures S1-S3). The effect of c.351 + 1G > A on splicing mechanisms was not determined because RNA from members of family 2 was not available. In family 3, RT-PCR analysis revealed two alternative spliced products, sp1 and sp2 that led to frameshift/truncation and in-frame deletion alterations at the transactivation domain of RUNX1, respectively. The sp1 RNA was expressed at higher level than the sp2 form.

The 13 individuals from the three families carrying the *RUNX1* mutations had mild thrombocytopenia (platelet count ranging from 70 to 130 $\times$ 10^9/L) and mild bleeding tendency (Table 1). The platelet size was normal in all the six cases analyzed. Consistent with the mild reduction in platelet count, patients' serum thrombopoietin levels were normal or moderately increased (Table 1). No specific morphological alteration of platelets was detected at the May–Grunwald–Giemsa staining, except for moderate reduction in the alpha-granule content in family 1, as confirmed by immunofluorescence analysis (data not shown).

Flow cytometry of platelets showed normal expression of the major surface glycoprotein (GP) complexes GPIIb-IIIa and GPIb-IX-V (Table 1). In family 1, we found moderate reduction (25-45%) of GPIa-IIa, whose alteration was independent of genotypes at the *ITGA2* locus.[3] In this family, platelet aggregation was defective after stimulation with low doses of collagen (4 μg/mL), a defect that could not be explained by the reduction of GPIa-IIa, as the specific reagent used for the platelet aggregation assay does not bind this receptor. No second wave aggregation was observed in response to low doses of ADP (2 μM) in the five individuals investigated, suggesting alterations of the platelet delta-granules (Table 1). Although we did not support this hypothesis through specific assays, deficiency, or defective release of the dense-granules is one prevalent defect in individuals with FPD/AML.[4] Increasing the concentration of ADP (5 μM), we found defective response only in family 1. Normal responses were observed using higher concentrations of both the agonists (collagen 20 μg/mL and ADP 20 μM) and with ristocetin (1.5 mg/mL), suggesting that the platelet dysfunction was mild.

Consistent with data from literature,[4] our findings indicate that multiple aspects of platelet structure and function are compromised in FPD/AML, which is expected considering that RUNX1 regulates expression of multiple genes and that their expression is modulated by many genetic factors.

Individuals with heterozygous mutations of *RUNX1* are at risk of hematological malignancies, which have been reported in almost 40% of patients with a median age of onset in the early 30s.[1] In our cohort of 14 carriers (individual II-3 of family 3 is an obligate carrier), only three individuals (one from family 2 and two from family 3) had developed AML (Supporting Information Materials and Methods). This low prevalence (20%) could depend on the relatively young age of some of the individuals studied, as well as on the fact that the probands were not all selected on the basis of their personal or family history of hematological malignancies. Indeed, families 1 and 2 were identified by diagnostic work-up of an IT of unknown origin, which was not associated with MDS or AML. Therefore, only systematic molecular genetic testing will provide the actual risk of malignancies in individuals with *RUNX1* mutations. Although AML remains the most prevalent neoplasm, we cannot exclude that mutations of *RUNX1* predispose also to solid tumors, as those (lung and uterine cancer) diagnosed in two members of family 3.

Although mutations of *RUNX1* are relatively rare (1% in our cohort), their identification in families with IT is important because of the associated risk of malignancies. However, it is difficult to discriminate FPD/AML from other forms of IT due to lack of pathognomonic signs. The most promising features could be the defects of the alpha-

NOVEL *RUNX1* MUTATIONS IN FAMILIES WITH INHERITED THROMBOCYTOPENIA

De Rocco D, **Melazzini F**, Marconi C, Pecci A, Bottega R, Gnan C, Palombo F, Giordano P, Coccioli MS, Glembotsky AC, Heller PG, Seri M, Savoia A, Noris P. Mutations of RUNX1 in families with inherited thrombocytopenia. Am J Hematol. 2017 Feb 27.

INTRODUCTION

Familial platelet disorder with propensity to acute myeloid leukemia (FPD/AML) is a rare autosomal dominant disease characterized by impaired megakaryopoiesis and moderate thrombocytopenia, with normal-sized and dysfunctional platelets. Despite quantitative and qualitative defects of platelets, bleeding tendency is usually mild to moderate (Liew & Owen, 2011). As the name suggests, an important hallmark of FPD/AML is the increased risk of myeloid neoplasms, such as MDS and AML.

FPD/AML is caused by mutations of *RUNX1*, a gene encoding the DNA binding subunit (also known as CBF-alpha) of the core binding factor (CBF) transcription complex (Song WJ *et al,* 1999). The CBF-beta component of the complex does not directly interact with DNA but it stabilizes RUNX1 enhancing its affinity to the TGT/cGGT consensus sequence of target genes. The runt-homologous domain, located at the N-terminus of RUNX1, mediates DNA binding and heterodimerization to CBF-beta. The other main C-terminus domains are responsible for transcription activation (TAD) and repression (RD). Different, usually private, mutations have been reported in over 60 unrelated FPD/AML pedigrees (Liew E & Owen C, 2011; Latger-Cannard V *et al*, 2016; Yoshimi A *et al*, 2016). They are spread through the entire gene and include missense, nonsense and frameshift mutations together with large genomic deletions (Béri-Dexheimer M *et al*, 2008).

In a systematic attempt aimed at unraveling the molecular basis of inherited thrombocytopenias (ITs), we investigated our series of consecutive patients with IT and identified three novel FPD/AML pedigrees carrying different mutations of *RUNX1*. The clinical and laboratory data reported here further improve our knowledge of this disorder.

PATIENTS & METHODS

Patients

We studied three families with autosomal dominant IT from a cohort of 274 consecutive unrelated probands with IT referred to the IRCCS Policlinico San Matteo Foundation of Pavia from 2003 to 2014 (Melazzini F *et al*, 2016).

Family 1. The proband was a 24 year-old man who was incidentally found to have mild thrombocytopenia at the age of 5 during hospitalization for enuresis. Bleeding symptoms were limited to easy bruising. The patient underwent major orthopedic surgery at age 14 for reduction of multiple fractures of left tibia, fibula and femur caused by a motorcycle accident. He received prophylactic recombinant coagulation FVIIa and did not develop any hemorrhagic complication. In the following years, he no bleeding complication occurred after several plastic surgeries carried out without antihemorrhagic prophylaxis. His mother (I-2) and brother (II-1) were also found to have mildly reduced platelet count (Figure 10). Individual I-2 had menorrhagia resulting in mild iron deficiency anemia; she referred abnormal bleeding after some dental extractions, whereas the two vaginal deliveries were not complicated by hemorrhages. Patient II-1 did not experienced bleeding symptoms, though he never underwent any surgical procedure. The proband was enrolled in a whole exome sequencing study to identify the disease-causing gene.

Family 2. The proband was a 52 year-old woman with thrombocytopenia incidentally detected at age 49 during routine medical check-up. She had a history of easy bruising since childhood without other bleeding manifestations. An inherited disorder was suspected because her 12 year-old son had thrombocytopenia and easy bruising when a diagnosis of immune thrombocytopenia was made at age of 4 (Figure 10). The proband underwent whole exome sequencing to identify the disease-causing gene.

Family 3. The proband was incidentally found to have mild thrombocytopenia at the age of 3. At age 6, he was diagnosed with type 1 diabetes. He had a history of rare epistaxis, easy bruising and ecchymosis at insulin inoculation sites; a surgical removal of Spitz nevus was performed without any hemorrhagic complications. His mother (III-4) had a lifelong history of mild thrombocytopenia and epistaxis (Figure 11). She developed AML at the age of 41 and one year later underwent HSCT

successfully. Since in this family thrombocytopenia was associated with AML, *RUNX1* was analyzed for mutational screening.

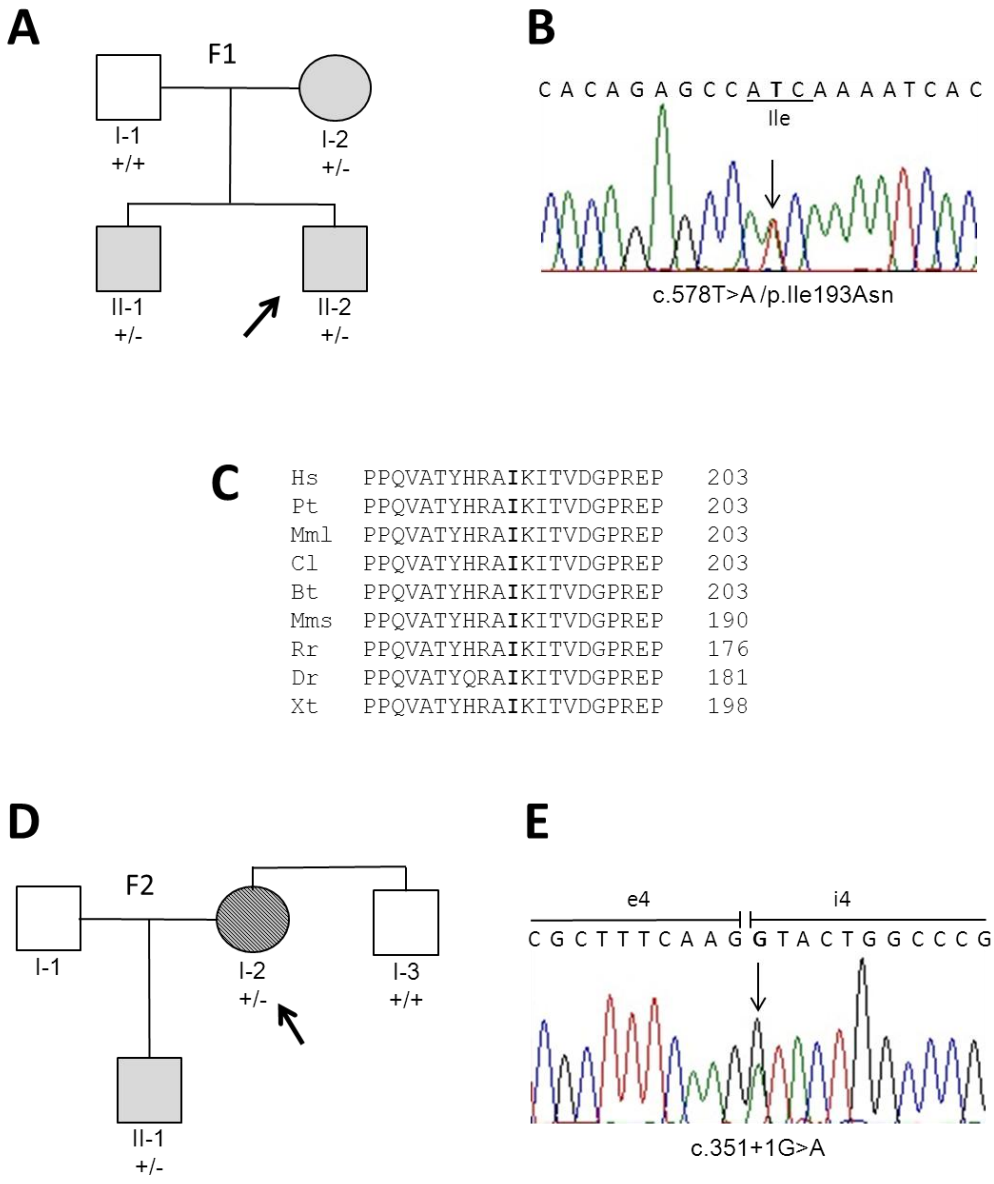

Figure 10. Mutations identified in families 1 and 2. Pedigrees from family 1 (**A)** and 2 (**D**) with probands designated by an arrow. Individual with thrombocytopenia are shown as grey symbols; dashed symbols indicate AML. Direct sequencing of PCR products showing the c.578T>A (p.Ile193Asn) and c.351+1G>A in family 1 (**B**) and family 2 (**E**), respectively. Nucleotide numbering reflects the *RUNX1* cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (RefSeq NM_001754.4). Therefore, the initiation codon is residue 1 in the amino acid sequence. C) Multiple-sequence alignment analysis of NBEAL2 orthologs from different species. Hs, H. sapiens (NP_001745.2); Pt, P. troglodytes (XP_003949210.1); Mml, M. mulatta (XP_002803189.1); Cl, C. lupus (XP_849375.2); Bt, B. taurus (NP_001243507.1); Mms, M. musculus (NP_001104491.1); Rr, R. norvegicus (NP_059021.1); Dr, (D. rerio (NP_571678.1); Xt, X. tropicalis (XP_002939387.2)

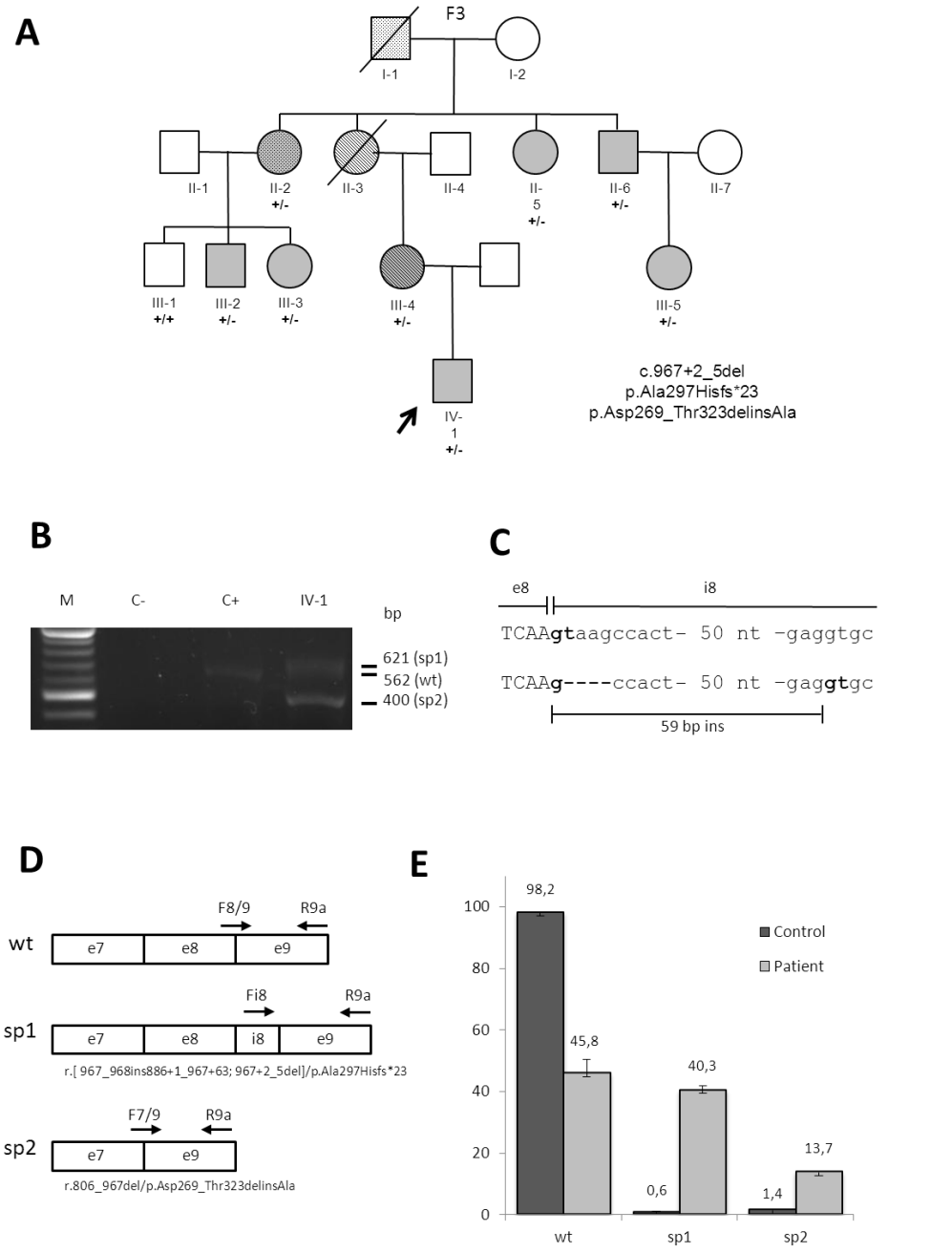

Figure 11. Identification of the c.967+2_5del in family 3. (A) Pedigree of family 3 carrying the c.967+2del mutation in intron 8. Individuals with thrombocytopenia are indicated as grey symbols; dashed and dotted symbols indicate AML and non-hematological cancers, respectively. A black arrow designates the proband. **(B)** RT-PCR carried out in the proband (IV-1) to determine the consequence of the c.967+2_5del mutation on splicing. C-, negative control; C+, wild type control. The analysis shows three fragments, the wild type (wt, 563 bp) and those derived from recognition of a cryptic donor site in intron 8 (sp1, 622 bp) and from skipping of exon 3 (sp2, 401 bp) **(C)** Schematic representation of the exon 8 and intron 8 boundary showing the localization of the "gt" cryptic donor site in intron 8. **(D)** Schematic representation of the wild type, sp1, and sp2 *RUNX1* mRNA indicating where the oligonucleotides used for Q-PCR were designed. **(E**) Q-PCR analysis of the wt, sp1 and sp2 forms of the *RUNX1* mRNA in peripheral blood from patient IV-1, compared to a healthy control. The mean and the standard deviation of three independent experiments are reported.

MOLECULAR ANALYSES AND OTHER LABORATORY INVESTIGATIONS

Mutation screening

Genomic DNA was extracted from peripheral blood. The RUNX1 gene was analyzed using whole exome (WES) and Sanger sequencing. WES was performed on DNA samples using the BGI exome kit (BGI Tech Solutions, Hong Kong) and sequenced as 100 bp paired-end reads on Illumina HiSeq2000 platform (Illumina Inc). Bioinformatic analysis from the sequences alignment to the variant annotation and filtration was performed. For Sanger sequencing PCR was carried out in 35 µl of total reaction volume with 50 ng of genomic DNA, 10 µM of each primer, and Kapa 2G Fast Hot Start ReadyMix 2X (Kapa Biosystems, Cape Town, South Africa). PCR products were sequenced using the ABI PRISM BigDye v3.1 Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3130xl sequencer (Applied Biosystems, Foster City, CA). Nucleotide numbering reflects the RUNX1 cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (RefSeq NM_001754.4). Therefore, the initiation codon is residue 1 in the amino acid sequence.

Analysis of RUNX1 mRNA

RNA was extracted from peripheral blood cells. cDNA was synthesized from 1 µg of RNA in 20 µL reaction volume using the Go Script Reverse Transcription System kit (Promega, Madison, WI, USA). To verify the effect of mutation c.967+2_5del on RNA we performed PCR using the following pairs of primers F7 (5'-TGCGGCGCACAGCCATGAG-3') in exon 7 and R9a (5'-TGGAGAACTGGTAGGAGC-3') in exon 9, using DNA polymerase KAPA2G Fast HS Ready Mix (Kapa Biosystems). Three independent experiments for quantitative determination (Q-PCR) of the RUNX1 transcripts were performed in a 12 µL reaction volume, containing 2 µL of the cDNA reaction, 300 nM of each primer, FastStart Universal SYBR Green Master (Roche) in an ABI PRISM 7900 sequence detection system (Applied Biosystem). Oligonucleotides for Q-PCR were F8/9 (5'-GTCGACTCTCAACGGCACC-3') and R9b (5'-GTAGGTGTGGTAGCGCGTG-3') for the wild type transcripts, Fi8 (5'-CTTTGCAGCTGAGCTGGGG-3') and R9b for the sp1 alternative splicing, and F7/9 (5'-AGAGTCAGATGCAGGCGGCACC-3') and R9b for the sp2 mRNA. Primers FTUBB1 (5'-GGATCATGAATTCCTTCAGC-3') and RTUBB1 (5'-ATGGTCAAGGACACTAGGTG-3') were used to amplify the reference TUBB1 gene. Quantification was obtained generating a calibration curve (using twofold dilution steps) for TUBB1 gene used for normalization. The amount of RUNX1

variants in the patient and control was interpolated from the calibration curve and normalized with TUBB1 amplification. Amplification efficiency of the standards was equivalent to that of the test samples.

Bioinformatic tools

Missense variants were evaluated using prediction programs, such as Combined Annotation Dependent Depletion (CADD; http://cadd.gs.washington.edu/home), Mutation Taster (http://www.mutationtaster.org/), Mutation Assessor (http://mutationassessor.org/), and SIFT (http://sift.jcvi.org). For splice-site mutations, in silico analyses were carried out at Splice Site Prediction by Neural Network (NNSplice; http://www.fruitfly.org/seq_tools/splice.html).

Platelet features and serum thrombopoietin

Platelet count and MPV were evaluated by electronic counters, while MPD were measured by software-assisted image analysis on peripheral blood smears (Noris P *et al*, 2014) as reported above. Immunofluorescence analysis against thrombospondin-1 was carried out as previously reported (Bottega *et al*, 2013).

The study of surface expression of platelet GPs and platelet aggregation were investigated as reported in the Materials & Methods section.

Serum THPO level was determined by a commercially available ELISA (Quantikine Human THPO Immunoassay, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. By this assay, THPO serum concentrations can be directly measured when in the range of 7-2000 pg/mL.

RESULTS

Identification of *RUNX1* mutations

Three different potentially pathogenic variants of *RUNX1* were identified. They were not reported in the SNP, 1000 Genome, and ExAC databases. In the three affected individuals of Family 1, we detected a novel heterozygous c.578T>A variant of exon 6, leading to the p.Ile193Asn substitution affecting the runt-homologous domain (Figure 10). It was predicted to be deleterious using different bioinformatic tools, such as CADD (score 23.4), Mutation Taster (disease-causing mutation), Mutation Assessor (score 3.195), and SIFT (not tolerated). Indeed, isoleucine at residue 193 is well conserved through evolution (Figure 10-C).

In the probands of the other two families, the screening identified heterozygous variants affecting the canonical "gt" dinucleotide of the donor splice sites of intron 4 (c.351+1G>A) and intron 8 (c.967+2_5del) (Figure 10, 11). The c.351+1G>A substitution detected in the two affected individuals of family 2 was previously reported in one family with mild thrombocytopenia associated with MDS and AML (Owen CJ *et al*, 2008). Inspection of the genomic DNA sequence detected a strong cryptic "gt" donor splice site at nucleotide 428-429 of intron 4 (score 0.83), suggesting that the variant could either cause skipping of exon 4 or recognition of the cryptic splice site. Both events would lead to a truncated protein at the runt-homologous domain. However, RNA was not available to determine consequences on the splicing mechanism.

In the proband of family 3 (IV-1), we found a novel c.967+2_5del deletion inherited from his mother (III-4). In addition to the wild-type product of 562 bp, RT-PCR revealed another two fragments of 621 (sp1) and 400 bp (sp2), which were not revealed in the control sample (Figure 2B, C). The first is caused by recognition of a cryptic "gt" site (splice score 0.43) at nucleotides 64-65 of intron 8, leading to an insertion of 59 bp (considering the 4 bp deletion of the mutant allele; r.[967_968ins886+1_967+63; 967+2_5del]; p.Ala297Hisfs*23). The other 401 bp fragment is instead generated by skipping of exon 8 (r.806_967del; p.Asp269_Thr323delinsAla). The two alternative spliced products result in a frameshift/truncation and in-frame deletion of the transactivation domain of RUNX1, respectively. Q-PCR performed revealed a higher expression of the sp1 isoform with respect to sp2 (Figure 11 - D, E).

Cancer development in family members

The identification of mutations in *RUNX1* led us to diagnose the three families as affected with FPD/AML. No hematological malignancy was initially reported in Families 1 and 2. However, the proband of family 2 was diagnosed with AML with trilineage dysplasia, FAB subtype M2 one year after she was recognized as a carrier of the c.351+1G>A mutation. Bone marrow examination showed 50% blasts CD34+, HLADR+, CD117+, CD13+, CD33+, CD7+, and negative to CD64, CD56, CD11b, CD61, CD14, CD15, and B/T cell antigens. No cytogenetic abnormalities were detected. She was treated with chemotherapy achieving complete remission and then underwent HSCT from her unaffected brother. She is currently undergoing second-line chemotherapy for relapse of AML six months after HSCT.

A fine anamnesis of family 3 allowed us to study seven additional relatives (six thrombocytopenic and one healthy members). The mutational screening showed that c.967+2_5del segregated with thrombocytopenia (Figure 11-A). Moreover, the anamnesis revealed that the proband's grandmother (II-3) died of AML after three HSCTs. One of the HSC donors was her thrombocytopenic daughter III-4, who eventually developed AML. Moreover, individual I-1 died of lung cancer and the thrombocytopenic member II-2 suffered from uterine cancer. Whereas individual II-3 was an obligate carrier, platelet count and/or DNA samples of I-1 were not available.

Laboratory features

In the individuals carrying heterozygous *RUNX1* mutations (N=13, from the three families), the number of platelets ranged from 70 to 130 $x10^9$/L. The platelet size was in the normal range in all the cases analyzed (N=6; Table 6). No specific morphological alteration of platelets was detected by MGG staining, except for a moderate reduction in alpha-granule content in the patients of family 1 (Table 6), feature that was confirmed by immunofluorescence analysis.

Flow cytometry of platelets showed that the expression of the two major surface GP complexes GPIIb-IIIa and GPIb-IX-V was normal in families 1 and 2 (Table 6). In family 1 we detected a moderate reduction of GPIa-IIa, whose expression level was independent of genotypes at the *ITGA2* locus (Noris P *et al*, 2006). In fact, the surface density of the GPIa-IIa receptor was reduced of 25-45% in all subjects (I-2 and II-2 heterozygous compounds for alleles 1 and 3; II-1 homozygous for the high receptor density allele 1) compared to a healthy control homozygous for the intermediate receptor density allele 3.

Analysis of *in vitro* platelet aggregation showed reduced response after stimulation with low doses of collagen (4 µg/mL) only in the patients of family 1. No second wave aggregation was detectable after stimulation with low doses of ADP (2 µM) in all the five individuals investigated. At higher concentration (ADP 5 µM), the response was defective only in affected individuals II-1 and II-2 of family 1. However, platelets from all the patients had normal responses to higher concentrations of both the agonists (collagen 20 µg/mL and ADP 20 µM) and to ristocetin (1.5 mg/mL), indicating a mild platelet functional defect.

The serum THPO level was normal or slightly increased in patients of family 1, while it was 6-fold higher compared to the upper limit of the normal range in the proband of family 3.

DISCUSSION

In three families with IT, we have identified different mutations of *RUNX1*. The novel missense (p.Ile193Asn) variant identified in family 1 is predicted to be deleterious. Like many other missense mutations of this gene, it affects the runt-homologous domain, a highly conserved region involved in both DNA and CBF-beta binding (Michaud J *et al*, 2002; Glembotsky AC *et al*, 2014). The mutations in the other two families destroy the donor splice site of introns 4 (c.351+1G>A) and 8 (c.967+2_5del). The effect of c.351+1G>A on the splicing process could not be determined. However, the same variant, as well as another affecting the same nucleotide (c.351+1G>T), was previously reported in other FPD/AML pedigrees (Owen CJ *et al*, 2008; Stockley J *et al*, 2013). Prediction analysis suggested that it could cause skipping of exon 4 and/or recognition of a cryptic donor splice site and consequent destruction of the runt-homologous domain. The c.967+2_5del variant generated two alternatively spliced isoforms, an in-frame deletion and a frameshift/truncated form that affect the trans-activating domain. Since the truncated isoform is expressed at higher level than the other, the c.967+2_5del mutation is likely to be associated with haploinsufficiency.

Consistent with data from the literature (Latger-Cannard V *et al*, 2016), the RUNX1 heterozygous carriers had mild thrombocytopenia and normal platelet size associated with mild bleeding tendency. Different and heterogeneous platelet abnormalities have been reported in patients with *RUNX1* mutations, including alpha- or dense- e deficiencies and reduced platelet aggregation and secretion (Michaud J *et al*, 2002; Aneja K *et al*, 2011). Accordingly, we found decreased alpha-granule content but only in the affected members of family 1. Moreover, platelet aggregation was reduced with low doses of ADP, suggesting alterations of delta-granules. Although in our study this hypothesis is not supported by specific assays, deficiency or defective release of the dense-granules is one prevalent defect in individuals with *RUNX1* mutations (Latger-Cannard V *et al*, 2016). Platelet aggregation defects were also observed with low doses of collagen but only in family 1, where a reduced membrane expression of GPIa-IIa was also detected. The low density of the receptor on platelet surface was independent of the genotypes of the family members (Noris P *et al*, 2006). Moreover, the GPIa-IIa defect cannot explain the defective aggregation of platelets after stimulation with collagen, as the specific reagent used for platelet aggregation does not bind the GPIa-IIa receptor. All together, these data indicate that multiple aspects of platelet structure

and function are compromised, as it is expected considering that RUNX1 regulates expression of multiple genes and that their expression is modulated by many genetic factors.

Individuals with heterozygous mutations of *RUNX1* are at risk of hematological malignancies, which have been reported in almost 40% of patients with a median age of onset in the early 30s (Liew & Owen, 2011) . In our cohort consisting of 14 carriers (individual II-3 of family 3 is an obligate carrier), only three individuals (one from family 2 and two from family 3) had developed AML. This low prevalence (20%) could depend on the relatively young age of some individuals. However, families 1 and 2 were selected for molecular screening for IT of unknown origin not associated with leukemia. Therefore, since the enrolment criteria might considerably influence the evaluation of the leukemic risk, a systematic screening of the *RUNX1* gene in the IT families will help determining the effective predisposition to leukemia of individuals with mutations of *RUNX1*.

Although AML remains the most prevalent neoplasia, an increased risk of T-ALL has also been reported in FPD/AML families (Latger-Cannard V *et al*, 2016). A predisposition to B-lymphoid cell malignancies may also be possible, as one individual with a large *RUNX1* intragenic duplication developed a non-Hodgkin lymphoma (Jongmans MC *et al*, 2010). We cannot exclude that mutations of *RUNX1* even predispose to solid tumors, as those (lung and uterine cancer) diagnosed in two members of family 3. A systematic collection of clinical data and an extensive follow-up will be fundamental to define the risk of any neoplasia in individuals carrying mutations of *RUNX1*.

Considering the high risk of developing malignancy, identification of *RUNX1* mutations is essential when a family member is considered as a donor for HSCT. Indeed, if a disorder predisposing to hematological malignancies is not recognized, family members carrying mutations could be chosen for HSCT, leading to insufficient engraftment, poor immune reconstitution, and development of donor-derived malignancies in recipients, as well as hematological malignancies in the donor after HSC mobilization (Churpek JE *et al*, 2016a; Churpek JE *et al*, 2016b). Consistently, individual II-3 of family 3 died of AML after three HSCT, one from her daughter (III-4), who was an unrecognized carrier of the c.967+2_5del mutation and eventually developed AML.

Despite the importance of an appropriate diagnosis, it is difficult to discriminate FPD/AML from other thrombocytopenia/familial malignancies because of the lack of pathognomonic signs at both clinical and laboratory level (Yoshimi A *et al*, 2016). Consistent with the literature, thrombocytopenia could be not completely penetrant and the diagnosis of hematological

malignancies may occur many years after the detection of thrombocytopenia. Although platelets display some morphological and/or in vitro functional defects, no systematic studies have been carried out in large cohorts of FPD/AML individuals to determine their diagnostic value. In terms of specific signs, the most promising feature could be the defects of alpha- and delta-granules, or the persistence of MYH10 protein expression in platelets (Antony-Debré *et al*, 2012). However, these investigations require specialized laboratories and cannot be performed on a routine basis.

Considering that mutations of not only *RUNX1* but also *ANKRD26* and *ETV6* are responsible for thrombocytopenia associated with predisposition to hematological malignancies, recognition of these disorders would provide patients with genetic counseling, clinical follow-up and appropriate treatment, especially in case of HSCT. Even if these autosomal dominant disorders are not associated with any specific clinical or laboratory features, all of them are characterized by normal platelet size, which is a relatively uncommon finding in IT (Noris P *et al*, 2014). Therefore, we suggest that all the individuals with autosomal dominant thrombocytopenia and normal platelet size should be tested for mutations in *RUNX1*, *ANKRD26*, and *ETV6*.

In conclusion, individuals with mutations of *RUNX1* are - at least in our cohort of IT - relatively rare (approximately 1%). At the initial recruitment, only family 3 was suspected to have FPD/AML because of a diagnosis of AML in one member. In the other two families, although a careful diagnostic work-up was carried out (Pecci A, 2016), definite diagnosis was made only after molecular genetic testing. Indeed, the proband of family 2 eventually developed AML. All these data highlight the need to search for mutations of *RUNX1* in family with autosomal dominant thrombocytopenia and normal-sized platelets.

Table 6. Clinical and laboratory finding of individuals carrying *RUNX1* heterozygous germline mutation.

Affected individuals (probands in bold)	Age (years)[1]	Platelet count ($x10^9$/L)[2]	MPV (fL)[3]	MGG-stained blood smear examination	Flow cytometry of platelet surface GPs	Platelet aggregation by collagen, ADP, and ristocetin	Serum THPO level (pg/mL)[4]	Bleeding symptoms	Hematological malignancies (age of onset)
F1/I-2	54	87	7.8	Normal platelet size; reduced alpha-granule content	Normal GPIIb-IIIa and GPIb-IX-V; reduction of GPIa-IIa	Collagen (4 µg/ml): reduced collagen; ADP (2 µM): no second wave	30.33	Menorrhagia, mild bleeding after dental extraction	No
F1/II-1	25	86	11.1	Normal platelet size; reduced alpha-granule content		Collagen (4 µg/ml): reduced collagen; ADP (5 µM): no second wave	14.57	No	No
F1/II-2	24	70-120	11.2	Normal platelet size; reduced alpha-granule content		Collagen (4 µg/ml): reduced collagen; ADP (5 µM): no second wave	14.73	Easy bruising, ecchymosis	No
F2/I-2	52	80-110	8.9	Normal platelet size and morphology	Normal GPIIb-IIIa and GPIb-IX-V	ADP (2 µM): no second wave	nd	Easy bruising	AML (53y)
F2/II-1	12	80-140	8.2	Normal platelet size and morphology			nd	Easy bruising	No
F3/IV-1[5]	3	110 - 130	8.4	Normal platelet size and morphology	nd	nd	110	Epistaxis, easy bruising, ecchymosis	No

Notes: [1]Age at the last evaluation. [2]When more than one platelet count is available, the lowest and highest counts are reported. [3]Data have been acquired by automated counters using different normal range for MPV; all the MPV described were within the normal range. [4] Normal range of serum THPO: 6.9 - 18 pg/mL. [5]Except for cancer development (in Results), no additional information is available for other family members. **Abbreviations:** MGG = May-Grünwald-Giemsa; GPs = glycoproteins. nd = not determined.

A new form of inherited thrombocytopenia due to monoallelic loss of function mutation in the thrombopoietin gene

Thrombopoietin (THPO) is an essential regulator of haemopoiesis that is required for the maintenance of haemopoietic progenitors and their differentiation into megakaryocytes (Mks). Moreover, it modulates the events that drive Mk maturation and allows the release of platelets into bone marrow sinusoids (Kaushansky, 2015). THPO plays these roles by binding the MPL receptor, which is expressed in bone marrow stem cells, Mks, platelets and many other human cells (Columbyova *et al*, 1995).

Until recently, no inherited THPO defect was known to cause thrombocytopenia or bone marrow aplasia. However, it was recently shown that microdeletions encompassing the *THPO* gene in chromosome 3 result in a complex clinical picture, including mild congenital thrombocytopenia (Mandrile *et al*, 2013; Dasouki *et al*, 2014). Moreover, a Micronesian family carrying the homozygous c.112C>T (p.Arg38Cys or p.Arg17Cys in the mature protein) missense mutation in *THPO* presents inherited bone marrow aplasia (Dasouki *et al*, 2013). No *THPO* mutation associated with isolated thrombocytopenia has been reported to date, but the application of whole exome sequencing (WES) in a cohort of patients with inherited thrombocytopenias (ITs) of unknown origin revealed that monoallelic changes in this gene identify a new form of IT.

WES was performed in 86 propositi with unknown IT. They formed part of our case series of 274 consecutive families, 151 of which remained without a definite diagnosis after a diagnostic workup performed according to the diagnostic algorithm of the Italian platelet study group (Balduini *et al*, 2003; Pecci, 2016). The investigation was approved by the Institutional Review Board of the IRCCS Policlinico San Matteo Foundation and all patients gave written informed consent. Target enrichment of the whole exome was performed on DNA from patients' whole blood using the BGI exome kit (BGI TECH SOLUTIONS, Hong Kong) and sequencing was carried out on the HiSeq2000 platform (Illumina Inc., San Diego, CA, USA). Bioinformatic analysis of raw data, including quality controls, reads alignment, variant calls and annotation was carried out as reported before (Marconi *et al*, 2016). Sanger sequencing was used for validation and segregation analysis following standard protocols. Variant names are referred to the *THPO* reference sequence ENST00000204615.

Platelet aggregation after stimulation with collagen (4 and 20 μg, Mascia Brunelli, Milan, Italy), ADP (5 and 20 μmol/l, Sigma Chemical Co, St. Louis, MO, USA), epinephrine (10 μmol/l, Sigma Chemical Co) and ristocetin (0·5 and 1·5 mg/ml, Mascia Brunelli) was investigated in platelet-rich plasma by the densitometric method, as previously reported (Noris *et al*, 2011). The surface expression of platelet glycoproteins (GPs) was investigated by flow cytometry, as previously described (Noris *et al*, 2011), and serum THPO levels were determined by a commercially available enzyme-linked immunosorbent assay (Quantikine Human TPO Immunoassay, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Out of 86 propositi, WES identified two unrelated individuals carrying the heterozygous variant c.91C>T (p. Arg31*), which is expected to result in mutant protein degradation and THPO haploinsufficiency. In each family the segregation with the disorder was confirmed by analysing one affected relative (Table I, Fig 1). This variant was not present in an in-house dataset, including WES data from 445 individuals either healthy or affected with different genetic disorders not associated with thrombocytopenia, and has been reported in only one of the 60 000 individuals in the Exome Aggregation Consortium dataset (ExAC, exac.broadinstitute.org/). Given that the bleeding symptoms in our families carrying this variant are very mild, we believe that the individual described in the ExAC is an asymptomatic thrombocytopenic patient. At least 4 fully penetrant alleles causative for different forms of dominant thrombocytopenia are reported in the ExAC, demonstrating that unrecognized IT patients may be present in this cohort. Altogether, these data support the pathogenic effect of the variant.

Patients with *THPO* mutations had no bleeding tendency and their thrombocytopenia was discovered incidentally. Patient I-2 and II-1 of Family B gave birth to one and two healthy children, respectively, without excessive bleeding.

Blood counts (Table I) show that mild thrombocytopenia with normal or slightly increased platelet size was the only abnormality. Blood film examination did not identify any morphological abnormality of platelets, except for some elements with slightly increased size in members of Family A (Fig 1). *In vitro* platelet aggregation and surface expression of GPIIb/IIIa and GPIb/IX were normal in the two patients of Family B (data not shown). The mild severity of thrombocytopenia and the absence of qualitative platelet defects are consistent with the absence of bleeding tendency.

As discussed, the *THPO* mutation identified in both our families is expected to result in haploinsufficiency, and THPO measurement in members of Family B confirmed this hypothesis. Serum THPO level was 7 and 6·9 pg/ml in

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A NEW FORM OF INHERITED THROMBOCYTOPENIA DUE TO MONOALLELIC LOSS OF FUNCTION MUTATION IN THE THROMBOPOIETIN GENE

Noris P, Marconi C, De Rocco D, **Melazzini F**, Pippucci T, Loffredo G, Giangregorio T, Pecci A, Seri M, Savoia A. A new form of inherited thrombocytopenia due to monoallelic loss of function mutation in the thrombopoietin gene. Br J Haematol. 2017 May 3

INTRODUCTION

Thrombopoietin (THPO) is an essential regulator of hemopoiesis being required for both the maintenance of hemopoietic progenitors and their differentiation into Mks. Moreover, it modulates the series of events that drive maturation of Mks and allow them to release platelets into bone marrow sinusoids (Kaushansky K, 2015). THPO plays these roles by binding to the MPL receptor, which is expressed in bone marrow stem cells, Mks and platelets, as well as in many other human cells (Columbyova L *et al*, 1995).

Different diseases are known to derive from inherited abnormalities of *MPL* and *THPO*. Gain of function mutations in *MPL* and *THPO* cause congenital thrombocytosis (Hong *et al*, 2014), while loss of function mutations in *MPL* result in congenital amegakaryocytic thrombocytopenia (CAMT) (Ballmaier & Germeshausen, 2011). Patients with the last disorder present at birth with isolated thrombocytopenia, which subsequently evolves into severe bone marrow aplasia, confirming the essential role of the THPO-MPL signaling pathway not only for Mks development but also for the long-term maintenance of hemopoietic progenitors.

Until recently, no inherited defect of THPO was known to cause thrombocytopenia or bone marrow aplasia. However, a few years ago it has been shown that microdeletions encompassing the THPO gene in chromosome 3 result in a complex clinical picture including mild, congenital thrombocytopenia (Mandrile G *et al*, 2013; Dasouki M *et al* 2014). Moreover, a Micronesian family carrying the homozygous c.112C>T (p.Arg38Cys or p.Arg17Cys in the mature protein) missense mutation in THPO presents inherited bone marrow aplasia (Dasouki MJ *et al*, 2013). No mutation in *THPO* was so far found associated with isolated thrombocytopenia, but application of

WES to our case series of patients with ITs of unknown origin revealed that monoallelic changes in this gene originate a new form of IT.

PATIENTS & METHODS

Patients

WES was performed in 86 propositi with an unknown IT. They were part of our case series of 274 consecutive families, 151 of which remained without a definite diagnosis at the end of the diagnostic workup carried out according to the diagnostic algorithm proposed in 2003 by the Italian platelet study group (Balduini CL *et al*, 2003) and subsequently updated to include recently discovered disorders (Pecci A, 2016).

Genetic studies

Target enrichment of whole exome was performed on DNA from patients' whole blood using the BGI exome kit (BGI Tech Solutions) and sequencing was carried out on Illumina HiSeq2000 platform (Illumina Inc). Bioinformatic analysis of raw data, including quality controls, reads alignment, variant calls and annotation was carried out as reported before (Marconi C *et al*, 2016). Sanger sequencing was used for validation and segregation analysis following standard protocols. Variant names are referred to the THPO reference sequence ENST00000204615.

Platelet and THPO studies

The study of surface expression of platelet glycoproteins (GPs) and platelet aggregation were investigated as reported in the Materials & Methods section.

Serum THPO levels were determined by a commercially available enzyme linked immunosorbent assay (Quantikine Human TPO Immunoassay) according to the manufacturer’s instructions.

RESULTS AND DISCUSSION

WES in 86 propositi with unknown IT identified two unrelated individuals carrying the heterozygous variant c.91C>T (p. Arg31*), which is expected to result in mutant protein degradation and THPO haploinsufficiency. In each family the segregation with the disorder was confirmed analyzing one affected relative (Table 7 and Figure 12).

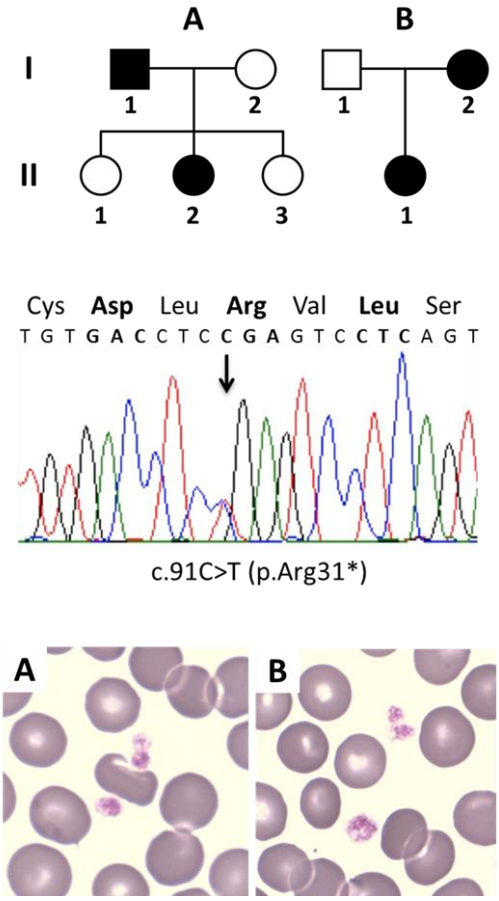

Figure 12. Upper line, pedigrees of the two families with monoalleic THPO mutations; Middle line, direct sequencing of PCR products showing the heterozygous c.91C>T (p.Arg31*) mutation of the THPO gene identified in families A and B; Lower line, representative pictures of May-Grünwald-Giemsa–stained blood films. Platelet morphology was completely normal in patients of family A (A), while a few slightly enlarged platelets were observed in patients of family B (B)

The variant is not present in an in house dataset including WES data from 445 individuals either healthy or affected with different genetic disorders not associated with thrombocytopenia. However, it is reported in a single individual of the about 60000 included in in the Exome Aggregation Consortium (ExAC , exac.broadinstitute.org/) dataset. Since the bleeding symptoms in

our families carrying this variant are very mild, we believe that the individual described in the ExAC is an asymptomatic patient affected with thrombocytopenia. As a matter of fact, at least 4 fully penetrant alleles causative for different forms of dominant thrombocytopenia are reported in the ExAC, demonstrating that unrecognized IT patients may be present in this cohort. Altogether, these data support the pathogenic effect of the variant.

Medical history of patients with THPO mutations revealed that they had no bleeding tendency and their thrombocytopenia was discovered incidentally on the occasion of blood counts performed for other reasons. Moreover, patient I-2 and II-1 of family B gave birth to one and two healthy children, respectively, without excessive bleeding.

Blood counts of affected subjects reported in Table 7 show that mild thrombocytopenia with normal or slightly increased platelet size was the only abnormality. Blood film examination did not identify any morphological abnormality of platelets, except for some elements with slightly increased size in patients of family A (Figure 12). In vitro platelet aggregation and surface expression of GPIIb/IIIa and GPIb/IX were investigated in the two patients of Family B and gave normal results. The mild severity of thrombocytopenia and the absence of qualitative platelet defects, at least in the two patients of family B, are consistent with the absence of bleeding tendency in affected subjects.

Table 7. Main characteristics of investigated patients.

Family/Individual	**A/I-1**	**A/II-2**	**B/I-2**	**B/II-1**
THPO mutation	p.Arg31*	p.Arg31*	p.Arg31*	p.Arg31*
Age, y/Gender	53/M	15/F	57/F	33/F
Platelets x 10^9/L	103	102	112	95
MPV, fL	11.5	11.9	10.4	9.4
Hgb, g/dL	14.1	12.7	14.5	14.3
MCV, fL	91.5	82.4	89.7	91.3
WBC x 10^9/L	6.57	5.09	4.76	4.3
Neutrophils x 10^9/L	3.10	2.55	2.75	2.35

As discussed, THPO mutation identified in both our families is expected to result in haploinsufficiency, and THPO measurement in patients of family B confirmed this hypothesis. In

fact, THPO serum level was 7 and 6.9 pg/mL in patients I-2 and II-1, respectively, thus at the lower limit of the normal range of 6.9-54.4 pg/mL obtained by the study of 50 healthy subjects.

The observation that haploinsufficiency of THPO may be responsible for mild thrombocytopenia is not surprising, since slightly reduced platelet count is one of the features of the syndromic disorders deriving from microdeletions in chromosome 3 including the THPO gene (Mandrile G *et al*, 2013; Dasouki M *et al*, 2014). Moreover, platelet count was mildly reduced ($91x10^9$/L) in the heterozygous father of the two children with inherited bone marrow aplasia due to homozygous c.112C>T THPO mutation (Dasouki MJ *et al*, 2013). Interestingly, two other family members with the same monoallelic change (mother and one sibling) had normal platelet count, suggesting that thrombocytopenia was not fully penetrant. Thus, data from the literature and the two families described here clearly indicate that biallelic *THPO* defects cause congenital bone marrow aplasia, while monoalleic mutations may cause mild thrombocytopenia.

In conclusion, THPO-related thrombocytopenia is a new, autosomal dominant form of IT with normal or slightly increased platelet size and no bleeding tendency. This innocuous disease has to be distinguished from the more severe autosomal dominant ITs with normal platelet size deriving from mutations in *ETV6*, *ANKRD26* and *RUNX1*, which predispose to the development of hematological malignancies. Because of the similarity of the clinical features and the lack of reliable laboratory markers, we suggest that all subjects with autosomal dominant thrombocytopenia and normal platelet size have to undergo genetic analysis to identify their disorders, define prognosis and organize an appropriate follow-up regimen.

ACTN1 MUTATIONS LEAD TO A BENIGN FORM OF PLATELET MACROCYTOSIS NOT ALWAYS ASSOCIATED WITH THROMBOCYTOPENIA

Melazzini F, Faleschini M, Marconi C, Noris P, Giangregorio T, Pecci A, Bottega R, Seri M, Savoia A. ACTN1 mutations lead to a benign form of platelet macrocytosis not always associated with thrombocytopenia. Submitted to British Journal of Haematology

INTRODUCTION

ACTN1-related thrombocytopenia (*ACTN*1-RT; OMIM 615193) is caused by heterozygous mutations in *ACTN1*, a gene encoding for one of the two non-muscle isoforms of α-actinin 1, mainly expressed in megakaryocytes and platelets (Haudek VJ *et al*, 2009). Indeed, 7 independent studies have identified 14 different mutations of *ACTN1* in 44 unrelated families (Kunishima S *et al*, 2013; Boutroux H *et al*, 2017; Yasutomi M *et al*, 2016, Gueguen P *et al*, 2013, Bottega R *et al*, 2015). The ACTN1 protein is organized in an actin binding domain (ABD) constituted of two calponin homology domains (CHD) at the N-terminus, four spectrin-like repeats (SLR), and a calmodulin-like domain (CaM) at the C-terminus (Otey CA *et al*, 2004; Sjoblom B *et al*, 2008). Antiparallel molecules dimerize in rod-like structures with the ABD at each extremity for cross-linking the actin filaments into bundles. Dimers of ACTN1 cross-link actin filaments, which interact with myosins and generate contractile forces. Of note, except for one variant (p.Leu395Gln) altering the rod domain (Yasutomi M *et al*, 2016), all the other mutations are within, or close to, the ABD and CaM domains. Although the exact role of ACTN1 in platelet biogenesis is poorly known, the observation that mouse megakaryocytes expressing ACTN1 variants caused defective proplatelet extension supports the hypothesis that this molecule is important for the latest phases of platelet production (Kunishima S *et al*, 2013).

We recently identified 20 new patients from eight unrelated families suffering from *ACTN1*-RT, who together with those previously reported by us (Bottega R *et al*, 2015), represent a cohort of 51 patients. In addition to mutations previously observed in other patients, we report two novel missense variants, whose pathogenic role has been confirmed by *in vitro* studies. Critical analysis of this large case series, as well as of the data reported in literature, allows us to propose a reliable clinical picture of *ACTN1*-RT.

MATERIALS & METHODS

Patients

We enrolled to the study 130 unrelated patients with IT who were observed at the IRCCS Policlinico San Matteo Foundation of Pavia. All of them had no definite diagnosis because they did not fit the criteria for any known disorder. Whenever *ACTN1* mutations were identified, the proband's available relatives were studied. Information on medical history (family history included), bleeding tendency, and outcome of possible surgeries and pregnancies were obtained from medical records or patients' interview.

Mutational screening

The *ACTN1* gene was screened using genomic DNA extracted from peripheral blood. Mutational analysis was performed by WES as reported in Marconi et al, 2016 or by target NGS of 22 IT genes as described by Nicchia *et al* (Nicchia E *et al*, 2016). Variants of the *ACTN1* gene were confirmed by Sanger sequencing.

Immunofluorescence analysis

A full-length *ACTN1* sequence was amplified from normal platelet cDNA and constructed into the pcDNA3.1-Myc tagged expression vector as previously described (Bottega R *et al*, 2015). Two *ACTN1* mutations (c.384G>C/p.Trp128Cys and c.698C>T/p.Pro233Leu) were generated by directed mutagenesis using specific 29 bp mutagenesis primers designed with the mutations (available upon request). For immunofluorescence assay, CHO cells were seeded on chamber slides and transfected with myc-tagged wild type or mutated *ACTN1* plasmids. After 16 hours, cells were fixed with 4% paraformaldehyde for 20 minutes and then permeabilized with 0.1% Triton X-100. For detection of ACTN1, primary antibody against c-myc (9E10; Santa Cruz Biotechnology, Dallas, Texas, U.S.A.) was used with anti mouse FITC secondary antibody (F0479; DakoCytomation; Glostrup, Denmark), while actin filaments were stained with AlexaFluor594 conjugated phalloidin (Invitrogen, Marseille, France). Images were obtained with a Nikon C1si confocal microscope, containing 488nm, argon laser line and 561 nm diode laser. Images were processed for z-projection (maximum intensity), brightness and contrast regulation using ImageJ 1.45 (NIH, Bethesda, USA).

Blood cell studies

Blood cell counts were evaluated by electronic counters. In some cases, platelet count was also measured by manual platelet counting with optical microscopy in a Neubauer chamber. Platelet diameters were measured on MGG stained blood films by software-assisted image analysis on blood smears as reported. The following parameters were computed: mean platelet diameter (MPD); platelet diameter distribution width (PDDW), as the 97.5th to 2.5th percentiles difference; platelet diameter large cell ratio (PDLCR), as the mean percentage of platelets above the 97.5th percentile of platelet diameter distribution in controls; and platelet diameter small cell ratio (PDSCR), as the mean percentage of platelets below the 2.5th percentile of platelet diameter distribution in controls (Noris P *et al*, 2014).

Surface expression of platelet GPs was investigated by flow cytometry. Platelet aggregation was evaluated by the densitometric method of Born (Noris P *et al,* 2009); both methods are reported at page 17.

RESULTS

Identification of novel *ACTN1*-RT families

In order to define the molecular diagnosis in our case series of patients affected by an IT of unknown origin, we search for mutations using a NGS approach by WES (89 probands) and a target panel (41 probands) of 22 IT genes.

We identified 7 variants of the *ACTN1* gene in 8 families (6.1%). Of these variants, 5 were mutations known to be associated with *ACTN1*-RT. Two variants, c.384G>C/p.Trp128Cys and c.698C>T/p.Pro233Leu, were novel amino acid substitutions at residues within the ABD domain and highly conserved during evolution, as well as in the other α-actinin isoforms (Fig. 13). The c.384G>C variants segregated with thrombocytopenia in 4 affected members of family 3. In family 2, segregation analysis of c.698C>T was not carried out as the proband was the only individual available for the study. Both the variants have a high CADD score of 34, suggesting that they are likely pathogenic (Kunishima S *et al*, 2013; Boutroux H *et al*, 2017; Yasutomi M *et al,* 2016; Gueguen P *et al*, 2013; Bottega R *et al,* 2015).

In order to confirm the pathogenetic role of p.Trp128Cys and p.Pro233Leu, we performed immunofluorescence analyses of the wild type and mutant forms of ACTN1 transiently overexpressed in CHO cell line (Fig. 14). When cells were transfected with the wild type construct, ACTN1 co-localized with actin along filaments in well-organized cytoskeleton. On the contrary, cells transfected with the mutant constructs and stained with phalloidin showed altered distribution of actin, which was not organized in filaments causing an evident disruption in the cytoskeletal structure. Moreover, as shown through the anti-myc antibodies, the mutant ACTN1 lost its specific localization with actin and was uniformly distributed within the cytoplasm. For this reason, according to the functional studies performed on previously reported mutations associated with thrombocytopenia, we concluded that p.Trp128Cys and p.Pro233Leu, are two novel mutations causing *ACTN1*-RT.

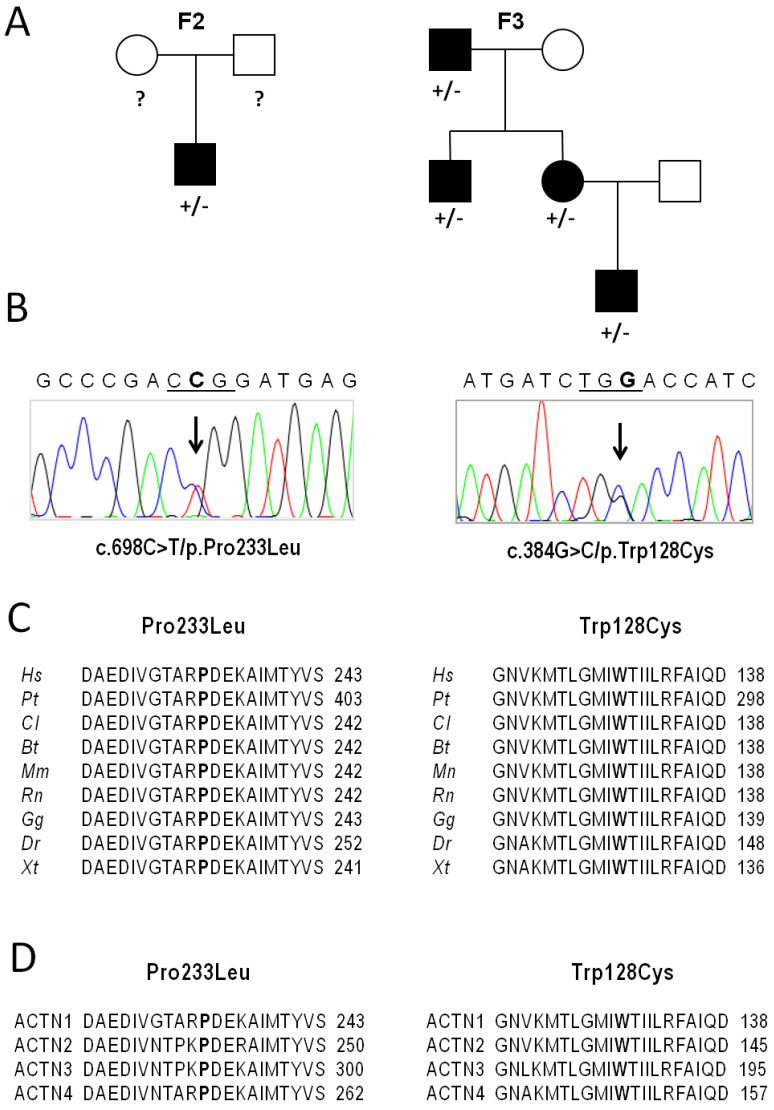

Figure 13. **Identification of two novel *ACTN1* mutations.** (A) Pedigrees of two families with heterozygous novel missense mutations of *ACTN1*. Symbols "+" and "-" indicate wild type and mutant alleles, respectively in the segregation analysis. In family members with question mark, both phenotype and genotype are unknown. (B) Direct sequencing of PCR products showing the heterozygous c.698C>T/p.Pro233Leu and c.384G>C/p.Trp128Cys mutations (C) Multiple-sequence alignment analysis of ACTN1 orthologs from different species, showing conservation of proline and tryptophane at residues 233 and 128, respectively. *Hs*, *H.sapiens* (NP_001123476.1); *Pt*, *P.troglodytes* (XP_001139826.3); *Cl*, *C.lupus* (XP_853103.1); *Bt*, *B.taurus* (NP_001030428.1); *Mm*, *M.musculus* (NP_598917.1); *Rn*, *R.norvegicus* (NP_112267.1); *Gg*, *G.gallus* (NP_989458.1); *Dr, D.rerio* (NP_001161758.1); *Xt*, *X.tropicalis* (NP_001072666.1). (D) Multiple-sequence alignment analysis of human α-actinin isoforms ACTN1 (NP_001123476.1), ACTN2 (NP_001094), ACTN3 (NP_001245300), and ACTN4 (NP_004915).

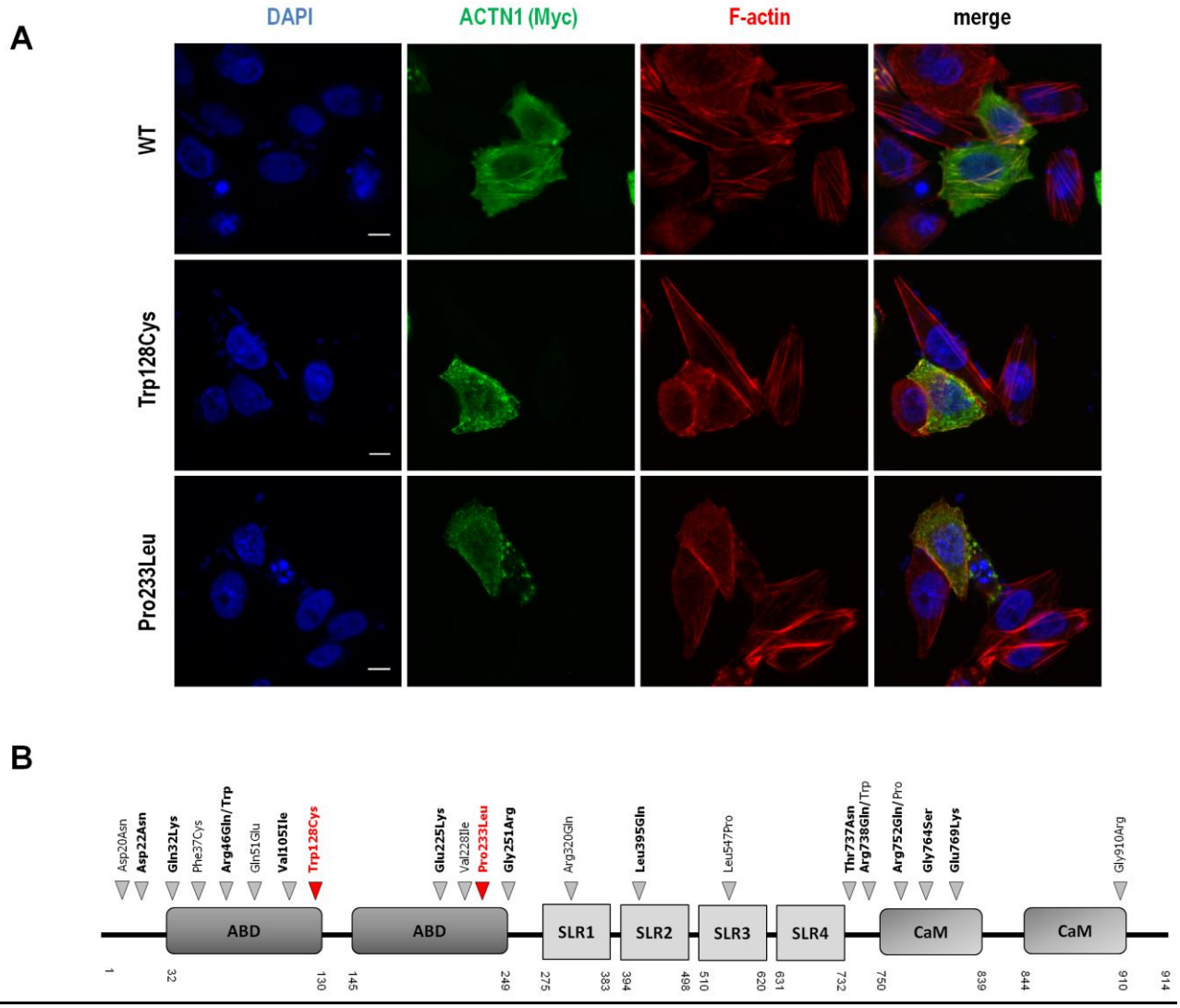

Figure 14. **Functional studies of novel ACTN1 variants.** (A) Immunofluorescence analysis of CHO cell line transiently transfected with Myc-tagged wild type (top panel) or mutant (lower panels) *ACTN1* cDNAs. The subcellular localization of the over-expressed α-actinin1 (green) was examined using a c-myc antibody while the actin filaments were stained with phalloidin (red). The cells shown are representative of three independent experiments. Scale bar, 10 µm. (B) Localization of 24 mutations reported so far in ACTN1(Kunishima S 2013; Boutroux H 2017; Yasutomi M 2016, Gueguen P 2013, Bottega R 2015, Johnson B, 2016, Westbury 2015). The novel mutations reported in this paper are in red. Pathogenicity of mutations in bold style is supported by *in vitro* functional studies (Kunishima S 2013; Yasutomi M 2016; Bottega R 2015). For mutations in regular style, the pathogenic effect is based on bioinformatics prediction (Boutroux H 2017; Johnson B, 2016; Westbury 2015). ABD, actin-binding domain; SLR, spectrin-like repeat; CaM, calmodulin-like domain.

Clinical features

Most of the families with pathogenetic variants in *ACTN1* were from Italy, while one was a Finnish family. Of the 20 mutated family members, 12 were females and 8 males. Family history reported 22 additional family members, dead or not available for this study, with low platelet count revealed in one or more occasions.

In all the propositi, thrombocytopenia was discovered incidentally as blood counts were performed for reasons other than bleeding tendency. Initially, one patient received a diagnosis of immune thrombocytopenia without receiving any treatment due to his mild thrombocytopenia.

Bleeding tendency was absent in 15 of the mutated subjects and very mild in 5, consisting of rare nosebleeds and easy bruising. Eleven patients received one or more surgeries or other invasive procedures without any prophylactic treatment and no excessive blood loss was reported. Seven women gave birth to 14 children (10 vaginal deliveries and 4 cesarean sections) without excessive bleeding. Only one delivery was covered by platelet transfusion.

History and examination of patients did not reveal any congenital or acquired phenotypic defect consistently associated with *ACTN1* mutations.

Blood cell counts and peripheral blood film examination

Hemoglobin and leukocyte values were within the normal range in all cases. Interestingly, platelet count was reduced in most, but not all cases. In fact, using the traditional lower limit of normal platelet count (150 x 10^9/L), 3 mutated cases were not thrombocytopenic (Table 8), while the number of subjects with normal platelet count increased to 5 (25% of cases) using the reference range personalized for age and gender recently proposed for the Italian population (Biino G *et al*, 2013). No genotype-phenotype correlation was found concerning the occurrence of thrombocytopenia.

When present, thrombocytopenia was mild to moderate and no subject had less than 70 x 10^9 platelets/L, while only 7 out of 20 patients had less than 100 x 10^9 platelets/L (Table 8).

Of note, in all the 8 subjects receiving platelet number evaluation by both cell counter and manual counting, we observed an underestimation of the platelet count through the electronic instrument (Table 8). This phenomenon is typical of macrothrombocytopenias, since electronic counters do not recognize very large platelets (Noris P *et al*, 2013). This also alters the MPV evaluation. In fact, measuring the platelet diameters by image analysis on blood smears we observed an increased

platelet size in all cases, including 4 cases with normal MPV reported by the electronic counter (Table 8).

Measurement of PDDW revealed that another constant feature of investigated subjects was platelet anisocytosis due to the presence of a high proportion of large platelets (PDLCR higher than normal in all cases). The percentage of small platelets was instead reduced or normal. Based on these findings, the only blood cell abnormality found in all patients was platelet macrocytosis with anisocytosis. Of note, careful evaluation of blood films revealed platelet poikilocytosis due to the presence of elongated, often curved elements that sometimes resemble closely the proplatelets that are released in vitro by cultured human megakaryocytes (Fig. 15) (Thon *et al*, 2010).

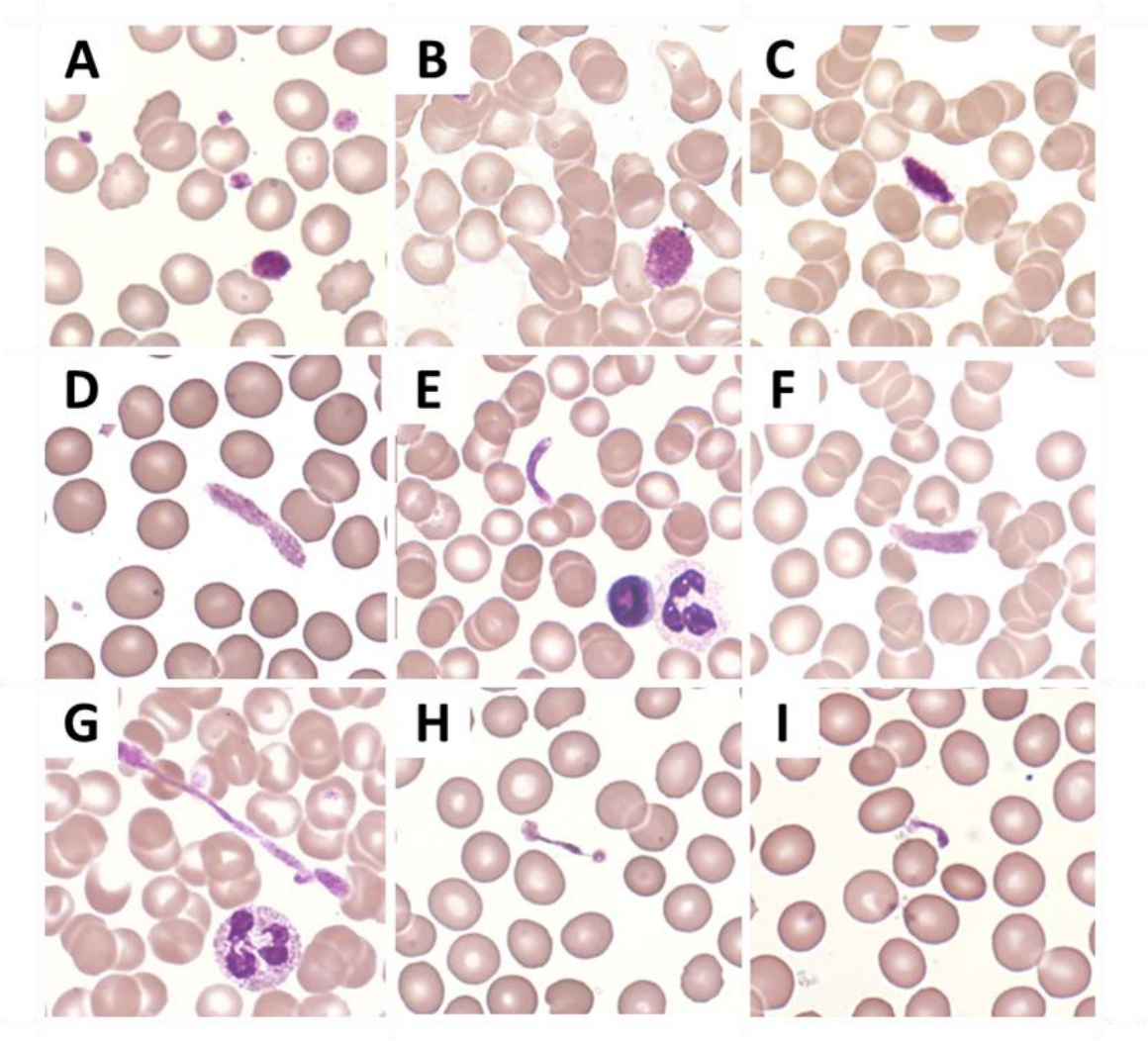

Figure 15. Platelet morphology on peripheral blood films of patients with *ACTN1*-RT. Platelet anisocytosis due to the presence of many large (A) and a few giant platelets (B) beside normal-size platelets is a constant feature of this condition, as well as of many other forms of IT. Another morphological abnormality observed in patients of this study was the presence of few elongated, often curved, elements (C-F) that sometime had the typical features of barbell-shaped proplatelets (G-I). These proplatelet-like elements are not observed in healthy subjects.

Table 8. Platelet characteristics of investigated subjects.

Family/ Patient	Platelet count x 10^9/L*		MPV (fL)**	MPD (μm)**	PDDW (μm)**	PDLCR (%)**	PDSCR (%)**	Aggregation Coll/ADP/Rist°	Glycoproteins IIb/IIIa/Ibα/IX°°
	counter	**manual**							
1/1	147	na	11.4	3.37	3.21	21	0	na	na
1/2	**155**	na	12.2	3.18	3.24	21	1	na	na
2/1	106	121	12.5	2.97	2.93	15	1	N/N/N	I/I/I/I
3/1	71	na	10.9	3.44	4.46	24	2	N/N/N	N/N/N/N
3/2	88	90	11.7	3.23	4.25	23	1	N/N/N	N/N/N/N
3/3	101	na	8.55	3.12	2.98	16	1	N/N/N	N/N/N/N
3/4	67	86	8.99	3.05	5.34	12	0	N/N/N	N/N/N/N
4/1	106	118	11.2	2.80	3.15	8	3	N/N/N	N/N/I/I
4/2	108	119	10.2	2.87	2.77	9	0	na	na
4/3	103	**125**	11.8	3.02	3.59	17	0	N/N/N	N/N/I/I
5/1	89	na	11.3	2.89	3	11	0	N/N/N	I/I/I/I
5/2	91	**124**	12.4	3.92	5.31	39	1	N/N/N	I/I/I/I
5/3	81	na	16.5	3.47	3.19	28	0	N/N/N	N/N/N/N
6/1	104	na	na	3.29	3.04	22	0	na	na
7/1	85	99	14.2	2.91	2.82	11	0	na	na
7/2	**203**	na	na	2.97	3.97	27	6	na	na
8/1	105	na	na	4.12	4.07	52	0	na	na
8/2	90	na	na	3.97	5.13	39	0	na	na
8/3	120	na	na	4.27	6.03	49	1	na	na
8/4	**190**	na	na	3.37	4.96	33	3	na	na

*The lower limit of normal range of platelet count according to the traditional reference range is 150x10^9/L. According to the personalized reference range, it varies according to sex and age: < 15 years, women and men, 165x10^9/L; 15-64 years, women 136x10^9/L, men 120x10^9/L; > 64 years, women 119x10^9/L, men 112x10^9/L. ** Platelet size parameters in 50 healthy subjects (95% Confidence interval): Mean Platelet Volume (MPV) 9.7-10.4 fL; Mean Platelet Diameter (MPD) 2.4-2.7 μM; Platelet Diameter Distribution Width (PDDW) 2.2-2.5 μM; Platelet Diameter Large Cell Ratio (PDLCR) 2.2-4.8%; Platelet Diameter Small Cell Ratio (PDSCR) 3.1-6.3%. ° N: within the normal range of the laboratory. °° N: normal, i.e. mean florescence intensity no more than 20% higher or lower with respect to a control run in parallel; I: increased, i.e. mean florescence intensity 20% higher than control. Platelet counts in bold and underlined are within the normal range of platelet count personalized for age and gender recently proposed for the Italian population (Biino G 2013).

Platelet aggregation and flow cytometry

In vitro platelet aggregation and platelet flow cytometry of the major platelet GPs were performed in 10 of 20 patients. Maximal extent of platelet aggregation after collagen, ADP and ristocetin stimulation was within the normal range of our laboratory. Components of the GPIb/IX/V and

GPIIb/IIIa complexes were not reduced in any patient with respect to controls run in parallel. In a few cases they were increased by more than 20% due to the presence of large platelets.

DISCUSSION

As a result of the recent advances, the number of known ITs is increasing rapidly and this chapter of hematology become very complex for the great variability of clinical symptoms and prognosis of affected subjects (Balduini CL *et al*, Platelets 2017). Some ITs, as WAS, CAMT and *PRKACG*-related thrombocytopenia, are typically characterized by very low platelet counts and severe bleeding tendency. Others, such as the biallelic BSS, have a bleeding risk much higher than expected on the basis of platelet count due to an associated severe defect of platelet function, while many ITs, as *TUBB1*- and *CYCS*-related thrombocytopenia, have mild thrombocytopenia and mild bleeding tendency, or even no bleeding tendency at all. Moreover, many ITs, as thrombocytopenia-absent radius syndrome and WAS, have additional congenital defects associated with thrombocytopenia, while many other forms are not syndromic. Finally, some mutations expose to the risk of acquiring additional defects that endanger patients' life much more than the low platelet count. This is the case of mutations in *RUNX1*, *ANKRD26* and *ETV6*, which expose to the risk of hematological malignancies, *MYH9*, which predispose to kidney failure, and *MPL* and *THPO*, which cause acquired bone marrow aplasia (Melazzini F *et al*, 2017).

Clinical approach to patients with ITs must take into account all these variables to institute personalized follow-up regimens, give appropriate genetic counseling and, when required, administer effective treatments. However, many recently identified ITs are poorly defined concerning their clinical characteristics and this hampers personalized management of affected subjects.

ACTN1-RT is a form of IT described in 2013 by Kunishima et al. in 6 families (Kunishima S *et al*, 2013). Subsequently, other 5 groups (Boutroux H *et al*, 2017; Yasutomi M *et al,* 2016; Gueguen P *et al*, 2013; Bottega R *et al,* 2015; Johnson *et al*, 2016), reported 28 additional families, but patients were not studied homogeneously, and full clinical-laboratory characteristics were not always described. We have previously reported 10 families including 31 affected individuals (Bottega R *et al*, 2015). Together with the 20 subjects from 8 families of this study, our case series

consists of 51 patients from 18 families homogeneously investigated that allowed us to define the clinical features of this condition.

Our work allows us to establish the relative frequency of *ACTN1*-RT. Our database includes 303 consecutive families with ITs. A definite diagnosis was made in 160 cases (including those reported in this paper), while the remaining pedigrees did not fit the criteria for any known disorders. Thus, at least in our experience, *ACTN1* mutations were responsible for thrombocytopenia in 11.2% of families with known diagnosis and 5.9% of all IT families. These data indicate that *ACTN1*-RT is the fifth most frequent form of IT in Italy, after the monoallelic form of BSS (21.9% of families with known disorders), *MYH9*-RD (19.4%), *ANKRD26*-RT (15%) and biallelic BSS (12.5%). Since the large majority of our patients with monoallelic BSS have the p.A156V variant of GPIbα, which is exclusive of the Italian population (Noris P *et al*, 2012), *ACTN1*-RT is expected to be the fourth most frequent form of IT worldwide.

Our case series confirms and extend the concept that *ACTN1*-RT is a mild bleeding disorders. Bleeding tendency, both spontaneous and in the event of hemostatic challenges, was absent or, at most, mild in all cases. Accordingly, platelet count was only slightly reduced, with the majority of patients presenting with a platelet count between 100 and 150 x 10^9/L. Interestingly, using the reference range of platelet count personalized for gender and age recently proposed for the Italian population (Biino G *et al*, 2013), we found that 5 of the 20 subjects investigated in this study had normal platelet counts. On this basis, we reevaluated our previous case series and found that 6 of 31 subjects had normal platelet count (Bottega R *et al*, 2015). Therefore, considering our 51 patients with ACTN1 mutations, penetrance for thrombocytopenia was incomplete, having more than 20% of the carriers normal platelet counts. On the contrary, macrocytosis and anisocytosis of platelets were present in all the mutated individuals, and this abnormality was therefore the only constant phenotypic consequence of the *ACTN1* mutations. Thus, we can conclude that *ACTN1*-RT presents variable expressivity causing the increase of platelet size in all the mutated individuals but manifesting with thrombocytopenia in less than 80% cases.

In our cohort, the *ACTN1* mutations do not associated with other congenital defects. Importantly, they do not predispose to acquire any additional disorder during life, in accordance with the

literature data (Kunishima S *et al*, 2013; Boutroux H *et al*, 2017; Yasutomi M *et al,* 2016; Gueguen P *et al*, 2013). Therefore, *ACTN1*-RT should be regarded as a benign disorder.

Together with the 2 novel variants described here, 24 *ACTN1* mutations have been described so far, all of them causing aminoacid substitutions (Kunishima S *et al*, 2013; Boutroux H *et al*, 2017; Yasutomi M *et al,* 2016; Gueguen P *et al*, 2013; Bottega R *et al,* 2015; Johnson B *et al*, 2016, Westbury *et al*, 2015). Most of the variants map within, or very close to, the ABD and CaM domains and are distributed in mutational hotspots. In fact, 8 variants account for 65% of the *ACTN1*-RT families. In particular, residue Arg46 is the most frequently mutated aminoacid and is substituted by Trp or Gln in 13 families from different populations, while variants affecting Glu225, Arg738 and Arg752 have been found in 5, 7, and 4 families, respectively, suggesting their fundamental role for the ACTN1 activity. Only 3 variants affect different SLR repeats within the rod domain even though it encompasses half of the actinin protein sequence (Yasutomi *et al*, 2016, Westbury *et al*, 2015). Since the ABD and CaM domains are responsible for the interaction with actin, the ACTN1 alterations are likely to impair the actin filament organization.
Indeed, the cytoskeleton structure has been investigated to support its defective organization in 15 variants of ACTN1 (Kunishima S *et al*, 2013; Yasutomi M *et al*, 2016, Bottega R *et al*, 2015), and similar results have been obtained for the two novel mutations p.Trp128Cys and p.Pro233Leu in this work (Figure 14-B). It has been recently reported (Murphy AC *et al*, 2016) that some of these mutant forms of ACTN1 exert a gain-of-function effect, resulting in an increased binding to actin, which is likely to affect its filament dynamics. Finally, another nine *ACTN1* variants have been identified in thrombocytopenic individuals, whose deleterious effect has been predicted only through bioinformatics tools and/or segregation analysis.

Although these observations suggest that the ACTN1 variants alter the cytoskeleton organization, the molecular mechanisms leading to the platelet defects observed in *ACTN1*-RT are not clear. Both low young platelet count and only slightly increased serum thrombopoietin levels indicated that thrombocytopenia derives from defects of the latest phases of megakaryocyte maturation (Bottega R *et al*, 2015). Consistent with this conclusion, MKs from mice expressing mutant forms of ACTN1 extended proplatelets but their tips were reduced in number and increased in size, suggesting a defect in the late phase of proplatelet formation (Kunishima S *et al*, 2013). Moreover, in the case series reported here we identified typical and atypical proplatelets in blood smears of

our patients. Considering that proplatelets are not observed in healthy control blood films, probably because of their quick transition to platelets, we hypothesize that defective ACTN1 impairs fission of proplatelets into platelets. Indeed, Mks originate platelets through the formation of long (up to millimeters in length), thin cytoplasmic extensions that are released into circulation and undergo further development into individual platelets. *In vitro* culture of Mks revealed that platelet biogenesis requires the formation of preplatelets (discoid giant elements) that have the capacity to convert into barbell-shaped proplatelets and undergo fission into platelets (Thon *et al*, 2010). Thus, it is conceivable that the disorganized actin-based cytoskeleton produced in megakaryocytes by ACTN1 variants does not only affect proplatelet extension, as demonstrated by in vitro studies (Kunishima S *et al*, 2013), but also hampers proplatelet fission into platelets.

In conclusion, mutations in *ACTN1* cause platelet macrocytosis in all affected subjects and mild thrombocytopenia in near 80% of cases, but they never result in clinically relevant bleeding tendency. No other defects, either congenital or acquired, have been reported in the 126 patients investigated so far. Therefore, *ACTN1*-RT is a benign disorder and the most important result of diagnosing this disease is the possibility to reassure affected subjects that their prognosis and their quality of life, as well as those of their progeny, is expected to be very good.

CONCLUSIONS

In no field of medicine the view of a group of disorders has changed to an extent as great as in the case of ITs. Until the end of the last century, we knew only a handful of disorders, all of them characterized by a severe bleeding tendency sometimes associated with other congenital defects. The most typical example of isolated thrombocytopenia was BSS, characterized by spontaneous and recurrent hemorrhages often endangering patients' life because of the association of thrombocytopenia with severely defective platelet function (Savoia A, 2014). The prototypical form of syndromic IT was WAS, with frequent and life threatening bleeding since birth associated with congenital, severe immunodeficiency (Buchbinder D *et al*, 2014).

Since the beginning of this century, the identification of dozens of new forms led to more than 30 the number of known ITs and revealed that most patients have mild bleeding tendency or no bleeding tendency at all, because the most prevalent disorders are usually characterized by moderate or mild thrombocytopenia and no severe defect of platelet function (Balduini CL *et al* 2017). In many cases thrombocytopenia is discovered incidentally long after birth, often in adulthood, and a major risk of affected subjects is that of being misdiagnosed with ITP and receiving unnecessary treatments, such as immunosuppressive drugs or splenectomy, which not only do not increase platelet count, but also may cause severe side effects. The hundreds of IT patients reported in literature who received a diagnosis of ITP and were treated accordingly demonstrate that this is a real risk and that suspecting these diseases is not always easy (Noris P *et al*, 2014).

A recently identified aspect of ITs is that some of them predispose to acquire in childhood or adult life specific disorders that may endanger the life of patients much more than thrombocytopenia itself. The most complex and better characterized of these forms is *MYH9*-RD. With more than 300 families reported, *MYH9*-RD is the most prevalent IT worldwide; it is caused by monoallelic mutations in *MYH9*, the gene for the heavy chain of non-muscle myosin IIA (NMMHC-IIA). All patients present at birth with giant platelets and thrombocytopenia, which may result in bleeding tendency of different degrees. In some cases, macrothrombocytopenia remains the only manifestation of the disease throughout life; however, about one-third of *MYH9*-RD patients develop proteinuric nephropathy often leading to end-stage renal disease. Moreover, most *MYH9*-RD individuals acquire sensorineural hearing loss and about 20% of patients develop presenile cataracts (Pecci A *et al*, 2014; Verver EJ *et al*, 2016). The investigation of wide series of consecutive

patients identified genotype-phenotype correlations that allow to predict the evolution of the disease in about 85% of *MYH9*-RD cases, thus providing a more accurate prognostic model (Pecci A *et al*, 2014; Noris P & Pecci A, 2017).

Another important news that emerged from the improved knowledge of this group of disorder was the finding that the consequences of mutations in *RUNX1*, *ANKRD26* and *ETV6*, may be very severe, because if on one hand they are responsible for causing congenital thrombocytopenia, on the other they facilitate the occurrence of hematological malignancies. Moreover, subjects with IT due to *MPL* mutations always develop bone marrow aplasia before the adult life and die when left untreated, while patient with *THPO* mutation, even showing an overlapping clinical picture to CAMT, have a good prognosis (Melazzini F *et al*, 2017). The same good prognosis as the patients affected by *ACTN1* mutation.

Thus, when we are dealing with a patient with an unknown IT, an important question we have to answer is whether his disease belongs to this group of predisposing syndromes or not.

How to suspect and diagnose inherited thrombocytopenias predisposing to other diseases

Whenever we approach a patients who might have an IT predisposing to potentially fatal diseases, we have to preliminarily explain him what predisposition syndromes are, and ascertain if he wants that we try to identify his disorder. In our experience, some patients prefer not to know whether they are at risk of developing, for instance, acute leukemia, because we cannot offer them any treatment or behavior for preventing this occurrence. If the patients wants to know about his disorder, we can use two different approaches for making diagnosis.

- Multi step diagnostic approach

The most powerful tool for suspecting ITs predisposing to the development of severe additional disorders is represented by the evaluation of platelet size, as *MYH9*-RD*,* CAMT*, RUNX1*-RT*, ANKRD26*-RT*, ETV6*-RT and the thrombocytopenia due to mutation in *THPO* represent an exception to the rule that ITs have moderately enlarged platelets.
In fact they are the most frequent non-syndromic ITs with normal (or near normal) platelet size (Balduini CL *et al*, 2017), excepting for *MYH9*-RD, characterized by giant platelet.

Thus normal size platelets indicate the possibility of an IT predisposing to hematological malignancies or to bone marrow aplasia. Mutational screening is required for making a diagnosis of certainty.

MYH9-RD is one of the few forms with giant platelets, defined as a mean platelet diameter higher than 4 µm and more than 50% of platelets larger than 4 µm (half the diameter of red cells) on peripheral blood films (Noris P *et al*, 2014). Thus, *MYH9*-RD must be suspected in all patients with this characteristic. Blood smear examination is very important for diagnosing this disorder also because Döhle-like bodies (Figure 16) are identified in 15-100% of neutrophils of 42-84% of patients (Savoia A *et al*, 2010). In case Döhle-like bodies are not identified, we can confirm or deny *MYH9*-RD using another simple test: the immunofluorescence evaluation of NMMHC-IIA distribution within neutrophils (Pecci A *et al*, 2014). In fact, this protein is always clumped in *MYH9*-RD, while it is distributed homogeneously within the cytoplasm in all the other ITs (Figure 16). Although the immunofluorescence test is absolutely specific and sensitive, mutation screening for *MYH9* is useful not only for a diagnostic confirmation, but also for defining patients' prognosis (see above).

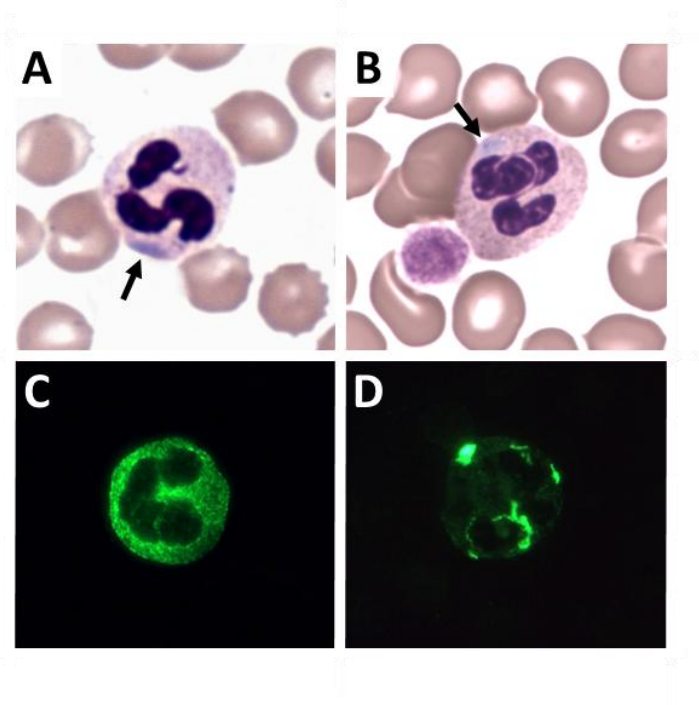

Figure 16. Peripheral blood films in *MYH9*-related disease. Evaluation platelet and neutrophil morphology is a powerful tool for suspecting *MYH9*-RD. The presence of many platelets as large as red cells and of faint, light blue, spindle shaped or round inclusion bodies (Döhle-like bodies), typically located at the cell periphery of neutrophils, is pathognomonic of this condition (A, B). However, in many patients Döhle-like bodies are not identified in May-Grünwald Giemsa stained blood smears. Instead, immunofluorescence analysis with antibodies against NMMHC-IIA reveals that the clumps of this protein that originate Döhle-like bodies are present in all neutrophils of all patients (D). Since NMMHC-IIA is homogeneously distributed in healthy subjects (C) as well as in all other forms of ITs, both specificity and sensitivity of this test are very high.

CAMT, FPD/AML, *ANKRD26*-RT and *ETV6*-RT are the most frequent non-syndromic ITs with normal (or near normal) platelet size (Balduini C & Melazzini F, 2017). Thus, as giant platelets must arouse the suspicion of *MYH9*-RD, normal size platelets indicate the possibility of an IT predisposing to hematological malignancies or to bone marrow aplasia. In case of young children, we have to perform bone marrow examination to identify CAMT (Babushok DV *et al*, 2016). In older patients, especially when thrombocytopenia is transmitted in an autosomal dominant fashion and family history is positive for hematological malignancies, we have to suspect FDP/AML, *ANKRD26*-RT and *ETV6*-RT. In all cases, mutation screening is required for making a diagnosis of certainty.

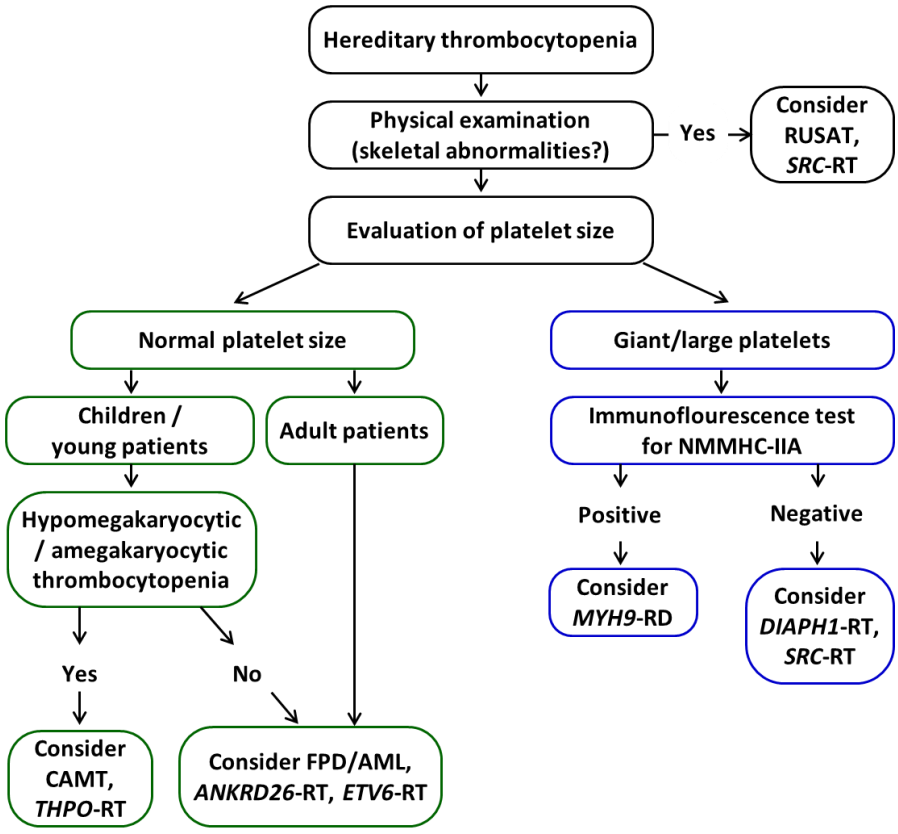

Figure 17. Diagnostic algorithm for IT predisposing to additional illnesses based on the evaluation of a few basic patients' clinical features.

Figure 17 proposes an updated diagnostic algorithm for ITs predisposing to additional illnesses based on the evaluation of a few basic patients' clinical features (Noris P & Pecci A, 2017). After excluding a syndromic IT by physical examination, evaluation of platelet size on peripheral blood smears can guide the diagnostic workup (Noris P *et al*, 2014; Pecci A *et al*, 2014). In patients with large platelets, the suspicion for *MYH9*-RD should be raised independently of the presence of

additional clinical features of the disease (Pecci A *et al*, 2014). In patients with normal MYH9 distribution in neutrophils, *DIAPH1*-RT and *SRC*-related thrombocytopenia should be considered. In children and young individuals with thrombocytopenia and normal-sized platelets, with or without anemia and/or neutropenia, a bone marrow examination should be performed to search for CAMT; very high serum THPO levels will support this diagnostic hypothesis while not being sufficient. In children in whom CAMT is excluded and in adults with normal platelet size, FPD/AML, *ANKRD26*-RT, and *ETV6*-RT should be considered, especially if family history is consistent with autosomal dominant transmission. Molecular analysis is required to confirm the diagnosis and provide patients with personalized management, counseling, and follow-up.

- Single step diagnostic approach

Recent advances in genetic techniques made feasible the targeted analysis of many different genes or even of the whole exome at once. Thus, it is now possible to search for mutations in all the genes whose mutations are known to be responsible for ITs as a single-step diagnostic procedure instead of prioritizing one or a few genes to be sequenced on the basis of patients' characteristics. However, massive sequencing invariably finds in each patient a number of changes in many genes, and distinguishing pathogenetic from non-pathogenetic variants may be difficult.

Single or multi step diagnostic approach?

The best diagnostic approach to ITs is still a matter of debate. The single step approach may be able to identify quickly patients' disorders in case massive sequencing identifies mutations that are already known to cause specific illnesses. Instead, in case no known mutation is found, there is the need of functional studies, which are time consuming, expensive and, much more importantly, require advanced technical skills that are available in only a few laboratories worldwide. At the opposite, the multi-step approach for limiting the number of candidate genes is time consuming, but has the advantage of being based on clinical finding and simple tests that can be performed in many laboratories.

Therefore, we believe that the skills available locally influence the choice, even if, in our opinion, combining the clinical approach with advanced sequencing techniques offers the best chances of success.

Making a definite molecular diagnosis is essential for giving informative genetic counselling and for planning the most appropriate follow-up regimen. Of note, specific treatments for specific disorders have been identified. For instance, it has been shown that bone marrow transplantation is the first line treatment for patients with CAMT due to *MPL* mutations or WAS due to *WAS* mutations, who always die when left untreated. At variance, drugs mimicking the effect of THPO (THPO receptor agonists, THPO-RA) are quite effective in increasing platelet count in patients with *MYH9*-RD and in the form of CAMT caused by *THPO* mutations. Moreover, THPO-RA cure and/or prevent the acquired bone marrow aplasia that patients with the latter condition always develop during childhood or early adult life. Finally, it has been shown that splenectomy increases survival in patients with WAS and has to be considered as an effective treatment in patients who cannot receive transplantation. Thus, making a definite diagnosis is essential for a proper management of patients with ITs, who will benefit from prognostic definition, personalized follow-up regimens and treatments.

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