

# 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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greatly and we presently recognize at least 26 disorders caused by mutations in 30 genes.<sup>1,2</sup> This advancement of knowledge revealed that most patients with inherited thrombocytopenias have only mild or moderate thrombocytopenia, with trivial bleeding episodes or no bleeding at all. However, it also became apparent that many patients are exposed to a threat of acquiring additional defects that worsen their quality of life or can even be fatal. Subjects with *MYH9*-related disease are predisposed to proteinuric nephropathy evolving into end-stage renal failure, those with congenital amegakaryocytic thrombocytopenia always develop bone marrow aplasia, while patients with *ANKRD26*-related thrombocytopenia (*ANKRD26*-RT) or familial platelet disorder with predisposition to acute myeloid leukemia (AML) due to *RUNX1* mutations (FPD/AML) have increased risk of AML and myelodysplastic syndromes. Thus, bleeding is no longer the unique problem of inherited thrombocytopenia patients.

In 2015, four independent studies showed that mutations in the *ETV6* gene are responsible for a new form of inherited thrombocytopenia and suggested that *ETV6*-related thrombocytopenia (*ETV6*-RT) predisposes to acute lymphoblastic leukemia (ALL).<sup>3-6</sup> However, only a few families have been reported so far and the clinical and laboratory features of *ETV6*-RT remain poorly defined.

In order to gain further information on this disorder, we screened 130 consecutive unrelated probands with inherited thrombocytopenia of unknown origin for *ETV6* mutations and identified seven affected families. Two of these pedigrees have been briefly reported in a previous paper.<sup>4</sup> Here we describe the features of 20 affected subjects, who form the largest cohort of *ETV6*-RT patients collected so far. As these patients were identified by screening a series of consecutive, unselected probands with familial thrombocytopenia, we could estimate the relative frequency of *ETV6*-RT among inherited thrombocytopenias and the risk of hematologic malignancies associated with this condition. By reporting the clinical and laboratory features of these patients in detail, we provide indications to raise the level of suspicion of the presence of this disorder from the findings of routine diagnostic workup of probands with inherited thrombocytopenia. Finally, we discuss the pathogenesis of *ETV6*-RT, having investigated, for the first time, megakaryocytes differentiated from hematopoietic progenitors of patients with *ETV6*-RT and functionally characterized the patients' platelets.

## Methods

### Patients

Between 2003 and 2014, we analyzed at the IRCCS Policlinico San Matteo Foundation of Pavia (Italy) 274 consecutive unrelated probands with familial thrombocytopenia. By applying a well-defined diagnostic algorithm for inherited thrombocytopenias,<sup>1</sup> 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 inherited thrombocytopenia. These 130 consecutive probands with inherited thrombocytopenia of unknown origin have been screened for mutations in *ETV6*. Whenever *ETV6* mutations were identified, the available relatives of probands were also investigated.

Bleeding tendency was measured using the International

Society on Thrombosis and Haemostasis bleeding assessment tool.<sup>7</sup>

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

### Mutation screening and reverse transcriptase polymerase chain reaction analysis

Genomic DNA and RNA were extracted from peripheral blood. The *ETV6* gene was analyzed using Sanger and whole exome sequencing. Methods of mutation screening and reverse transcriptase polymerase chain reaction analysis are detailed in the *Online Supplementary Information*.

### Bioinformatic tools and analysis of *ETV6* structure

The bioinformatic tools used to evaluate missense variants together with the methods used to analyze *ETV6* structure are reported in the *Online Supplementary Information*.

### Basic blood cell studies

Blood cell counts were evaluated by electronic counters. Parameters relative to platelet diameter were measured by software-assisted image analysis on blood smears, as reported elsewhere.<sup>8</sup> The following previously defined parameters were computed: mean platelet diameter, platelet diameter distribution width, platelet diameter large cell ratio, and platelet diameter small cell ratio.<sup>8</sup> The percentage of large platelets was also estimated empirically, as previously reported<sup>8</sup> and detailed in the *Online Supplementary Information*. Surface expression of platelet glycoproteins (GP) was investigated by flow cytometry as reported, whereas platelet aggregation was evaluated using the densitometric method described by Born.<sup>9</sup> The antibodies and platelet agonists used are listed in the *Online Supplementary Information*.

### Platelet activation

Platelet activation in response to ADP or TRAP was investigated by flow cytometry as reported previously.<sup>10</sup> The protocol is described in detail in the *Online Supplementary Information*.

### Platelet adhesion and spreading

Platelet adhesion and spreading on the subendothelium components of the extracellular matrix, type I collagen, von Willebrand factor, or fibrinogen, were investigated as previously described<sup>11,12</sup> and as detailed in the *Online Supplementary Information*.

### Investigation of megakaryocytes

Megakaryocytes were differentiated *in vitro* from peripheral blood CD45<sup>+</sup> cells as previously reported.<sup>13,14</sup> Morphological analysis of megakaryocytes was performed by phase-contrast and fluorescence microscopy, while the percentage of fully differentiated megakaryocytes and megakaryocyte ploidy at the end of the culture were investigated by flow cytometry.<sup>14,15</sup> Proplatelet yields were evaluated both in suspension and following adhesion on fibrinogen at the end of the culture, as previously described.<sup>15,16</sup> Methods are reported in the *Online Supplementary Information*.

### Statistical analysis

Data are presented as means and standard deviations or ranges. Statistical comparisons were performed by the 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.

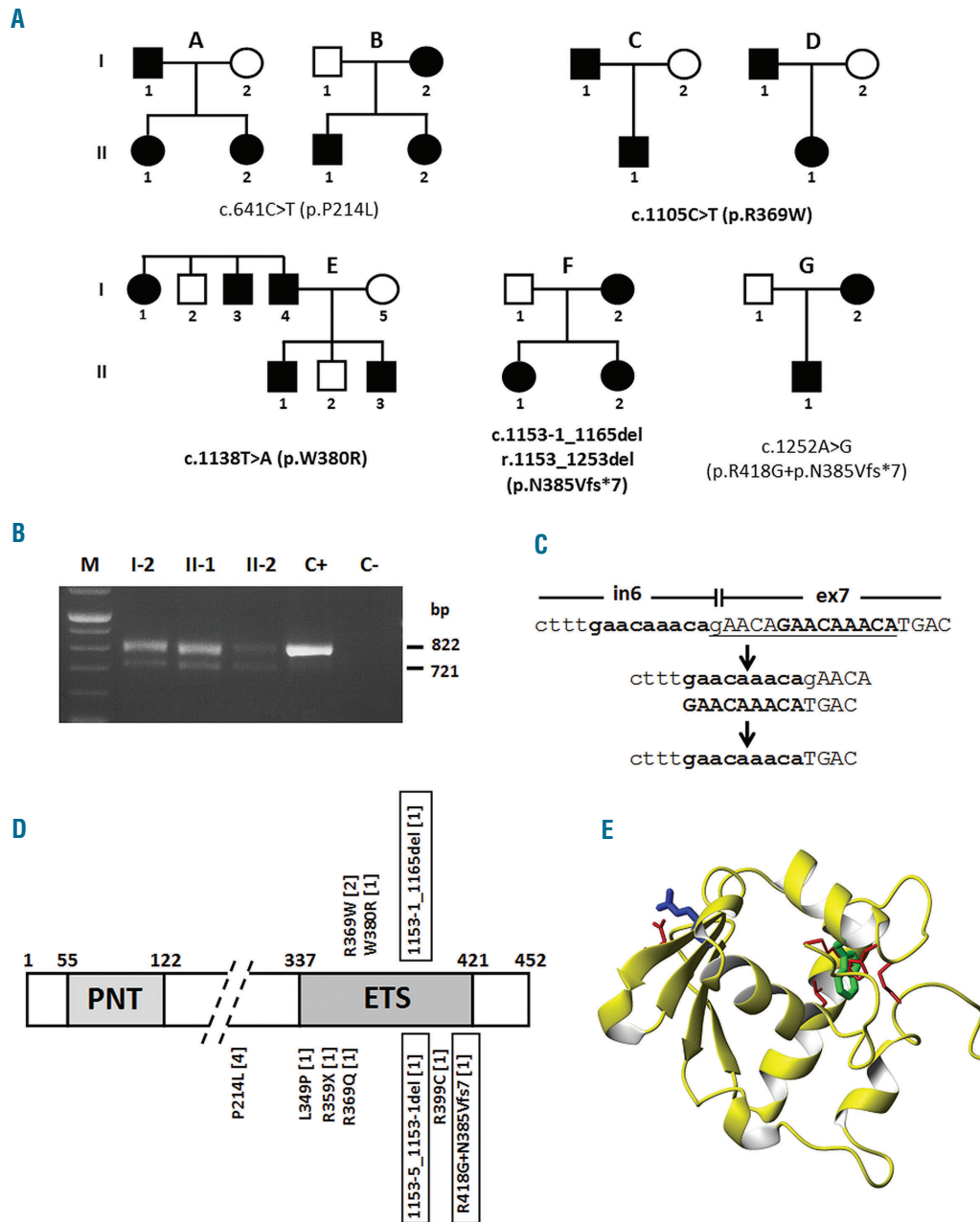
## Results

### Mutation screening

Analysis of the *ETV6* gene allowed us to identify five different heterozygous variants in seven unrelated pedigrees. Two variants (c.641C>T/p.P214L and c.1252A>G/p.R418G+p.N385Vfs\*7) have been reported previously in

two families (Figure 1A, families B and G).<sup>4</sup> The remaining three novel variants are two missense alterations and one deletion.

The two missense variants, c.1105C>T (p.R369W) and c.1138T>A (p.W380R) segregate in the affected family members and are not present in healthy relatives (Figure 1A). They are absent in public genomic databases, such as



**Figure 1. Mutations identified in the *ETV6* gene and their effect on protein structure.** (A) Pedigrees of families enrolled in this study carrying different mutations as indicated (novel mutations in bold). 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 have been previously reported (Noetzi et al.<sup>4</sup>). (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, pointed N-terminal domain; ETS, C-terminal DNA binding domain), with mutations identified in *ETV6*, already reported or identified in this study (top). The numbers 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.

dbSNP ([www.ncbi.nlm.nih.gov/SNP](http://www.ncbi.nlm.nih.gov/SNP)), 1000 genomes ([www.1000genomes.org](http://www.1000genomes.org)), and Exome Aggregation Consortium ([www.exac.broadinstitute.org](http://www.exac.broadinstitute.org)). Multiple-sequence alignment indicated that they affect highly conserved amino acid residues (*data not shown*). They are predicted to be deleterious for protein function according to different tools (*Online Supplementary Table S1*). Moreover, the CADD scores were 26.1 and 23.1 for p.R369W and p.W380R, respectively. Both mutations map in the E26 transformation-specific (ETS) domain which is in the C-terminal half of the protein (residues 337-421). Analysis of the coordinates of the ETS domain of ETV6 (2DAO) (numbered in pdb C8 and L112, so that R369 and W380 correspond to R39 and W50) showed that 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 1E). W380 is also close to the side chains of H383 and K384 (H53 and K54). Its substitution to an arginine will greatly destabilize the structure by creating both an uncompensated cavity in the hydrophobic core and electrostatic repulsion of nearby positively charged residues. Residue R369 is well exposed

on the protein surface and is predicted to form an electrostatic interaction with the spatially nearby E361 (E31). Its substitution by a tryptophan could destabilize the fold by abolishing this interaction. Alternatively, this residue could be implicated in protein-protein interactions. In this case, its substitution by a much bulkier and uncharged residue could 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. To investigate the effect of this deletion, we carried out reverse transcriptase polymerase chain reaction analysis 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 1B) resulting in truncation of the ETS domain. Since the 721 bp band was fainter than the wild-type product (822 bp), we cannot exclude that the alternatively spliced mRNA was partially degraded. Inspection of the intron 6/exon 7 genomic boundary revealed repeats that are likely to be involved in non-allelic homologous recombination leading to micro-deletions/duplications (Figure 1C).

The seven families reported in Figure 1A formed our

**Table 1.** Main characteristics of the investigated patients.

| Family/<br>Individual | ETV6 mutation <sup>b</sup>                          | Age <sup>c</sup> , y/<br>Gender | Age at<br>diagnosis <sup>d</sup> , y | ISTH BAT<br>score <sup>e</sup> | Platelets,<br>x10 <sup>9</sup> /L | MPV,<br>fl <sup>f</sup> | MPD,<br>µm <sup>g</sup> | Hb,<br>g/dL | MCV,<br>fl <sup>h</sup> | WBC, Neutrophils<br>x10 <sup>9</sup> /L x10 <sup>9</sup> /L | Hematological<br>malignancies |
|-----------------------|---|---------------------------------|--------------------------------------|--------------------------------|-----------------------------------|-------------------------|-------------------------|-------------|-------------------------|---|-------------------------------|
| A/I-1                 | c.641C>T<br>p.Pro214Leu                             | 57/M                            | 30                                   | 3                              | 115                               | 8.8                     | 2.44                    | 14.6        | 99                      | 7.13 4.9  |                               |
| A/II-1                |   | 20/F                            | birth                                | 7                              | 59                                | 8.6                     | 2.24                    | 10.4        | 68                      | 4.98 2.3  |                               |
| A/II-2                |   | 27/F                            | birth                                | 3                              | 82                                | 8.2                     | 2.23                    | 13.6        | 98                      | 5.5 3.39  | Common ALL at age 7 years     |
| B/I-2'                |   | 43/F                            | 14                                   | 0                              | 115                               | 10                      | 2.82                    | 11.1        | 88                      | 5.02 1.75   | B-cell ALL at age 15 years    |
| B/II-1'               |   | 15/M                            | birth                                | 3                              | 66                                | 10.4                    | 2.89                    | 14.0        | 91                      | 5.36 1.18   |                               |
| B/II-2'               |   | 18/F                            | 2                                    | 0                              | 44                                | 10.1                    | 3.26                    | 13.1        | 97                      | 4.04 1.42   |                               |
| C/I-1                 | c.1105C>T<br>p.Arg369Trp                            | 48/M                            | 38                                   | 3                              | 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<br>p.Trp380Arg                            | 37/F                            | 8                                    | 0                              | 105                               | 8.1                     | na                      | 14.2        | 97                      | 7.50 5.2  |                               |
| E/I-3                 |   | 42/M                            | 5                                    | 4                              | 55*                               | 9.1*                    | na                      | 14.9*       | 94*                     | 8.0* 6.1*   | JAK2V617F+ PV at age 37 years |
| E/I-4                 |   | 45/M                            | 20                                   | 0                              | 93                                | 7.9                     | na                      | 16.9        | 101                     | 8.30 4.24   |                               |
| E/II-1                |   | 20/M                            | 4                                    | 2                              | 60*                               | 8.0*                    | 2.73*                   | 14.8*       | 86*                     | 5.11* 1.97*   | Common ALL at age 7 years     |
| E/II-3                |   | 13/M                            | birth                                | 4                              | 99                                | 7.4                     | na                      | 14.0        | 90                      | 6.15 2.45   |                               |
| F/I-2                 | c.1153-1_1165del<br>r.1153_1253del<br>p.Asn385Vfs*7 | 49/F                            | 7                                    | 2                              | 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                                | 2                              | 70                                | 8.7                     | 2.36                    | 14.4        | 97                      | 8.24 5.3  | Common ALL at age 3 years     |
| G/I-2*                | c.1252A>G<br>p.Arg418Gly +<br>p.Asn385Vfs*7         | 51/F                            | 20                                   | 0                              | 101                               | 7.6                     | 3.17                    | 13.6        | 97                      | 4.71 2.02   |                               |
| G/II-1*               |   | 28/M                            | 3                                    | 2                              | 101                               | 7.8                     | 2.99                    | 15.9        | 97                      | 5.3 2.39  |                               |

\*Previously reported patients. <sup>b</sup>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. <sup>c</sup>Age at the last evaluation: the blood parameters and bleeding score reported here were measured at the last evaluation, unless otherwise specified. <sup>d</sup>Age at diagnosis of thrombocytopenia. <sup>e</sup>International Society on Thrombosis and Haemostasis (ISTH) bleeding assessment tool (BAT) score was calculated as previously reported (Lowe et al.). <sup>f</sup>Normal range: 8-13.4 fL. <sup>g</sup>Normal range: 1.9-3.4 µm. <sup>h</sup>Normal range: 82-98 fL. <sup>i</sup>Parameters measured at the last available examination before the development of PV (age 35). <sup>j</sup>Parameters measured before chemotherapy and hematopoietic stem cell transplantation for the development of ALL. Na: not available; ALL: acute lymphoblastic leukemia; PV: polycythemia vera.



cohort of 20 affected individuals who have been studied to characterize the phenotype of *ETV6*-RT.

### Clinical picture

A mild bleeding tendency was present in 12 patients, whereas eight subjects did not have any significant bleeding diathesis (Table 1). The more common bleeding symptoms were petechiae, ecchymoses, gum bleeding, epistaxis, and menorrhagia. Thrombocytopenia was discovered in adulthood in five patients, whereas it was identified at birth in six patients because of the family history of low platelet count. One patient (E/I-3) was initially misdiagnosed with immune thrombocytopenia, and received steroids and underwent splenectomy at the age of 9 years without this producing an increase in his platelet count. Ten patients had undergone 17 operations and six had had teeth extracted without excessive bleeding. Four women had given birth to six 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 (patient F/I-2) who had given birth vaginally. We have no information on her platelet count or function at the time of the delivery; however, it is interesting to note that she had a defective platelet response to low doses of collagen and ADP when she was investigated at our institution (see below).

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

Four patients from four families developed B-cell ALL during childhood (common ALL in three cases, not better defined in one). Conventional cytogenetic analysis resulted normal in three cases, while patient E/II-1 had hyperdiploid ALL; the search for the *ETV6-AML1* transcript was performed in one patient (E/II-1) with normal findings. The incidence of ALL in our case series was 731.3 per 100,000 (95% CI, 274.5-1948.4), while it is 1.4 per 100,000 in the general population according to the National Cancer Institute.<sup>17</sup> Three patients obtained remission after conventional chemotherapy, and one after hematopoietic stem cell transplantation from an unrelated donor. Patient E/I-3, who had a history of isolated thrombocytopenia

since childhood (Table 1), at the age of 37 developed an increased hemoglobin level (19.0 g/dL, hematocrit 56%), with mild leukocytosis and thrombocytosis. The *JAK2V617F* mutation was identified and a diagnosis of polycythemia vera was made.

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

### Blood cell counts and peripheral blood film examination

Table 1 reports the blood cell counts obtained at the last examination for 18 patients, and at the last available examination before the development of polycythemia vera and before hematopoietic stem cell transplantation for patients E/I-3 and E/II-1, respectively. Eleven patients had fewer than  $100 \times 10^9$  platelets/L and only one fewer than  $50 \times 10^9$ /L. For most patients we had platelet counts measured at different ages prior to the evaluation for this study (*Online Supplementary Table S2*). There was some fluctuations in the patients' platelet counts over time, but none of the patients showed a definite trend toward improvement or worsening of thrombocytopenia during their life.

The mean platelet volume was slightly reduced in four cases and normal in the other 14 evaluable patients (Table 1). Peripheral blood film examination in 16 patients showed that mean platelet diameter was similar to that of healthy subjects, confirming that average platelet size is consistently normal in *ETV6*-RT patients (Table 2). We found very mild but significant increases in platelet diameter distribution width and platelet diameter large cell ratio, which indicate that a mild platelet anisocytosis and a slightly increased proportion of large platelets were frequent features of the investigated patients. In agreement with previous findings,<sup>8</sup> empirical measurement of the percentage of platelets larger than half an erythrocyte gave similar results to the assessment of platelet diameter large cell ratio by image analysis (*data not shown*). Conversely, the increased mean platelet diameter distribution width detected by image analysis did not correspond to increased mean platelet distribution width values

**Table 2. Parameters of platelet diameters measured on peripheral blood films in investigated patients.**

|                                      | N. <sup>a</sup> | MPD, $\mu$ m<br>mean (SD) | PDDW, $\mu$ m<br>mean (SD) | PDLCR, %<br>mean (SD) | PDSCR, %<br>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)            |
| <b>Total <i>ETV6</i>-RT patients</b> | <b>16</b>       | <b>2.63 (0.17)</b>        | <b>2.70 (0.29)*</b>        | <b>9.23 (4.47)*</b>   | <b>2.85 (1.77)</b>    |
| <b>Healthy subjects<sup>b</sup></b>  | <b>55</b>       | <b>2.49 (0.32)</b>        | <b>2.18 (0.58)</b>         | <b>3.64 (4.93)</b>    | <b>4.35 (5.9)</b>     |

<sup>a</sup>Number of investigated subjects. <sup>b</sup>Values of healthy subjects previously measured in a cohort of 55 healthy volunteers (Noris et al.<sup>8</sup>). \* $P < 0.01$  with respect to healthy subjects. MPD: mean platelet diameter; PDDW: platelet diameter distribution width = difference from the 2.5 to the 97.5 percentile of platelet diameter distribution; PDLCR: platelet diameter large cell ratio = proportion of platelets larger than the 97.5 percentile of MPD of healthy subjects (3.9  $\mu$ m); PDSCR, platelet diameter small cell ratio = proportion of platelets smaller than the 2.5 percentile of the MPD of healthy subjects (1.6  $\mu$ m) (Noris et al.<sup>8</sup>).

**Table 3.** *In vitro* platelet aggregation and surface expression of major platelet glycoproteins in investigated patients.

| Family | Platelet aggregation, maximal extent, % <sup>a</sup> - mean (range) |                   |            |                       | Surface expression of platelet glycoproteins, % of controls - mean (range) |                     |               |                 |
|--------|---|-------------------|------------|-----------------------|--|---------------------|---------------|-----------------|
|        | N. of investigated subjects   | Collagen, 4 µg/mL | ADP, 5 µM  | Ristocetin, 1.5 mg/mL | N. of investigated subjects  | GPIb $\alpha$ (SZ2) | GPIX (SZ1)    | 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)   | 98.7 (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)      |

<sup>a</sup>Normal ranges: collagen 66-88; ADP 43-76; ristocetin 67-90.

obtained by automated cell counts (*data not shown*).

Mild anemia was observed in one patient with iron deficiency (A/II-1). Mean corpuscular volume was reduced in this subject, increased without any apparent cause in five subjects, and within the normal range in the remaining patients. White blood cell count was normal in all the cases.

### 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 µg/mL and ADP 5 µM, while individual C/II-1 showed a slightly reduced response to ristocetin 1.5 mg/mL (Table 3). 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.

#### Platelet flow cytometry

As shown in Table 3, flow cytometry performed in 11 patients did not identify any consistent defect of the major glycoproteins of the platelet surface.

#### Platelet activation

Overall, the surface expression of activated GPIIb-IIIa and P-selectin and the reduction of GPIb $\alpha$  upon stimulation of platelets with ADP or TRAP, were not significantly different in 11 *ETV6*-RT patients with respect to those in controls (*Online Supplementary Figure S1*). A mild reduction of activated GPIIb-IIIa expression after stimulation with TRAP (52% to 65% of the expression in controls) was observed in three patients.

#### Platelet adhesion and spreading

*In vitro* adhesion of platelets from seven 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 4).

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

Megakaryocytes from eight patients and eight healthy subjects were cultured *in vitro*. After 14 days of culture, expression levels of the major megakaryocyte differentia-

**Table 4.** *In vitro* platelet interaction with subendothelium molecules in seven *ETV6*-RT patients.

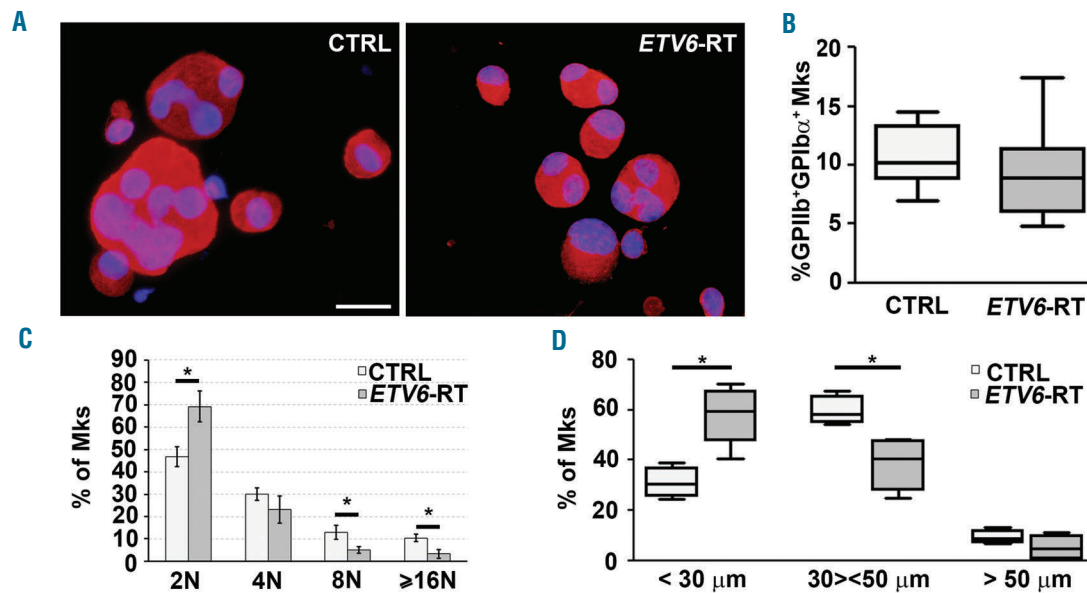
|                       | Platelet adhesion and spreading, % of controls - mean (SD) |                       |                                   |
|-----------------------|--|-----------------------|-----------------------------------|
|                       | N. of adhering platelets                                   | % 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)                      |

\* $P < 0.01$  with respect to controls.

tion surface markers (GPIIIa, GPIIb and GPIb $\alpha$ ) were similar to those of healthy controls (Figure 2A,B). Conversely, megakaryocyte ploidy was significantly lower in patients than in controls (Figure 2C), and this was paralleled by differences in megakaryocyte diameters (Figure 2D). The analysis of proplatelet formation revealed that, compared to megakaryocytes from controls, megakaryocytes from patients had elongated proplatelet shafts of shorter length and with decreased number of branches. Furthermore, the percentage of proplatelet-forming megakaryocytes was significantly reduced in patients. In contrast, the size of proplatelet tips was similar in patients and in controls (*data not shown*). Similar results were obtained with megakaryocytes in suspension (Figure 3A,B) and following adhesion on fibrinogen (Figure 3C,D).

## Discussion

Here we report the molecular and phenotypic characterization of seven families with germline mutations in *ETV6*. In addition to the variants previously reported,<sup>4</sup> we identified three novel alterations, which are likely to be pathogenic. The two novel missense variants (p.R369W and p.W380R) segregated within the families, are absent in public genomic databases, and are expected to be deleterious for protein function according to bioinformatic tools and analysis of protein conformational structure. *ETV6* is a modular protein which contains a PNT and an ETS domain sandwiched between regions of potential intrinsically unstructured nature. Both p.R369W and p.W380R affect the ETS domain, a conserved region that interacts directly with DNA consensus sequences. We have shown that the role of W380 is structural, it being surrounded by hydrophobic residues in the domain hydrophobic core. Its substitution by an arginine will



**Figure 2. Normal differentiation but decreased ploidy of ETV6-RT megakaryocytes.** Hematopoietic progenitors from peripheral blood samples of healthy controls (CTRL) and patients (ETV6-RT) were differentiated *in vitro* into megakaryocytes in the presence of thrombopoietin, interleukin-6 and interleukin-11. (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 GPIIbα expression revealed comparable percentages of double-stained populations 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 from ETV6-RT patients (\* $P < 0.05$ ). (D) Diameters of megakaryocytes were also significantly lower in ETV6-RT patients (total number of cells analyzed: 1,100, \* $P < 0.01$ ).

therefore severely destabilize the domain structure. Residue R369 is involved in an electrostatic interaction and possibly in protein-protein interactions.

It is important to note that the somatic p.R369W has previously been associated with chronic myelomonocytic leukemia, colorectal cancer, and childhood leukemia.<sup>5,6</sup> Moreover, Zhang *et al.* previously reported one ETV6-RT pedigree carrying a different germline missense variant affecting the same residue (p.R369Q). Similarly to our patients with the p.R369W, the subjects with p.R369Q had mild thrombocytopenia and normal platelet morphology.<sup>6</sup> Among the eight members of the p.R369Q pedigree, one had chronic myelomonocytic leukemia at the age of 82 and one had colorectal cancer at the age of 43,<sup>6</sup> whereas we did not observe neoplasms in our p.R369W patients at a median age at evaluation of 30 years. Finally, both p.R369Q and p.R369W have been associated with genetic predisposition to childhood ALL.<sup>3</sup> These observations suggest that arginine 369 is a mutational hot spot.

With regards to the c.1153-1\_1165del variant, reverse transcriptase polymerase chain reaction analysis demonstrated that it affects the splicing process leading to skipping of exon 7 (p.N385Vfs\*7). The same alternative splicing was also caused by the c.1153-5\_1153-1del mutation,<sup>5</sup> which, as c.1153-1\_1165del, is likely to derive from non-allelic homologous recombination between repetitive sequences present at the intron 6/exon 7 boundary. The skipping of exon 7 is also determined by the c.1252A>G substitution.<sup>4</sup> Affecting the second to last nucleotide of exon 7, this allele is associated with both correctly (p.R418G) and alternatively (p.N385Vfs\*7) spliced mRNA.<sup>4</sup>

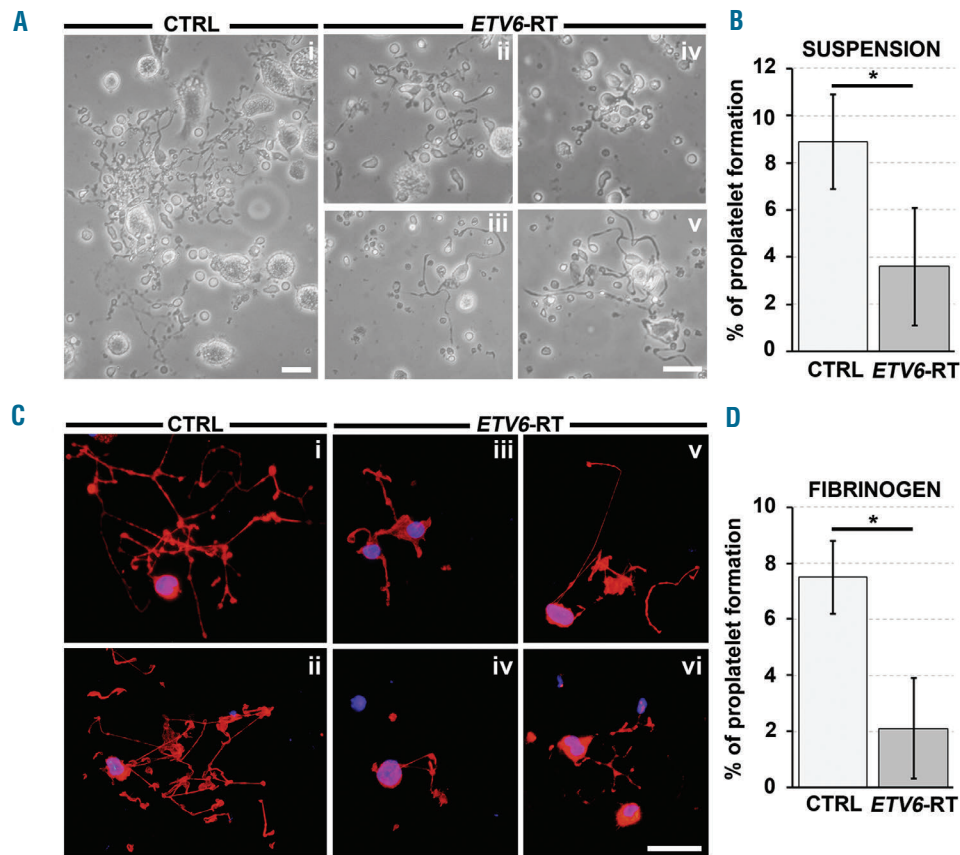
Of the ten different mutant forms identified so far in ETV6-RT families, p.P214L is the only one that does not affect the ETS domain (Figure 1D), but instead alters a less

conserved central domain that interacts with several transcription repressors further controlling expression of the target genes. Unlike the other germline mutations, which are mainly private, this substitution was responsible for ETV6-RT in four of the 14 families characterized so far, indicating that it represents another potential mutational hot spot.

ETV6 is a transcriptional repressor involved in embryonic development and hematopoietic regulation.<sup>18</sup> 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 increased greatly at the end of the last century after demonstration that its deregulation due to rearrangements, fusions or deletions is involved in hematologic malignancies.<sup>19,20</sup> Moreover, somatic mutations in ETV6 were recently found in a variety of hematologic neoplasm, including AML, T- and B-cell ALL, mixed-phenotype acute leukemia, myelodysplastic syndromes, chronic lymphocytic leukemia and chronic myelogenous leukemia.<sup>21</sup> Even more recently, targeted sequencing of ETV6 in 4405 childhood ALL cases identified 31 germline variants potentially related to leukemia in 35 cases.<sup>3</sup> Based on this evidence, it is not surprising that the four studies that identified ETV6-RT in 41 subjects from nine families found that 16 patients (39%) had hematologic malignancies, with 12 patients (29%) developing ALL.<sup>3-6</sup> Of note, 11 of the 12 subjects with ALL were children. The other blood neoplasms observed in ETV6-RT patients were mixed-phenotype acute leukemia, multiple myeloma, myelodysplastic syndromes and chronic myelomonocytic leukemia.

We found that four of 20 consecutive patients with ETV6-RT (20%) developed ALL during childhood, thus





**Figure 3. Aberrant proplatelet formation by ETV6-RT megakaryocytes.** (A) Representative light microscopy analysis of proplatelet formation and structure from control (CTRL, i) and patient (ETV6-RT, ii-v) megakaryocytes cultured for 16 h in suspension (scale bar=50  $\mu$ m). (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 h with adhesion on fibrinogen. The pictures clearly show defective proplatelet elongation in ETV6-RT (red= $\beta$ 1-tubulin; blue=nuclei; scale bar=30  $\mu$ m). (D) The percentage of proplatelet-forming megakaryocytes was calculated as the number of  $\beta$ 1-tubulin-positive cells displaying at least one pseudopod with respect to total number of round megakaryocytes per analyzed field ( $*P<0.01$ ).

confirming that early leukemic transformation is a major risk in these patients. Moreover, we observed that one patient developed *JAK2*-positive polycythemia vera at the age of 37, supporting the previous hypothesis that *ETV6*-RT predisposes not only to ALL, but also to other blood neoplasms. 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,<sup>3,6</sup> 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 inherited thrombocytopenia of unknown origin. This approach appears more suitable for providing 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 our figure.

Similarly to this study, we previously searched a large series of unselected patients for *ANKRD26* mutations and discovered that ten of 118 (8%) subjects with *ANKRD26*-RT had developed myeloid malignancies.<sup>22</sup> Thus, hematologic malignancies seem much more frequent in *ETV6*-RT than in *ANKRD26*-RT. The risk of malignancies appears even higher in FPD/AML, since over 40% of such patients had myeloid neoplasms.<sup>23</sup> However, as discussed for *ETV6*-RT, the *RUNX1* mutational screening was also generally performed in pedigrees with hematologic malignancies,<sup>24</sup> and it is therefore likely that the incidence of transformation has been overestimated. However, each patient with an inherited thrombocytopenia caused by mutations in *ETV6*, *RUNX1* or *ANKRD26* has a relevant risk of hematologic malignancies, and recognizing these patients

is important not only to provide effective genetic counseling and appropriate follow-up, but also to give appropriate treatment to patients who develop blood neoplasms and need hematopoietic stem cell transplantation. In fact, as shown in different disorders predisposing to myeloid malignancies,<sup>25</sup> 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 inherited thrombocytopenia. In fact, in our series of 274 consecutive probands, *ETV6*-RT was identified in seven 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 inherited thrombocytopenia (7/151). In our cohort, the frequency of *ETV6*-RT was lower only to that of monoallelic Bernard-Soulier syndrome (12.2% in the whole series), *MYH9*-related disease (11.4%), *ANKRD26*-RT (9.4%), and biallelic Bernard-Soulier syndrome (5.7%). Since most of our patients with monoallelic Bernard-Soulier syndrome had the Ala156Val mutation of GPIIb $\alpha$  (Bolzano mutation), which is exclusive to the Italian population,<sup>26</sup> 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 from the routine diagnostic workup and the diagnosis does, therefore, remain difficult. A previous investigation, which reported five patients who have been re-evaluated in this study, suggested that red blood cell macrocytosis is a feature of the *ETV6*-RT phenotype.<sup>4</sup> In that investigation, the percentage of patients with increased mean corpuscular volume was 40%,<sup>4</sup> whereas it was 25% in the present study.



With regards to the five patients reported in both studies, red blood cell macrocytosis was found in two individuals in the previous examination but was not confirmed in the present evaluation. Of note, the absolute mean corpuscular volumes were similar in the two studies (mean 94.5 fL with SD 3.8 *versus* mean 93.3 fL with SD 8.9) and the discrepancy in the percentage of patients with red cell macrocytosis resulted from the different upper limits of normal range used in the two investigations (95 fL in the previous study and 98 fL in the present one, according to the normal ranges of the different laboratories). On the whole, these findings indicate that red blood cell macrocytosis is present in a minority of patients with *ETV6*-RT, and suggest that it may be inconstantly found in the same patients over time. Thus, red cell macrocytosis seems to have limited diagnostic value for recognizing this condition. Moreover, we did not identify any distinguishing defect of major platelet glycoproteins or *in vitro* platelet aggregation and evaluation of peripheral blood films did not reveal any morphological abnormalities, except for mild platelet anisocytosis. However, at variance with most inherited thrombocytopenias, mean platelet diameter and mean platelet volume were consistently normal in *ETV6*-RT, and it is precisely the normal size of platelets that should raise suspicion of this condition in subjects with an autosomal dominant thrombocytopenia. The other dominant inherited thrombocytopenias 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,<sup>1</sup> whereas the other two disorders are more frequent and, like *ETV6*-RT, predispose to hematologic malignancies. Thus, we suggest that all subjects with a dominant inherited thrombocytopenia and normal platelet size should be tested for mutations in *ETV6*, *RUNX1*, and *ANKRD26*, in order to identify one of these predisposition syndromes.

The psychological impact of receiving a diagnosis of *ETV6*-RT, as well as of FPD-AML or *ANKRD26*-RT, should be carefully considered by physicians. We suggest that all patients are correctly informed, before undergoing diagnostic workup for thrombocytopenia of suspected genetic origin, about the possibility of receiving a diagnosis that implicates the risk of malignancies, and have the chance to state in advance whether they want to receive information about the risk of neoplasms, for themselves as well as for their progeny.

In this study, the *in vitro* megakaryopoiesis of *ETV6*-RT patients was investigated for the first time. We showed that *ETV6* pathogenic variants impair megakaryocyte maturation, as demonstrated by the production of smaller megakaryocytes with decreased ploidy. The ability of

these immature megakaryocytes to extend fully developed proplatelets was impaired, providing an explanation for the patients' thrombocytopenia. These findings seem consistent with the results of studies in mice, which suggested a role for *ETV6* in terminal megakaryocyte maturation,<sup>18</sup> and with the findings obtained with megakaryocyte differentiated from human CD34<sup>+</sup> cells transduced with some *ETV6* variants.<sup>4</sup> We also had the possibility to study platelet function in detail in a substantial number of patients. Although we did not identify any consistent defect of *in vitro* platelet aggregation, activation or 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 signaling 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 (60%) 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 inherited thrombocytopenia and confirmed that affected subjects have a mild bleeding tendency but propensity to hematologic malignancies, in particular ALL. Since *ETV6*-RT is one of the few autosomal dominant forms of inherited thrombocytopenia without platelet macrocytosis, screening for *ETV6* mutations is recommended in all patients with these characteristics.

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