Published Ahead of Print on November 9, 2012, as doi:10.3324/haematol.2012.075861.
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haematologica
the hematology journal

Early Release Paper

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Haematologica 2012 [Epub ahead of print]

Citation: Bottega R, Pecci A, De Candia E, Pujol-Moix N, Heller PG, Noris P, De Rocco D, Podda GM, Glembotsky AC, Cattaneo M, Balduini CL, and Savoia A. Correlation between platelet phenotype and NBEAL2 genotype in patients with congenital thrombocytopenia and α-granule deficiency. Haematologica. 2012; 97:xxx
doi:10.3324/haematol.2012.075861

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Official Organ of the European Hematology Association
Published by the Ferrata Storti Foundation, Pavia, Italy
www.haematologica.org

DOI: 10.3324/haematol.2012.075861

Correlation between platelet phenotype and *NBEAL2* genotype in patients with congenital thrombocytopenia and α-granule deficiency

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Funding

This work was supported by grants from the Italian Ministry of Education, University and Research (PRIN 2008), Italian ISS (Istituto Superiore di Sanità), and Grant 16/12 of IRCCS Burlo Garofolo.

Acknowledgments The authors would like to thank Dr. Daniela Santon and Dr. Chiara Gnan for technical support.

The online version of this article contains a Supplementary Appendix

ABSTRACT

Background. The gray platelet syndrome is a rare inherited bleeding disorder characterized by macrothrombocytopenia and deficiency of α-granules in platelets. The genetic defect responsible for gray platelet syndrome was recently identified in biallelic mutations in the *NBEAL2* gene.

Design and Methods. We studied 11 consecutive families with inherited macrothrombocytopenia of unknown origin and α-granule deficiency. All of them underwent *NBEAL2* DNA sequencing and evaluation of the platelet phenotype, including a systematic assessment of the α-granule content by immunofluorescence analysis for α-granule secretory proteins.

Results. We identified 9 novel mutations hitting the two alleles of *NBEAL2* in 4 probands. They included missense, nonsense and frameshift mutations, as well as nucleotide substitutions that altered the splicing mechanisms as determined at the RNA level. All the individuals with *NBEAL2* biallelic mutations showed almost complete absence of platelet α-granules. Interestingly, the 13 individuals assumed to be asymptomatic because carriers of a mutated allele had platelet macrocytosis and significant reduction of the α-granule content. However, they were not thrombocytopenic. In the remaining 7 probands, we did not identify any *NBEAL2* alterations, suggesting that other genetic defect(s) are responsible for their platelet phenotype. Of note, these patients were characterized by a lower severity of the α-granule deficiency than individuals with two *NBEAL2* mutated alleles.

Conclusions. Our data extend the spectrum of mutations responsible for gray platelet syndrome and demonstrate that macrothrombocytopenia with α-granule deficiency is a genetic heterogeneous trait. In terms of practical applications, the screening of *NBEAL2* is worthwhile only in patients with macrothrombocytopenia and severe reduction of the α-granules. Finally, individuals carrying one *NBEAL2* mutated allele have mild laboratory abnormalities, suggesting that even haploinsufficiency has an effect on platelet phenotype.

INTRODUCTION

The gray platelet syndrome (GPS, OMIM #139090) is a rare inherited bleeding disorder characterized by macrothrombocytopenia and reduction of α-granules in platelets and in megakaryocytes.[1] Alpha-granules are the most abundant granules in platelets and store proteins that promote platelet adhesiveness, blood coagulation and wound healing when secreted during platelet activation. Some α-granule proteins are synthesized in megakaryocytes and packed into these vesicles, whereas others are either passively or actively taken up from the plasma by receptor-mediated endocytosis.[2,3] Their defective maturation causes a continuous leakage of growth factors and cytokines into the bone marrow, which leads to the development of myelofibrosis.[2-4]

Genome-wide linkage analysis mapped the GPS locus on chromosome 3p21.[5,6] More recently, three independent next generation approaches identified *NBEAL2* as the gene responsible for GPS.[1,7,8] *NBEAL2* encodes a 2,754 amino acid polypeptide, neurobeachin-like-2, similar to the lysosomal trafficking regulator (LYST). Interestingly, LYST is responsible for Chediak-Higashi syndrome (CHS), which is characterized by deficiency of platelet δ-granules and abnormalities of other cells.[9] Like LYST, *NBEAL2* contains the BEACH (beige and CHS), ARM (Armadillo) and WD40 domains, highly conserved regions that are crucial for protein-protein interactions, membrane dynamics and vesicle trafficking.[10]

In this article, we describe 11 probands with large platelets and α-granule reduction, and therefore, with a diagnostic suspicion of GPS. The search for mutations in *NBEAL2* allowed us to confirm the diagnosis in 4 families. The clinical and laboratory characteristics observed in biallelic patients and their monoallelic family members, as well as those of the remaining 7 families without *NBEAL2* mutations, better define the picture of GPS. The data also helps to improve the criteria for differential diagnosis of inherited macrothrombocytopenias with α-granule deficiency.

DESIGN AND METHODS

Patients

We studied 11 consecutive unrelated probands with congenital macrothrombocytopenia associated with deficiency of α-granules on evaluation of peripheral blood smears. All the subjects or their legal guardians gave written informed consent according with the Declaration of Helsinki. Protocols were approved by the Ethics

Review Boards at the Institutions that enrolled the patients. On examination of May-Grünwald-Giemsa (MGG)-stained slides, all the patients had a remarkable, although variable, percentage of platelets (about 30% to 95%) with no or very few azurophilic granules, and therefore presenting a gray appearance.[11] A clinical and cytopathological diagnosis of GPS was made in 6 of these probands (families 1-5 and 11), 4 of which (families 1, 3, 4, and 5) were previously reported.[12-14] All the patients underwent immunofluorescence analysis of α-granule secretory proteins and DNA sequencing of *NBEAL2* (see below). Their clinical and laboratory findings are reported in more detail in Table 1S, Table 3S and Supplementary Appendix.

Immunofluorescence analysis of platelet α-granule content

We quantified the platelet α-granule content by immunofluorescence labeling for the α-granule proteins thrombospondin-1 (TSP1) and platelet factor 4 (PF4), according to a previously published method.[12,15] Blood slides were prepared without anticoagulant by prick of the fingertip and immediately air-dried. Slides were then fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes at room temperature (RT), washed with PBS, and permeabilized with 0.1% Triton-X-100 (Sigma, St. Louis, MO) in PBS for 5 minutes. After washing with PBS, slides were incubated with the P12 antibody anti-TSP1 (Sigma) or a rabbit anti-PF4 (Chemicon, Temecula, CA) for 1 hour at RT. The appropriate goat Alexa Fluor 594-conjugated anti-mouse or anti-rabbit was used as secondary antibody (Invitrogen, Carlsbad, CA). In all the cases, the TSP1 and PF4 reactions resulted in a well-defined granular staining pattern; in normal platelets, a large number of granules were aggregated in the structure of granulomere, while in defective platelets the single TSP1- or PF4-positive granules could be identified and counted. For each subject, we calculated the percentage of platelets with less or more than 5 TSP1 or PF4-positive granules, as previously reported.[12,15] The cut-off of 5 granules was fixed empirically to categorize exclusively those platelets that presented a very marked reduction in TSP1- or PF4-positive granules. At least 300 platelets were observed and classified for each specimen. Results obtained in patients were compared with those obtained in 20 consecutive healthy subjects that were stained in parallel, as well as with those deriving from a series of 50 healthy subjects analyzed previously.[12] Analysis of cases and controls was performed blind.

Transmission electron microscopy

A systematic morphometric analysis of the granule content of platelets was performed in the proband of family 1 and in his three relatives carrying one mutated *NBEAL2* allele by electron microscopy. Fifteen consecutive healthy controls were also included in the study. Platelets were immediately fixed with 1.25% glutaraldehyde in White's buffer saline prior to obtaining platelet-rich plasma, and postfixed with 1% osmium tetroxide. The samples were dehydrated in alcohol and embedded in Epon 812®. Ultrathin sections were stained using standard solutions of uranyl acetate and lead citrate, and examined by transmission electron microscopy at 80 Kv accelerating voltage.[16] For each specimen, random microscopic fields were taken and at least 100 platelet sections were analyzed. The granule content was expressed as the mean number of granules per unit of platelet section (μ^2).

Evaluation of mean platelet diameter

Mean platelet diameters were assessed on MGG-stained blood smear by software assisted image analysis (Axiovision 4.5, Carl Zeiss, Göttingen, Germany) as previously described.[17]

DNA sequencing

NBEAL2 was screened for mutations using genomic DNA extracted from peripheral blood. Mutational analysis was performed by polymerase chain reaction (PCR) amplification using primers (available upon request) covering exons and the exon/intron boundaries of the gene. PCR was carried out in 35 μL of total reaction volume with 25 ng of genomic DNA, 10μM of each primer, and Kapa 2G Fast Hot Start ReadyMix 2X (KapaBiosystems, Cape Town, South Africa). After initial denaturation at 95°C for 1 min, amplification was performed for 30 cycles (denaturation at 95°C for 10 sec, annealing at 62°C for 10 sec, and elongation at 72°C for 1 sec). PCR products were bidirectionally sequenced using the ABI PRISM BigDye v3.1 Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3130xl sequencer (Applied Biosystems, Foster City, CA).

RT-PCR

Total RNA was extract from patient's derived lymphoblastoid cell lines or peripheral blood using TRIzol® (Life Technologies, Carlsbad CA, USA) and cDNA was synthesized with Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Foster City, CA). In the probands of families 1 and 2, the amplification reactions were performed using the following pairs of primers: 33F (5'-GCGCCGAGACATATTCACGC-3') - 37R (5'-AAGAGCTCAAGTGCTGAACG-3'), and 41F (5'-GATGTGAAGGAGCTCATCCC-3') - 43R (5'-AGAAGACATTGAGGGCCTCC-3'), respectively. RT-PCR was carried out for 30 cycles, 40 sec at 95°C, 45 sec at 60°C and 45 sec at 72°C.

Bioinformatic analysis

The effect of the missense variations on protein function was evaluated using a variety of patogenicity prediction programs, such as PoliPhen-2 (POLYmorphism PHENOtyping 2, *http://genetics.bwh.harvard.edu/pph2/*), MutationAssessor (*http://mutationassessor.org/*) and MutationTaster (*http://www.mutationtaster.org/*). Regarding splice-site mutations, in silico analysis were carried out using Splice Site Prediction by Neural Network (*NNSplice; http://www.fruitfly.org/seq_tools/splice.html*) and Human Splicing Finder Version 2.4.1 *(http://www.umd.be/HSF/).*

RESULTS

Identification of *NBEAL2* mutations in four families

Direct sequencing of the *NBEAL2* gene revealed 9 different novel mutations in the probands of families 1 to 4 (Table 1). The identified alterations were not reported in the dbSNP database (http://www.ncbi.nlm.nih.gov/snp/).

Molecular genetic analysis of the proband of family 1 detected a paternal deletion of 4 nucleotides (c.1504_1507del) in exon 13, which produces a frameshift and a premature stop codon 4 amino acids downstream of the mutation (Figure 1C). The second mutation, a nucleotide substitution (c.6801+7A>T) in intron 42, was inherited from the mother and was expected to generate a donor splice-site (GAGgtgag) stronger than the natural site (CTGgtgag). In fact, the splicing prediction tool NNSplice attributed scores 0,83 and 0,75 to the mutated and the natural splice-site, respectively (Figure 1D).

The proband of family 2 (II-1) carried three mutations, a maternal missense mutation (c.7033C>T, p.Arg2345Trp) in exon 45 and two paternal nonsense mutations: c.5572C>T (p.Arg1858*) and c.6652G>T (p.Glu2218*). His brother inherited the paternal mutated allele.

Consistent with their parents' consanguinity, the two affected brothers of family 3 were homozygous for a nonsense mutation (c.2187C>A), which is expected to truncate the protein at amino acid 728.

Finally, we identified three different mutations in family 4, a nucleotide deletion in exon 12 (c.1253del), which introduces an in-frame premature stop codon (p.His418Leufs*54), a splice variant (c.5720+1G>A) in intron 35 and a missense mutation, c.3584G>A (p.1195Arg>Gln) (Figure 2). The c.1253delA deletion was a maternal mutation while the other two mutations were inherited from the father. The paternal allele was also detected in individuals II-4, III-5 and IV-3 but not in the other family members (II-2 and in his descendants) though they had partial reduction of α-granules. The disruption of the natural donor splice-site by the c.5720+1G>A mutation was expected to lead to recognition of putative cryptic sites. In fact, NNSplice prediction recognized three "gt" splice sites located at nucleotide positions 30 (score 83), 35 (score 81) and 149 nt (score 93) of intron 35.

Splicing mutations lead to frameshift alterations

To test the effect of the two splicing variants, we performed RT-PCR using total RNA extracted from peripheral blood (family 1) or lymphoblastoid cell line (family 4). As predicted by the bioinformatics tools, in family 1 RT-PCR and sequencing analysis confirmed that the amplified product consisted of both wild-type and mutated alleles (Figure 1D and Table 1). The latter was characterized by an alternative splicing due to an out of frame insertion of the first 5 nucleotides of intron 42 (p.Glu2268Valfs*44).

In presence of the c.5720+1G>A mutation (family 4), the splicing machinery recognized the cryptic site with the highest score. Indeed, the analysis revealed products characterized by the insertion of 148 nucleotides (r.5720_5721ins148) of intron 35 (Figure 2D and Table 1). The first inserted codon is a stop codon leading to a premature truncated product (p.Met1908*).

Potential pathogenetic effect of missense mutations

Missense mutations were identified in families 2 and 4 (Table 1). The potential effect of the two missense mutations on protein function was evaluated using different pathogenicity prediction programs. Regarding p.1195Arg>Gln, the significance of this variant is unclear because of its low pathogenicity scores (PoliPhen-2, Score=0,398; MutationTaster, Polymorphism p=0,995; MutationAssessor, FI score=1,04). However, this alteration is likely not to exert any effect because localized *in cis* of p.Met1908*.

On the other side, the p.Arg2345Trp variant is likely to be pathogenetic because of its high prediction scores (PolyPhen2, Score=1; MutationTaster, Disease Causing Mutation p=0,997; MutationAssessor, FI score=3,55). It alters a highly conserved amino acid of the BEACH binding domain. However, since it is the only mutation carried by the maternal allele in family 2, only functional studies would be able to confirm the hypothesis.

Platelet features of patients with biallelic mutations of *NBEAL2*

All the 5 subjects with biallelic *NBEAL2* mutations had thrombocytopenia (Table 1S and Table 2). At examination of MGG-stained blood smears, all of them had platelet macrocytosis and giant platelets; the mean platelet diameter was 3.9 μm (p<0.001 with respect to healthy subjects; Table 2). Immunofluorescence analysis for TSP1 showed a severe reduction of platelet α-granule content in all the patients, as the percentage of platelets with >5 TSP1 positive granules ranged from 3 to 12% (p<0.0001 compared to healthy subjects) (Figure 3 and Table 2). The PF4 content evaluation gave similar values (data not shown). This data was consistent with that from electron microscopy, showing severe reduction platelet α-granules in the proband of family 1 (Figure 1B and Table 3). Specifically, the mean α-granule number per unit of platelet area was 10% with respect to controls. The content of δ-granules was instead in the normal range (Table 3). Similarly, a severe reduction of α-granules was reported for the proband of family 4.[12]

Platelet features of *NBEAL2* monoallelic individuals

None of the 13 relatives with one mutated *NBEAL2* allele had thrombocytopenia, suggesting a recessive transmission of this trait (Table 2). However, 9 individuals had evident platelet macrocytosis, while the other 4 had mean platelet diameter near to the upper limit of the normal range. Considering all the *NBEAL2* monoallelic individuals, their mean platelet diameter was significantly increased with respect to controls (3.1 vs. 2.4, p<0.001) (Table 2).

Moreover, a careful examination of their MGG-stained slides demonstrated that most of these patients presented with some distinct gray platelets. Consistently, immunofluorescence analysis showed that all but one (III-8 of family 4 who had 90% of platelets with >5 TSP1 positive granule) had moderate reduction in the α-granule content. The mean percentage of platelets with >5 TSP1 positive granules was 71% and it was significantly reduced with respect to healthy controls (p<0.0001) but higher than that observed in *NBEAL2* biallelic individuals (p<0.0001) (Figure 3 and Table 2). The statistical significance (p<0.0001) was maintained even excluding the four carriers from family 4, where the partial α-granule defect did not completely segregate with the *NBEAL2* mutations.

In order to confirm the partial α-granule deficiency observed by immunofluorescence analysis, we carried out electron microscopy in the two *NBEAL2* carriers belonging to family 1 (Table 3). While the δ-granule content was normal, that of the α-granules was partially reduced in both subjects. In more details, the mean α-granule number per unit of platelet area was 71% and 61%, of controls in the father (I-1) and the mother (I-2), respectively (Table 3). The electron microscopy also confirmed the platelet macrocytosis in these two individuals.

Finally, none of the *NBEAL2* carriers had other clinical or laboratory signs of GPS (Table 2S).

Platelet features in individuals with thrombocytopenia and no NBEAL2 mutations

Direct sequencing of *NBEAL2* did not identify any mutations in the probands of the remaining families 5-11, though two of them were previously diagnosed as GPS.[12-14] In these subjects, macrothrombocytopenia and α-granule deficiency was sporadic or inherited as an autosomal dominant trait (Table 3S). All these patients had moderate to severe thrombocytopenia and most of them had increased MPV. The mean values of their platelet count and volume were not different from those of the patients with biallelic mutations. However, their α-granule reduction was significantly less severe than that of individuals with *NBEAL2* biallelic mutations ($p<0.0001$; Table 2).

DISCUSSION

After the recent identification of mutations of *NBEAL2* in patients with GPS, we hypothesized that this gene was responsible for low platelet count and α-granule deficiency in 11 unrelated probands with inherited thrombocytopenia. The analysis allowed us to identify mutations hitting both *NBEAL2* alleles in 4 families. The 5 affected individuals from these pedigrees had a very marked reduction in the α-granule content (88% to 97% of platelets devoid of α-granules), which was associated with platelet macrocytosis and giant platelets. These features are similar to those of the patients with *NBEAL2* biallelic mutations identified so far.[1,7,8]

Moreover, we observed for the first time that the monoallelic family members had laboratory abnormalities, such as a moderate reduction in the α-granule content and platelet macrocytosis. These results confirm previous observations of moderate laboratory signs in obligate carriers.[12] These modest anomalies are likely to have no clinical relevance in many cases, since only one monoallelic individual (III-7 of family 4) had prolonged bleeding time and mild bleeding diathesis with platelet counts at the lower limit (170 x 10^9/L) of the normal range.[12] Even if an extensive clinical and laboratory characterization could rule out alternative causes of the bleeding diathesis, whether the *NBEAL2* mutated allele or other unrecognized factors are responsible for the bleeding tendency of this subject is presently unknown.

The finding of platelets abnormalities in the *NBEAL2* carriers was unexpected, particularly for what concerns the α-granule phenotypes. At first, the estimation was carried out by immunofluorescence analysis, which, although conducted with a standardized approach,[12,18] is not a validated procedure to study α-granule deficiency. Thus, we measured the α-granule number per unit of platelet area by electron microscopy in the two carriers (family 1), whose biological samples were available for the analysis. The evaluation was carried out in parallel with the proband of the same family. The analysis confirmed not only the severe reduction of platelet α-granules in the proband but also the mild α-granule defect in the two *NBEAL2* carriers. Interestingly, similar degrees of deficiency were measured by electron microscopy and immunofluorescence in the same individual. Although the immunofluorescence protocol has to be validated in a large number of samples, the two methodologies provided similar results in quantifying the extent of α-granule deficiency.

Since mutations affecting only one *NBEAL2* allele were associated with platelet abnormalities, we hypothesized that mutations in this gene could be responsible for autosomal dominant thrombocytopenia with α-granule deficiency. However, the analysis of another 7 probands (including autosomal dominant and sporadic forms) with congenital thrombocytopenia and α-granule defect did not identify any *NBEAL2* mutations. Since the other inherited thrombocytopenias characterized by α-granule deficiency, such as those caused by mutations of *ANKRD26* or *GATA1*, were excluded, we conclude that one or more unknown genes are responsible for the congenital defects in these cases. Of note, the probands without *NBEAL2* mutations had lower severity of the α-granule deficiency (31% to 63% of platelets devoid of granules) compared to those with biallelic mutations (p<0.0001). Based on these findings, it is reasonable to assume that the α-granule deficiency is a genetic heterogeneous trait with variable expressivity and that the diagnosis of GPS associated with mutations of *NBEAL2* has to be suspected only in subjects with macrothrombocytopenia and at least 85% of platelets devoid of α-granules.

Therefore, the comparison of the phenotypes of subjects with *NBEAL2* monoallelic and biallelic mutations suggests a dosage effect. In carriers, haploinsufficiency of NBEAL2 affects the size and the α-granule content of platelets. In addition to these platelet defects, loss of function due to biallelic mutations of NBEAL2 leads to thrombocytopenia. These results contribute to understand the effect of *NBEAL2* mutations on platelet dysfunction/biogenesis.

DNA sequencing of *NBEAL2* allowed us to identify 9 novel alterations of the gene, including 2 missense, 3 nonsense, 2 frameshift, as well as 2 splicing alterations confirmed at the RNA level. Together with those previously reported, they increase to 35 the number of different mutations identified in 27 GPS unrelated families.[1,7,8] Although *NBEAL2* encodes a protein of 2,754 amino acids characterized by specific domains, mutations are spread throughout the entire gene without evidence for any founder effect, as 31 out of 35 of the mutations are private. Moreover, despite the rarity of the disease, 10 out of 27 probands are compound heterozygous, suggesting that the disease might be more frequent than expected.

The role of *NBEAL2* is unknown, as well as the pathogenetic mechanisms responsible for α-granule deficiency, macrothrombocytopenia and the other features of the disease. Investigations focused on understanding these aspects, as well as studies on large series of cases, will be of fundamental importance to improve our knowledge on this disease.

AUTHORSHIP AND DISCLOSURES

R.B., A.P., and A.S. designed the research, interpreted results and wrote the manuscript.

R.B. performed molecular analysis and D.D.R. technically supported the analysis.

A.P. performed immunofluorescence analysis.

N.P.M. performed TEM analysis.

A.P., E.D.C., N.P.M., P.G.H., P.N., G.M.P., A.C.G., M.C., and C.L.B. enrolled patients and acquired clinical data.

All the authors had access to primary clinical data and approved the final manuscript.

All the authors disclose any conflicts of interest.

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Table 1. Mutations in *NBEAL2* identified in four unrelated families.

Family	cDNA[a] (RNA for splicing mutations)	Exon/Intron	Protein	Type of mutation	Transmission
1[13]	c.1504_1507del	13	p.Leu502Alafs*4	Frameshift	Paternal
	c.6801+7A>T (r.6801_6802insGTGAG)	i42	p.Glu2268Valfs*44	Splice-site Frameshift	Maternal
2	c.5572C>T	34	p.Arg1858*	Nonsense	Paternal
	c.6652G>T	41	p.Glu2218*	Nonsense	Paternal
	c.7033C>T	45	p.Arg2345Trp	Missense	Maternal
3[14]	c.2187C>A	16	p.Tyr729*	Nonsense	Maternal and paternal
4[12]	c.1253del	12	p.His418Leufs*54	Frameshift	Maternal
	c.3584G>A	25	p.Arg1195AGln	Missense	Paternal
	c.5720+1G>A (r.5720_5721ins148)	i35	p.Met1908*	Splice-site Frameshift	Paternal

[a] Nucleotide A of the ATG translation initiation start site of the *NBEAL2* cDNA in GenBank sequence NM_015175.1 is indicated as nucleotide +1.

DOI: 10.3324/haematol.2012.075861

Table 2. Platelet phenotypes (quantification of α-granule defect, platelet macrocytosis and platelet count) in relation to the *NBEAL2* genotypes.

***NBEAL2* genotype (No. of individuals)**	**Alpha-granule defect (% of platelets with >5 TSP1 positive granules) Mean [range]**	**Mean Platelet Diameter (μm) Mean [range]**	**Platelet count ($x10^9$/L) Mean [range]**
- / - (5)[a]	7 [3-12][e,f]	3.9 [3.5-4.3][g,h]	58 [30-85]
+ / - (13)[b]	71 [57-90][e]	3.1 [2.5-4.0][g]	233 [170-320]
+ / + (7)[c]	60 [37-69][e]	3.9 [3.3-4.8][g]	62 [38-110]
Controls (20)[d]	95 [92-99]	2.4 [2.0-2.7]	245 [180-347]

[a] Individuals from families 1-4 with *NBEAL2* biallelic mutations.

[b] Individuals from families 1-4 with *NBEAL2* monoallelic mutations.

[c] Probands of families 5-11 (macrothrombocytopenia and α-granule deficiency but no *NBEAL2* alterations).

[d] Healthy subject whose smears were studied in parallel with the analyzed patients. In a previous series of 50 healthy subjects the median proportion of platelets with >5 granules was 96.2% (range 94.2-98.0)

[e] $p<0.0001$ with respect to healthy controls (two-tailed Student t test).

[f] $p<0.0001$ with respect to individuals with *NBEAL2* genotypes +/- and +/+.

[g] $p<0.001$ with respect to healthy controls.

[h] $p<0.01$ with respect to individuals with *NBEAL2* genotype +/-.

Table 3. Platelet features determined by electron microscopy in members of family 1 (Figure 1).

Platelet features	**I-1 (Father)**	**I-2 (Mother)**	**II-1 (Proband)**	**Healthy controls** (n=15), mean [range]
Section area[a]	2.5	2.6	2.7	1.6 [1.5-2.0]
α-granules[b]	2.2	1.9	0.3	3.1 [2.8-3.3]
δ-granules[b]	0.3	0.2	0.3	0.3 [0.2-0.4]

[a] Mean area of platelet sections measured in μ^2.

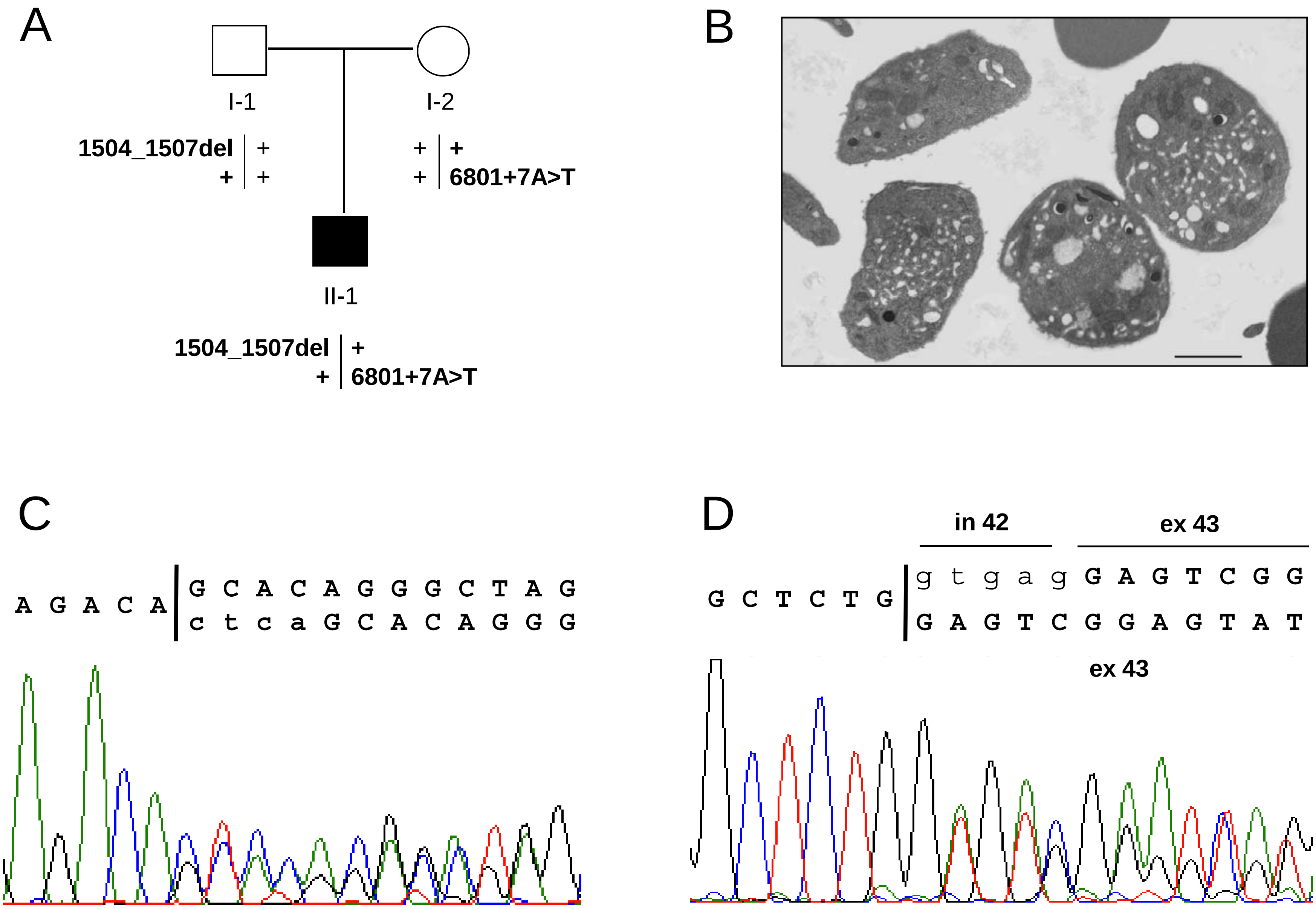
[b] Granule content was expressed as the mean number of granules per unit (μ^2) of platelet section.

LEGENDS TO FIGURES

Figure 1. Mutation analysis of *NBEAL2* in family 1. (A) Pedigree of family 1 with segregation of mutations; (B) Electron microscopy of platelets from proband II-1, showing a severe reduction of α-granules. A few very small α-granules are visible. Dense bodies are normal in number and morphology (bar = 1 μm); (C) Chromatograms of PCR product of exon 13 showing an heterozygous 4-bp frame-shifting deletion c.1504_1507del; (D) Sequence analysis of the RT-PCR product showing the alternative splicing r.6801_6802insGTGAG due to the c.6801+7A>T mutation in intron 42.

Figure 2. Mutation analysis of *NBEAL2* in family 4. (A) Pedigree of the family showing genotypes of 10 individuals. The proband IV-4 is indicated by a black symbol while the other individuals with α-granule deficiency and/or thrombocytopenia are in gray symbols. The proband carries three mutations, a maternal frame-shifting deletion (c.1253del) and two paternal (c.3584G>A and c.5720+1G>A) mutations. (B) Chromatogram of the heterozygous splice-site c.5720+1G>A mutation in the proband. (C) RT-PCR analysis of the proband's RNA extracted from his lymphoblastoid cell line. As confirmed by direct sequencing, the mutation causes an alternative splicing of a 629 bp product instead of the wild type 481 bp fragment due to the recognition of a cryptic splicing donor site 148 nucleotide downstream of the natural one.

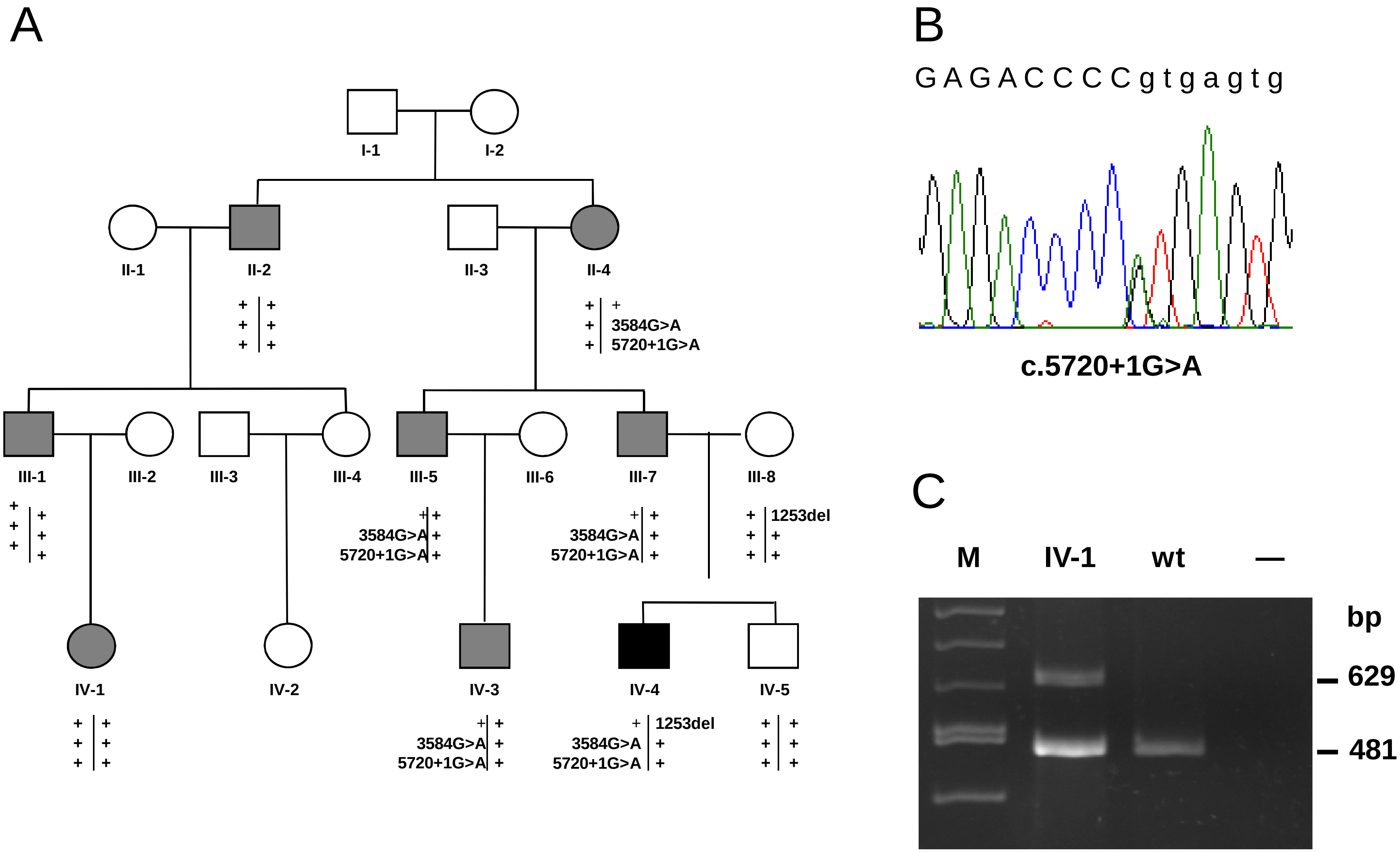
Figure 3. Immunofluorescence analysis for platelet TSP1 in the members of family 2. Platelets were identified on the blood smear by labelling for F-actin (green), while TSP1 was analysed in the red channel. **(II-1)**: the proband with biallelic *NBEAL2* mutation. Marked platelet macrocytosis with giant platelets and almost complete absence of TSP1 positive granules; the insert shows a giant platelet with a single TSP1 positive granule. **(I-1 and I-2)**: father (I-1) and mother (I-2) of the proband with monoallelic *NBEAL2* mutations. Representative fields of the finding of platelets with normal TSP1 positive granule content (very large number of TSP1+ granules packed in the structure of granulomere) together with platelets with no granules at all or with marked reduction of TSP1 positive granules (< 5 granules). Both monoallelic individuals also presented moderate platelet macrocytosis, which was more evident in the mother (H). In **(H)** platelets from a



c.1504_1507delCTCA

r.6801_6802insGTGAG

DOI: 10.3324/haematol.2012.075861



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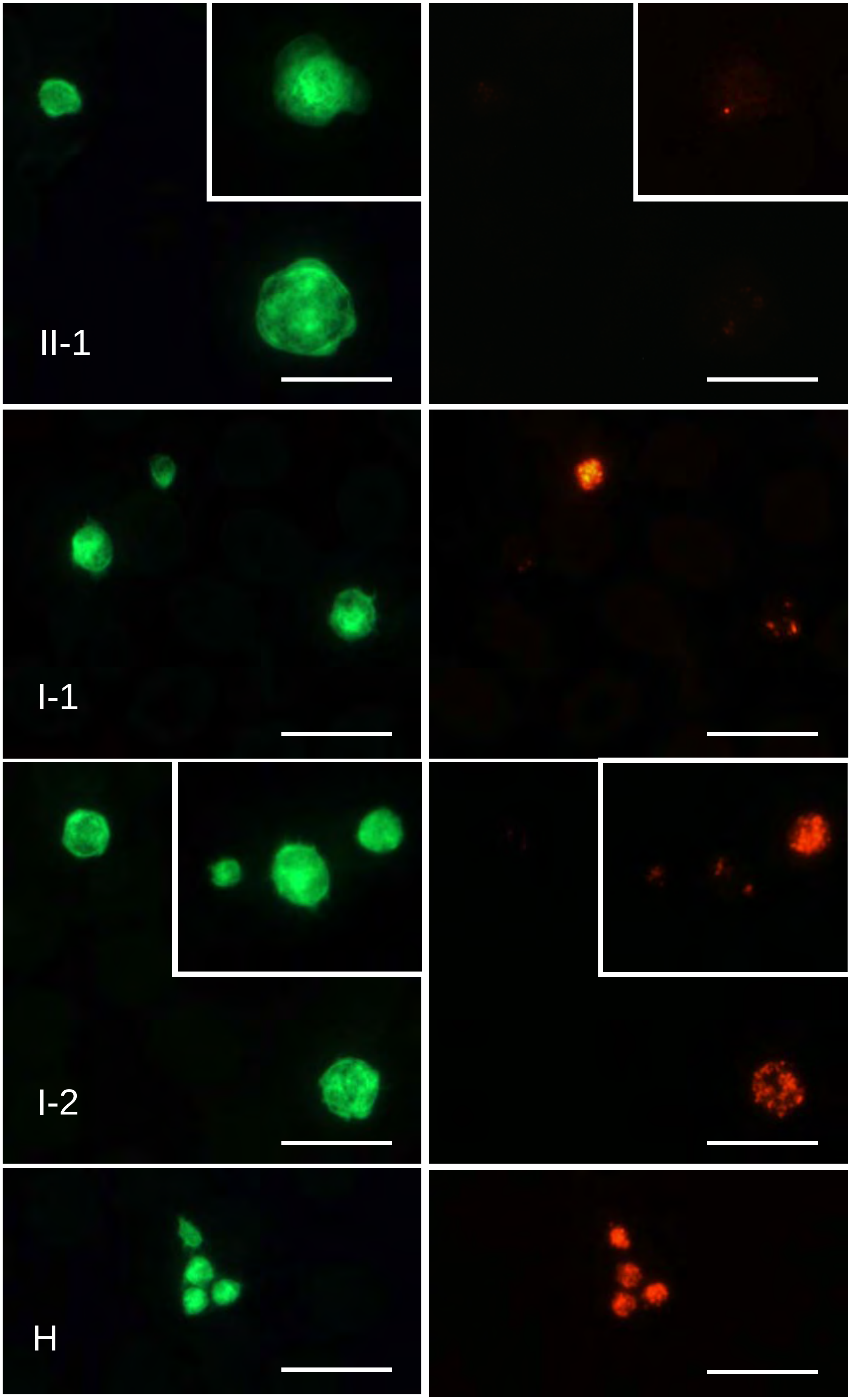

DOI: 10.3324/haematol.2012.075861

SUPPLEMENTARY APPENDIX

Phenotipic features of patients

The clinical and laboratory findings of the 4 families with *NBEAL2* mutations are reported in more detail (Table 1S) as follows.

Family 1. The proband was a male evaluated at the age of 28 years for a spontaneous severe epistaxis. He had a previous history of mild epistaxis and easy bruising.[1,2] Leukocyte count was 3.7 x 10^9/L with normal differential, hemoglobin was 147 g/L and platelet count was 47 x 10^9/L with a mean platelet volume (MPV) of 10.5 fL. Blood smears, prepared without anticoagulant and stained with MGG, showed that most platelets lacked azurophilic granules and therefore had a grayish tonality. Platelet macrocytosis and giant platelets were also evident (Table 1S). The patient also had moderate (28%) reduction of plasma levels of coagulation factor V.[2] At present (age 42 years), the proband has moderate splenomegaly (15.5 cm in diameter), high vitamin B_{12} levels and a slight increase in the bone marrow reticulin network. We included in the analysis both patients' parents, who had no thrombocytopenia or bleeding (Table 2S). The proband's mother, brother and two children had a borderline low level of plasma factor V (41%, 52%, 47% and 43%, respectively).

Family 2. A 5 year-old boy from southern Italy was referred for moderate bleeding diathesis, characterized by easy bruising and frequent epistaxis. His platelet count ranged from 58 to 100 x 10^9/L; MPV was 11.1 fL. Prothrombin time and activated partial thromboplastin time were normal, while his bleeding time was prolonged (11 minutes, normal values ≤7.5 min) (Table 1S). Analysis of the MGG-stained blood smears showed gray platelets, giant platelets, and anisopoikilocytosis. The proband's father, mother and 2 year-old brother had no history of pathological bleeding or thrombocytopenia. Their main clinical features are detailed in Table 2S.

Family 3. The proband was a 72 year-old male (II-1) with personal history of thrombocytopenia that was first identified during routine laboratory examination at the age of 53.[3] His 68 year-old brother (II-2) had a diagnosis of immune thrombocytopenia at the age of 42, which was never treated. Both had a life-long bleeding diathesis characterized by epistaxis, easy bruising and excess bleeding from minor wounds, after tooth extraction and after surgery. Their parents, who were first cousins, and other family members had normal platelet counts and no bleeding tendency. Platelet counts were 65 x 10^9/L for the proband and 30x10^9/L for his brother. Although MPV was normal, large platelets with gray appearance were evident on blood smears (Table 1S). Platelet aggregation revealed a mild defect in platelet response to ADP, epinephrine, collagen and arachidonic acid in the proband. Both patients developed moderate leukopenia, had elevated serum vitamin B_{12} levels and bone marrow biopsy revealed grade 3 and grade 2 fibrosis (scale 0 to 3), for II-1 and II-2, respectively. Individual II-2 only showed an enlarged spleen. Proband's two daughters and son had no history of abnormal bleeding, thrombocytopenia, or other signs of the disease (Table 2S).

Family 4. The proband (IV-4), who was an 11 years-old boy at diagnosis, and other 6 members (IV-5, III-7, III-8, II-4, II-2, III-1) were previously described.[4] Briefly, the proband had thrombocytopenia, severe α-granule deficiency, giant platelets and bleeding diathesis. Individuals III-1 and III-7 had personal history of epistaxis, platelet macrocytosis and prolonged bleeding time (Table 1S and 2S). In addition, patient III-1 had thrombocytopenia. All the other family members had normal platelet counts and no history of bleeding diathesis. Moreover, individuals III-1, II-2, II-4, III-7 all displayed mild reduction of platelet α-granule content.[4] We also enrolled five additional family members, III-5, IV-1 and IV-3, who had mild reduction of platelet α-granule content, as well as III-4 and IV-2, who had no platelet abnormalities.

The main clinical and laboratory features of the probands of families 5-11 are summarized in Table 3S. On examination of MGG-stained blood smears, all probands showed α-granule deficiency and a variable percentage of gray appearing platelets. Moreover, all of them had macrothrombocytopenia and 4 of them also had giant platelets. In 4 cases thrombocytopenia and the α-granule defect were inherited as an autosomal-dominant trait, while the remaining 3 patients had no familial history of thrombocytopenia and/or abnormal bleeding. Using a validated clinical and laboratory algorithm,[3,5] we could rule out the diagnosis of any known forms of inherited thrombocytopenia. In particular, the laboratory work-up of these patients included the study of *in vitro* platelet function (Born's method),[6] flow cytometry evaluation of platelet surface glycoproteins,[6] the immunofluorescence screening test for *MYH9*-related disease,[7] and the screening for mutations of the 5'UTR of the *ANKRD26* gene.[8] None of these examinations was consistent with the diagnosis of any known form of congenital thrombocytopenia (Table S3). Moreover, the diagnosis of the X-linked thrombocytopenia associated with mutations of *GATA-1* was also excluded because of the inherited transmission pattern and/or the absence of dyserythropoiesis or imbalanced hemoglobin chain synthesis.

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DOI: 10.3324/haematol.2012.075861

Table 1S. Clinical and laboratory features of 5 patients carrying *NBEAL2* biallelic mutations.

Family (Patient)	**Gender/Age at diagnosis of GPS**	**Previous diagnosis of ITP**	**Platelet count ($x10^9$/L) [Cell counter - Microscopy]**	**Mean Platelet Volume (fL)**	**Bleeding score[b]**	**Bleeding Time**	**Splenomegaly / diameter (cm)**	**Myelofibrosis[e]**	**Other findings**
1 (II-1)[2]	M/28	No	48 - 57	10.5	2	>10 min[c]	Yes / 15.5	Slight	High vitamin B_{12} level Leukopenia Deficiency of coagulation factor V (28%)
2 (II-1)	M/5	No	60 - nd	11.1	2	11 min[d]	Yes /10.3	nd	High Vitamin B_{12} level
3 (II-1) [3]	M/72 (53)[a]	No	56 - 65	8.4	2	nd	No	Yes	High vitamin B_{12} level Leukopenia
3 (II-2)[3]	M/68 (42)[a]	Yes	26 - 30	8.6	2	nd	Yes /15.9	Yes	High vitamin B_{12} level Leukopenia
4 (IV-4)[4]	M/11	nd	85 - nd	13.7	2	>20 min[c]	Yes /13	Yes	High Vitamin B_{12} level

[a] In brackets age at diagnosis of thrombocytopenia.

[b] World Health Organization (WHO) bleeding score. Grade 0: no bleeding; Grade 1: petechiae; Grade 2: mild blood loss (no need for hospital admission or access to emergency); Grade 3: gross blood loss (hospital admission or access to emergency or iron therapy required or red cell transfusions after surgery or delivery); Grade 4: debilitating blood loss (red blood cell transfusion required for spontaneous haemorrhages).

[c,d] Bleeding time evaluated by the following methods: Ivy (c) or Symplate II (d).

[e] As evaluated by bone marrow biopsy.

Table 2S. Clinical and laboratory features of 13 subjects carrying *NBEAL2* monoallelic mutations.

Family	**Familial relationship with the proband** [a]	**Gender/ Age (yrs)**	**Platelet count (x10^9/L) ［Cell counter］**	**MPV (fL)**	**Bleeding score** [b]	**Bleeding Time**	**Splenomegaly**	**PB signs of myelofibrosis** [d]	**Other findings**
1[2]	Father (I-1)	M/58	320	9.9	0	Normal [c]	No	No	None
1[2]	Mother (I-2)	F/56	259	10.2	0	Normal [c]	No	No	Borderline level of coagulation factor V (48%)
2	Father	M/37	207	8.0	0	n.d.	No	No	None
2	Mother	F/32	183	11.0	0	Normal [d]	No	No	None
2	Brother	M/2	192	9.0	0	Normal [d]	No	No	None
3[3]	Daughter	F/33	320	7.0	0	n.d.	No	No	None
3[3]	Daughter	F/38	210	7.2	0	n.d.	No	No	None
3[3]	Son	M/39	212	7.9	0	n.d.	No	No	None

4[4]	Father (III-7)	M/40	170	9.7	2	11min (v.n.< 9) [c]	No	No	Recurrent epistaxis, easy bruising
4[4]	Mother (III-8)	F/38	237	7.3	0	Normal [c]	No	No	None
4[4]	Cousin (IV-3)	M/20	312	7.3	0	Normal [c]	No	No	None
4[4]	Uncle (III-5)	M/38	227	7.5	0	Normal [c]	No	No	None
4[4]	Grandmother (II-4)	F/69	180	7.3	0	Normal [c]	No	No	None

[a] In brackets the identification of patient in the pedigree (shown in Figures 1 and 2)

[b] World Health Organization (WHO) bleeding score (please see note to Table S1)

[c,d] Bleeding time evaluated by the following methods: Ivy (c) or Symplate II (d).

[e] Presence of anisopoikilocytosis with dacryocytes and/or immature erythroid or granulocytic cells at evaluation of PB smears.

Abbreviations: PB: peripheral blood. n.d. = not determined.

Table 3S. Main clinical and laboratory features of 7 unrelated probands with macrothrombocytopenia, α-granule deficiency, and gray platelets but without any *NBEAL2* mutations.

Family	**Gender/Age at diagnosis of GPS or thrombocytopenia**	**Inheritance pattern**	**Platelet count ($x10^9/L$) [Cell counter - Microscopy]**	**MPV (fL)**	**Bleeding diathesis** [a]	***In vitro* platelet function** [b]	**Platelet surface GP expression** [c]	**Other findings**
5[2]	M/3	Sporadic	20-55	8.0	2	n.d.	normal	Deficiency of coagulation factor V (59%); normal vitamin B12 level; 65% decrease in platelet PF4 and β-thromboglobulin by ELISA assay
6	M/44	AD	26-38	13.8	3	normal	normal	None
7	F/25	AD	104-110	13.9	0	normal	normal	Mild neutropenia, normal vitamin B12 level
8	M/20	AD	67-55	10.4	0	normal	Mild reduction of GPIa-IIa	None
9	F/5	AD	37-58	12.5	4	Reduced after 5 µM ADP and 4 µg/mL collagen	normal	Interventricular membranous defect, mild von Willebrand disease type 1
10[3]	F/11	Sporadic	65-42	10.6	3	n.d.	normal	Mitral valve dysplasia, annular pancreas, psychomotor retardation Mild reduction of δ-granules by TEM
11	F/58	Sporadic	61-nd	14.5	1	normal	normal	Normal vitamin B_{12} level Normal δ-granule content by TEM

[a] World Health Organization (WHO) bleeding score (see note to Table 1S).

[b] Platelet aggregometry (Born's method) on platelet rich plasma after stimulation with ADP (5 and 20 µM), collagen (4 and 20 µg/mL), and ristocetin (0.5 and 1.5 mg/mL) (reference 15).

[c] Flow cytometry for GPIb-IX-V, GPIIb-IIIa, and GPIa-IIa (reference 15).

Abbreviations: MPV = mean platelet volume; GP = glycoprotein; n.d.: not determined. TEM = transmission electron microscopy on platelet preparation.