A *de novo* splice donor mutation in the thrombopoietin gene causes hereditary thrombocythemia in a Polish family

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ABSTRACT

Background

Hereditary thrombocythemia is an autosomal dominant disorder with clinical features resembling sporadic essential thrombocythemia. Germline mutations in families with hereditary thrombocythemia have been identified in the gene for thrombopoietin (*THPO*) and its receptor, MPL.

Design and Methods

Here we characterized a *THPO* mutation in a hereditary thrombocythemia pedigree with 11 affected family members.

Results

Affected family members carry a G→C transversion in the splice donor of intron 3 of *THPO* that co-segregated with thrombocytosis within the pedigree. We previously described the identical mutation in a Dutch family with hereditary thrombocythemia. Haplotype analysis using single nucleotide polymorphisms surrounding the mutation indicated that the mutations arose independently in the two families. MPL protein levels, but not mRNA levels, were low in platelets from affected family members. Bone marrow histology showed features compatible with those of essential thrombocythemia, but the megakaryocytes were unusually compact, as assessed by planimetric analysis. Impaired microcirculation resulting in brief episodes of fainting and dizziness that responded well to aspirin were the predominant clinical features in a total of 23 affected family members studied. Disease onset is earlier in patients with hereditary thrombocythemia than in those with essential thrombocythemia, but the frequencies of thrombotic, vascular and hemorrhagic events are similar in the two groups.

Conclusions

A mutation in *THPO* occurred *de novo* in the same position as in a previously described family with hereditary thrombocythemia. Patients with this mutation have elevated serum levels of thrombopoietin and a phenotype that responds to aspirin and does not require cytoreductive treatment.

Key words: hereditary thrombocythemia, *de novo* mutation, founder effect, single nucleotide polymorphism analysis.

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Introduction

Hereditary thrombocythemia, also known as familial thrombocytosis or familial essential thrombocythemia, is an autosomal dominant disorder with clinical features resembling those of sporadic essential thrombocythemia.[1] Hereditary thrombocythemia is characterized by active proliferation of megakaryocytes and overproduction of platelets. The key regulators of platelet production are thrombopoietin and its receptor, MPL.[2,3] To date, four different germ line mutations in the thrombopoietin gene (*THPO*) have been identified and all of them alter the 5' untranslated region (5'-UTR) of the *THPO* mRNA, which contains upstream open reading frames (uORF) that inhibit the translation of *THPO* mRNA.[4-10] The mutations remove the inhibitory upstream open reading frames and lead to increased translation of the *THPO* mRNA, causing elevated serum levels of thrombopoietin and overproduction of platelets.[4,6] A missense mutation in the transmembrane domain of MPL has been identified in one family with hereditary thrombocythemia.[11] This mutation generates a hyperactive MPL protein and results in excessive platelet production. Recently, mutations in the juxtamembrane domain of MPL have been found in patients with chronic myeloproliferative disorders, in particular idiopathic myelofibrosis and essential thrombocythemia,[12,13] but *THPO* mutations have not been detected in patients with sporadic essential thrombocythemia.[14] In some families with hereditary thrombocythemia, both *THPO* and *MPL* genes can be excluded as the cause of thrombocytosis and thus, other as yet unknown genes must be involved in causing the phenotype.[15,16]

In this study, we analyzed a Polish family with hereditary thrombocythemia and identified a G→C transversion in the splice donor of intron 3 of the *THPO* gene. We previously described the identical mutation in a Dutch family with hereditary thrombocythemia.[4] Here we present the analysis of the clinical and pathomorphological features of 23 affected family members with the same *THPO* mutation and compare these features with those of 107 patients with sporadic essential thrombocythemia.

Design and Methods

Patients and clinical features

The proposita (PL09) was referred to the hematology clinic at the Ludwik Rydygier Memorial District Hospital in Kraków, Poland, in 2000 (age at diagnosis, 19 years) because of significant thrombocytosis (platelet count1455×10^9/L) detected in a routine blood test. At presentation, the patient was asymptomatic and without physical signs. Abdominal ultrasound revealed minimal splenomegaly (length of long axis, 124 mm). The peripheral blood values were: platelets 1032×10^9/L, white blood cells 7×10^9/L, red blood cells 5.1×10^{12}/L, hemoglobin 136 g/L, hematocrit 39.7%, mean corpuscular volume 78.5 fL; mean cell hemoglobin 26.9 pg; mean corpuscular hemoglobin concentration 342 g/L. No cause of reactive thrombocytosis was found and the histology of the bone marrow was compatible with the diagnosis of myeloproliferative disease other than chronic myeloid leukemia, most probably essential thrombocythemia. Since the patient fulfilled the Polycythemia Vera Study Group (PVSG) and the World Health Organization (WHO) criteria for essential thrombocythemia,[17-21] and her platelet levels on follow-up constantly exceeded 1000×10^9/L, treatment with hydroxyurea at a dose of 1 g/day was initiated and continued for 2 years. During this period she suffered from brief episodes of transient unconsciousness, initially interpreted with the aid of electroencephalography as epileptic in origin, which were treated with carbamazepine for 1 month. After the familial background of the disease became evident, hydroxyurea was stopped and treatment was changed to low-dose aspirin (75 mg/day). Currently, she is maintained on low-dose aspirin and her platelet count has stabilized around 800×10^9/L. The spleen is not palpable, and she does not manifest any other signs or symptoms of disease.

At the end of 2001, thrombocytosis was diagnosed in her two sisters (PL07 and PL08). The older sister, PL07, suffered from Raynaud's phenomenon and brief episodes of fainting and dizziness. In addition she had a persistent pain in her right elbow, without any detectable local radiological or vascular abnormalities. She manifested mild splenomegaly (length of long axis, 130 mm on ultrasound). She was treated with low-dose aspirin and ticlopidine, and is currently asymptomatic. At presentation, PL08, the dizygotic twin of the proposita, complained of bilateral paresthesia in her fingers, and reported an episode of superficial vein thrombosis in her left hand. Treatment with low-dose aspirin resulted in complete remission of the paresthesia. Her platelet counts are stable at levels below 700×10^9/L. Soon after, another young thrombocythemic female patient treated in another institution for headaches, arterial hypertension and obesity, was identified as their great-grandparental cousin (PL04). Similar to the other family members, her symptoms responded to low-dose aspirin. The clinico-pathological picture found in several members of the youngest generation prompted a wide screening of their extended family, revealing altogether 11 affected family members.

To compare the clinical course and the rate of complications, a cohort of 107 patients with sporadic essential thrombocythemia was studied. The diagnosis in these patients was made according to WHO criteria.[19,20] The collection of patients' samples was approved by the local ethics committees. Written consent was obtained from all patients.

Separation of blood cells and extraction of DNA and RNA

Blood cells were separated by standard protocols using Histopaque (Sigma, St. Louis, MO, USA) gradient centrifugation. Granulocytes and peripheral blood mononuclear cells were collected. Platelets were col-

lected using the Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gel filtration method.[22] DNA was extracted using a standard proteinase K (Promega, Madison, WI, USA)/phenol (Fluka Chemie AG, Buchs, Switzerland) extraction protocol. RNA was isolated using the TRIfast reagent (peqLab Biotechnology GmbH, Erlangen, Germany).

Pathology of bone marrow

Diagnostic trephine bone marrow biopsies were obtained from five members of the family after their informed consent, fixed in 4% buffered formaldehyde and decalcified in Shandon TBD-1 Rapid Decalcifier (Anatomical Pathology International, Runcorn, UK). The 4-μm dewaxed slides were stained with routine tinctorial stains. Reticulin fibers were assessed in trephine biopsies stained with Gomori silver and graded on a scale ranging from 0 to +4.[23] Blasts were highlighted using CD34 (DakoCytomation, Glostrup, Denmark). Objective, computer-assisted analysis of megakaryocyte planimetric parameters was performed as described previously.[24] Briefly, the high-power/high-resolution electronic images of representative megakaryocytes were transformed into two-color bitmaps depicting the cytoplasmic and nuclear shapes. Standard planimetric parameters (linear sizes, areas, shape factors, etc.) were analyzed using a computer image analysis system Analysis pro v. 3.2 (Soft Imaging System GmbH, Münster, Germany). The results were compared to those of ten control trephines representing normal marrows and 20 cases of classical, sporadic essential thrombocythemia, diagnosed according to the WHO criteria.[19,20]

Quantitative polymerase chain reaction for PRV-1 and MPL

Total RNA (2 μg) was reverse transcribed after random hexamer priming. The primers for ribosomal protein L19 (*RPL19*), and polycythemia rubra vera-1 (*PRV-1*) were described previously.[25] The SYBR detection primers for *MPL* were AGCCCTGAGCCCGCC and TCCACTTCTTCACAGGTATCTGAGA. The ΔCT values were derived by subtracting the threshold cycle (C_T) values for *PRV-1* and *MPL* from the C_T value for *RPL19*, which served as an internal control.[26] A non-affected family member (PL15) was chosen as a calibrator for calculating the $\Delta\Delta C_T$ values.[25,27] All reactions were run in duplicate using the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Erythropoietin-independent colony formation assay

The clonogenic cultures for erythropoietin-independent colony formation were performed as previously described using Methocult H4531 media (Stem Cell Technologies Inc, Vancouver, BC, Canada).[26]

Analysis of genetic linkage

DNA was amplified by PCR using dye-labeled primers for microsatellite markers. The conditions were 94°C for 15 s, 55°C for 15 s, 72°C for 30 s for 10 cycles, 89°C for 15 s, 55°C for 15 s and 72°C for 30 s for 20 cycles. The PCR products were analyzed using the ABI 3100 genetic analyzer and the Genemapper software package version 3.5 (Applied Biosystems, Foster City, CA, USA). Linkage analysis was carried out with FASTLINK software package version 4.1p assuming equal allele frequencies for the marker alleles and an autosomal dominant inheritance model with 100% penetrance.

Genomic DNA sequencing

The entire coding region including intron/exon boundaries of the *THPO* gene was sequenced from PCR fragments, amplified from genomic DNA of the affected family member PL10. The primer sequences for PCR are shown in *Online Supplementary Table S1*. The PCR conditions were 95°C for 2 min, 94°C for 30 s, 58°C for 30 s and 72°C for 1 min for 35 cycles. Sequencing was performed on an Applied Biosystems 3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols.

Restriction fragment length polymorphism analysis

For co-segregation analysis, a 951 bp PCR fragment was amplified using the primers AGCCTAAGCCGCCTCCATG (exon 3, sense) and GGTGGCCAAGCTGAAGGTG (intron 5, antisense) from genomic DNA of all family members and digested with *BsrI* restriction enzyme at 65°C overnight. Fragments of 460 bp for the mutant allele and 359 bp for the normal allele were visualized by ethidium-bromide staining after agarose gel electrophoresis.

Haplotype analysis

To examine a potential founder effect, six microsatellite markers located in the vicinity of the *THPO* gene were chosen (*Online Supplementary Table S2*). The haplotypes were determined based on the segregation within the pedigrees and the sizes of the PCR products of the co-segregating microsatellite markers were compared between affected members of the two families. In addition, ten single nucleotide polymorphisms located within *THPO* (*Online Supplementary Table S3*) were selected from the dbSNP at the NCBI homepage (*http://www.ncbi.nlm.gov/projects/snp/*) and genotyped by sequencing.

Human thrombopoietin enzyme-linked immunosorbent assy (ELISA) and immunoblot assay of MPL

Thrombopoietin serum levels were measured using the TPO-Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. MPL protein expression in platelets was determined by immunoblot analysis using the polyclonal rabbit antibody (CTP7) specific for the C-terminus of human MPL (*kindly provided by Dr. Jerry L. Spivak, and Dr. Alison Moliterno, John Hopkins University, Baltimore, USA*). The membranes were re-probed using a monoclonal antibody against human CD61 (BD Biosciences, San Jose, CA, USA) serving as a loading control.

Statistical analyses

The characteristics of patients with hereditary thrombocythemia and sporadic essential thrombocythemia were compared by Pearson's χ^2 test and the Mann-Whitney U test. Incidences of complications were calculated per 100 years of follow-up and compared in a univariate fashion by the Mann-Whitney U test and in multivariate fashion by logistic regression.

Results

The clinical features of the 11 affected family members are summarized in Table 1. Thrombocytosis was detected in many of the patients in childhood or adolescence. Five of the 11 affected family members had symptoms potentially related to thrombocytosis, including hypertension, headaches, Raynaud's phenomenon, limb paresthesia, venous thrombosis, transient ischemic attacks, miscarriage and thrombangiitis obliterans (Buerger's disease). Most of these symptoms, except Buerger's disease, were manageable using low-dose aspirin (75 mg/day). In contrast, attempts to relieve the symptoms by cytoreductive therapy with hydroxyurea in the proposita (PL09) were ineffective. Patient PL13 died of thromboembolic complications of Buerger's disease at the age of 57.

Linkage analysis revealed co-segregation of thrombocytosis with two microsatellite markers (THPO1 and THPO2) located in close vicinity of the *THPO* locus. The logarithm of odds (LOD) score was 3.3 at Θ=0. Sequencing of the *THPO* gene revealed a G→C transversion in the splice donor of intron 3 (Figure 1A). The mutation generates a *BsrI* restriction fragment length polymorphism that was used to confirm the co-segregation of the mutation within the pedigree (Figure 1B). We previously described the identical mutation in a Dutch family with thrombocytosis.[4] This mutation destroys the splice donor site in intron 3 and results in exon 3 skipping (Figure 1A). The resulting shortened 5'-UTR leads to overproduction of thrombopoietin by a mechanism of increased efficiency of *THPO* mRNA translation.[4] We did not detect this mutation in 76 analyzed patients with sporadic essential thrombocythemia (*data not shown*) and the mutation was not found in a previously published series of 50 patients with sporadic essential thrombocythemia.[14] No somatic mutations in *JAK2* or *MPL* were detected in affected family members (*not shown*).

To determine whether the mutation in the two families arose in a common founder or *de novo*, we examined polymorphic DNA sequences in the vicinity of the mutation. A founder effect, i.e. descent of both families from a common affected ancestor, is expected to result in sharing of allelic sequence polymorphisms in the vicinity of the *THPO* mutation in affected members from the two families. First, we compared six microsatellite markers located between 4 kb to 40 kb from the *THPO* mutation, but all PCR products that represent the haplotype of the affected allele in the two families had different sizes (Figure 2A). This suggests that the mutation occurred independently in these two families. However, as the mutation rate of microsatellites is in the range of 10^{-3} to 10^{-4} per locus per genera-

Table 1. Summary of clinical data of the 11 affected members of the Polish family with thrombocytosis.

UPN	*Sex*	*Date of birth*	*Date of diagnosis*	*Date of last follow-up or DOD*	*Platelets 150-450 ×10⁹/L*	*WBC 3.5-10.0 ×10⁹/L (2003)*	*RBC 4.2-6.3 ×10¹²/L (2003)*	*Hemoglobin 120-140 F 140-180 M g/L (2003)*	*Splenomegaly (last follow-up)*	*Hepatomegaly (last follow-up)*	*Thrombocytosis-associated symptoms*	*Important co-morbidity*
PL02	F	1956	3/2003	3/2003	545-560	5.9	4.5	131	na	na	none	not known
PL04	F	1986	8/2001	6/2006	595-1300	8.1	4.7	123	(+)	(-)	hypertension headaches	obesity
PL06	M	1950	3/2003	3/2006	408-420	6.5	5.0	145	(-)	(-)	none	none
PL07	F	1978	10/2001	9/2005	760-960	6.1	4.7	132	(+)	(-)	Raynaud's phenomenon, transient ischemic attacks, miscarriage, persistent pain in the right elbow	
PL08	F	1982	10/2001	92004	750-890	7.1	4.7	135	(-)	(-)	limb paresthesia, venous thrombosis	none
PL09	F	1982	11/2000	12/2005	740-1340	6.7	4.1	127	(-)	(-)	transient ischemic attacks	none
PL10	F	1991	3/2003	3/2003	960	10.6	5.2	142	na	na	none	none
PL11	F	1922	3/2003	3/2003	510	7.7	5.0	150	na	na	na	na
PL12	M	1947	10/1998	3/2003	550-560	5.3	5.5	156	(+)	na	na	melanoma
PL13	M	1948	3/2003	4/2003	910-1190	10.7	4.7	139	(+)	(-)	Buerger's disease, died in 2005	viral hepatitis type B
PL14	M	1992	3/2003	3/2003	460	6.2	4.6	123	na	na	na	none

UPN, unique patient number; DOD, date of death; WBC, white blood cells; RBC, red blood cells; Reference values are given for platelets, WBC, RBC, and hemoglobin; F, female, M, male; the year of analysis is given in parentheses; lowest and highest platelet values are given where available; na, data not available.

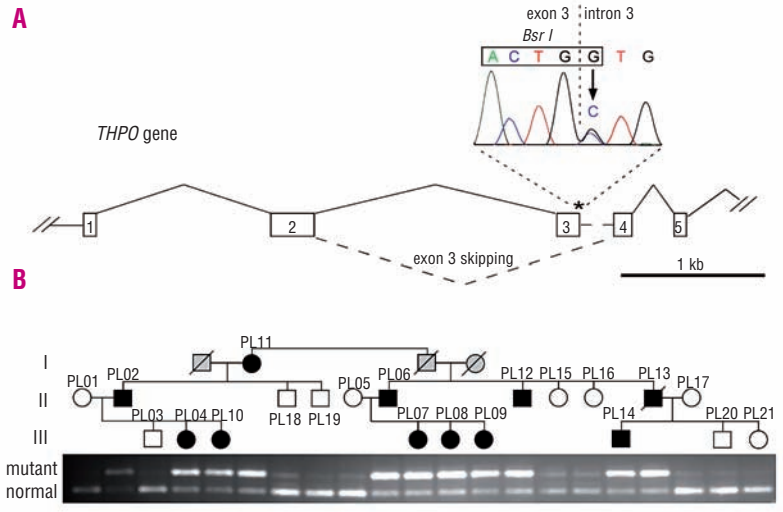

Figure 1. The *THPO* gene mutation. (A) The sequencing chromatogram of the boundary between THPO exon 3 and intron 3 (dashed vertical line) from an affected individual is shown. The arrow points to the G→C transversion in the sequence. The recognition sequence for the *BsrI* restriction endonuclease is boxed. This recognition sequence is destroyed by the G→C transversion. The *THPO* locus is shown below. The asterisk marks the position of the G→C transversion. Open boxes represent exons. Exons connected by solid lines represent normal splicing and dashed lines indicate the expected consequence of the *THPO* mutation on splicing. (B) Co-segregation of the *THPO* mutation and thrombocythemia within the pedigree. The BsrI restriction fragment length polymorphism, caused by the presence or absence of the G→C transversion, was used to follow the inheritance of the *THPO* mutation. Individuals within the pedigree are positioned above the corresponding lanes.

tion,[28] we cannot exclude that some of the differences could be due to the inaccuracy in the replication of repetitive elements. We, therefore, genotyped single nucleotide polymorphisms, which are genetically more stable and display a lower mutation rate (10^{-8} per locus per generation).[29] By screening ten such polymorphisms located within the *THPO* gene we found that three informative single nucleotide polymorphisms, representing the haplotype of the co-segregating mutant allele, differed in their sequences between the two families (Figure 2B). One single nucleotide polymorphism (rs956732) is located 150 nucleotides upstream of the mutation, while the other two (rs2280740 and rs10513797) are located 507 and 1553 nucleotides downstream of the mutation, respectively (Figure 2B). Due to the very short physical distance between these polymorphisms and the G→C mutation, it is very unlikely that the differences in the sequence between these two families are the consequence of recombination, indicating that the mutation in these two families occurred *de novo*.

To explore how the *THPO* mutation affects the regulation of platelet production, we measured thrombopoietin serum concentrations and MPL protein expression levels on platelets and compared them with the platelet counts in all family members (Figure 3A). Two affected family members (PL12 and PL13) showed highly elevated serum thrombopoietin, the other nine affected family members had only slightly elevated or normal thrombopoietin serum levels. Thrombopoietin concentrations showed no clear correlation with the platelet counts (Figure 3A). The MPL protein expression levels were determined in platelet lysates and normalized ratios against CD61 were used to determine the relative MPL protein amount. Nine of 11 patients showed decreased expression of MPL amount compared to the normal. There were significant differences in mean values for platelet counts ($p<0.001$), serum thrombopoietin concentration and MPL expression ($p<0.05$) between affected and non-affected family members (Figure 3B). The low amount of MPL protein

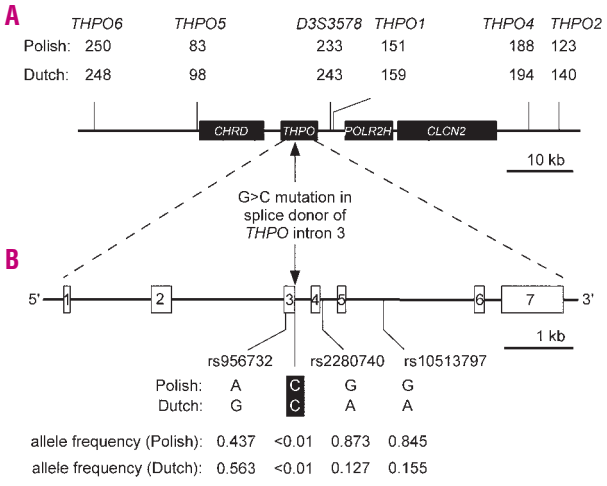

Figure 2. Haplotype analysis for the *THPO* locus in the Polish and Dutch families. (A) The chromosomal locus containing *THPO* is shown. Black boxes represent genes: *THPO*, thrombopoietin; *CHRD*, chordin; *POLR2H*, polymerase RNA II DNA directed polypeptide H; *CLCN2*, chloride channel 2. Microsatellite markers are shown above the locus, numbers indicate the sizes in nucleotides of the PCR products of the co-segregating mutated alleles in the Polish and Dutch families. Note that none of the allele sizes is identical in the two families. (B) The *THPO* locus, with the positions of three informative single nucleotide polymorphisms (SNP) is shown. The sequences at each of the SNP positions are shown for the co-segregating mutated allele only. The allele frequencies for each SNP are listed below. Note that the sequences of the three SNP located in the vicinity of the G→C transversion (black box with white letters) differ in the two families.

was not due to decreased mRNA levels, as shown by real-time PCR. Rather, there was a slight, but non-significant increase in *MPL* mRNA in the affected individuals (Figure 3B). Interestingly, the individual with the highest thrombopoietin serum concentration (PL12) had the lowest MPL protein level and showed the highest *MPL* mRNA expression (Figure 3A). Similar data were obtained in affected Dutch family members carrying the same *THPO* mutation.[26] All 11 affected mem-

bers of the Polish family had normal levels of *PRV-1* mRNA in granulocytes and none had growth of erythropoietin-independent colonies (*data not shown*).

Since thrombocytosis in this family is caused by a known mechanism, i.e. overproduction of thrombopoietin, we compared the histopathology of bone marrow trephines from affected family members with trephines from patients with sporadic essential thrombocythemia (Table 2). The megakaryocyte densities were comparable to the lower limits of the values encountered in chronic myeloproliferative disorders, but were higher than the age norms,[30,31] and did not correlate with platelet counts. Some tendency to clustering was noted, but rarely did the clusters contain more than three cells. The megakaryocytes were strikingly compact (Figure 4), which was corroborated by the planimetric analysis. The compactness factor, which ranges from 1 (for a circle) to 0 (for an extremely long and thin object), of hereditary thrombocythemia megakaryocytes (0.752±0.102) significantly surpassed that of megakaryocytes from both normal controls (0.726±0.111, p=0.0078) and the cases of essential thrombocythemia (0.706±0.122, $p<1x10^{-6}$). Furthermore, the compactness factor for megakaryocyte nuclei was significantly higher in hereditary thrombocythemia (0.697±0.116) than in normal controls (0.675±0.123, p=0.026), but only marginally higher than in essential thrombocythemia (0.686±0.118, p=0.15). Descriptors of cellular and nuclear size, nucleo-cytoplasmic ratio and the degree of nuclear segmentation of hereditary thrombocythemia megakaryocytes were comparable to the values typical for normal megakaryocytes. There was no increase or clustering of blasts. The reticulin fiber meshwork was compatible with the upper limit of the semiquantitative norm,[23] but the histological features of hereditary thrombocythemia did not resemble those of *pre-fibrotic* idiopathic myelofibrosis, as defined by Thiele *et al.*[32] In

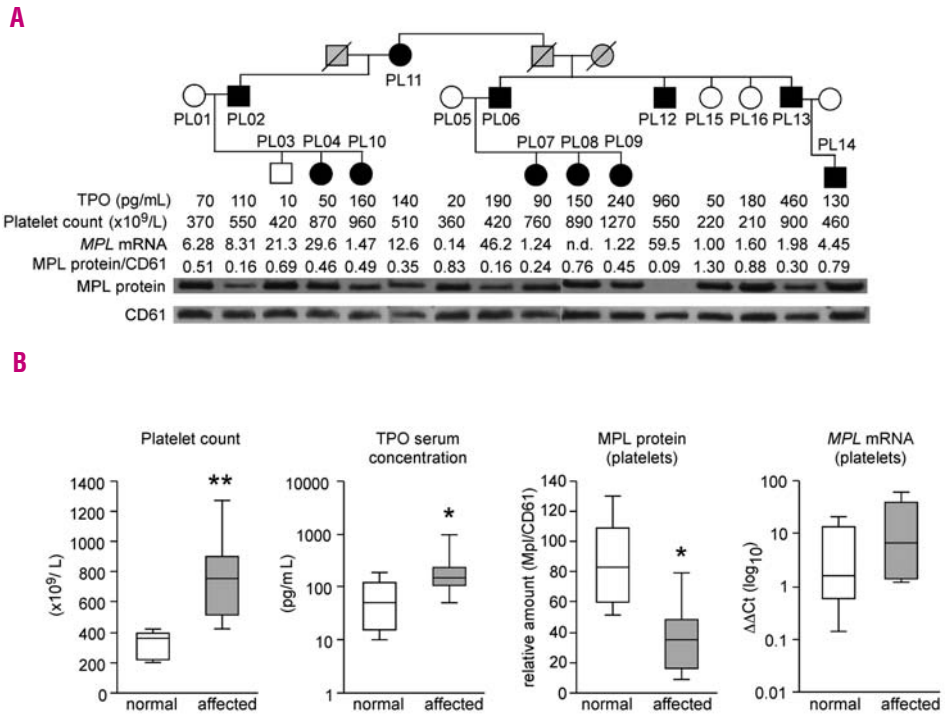

Figure 3. The correlations between the platelet count, thrombopoietin serum concentration and MPL expression. (**A**) Western blotting of MPL and CD61 protein in platelets in shown. The individuals within the pedigree are placed above the corresponding lanes. Platelet counts, serum concentrations, *MPL* mRNA expression (determined by real time PCR), and the ratios of MPL protein against CD61 (determined by densitometry) are shown. (**B**) Boxes represent the interquartile range that contains 50% of the values, the horizontal line in the box marks the median and bars indicate the range of values. *p* values were calculated by a one-side *t* test for independent samples (*p value <0.05, **p value <0.001). The relative expressions of *MPL* mRNA ($\Delta\Delta C_T$ values) are shown on a logarithmic scale. The values are relative to those of a non-affected family member (PL15).

Table 2. Quantitative aspects of bone marrow histology in familial thrombocytosis.

UPN	*Sex*	*Date of birth*	*Year of biopsy*	*Platelets x10⁹/L*	*Cellularity [%]*		*Megakaryocytes per mm²*		*Myeloid/ erythroid ratio*	*CD34 blast cells*	*Clusters of megakaryocytes*	*Increase in eosinophils*	*Reticulin fibers***	*Distended sinuses*	*CD34+ megakaryocytes*
					Cases	*Age norm**	*Cases*	*Age norm**							
PL08	F	1982	2001	887	90	72±11	90	16±4	4:1	<1%	+	+	0	+	+
PL09	F	1982	2000	1340	90	72±11	58	16±4	2:1	<1%	+	+	0/+1	+	-
PL07	F	1978	2001	956	80	70±12	60	15±6	3:1	<1%	+	+	+1	+	-
PL04	F	1986	2003	868	95	72±11	78	16±4	4:1	2%	+	+	0	+	+
PL13	M	1948	2003	905	45	59±13	38	14±5	3:1	nd	+	+	+1	+	nd
II-3	M	1934	1991	701-1200	60	59±13	88	14±5	4:1	nd	+	+	+1	+	nd

**Age norms based on the study by Thiele et al.[30]; ** According toThiele et al.[21]*

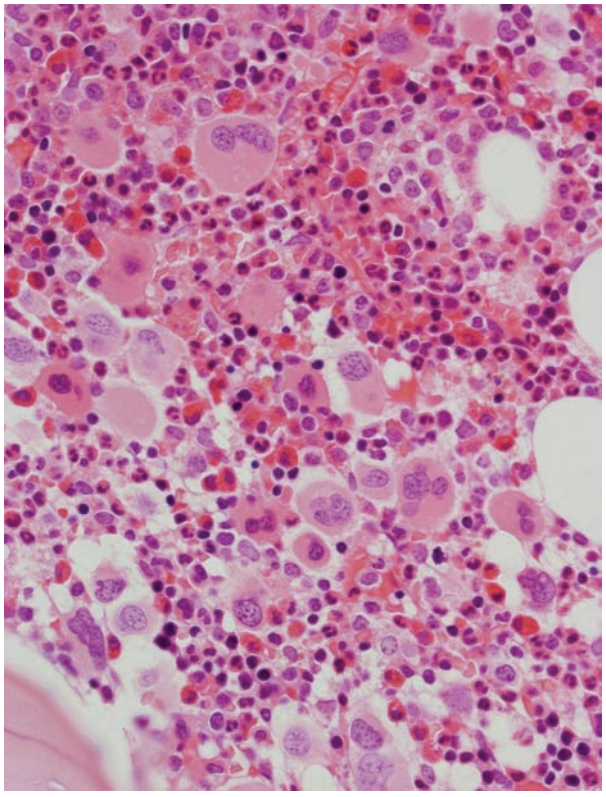

Figure 4. Trephine bone marrow biopsy of patient PL08 stained with hematoxylin and eosin, showing high marrow cellularity, a markedly increased number of megakaryocytes, occasional loose clustering of megakaryocytes and moderate increase in dispersed mature eosinophils. Note the very regular shapes of the megakaryocytes. Original objective magnification 60x.

summary, bone marrow histology mimicked that of a "true" myeloproliferative disorder; however, the megakaryocytic morphology, with their very compact (*hyper-normal*) shapes and normal sizes differed notably from that of megakaryocytes from patients with myeloproliferative disorders.

We compared the clinical course in 23 affected members of the Polish and Dutch families (Table 1 and *Online Supplementary Table S4*) with that in 107 patients with sporadic essential thrombocythemia. General features between the two groups were comparable, except for age at diagnosis (Table 3). As expected, hereditary thrombocythemia was diagnosed at an earlier age than sporadic essential thrombocythemia. The frequencies of venous and arterial thromboembolic events, hemorrhage and major vasomotor complications were comparable in the two groups (Table 3). The values were normalized per 100 years of patient follow-up. After adjusting for the patients' age in multivariate linear regression models, the relative risks of developing complications remained statistically non-significant (p=0.78).

Discussion

We described a Polish family carrying a splice donor mutation in intron 3 of the *THPO* gene (Figure 1), identical to that previously identified in a Dutch family with hereditary thrombocythemia.[4] The mechanism by which this mutation increases thrombopoietin production is loss of translational inhibition from the 5'-UTR of *THPO* mRNA.[4,10] Although there is no obvious relat-

Table 3. Comparison of characteristics of patients with familial and sporadic thrombocythemia.

	Familial thrombocythemia (n= 23)	*Sporadic essential thrombocythemia (n= 107)*	*p value*
Sex (male/ female)	8/15	46/61	0.47
Age at diagnosis (years), median (range)	22 (0.5- 81)	55 (16-80)	<0.001
Time of follow up (months), median (range)	62 (0-242)	155 (0-284)	<0.001
Platelets (x10^9/L); median (range)	890 (414-1400)	874 (321-2669)	0.40
Number of patients with palpable splenomegaly	6	23	0.88
Rate and localization of clinical complications:			
Total number of events	11	122	na
Number of events per 100 patient-years	6.7	9.2	0.20
Thrombotic events (per 100 patient-years)	6.1	7.0	0.31
Venous thromboembolic events	0.6	2.8	0.14
Vasomotor symptoms/functional symptoms	1.8	0.8	0.33
Arteriovascular events	3.7	3.4	0.84
Hemorrhage (per 100 patient-years)	0.6	1.8	0.26
Myelofibrosis (per 100 patient-years)	0	6.7	0.40
Transformation to acute leukemia	0	1.7	0.68
Causes of death:			
Total numbers of deaths	4	27	na
Thrombocytosis-related deaths	2	6	na
Stroke	2	2	na
Pulmonary embolism	0	2	na
Hemorrhage	0	2	na
Other causes of death	0	6	na
Unknown causes of death	2	15	na

When multiple events per patients occurred, every event was scored. Vasomotor symptoms: only Raynaud's phenomenon and erythromelalgia were scored. Cold tip feeling, acral paresthesia and headache were excluded. Limb paresthesia was included in arterial events. Other causes of death: sepsis (n=2), neoplasia (n=2), suicide (n=1), and left ventricular failure (n=1).

edness between members of the two families, it was conceivable that the mutation may have originated from a common ancestor (founder effect). However, haplotype analysis for the two families showed differences in genetic polymorphisms flanking the mutation, indicating that the mutation in the two families arose *de novo* (Figure 2).

The thrombopoietin serum concentrations in most affected family members were only slightly elevated or even normal, as in the Dutch family.[4] A possible explanation is that the increase in platelet count and megakaryocyte mass lowers the serum concentration by binding thrombopoietin through its receptor MPL,[33,34] reaching a new equilibrium at levels close to normal. Perhaps as a consequence of the increased internalization and degradation of the MPL-thrombopoietin complex,[35,36] MPL protein was decreased in platelets from most of the affected family members. *MPL* mRNA levels in platelets were normal or even slightly elevated in affected family members, indicating that the low MPL protein levels were not due to a decrease in mRNA expression.

Histological appearances of bone marrow from affected Polish family members and from the member of the Dutch family (II/3) showed some similarities to a chronic myeloproliferative disorder, e.g. increase and clustering of megakaryocytes, marrow hypercellularity and occasional mild increase in reticulin fibers. However, in contrast to essential thrombocythemia, the megakaryocytes from patients with hereditary thrombocythemia assumed compact shapes and were even more regular than megakaryocytes from normal controls. These nuances of histology, particularly the specific features of megakaryocytes, may be useful in the differential diagnosis from true sporadic essential thrombocythemia.

The clinical course in patients with familial thrombocytosis is generally believed to be milder than that of patients with sporadic essential thrombocythemia. We studied the consequences of increased thrombopoietin production and elevated platelet counts in a total of 23 patients with hereditary thrombocythemia and compared the rate of complications with that in a cohort of 107 patients with sporadic essential thrombocythemia. All complications investigated, such as venous thrombotic events, major vasomotor events, arterio-vascular events and hemorrhage occurred at a comparable rate in both groups (Table 3). A previous study from our group showed similar incidences of complications in patients with sporadic essential thrombocythemia,[37] whereas one recent study showed a lower incidence of thrombotic events in a population of young patients with sporadic essential thrombocythemia.[38] On the other hand, other studies found a higher frequency of thrombotic complications in essential thrombocythemia patients.[39-42] The major thrombotic events in hereditary thrombocythemia occurred in patients over 70 years old. As in essential thrombocythemia, minor vasomotor symptoms such as cold tip feeling and acral paresthesia responded well to aspirin in all affected hereditary thrombocythemia patients. Progression to myelofibrosis or acute leukemia has not been observed in hereditary thrombocythemia without cytoreductive treatment. Although this is the largest group of patients with hereditary thrombocythemia studied so far, our conclusions concerning the rate of complications in this condition need to be verified in a larger cohort of patients.

Authorship and Disclosures

KL performed research, analyzed data and wrote the paper, RK performed research and analyzed data, ZR analyzed histopathology, BG, ASB, KO and APCvdM analyzed clinical data, RT and PF performed research, RCS designed research, analyzed data and wrote the paper. The authors reported no potential conflicts of interest.

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