

Evidence for a founder effect of the *MPL*-S505N mutation in eight Italian pedigrees with hereditary thrombocythemia

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ABSTRACT

Background

Hereditary thrombocythemia is a rare disease characterized by increased megakaryopoiesis and overproduction of platelets. Germ line mutations have been identified in the genes for thrombopoietin (*THPO*) and its receptor, *MPL*. A clustering of familial cases with the *MPL*-G1073A mutation that results in a serine to asparagine substitution (S505N) has been recently reported in Italy. Here we performed haplotype analysis in nine families (eight Italian and one Japanese) with hereditary thrombocythemia carrying the *MPL*-S505N mutation in the *MPL* gene.

Design and Methods

The *MPL* gene was examined by genomic DNA sequencing. Haplotype analysis was performed using microsatellites and single nucleotide polymorphisms.

Results

Analysis of microsatellite markers and single nucleotide polymorphisms in the eight Italian families with hereditary thrombocythemia revealed the presence of a common haplotype compatible with a founder effect, which may have originated 23 generations ago. This haplotype was rarely observed in 132 unrelated individuals and was absent in a Japanese family with the *MPL*-S505N mutation.

Conclusions

The recurrent *MPL*-S505N mutation found in the eight Italian families with hereditary thrombocythemia is likely due to a founder effect.

Key words: hereditary thrombocythemia, *MPL*, mutation, founder effect, TPO.

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The online version of this article contains a supplementary appendix.

Introduction

Hereditary thrombocythemia (HT) is a rare disease characterized by sustained megakaryopoiesis with overproduction of platelets, with clinical features resembling those of sporadically occurring essential thrombocythemia.¹ Thrombopoietin (THPO) and its receptor MPL regulate platelet production by stimulating proliferation and maturation of megakaryocytes.² Activating germ line mutations in the *THPO* and *MPL* genes have been described in HT families. The four *THPO* gene mutations known to date are all located in the 5' untranslated region of the mRNA sequence,³⁻⁸ which contains upstream open reading frames that inhibit the translation of the *THPO* mRNA. 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.^{3,6,9} A G>A transition in position 1073 of the *MPL* gene (G1073A), which changes a serine to an asparagine at amino acid position 505 (S505N) in the transmembrane domain of MPL protein, was first identified in a Japanese family with HT.¹⁰ This mutant MPL protein is hyperactive and stimulates megakaryopoiesis resulting in excessive platelet production.¹⁰ Interestingly, the identical activating mutation was found in mouse *Mpl* in a retroviral mutagenesis screening.¹¹ *THPO* mutations have not been identified in patients with sporadically occurring thrombocythemia,¹² but recently the *MPL*-S505N mutation was described also in patients with non-familial thrombocythemia.¹³ Furthermore, mutations altering the codon encoding tryptophan in position 515 (W515) located in the juxtamembrane domain of the MPL protein have been found in patients with sporadic myeloproliferative disorders, in particular primary myelofibrosis and essential thrombocythemia.^{14,15} However, no familial cases of *MPL*-W515 mutations have been reported to date. Recently, a recessive germ-line mutation changing a proline to leucine at amino acid 106 (P106L) in the *MPL* gene was reported in families with thrombocytosis.¹⁶

We screened for mutations in the *MPL* gene in 14 families with thrombocytosis and low or normal serum levels of thrombopoietin and found one Italian family with the *MPL*-S505N mutation. In contrast, in another study, the *MPL*-S505N mutation was detected in four of five families of Italian descent.¹⁷ The clustering of the *MPL*-S505N mutation in Italian HT families suggested that the mutation may have originated from a single mutation that occurred many generations ago and that these families are distantly related and inherited the *MPL*-S505N mutation from the same individual. A founder effect for a disease-causing germline mutation can arise when a new population is established by a very small number of individuals who include a carrier of the mutation. One example is the *VHL* 598C>T mutation that causes Chuvash polycythemia and has been reported to originate from a single founder event.¹⁸ Here we tested the hypothesis that the G>A transition in the Italian families with *MPL*-S505N is the result of a founder effect.

Design and Methods

Patients and clinical features

Blood samples were collected at the study centers in Basel (Switzerland), Rome (Italy), and Nagoya (Japan) following approval by the local ethics committees (*Ethik Kommission Beider Basel*, and the institutional boards of Rome and Nagoya). Written consent was obtained from all patients in accordance with the Declaration of Helsinki. Five of the families (A, B, C, D and J) have been previously described.^{10,17} In family E with the *MPL*-S505N mutation, the proposita, a 5-year-old girl, was referred to us because of asymptomatic thrombocytosis since birth and the suspicion that she had essential thrombocythemia. However, clonogenic assays were negative, and family history and blood cell counts revealed that the father and grandmother also had high platelet counts on repeated occasions. Initial screening for the *MPL* mutation was performed on DNA from 13 additional Caucasian families with thrombocytosis originating from the UK (5 pedigrees), Italy (2), Spain (2), Switzerland (1), Israel (1), Germany (1) and USA (1). Family F consisted of two sisters (23 and 21 years old) with asymptomatic thrombocytosis, referred to us because of the suspicion of essential thrombocythemia. Family G consisted of three female patients aged 23, 42 and 61 years at diagnosis. Family H included two patients (father and son) aged 71 and 31 years, respectively. DNA from 132 unrelated patients with sporadic myeloproliferative disorders (kindly provided by Prof. Mario Cazzola and Dr. Francesco Passamonti) was used to determine the frequency of the *MPL*-S505N haplotype.

Table 1. Sequencing primers for the *MPL* gene.

Exon	Primer sequences
Exon 1+2	5'-CCAGTGTGGTCTGGATGGG-3' 5'-GGCCACATACATCCAAG-3'
Exon 3	5'-CCTATCCCAGGCAGTGAGAAG-3' 5'-ACAAGGGAGTCTTGGGGTG-3'
Exon 4	5'-CCAGAGGCTGAGCCATAGAC-3' 5'-TGGGGCAAGATTGAAGGTAG-3'
Exon 5	5'-CTTAGCCCTGGTCTGTGTG-3' 5'-GTCCAGTGGCAAGTTCGAG-3'
Exon 6	5'-GGACAGGAATATGTTCCAGGG-3' 5'-CATTGAAAGCTGTATGGTTTG-3'
Exon 7	5'-AAGACAGAGGAAAGCTCTGG-3' 5'-CTACAGGGATGTCAGCAGGG-3'
Exon 8	5'-CTTCCCACATAGTTCACC-3' 5'-TGCCCAAGAGAGACTAAGATACC-3'
Exon 9	5'-GGATTTGGGTCAAACAGACG-3' 5'-AGCCAGCCCTACTCTGG-3'
Exon 10	5'-CAACTAGGGTGGAGACCGC-3' 5'-AGGTGACGTGCAGGAAGTG-3'
Exon 11	5'-TGCCCAACTTTATTGCCTTG-3' 5'-AGTGCTGGGGTAGTGCCCTG-3'
Exon 12	5'-CTACCAGTGTCCATCCC-3' 5'-TGAAGCCTAATTGTGAGGGC-3'

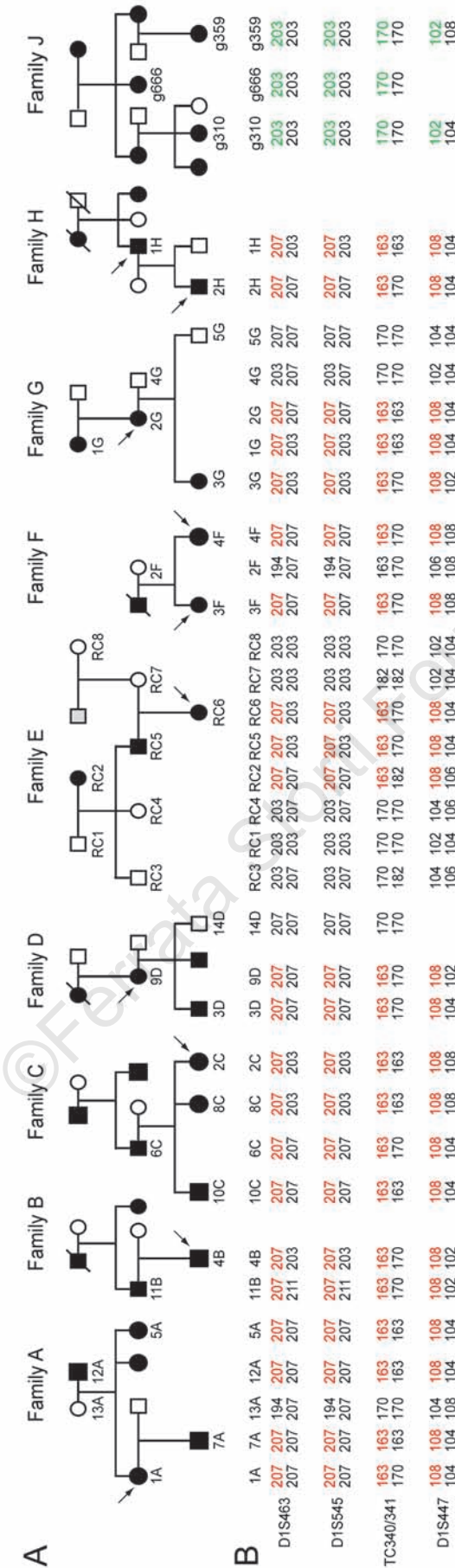


Figure 1. Families with the MPL-S505N mutation. (A) The pedigrees of the eight Italian families (pedigrees A-H) and one Japanese family (pedigree J) with the MPL-S505N mutation. Pedigree E is the index family that initiated this study. Squares represent males and circles females. Black symbols, represent affected individuals, open symbols, unaffected individuals, gray symbols, not studied. Unique patient numbers are shown below the symbols. Arrows mark the probands who initiated the family studies in each pedigree. (B) The genotypes of four informative microsatellite markers located closest to the MPL gene are shown below the corresponding family members in the pedigree. Numbers indicate the allele size in nucleotides. Affected alleles were determined by segregation analysis in family E and are marked in red (Italian families) and green (the Japanese family).

Table 2. Summary of laboratory data of affected family members with the *MPL*-S505N mutation.

UPN	Gender	Age at diagnosis	Platelets $\times 10^9/L$ (n.v. 150-450)	WBC $\times 10^9/L$ (n.v. 3.5-10.0)	Hemoglobin g/dL (n.v. 12-14 [F]) (n.v. 14-18 [M])	HUMARA clonality assay
Family A						
1A	F	14	1030*	9.6*	13.5*	Polyclonal
5A	F	10	602*	6.4*	13.7*	Polyclonal
7A	M	1	2506*	14.2*	11.2*	-
Family B						
11B	M	18	842*	6.8*	14.4*	-
4B	M	1	1459*	16.1*	11.1*	-
Family C						
6C	M	18	1006*	11.0*	15.0*	-
10C	M	11	783*	8.2*	14.3*	-
8C	F	17	695*	9.5*	13.7*	Polyclonal
2C	F	16	963*	7.3*	11.1*	Polyclonal
Family D						
9D	F	43	556	8.0	12.9	Polyclonal
3D	M	17	776*	7.5*	15.0*	-
Family E						
RC02	F	64	926	7.2	12.9	ND
RC05	M	35	625*	5.1*	16.2*	-
RC06	F	5	1092	7.7	13.4	ND
Family F						
3F	F	16	1019	8.7	14.7	Polyclonal
4F	F	20	738	4.6	11.1	Polyclonal
Family G						
3G	F	23	1020	7.3	12.1	Polyclonal
Family H						
1H	M	61	605	9.0	12.7	-
2H	M	31	795	16.8	16.4	-
Family J						
g310	F	24	932*	9.5*	15.1*	ND
g666	F	48	959*	6.3*	12.8*	ND
g359	F	14	919*	5.0*	13.3*	ND

UPN: unique patient number; WBC: white blood cells; n.v.: normal values; *: values obtained at diagnosis; F: female; M: male. HUMARA clonality assay is only applicable in female patients; ND: not determined.

MPL gene sequencing

Genomic DNA was extracted from blood or buccal swabs using the DNeasy Blood & Tissue Kit from QIAGEN (QIAGEN Spa, Milan, Italy) or DNAzol (Invitrogen, Milan, Italy), after isolation of peripheral granulocytes.¹⁵ All exons including the intron/exon boundaries of the *MPL* gene were sequenced from polymerase chain reaction (PCR) fragments amplified from genomic DNA. The primer sequences are shown in Table 1. The PCR conditions were 95°C for 2 min, 94°C for 30 s, 60°C for 30 s and 72°C for 1 min for 35 cycles. Sequencing was performed on an Applied Biosystems 3130 DNA sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols.

Haplotype analysis

Family E was genotyped using 12 microsatellite markers on chromosome 1p. One marker, TC340/341, was newly derived from the genomic sequence of chromosome 1 (FAM-CATGATGGGATAAGTGTCTTCG and GTTCTTCCTGGTGATGGCTTTC). One marker (CA214/215) has been described previously,³ the others (D1S493, D1S2676, D1S2830, D1S463, D1S1882E, D1S1758E, D1S545, D1S447, D1S1808E, D1S2737) were

derived from the UniSTS database. The PCR products were analyzed using an Applied Biosystems 3130 genetic analyzer and Genemapper software package version 3.5 (Applied Biosystems, Foster City, CA, USA). A haplotype co-segregating with thrombocytosis was derived from the segregation of markers within pedigree E. The sizes of the PCR products of the co-segregating microsatellite markers were compared between affected members of the nine families. Among the 12 microsatellite markers we tested, nine were informative in family E and allowed us to define the disease haplotype. Of these nine markers, four were informative (D1S463, D1S545, TC340/341, D1S447) and enabled us to define the smallest co-segregating haplotype shared in the eight Italian families. Genotyping with the four microsatellite markers shared by all eight Italian families was performed on DNA from 132 unrelated Italian control individuals. To identify haplotypes and their frequencies we used the Haplore program, which effectively analyzes data from tightly linked microsatellite loci.¹⁹

Single nucleotide polymorphism genotyping and association analysis

Genome-wide single nucleotide polymorphism (SNP) genotyping was undertaken for one affected family mem-

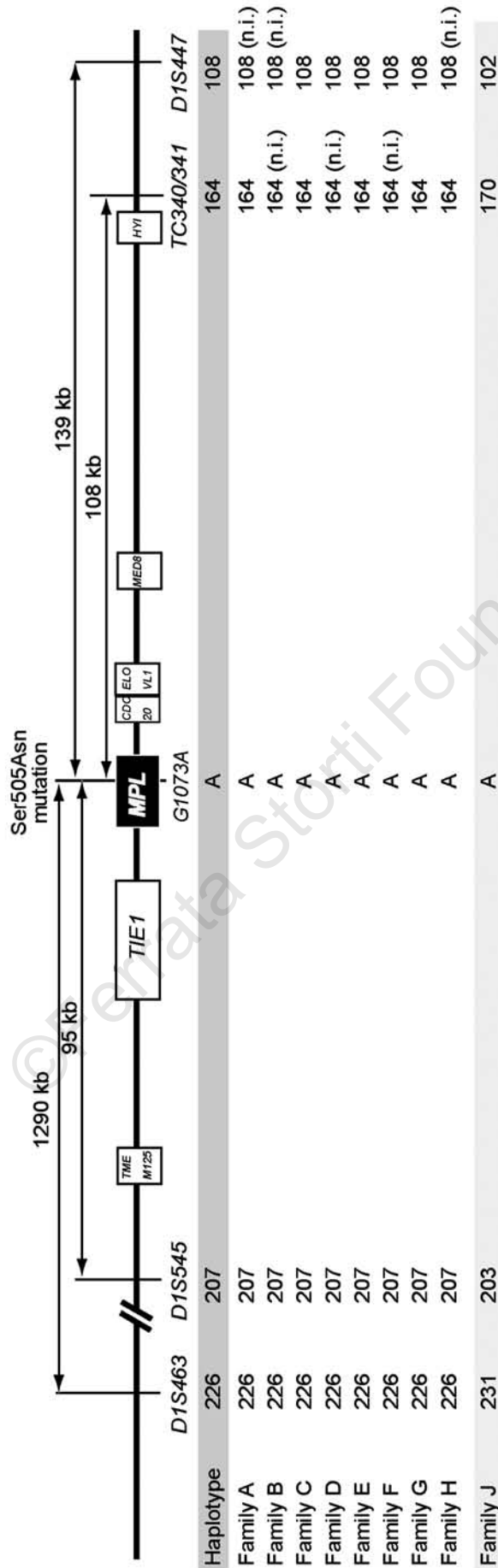


Figure 2. Haplotype analysis of the *MPL* locus in the eight Italian HT families and one Japanese family with the *MPL*-S505N mutation. The chromosomal region containing the *MPL* gene is shown. Arrows indicate the distances of microsatellite markers from the location of the *MPL* mutation. Names of microsatellite markers are shown below the locus, and numbers below the markers indicate the sizes in nucleotides of the PCR products of the co-segregating mutated alleles in each of the nine families. Families in which the marker was non-informative (n.i.) are marked. The two different haplotypes in the Italian and Japanese families are highlighted in gray.

ber from each family with the Affymetrix GeneChip Human Mapping 500K Nsp I according to the Affymetrix GeneChip Mapping Assay Manual (Affymetrix Inc., Santa Clara, CA, USA). The SNP calls were generated by GeneChip DNA Analysis Software. The association between SNP alleles and thrombocytosis was assessed using simple Pearson's χ^2 tests, implemented in SPSS v. 15 for Windows.

Dating the origin of the S505N mutation

The time of the origin of the mutation was calculated using the formula: number of generations = $\log((1-Q)/(1-P_n))/\log(1-\theta)$, as described before.¹⁸ Q is the proportion of haplotypes in diseased subjects that are not the disease haplotype. P_n is the allele frequency of the minor allele for each marker, and θ is the recombination fraction assuming that 1 Mb = 1 cM. In this analysis, Q = 0 for all of the Italian families.

Results

We screened for the presence of mutations in the *MPL* gene in 14 families with thrombocytosis and low or normal serum levels of thrombopoietin. All exons and intron/exon boundaries of the *MPL* gene were sequenced and the *MPL*-S505N mutation was detected in one Italian pedigree (Figure 1A, family E). Since the frequency of the *MPL*-S505N mutation in our series (1/14) contrasted with the high frequency recently reported in another series of familial cases from Italy (4/5),¹⁷ and our family as well as these four families came from the same region in the vicinity of Rome (Figure 1A, families A-D), we hypothesized that the high frequency of the *MPL*-S505N may represent a founder effect. We, therefore, initiated a collaborative study. In addition to these five families with *MPL*-S505N, we included three new families from the same region (Figure 1A, pedigrees F-H) that were identified after the report on clustering of *MPL*-S505N had been published.¹⁷ For comparison, we also analyzed the DNA from affected family members of a Japanese family, in which the *MPL*-S505N was first described (Figure 1A, pedigree J).¹⁰ The laboratory findings of affected family members with the *MPL*-S505N mutation are summarized in Table 2. *JAK2*-V617F was not detected in any of the affected family members (*data not shown*).

We performed haplotype analysis in affected family members using microsatellite markers located in the vicinity of the *MPL* mutation. A founder effect is expected to result in sharing of allelic sequence polymorphisms in the vicinity of the *MPL* mutation (linkage disequilibrium due to a common ancestor). We examined 12 microsatellite markers within 7-18 Mb of the *MPL* gene locus and found nine markers that were informative and allowed us to determine the disease haplotype that cosegregated with thrombocytosis in family E. We then tested whether the same haplotype is also present in affected family members of the other pedigrees. For the four microsatellite markers that are closest to the location of the *MPL*-S505N mutation (D1S463, D1S545, TC340/341 and D1S447), we found that at least one PCR product of the identical size as the disease allele in fami-

ly E was present in all 19 affected family members of the other pedigrees (Figure 1B). These four microsatellite markers are located between 95 kilobases (kb) and 1,290 kb from the *MPL* mutation (Figure 2). In contrast, the affected family members in the Japanese pedigree displayed differently sized alleles for the same markers (Figure 1B and 2). These results are in favor of a founder effect in the eight Italian families. To determine how frequently alleles of the same size can be obtained by chance in a general population, we genotyped DNA from 132 unrelated patients with sporadic myeloproliferative disorders from Italy using primers for the same four microsatellite markers (*Online Supplementary Table S1*). We found allele sizes corresponding to the disease haplotype with all four microsatellite markers in only 6 of these 132 individuals compared to in 22/22 familial cases of Italian descent ($p=6\times 10^{-27}$). Logical inference of haplotypes using Haplore showed that no controls inherited the same haplotype as was observed in all cases. If only the two microsatellite markers located most proximal to the mutation were considered, 38/132 of unrelated individuals showed allele sizes corresponding to the disease haplotype, compared to 22/22 familial cases ($p=2.3\times 10^{-10}$). The allele frequencies in all individuals tested are listed in *Online Supplementary Table S1*. To further confirm the strength of this association, we performed GeneChip Human Mapping 500K Nsp I for one affected family member from each of the nine families with HT. The genotypes of the 45 SNP within the region defined by the microsatellites showed a possible common haplotype in all eight Italian families but not the Japanese family (*data not shown*). However, the SNP data were less informative than the microsatellite analysis, because the SNP were less polymorphic and fewer family members were tested. Together, these results strongly suggest that a founder effect is responsible for the increased frequency of *MPL*-S505N mutation in familial thrombocytosis in Italy.

To estimate the time of origin of the *MPL*-S505N mutation in the Italian families, we used an approach suggested by Risch.²⁰ For the four microsatellite markers D1S463, D1S545, TC340/341 and D1S447, the number of generations to a common ancestor are 22.16, 22.74, 23.30 and 30.74, respectively. Since the distance between the markers is small and there are no haplotypes that are not shared between all the carriers, reporting about the number of generations is primarily driven by the population allele frequency of the markers and not by the shared haplotype in the affected cases. Despite this limitation these results are surprisingly consistent and suggest that the families had a common ancestor about 23 generations ago.

Discussion

In this study we demonstrated that a clustering of the *MPL*-S505N mutation in eight Italian families with HT is due to a common founder ancestor approximately 23 generations ago. Since this germ-line mutation is rare in the general population, the founder effect explains the high frequency of this mutation in the Italian families. The *MPL*-S505N mutation in a Japanese family with HT

appears to have originated independently from that of the Italian HT families. Mutations that persist following their occurrence in a single founder may reflect selection favoring heterozygotes, e.g. by providing the carriers with a survival advantage. However, there is no obvious reason why thrombocytosis should be an advantage. Our previous study on two families with thrombocytosis carrying an identical mutation in the splice donor of *THPO* intron 3 showed no evidence for a founder effect.⁸ These families were from Holland and Poland and affected family members displayed a clinical phenotype very similar to that of patients with the *MPL*-S505N mutation. Recently, several Arabic families with HT have been found to carry a homozygous or heterozygous P106L mutation in the *MPL* gene,¹⁶ with clinical phenotypes indistinguishable from those of other HT families. The possibility of a founder effect in these families with a P106L mutation has not yet been examined.

Other mutations in *MPL*, showing a less stringent association with thrombocytosis, have been described, including *MPL*-K39N (also called *MPL* Baltimore) and *MPL*-S204P.²¹⁻²³ Thus, HT is a disease-phenotype caused by a variety of disease-causing mutations and in a large proportion of HT families the disease-causing mutation remains to be determined.

Authorship and Disclosures

KL performed the research, analyzed data and wrote the paper, MM performed the research and analyzed data, BR analyzed clinical data, CIA performed genetic analyses, LT, FG, JD, HK and LML analyzed clinical data, RCS designed the research, analyzed data and wrote the paper.

The authors reported no potential conflicts of interest.

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