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A founder mutation in the *MPL* gene causes congenital amegakaryocytic thrombocytopenia (CAMT) in the Ashkenazi Jewish population

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ABSTRACT

Congenital amegakaryocytic thrombocytopenia (MIM #604498) (CAMT) is a rare inherited disease presenting as severe thrombocytopenia in infancy. Untreated, many CAMT patients develop aplastic anemia within the first decade of life; the only effective treatment of CAMT is bone marrow transplantation. CAMT is the result of the presence of homozygous or compound heterozygous mutations in the thrombopoietin receptor-encoding gene, *MPL*. We report here the identification and characterization of a founder mutation in *MPL* in the Ashkenazi Jewish (AJ) population. This mutation, termed c.79 + 2T>A, is a T to A transversion in the invariant second base of the intron 1 donor splice site. Analysis of a random sample of 2018 individuals of AJ descent revealed a carrier frequency of approximately 1 in 75. Genotyping of six loci adjacent to the *MPL* gene in the proband and in the 27 individuals identified as carriers of the c.79 + 2T>A mutation revealed that the presence of this mutation in the AJ population is due to a single founder. The observed carrier frequency predicts an incidence of CAMT in the AJ population of approximately 1 in 22,500 pregnancies. The identification of this mutation will enable population carrier testing and will facilitate the identification and treatment of individuals homozygous for this mutation.

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Introduction

Congenital amegakaryocytic thrombocytopenia (MIM #604498) (CAMT) is an autosomally inherited recessive bone marrow failure disorder that usually presents as thrombocytopenia and megakaryocytopenia in infancy, often evolving into pancytopenia and the development of aplastic anemia and leukemia [1–3]. Due to the thrombocytopenia, many affected infants display cutaneous purpura or experience serious hemorrhagic events shortly after birth [4–6]. Bone marrow evaluation of newborns with CAMT usually reveals the absence of megakaryocytes, though accurate diagnosis may require analysis of a series of bone marrow aspirations [7–9]. Hematopoietic stem-cell transplantation (HSCT) is presently the only curative treatment for CAMT [10–13]. CAMT is caused by mutations in the gene encoding the thrombopoietin receptor, *MPL*, that compromise the activity of this receptor [4,14–16]. Mutations in *MPL* have been classified as being either type I or type II. The type I mutations cause a complete loss of receptor activity and the type II mutations allow for the maintenance of some receptor function. Patients bearing the type I mutations exhibit bone marrow failure earlier than those with the type II mutations [5,16,17]. Mortality associated with this disorder is significant and attributable to various causes including bleeding, leukemias and HSCT complications [1–3,5,8].

Thrombocytopenia is a relatively common clinical problem in neonates and can result from a variety of etiologies [18–21]. Differentiating between those with a genetic defect from those with an acquired form of thrombocytopenia is critical to the care of the affected individual. The identification of mutations in *MPL* that cause CAMT enables clinicians to rapidly determine whether a newborn exhibiting thrombocytopenia has CAMT.

As a result of historical founder effects, individuals of AJ descent are subject to a variety of genetic disorders [22,23]. This increased risk has resulted in the establishment of carrier screening programs designed to reduce the incidence of these diseases. These screening programs have benefited from the relatively homogeneous nature of the AJ community and the often limited number of causative mutations in this population [24–26]. A recent comprehensive carrier frequency study on individuals of AJ descent has suggested an expansion of the number of disorders that should be routinely tested for and has provided valuable carrier frequency information on 16 diseases prevalent in this population [27].

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The identification of a child of AJ descent with suspected CAMT prompted a study of the genetic cause of this disorder. We demonstrate the presence of a founder mutation in the *MPL* gene that is responsible for CAMT in a child born to parents of AJ descent. Screening of a random population of individuals of AJ descent reveals the carrier frequency of this mutation to be 1 in 75. The identification of this mutation and its prevalence in the AJ population strongly supports its inclusion in carrier screening programs provided to this population. Furthermore, the identification of this mutation will facilitate the diagnosis and treatment of CAMT in individuals of AJ descent.

Materials and methods

Subjects and population studied

A full-term male child of nonconsanguineous AJ parents was the fourth viable child born to a mother who had had five previous miscarriages. At birth, the child presented with a vascular lesion on his right leg. Laboratory data revealed thrombocytopenia with a platelet count of $<10\times10^9$/L and a normal number of megakaryocytes in a bone marrow aspirate. The child did not exhibit any physical anomalies. At 5 weeks of age, a cranial MRI revealed a right parieto-frontal hemorrhage, prompting a platelet transfusion. An initial increase in the platelet count to 108×10^9/L was observed, but 2 days later the platelet count was 25×10^9/L. Bone marrow examination performed at this time revealed a normal number of megakaryocytes. At 5 months, the child continued to exhibit thrombocytopenia (19×10^9/L) and a bone marrow aspirate examined at this time revealed an absence of megakaryocytes. The early onset of hypomegakaryocytic thrombocytopenia suggested a diagnosis of CAMT.

Blood samples were obtained from the affected child and his parents. Anonymous blood samples from individuals of AJ descent were obtained from the Dor Yeshorim screening program [25]. This work was performed with the approval of the Fordham University institutional review board (IRB). Genomic DNA was purified from blood using QIAamp DNA Blood Kits (Qiagen, Germantown, MD).

DNA sequencing

DNA sequencing was performed by fluorescent dye terminator detection on an ABI 3730xl Analyzer (GENEWIZ, South Plainfield, NJ).

PCR and sequencing of MPL exons and flanking intron boundaries

All *MPL* exons with their flanking intron boundaries were amplified from the DNA of the CAMT-affected child by PCR as described [4] and sequenced.

PCR detection of the c.79 +2T>A mutation

For detection of the c.79 + 2T>A-containing allele, genomic DNA was amplified by mutant allele-specific PCR (95 °C × 3 min, then 40 cycles of 94 °C × 30 s, 55 °C × 30 s, 72 °C × 30 s) using three primers in combination: a mutant allele-specific primer, MPL-mut (30 μM); a second primer, MPL-1 (10 μM), located in exon 1; and a third primer, MPL-2 (2.5 μM), located in the 3′ region of intron 1 (see Table 1 for primer sequences). A 140 bp PCR product was amplified by the MPL-mut/MPL-1 primers from the mutant allele containing the c.79 + 2T>A mutation and a 287 bp PCR product was amplified by the MPL-1/MPL-2 primers from both the mutant and wild-type alleles; thus, all DNA samples gave PCR products of 287 bp while those containing a mutant allele gave an additional PCR product of 140 bp. The PCR products were run on 2% agarose gels containing ethidium bromide and the PCR products were visualized and photographed under UV light. The presence of the mutation was verified by PCR amplification of a 287 bp fragment that included the mutation, using MPL-1 and MPL-2 primers (described above), followed by purification and DNA sequencing.

Table 1
PCR primers used.

MPL-1	5′-AGGGAGGATGGGCTAAGGCA-3′
MPL-2	5′-AGGAACAGCTGGTGAGTTGG-3′
MPL-6	5′-CACCATTTGGCATGGTTGTGG-3′
MPL-mut	5′-TCCACCCTCTGTGCAACTCT-3′
D1S211-F	5′-AGCTACATGGCAGGATCAGA-3′
D1S211-R	5′-GGATTCCTTGCTCTGGAAG-3′
D1S443-F	5′-AGCTGAGATCATGGCACT-3′
D1S443-R	5′-TCTTCCAGAGCAAAAGTTGA-3′
D1S447-F	5′-TAGTCTGAGTTTGTGGGGG-3′
D1S447-R	5′-GTTTTAACTTCATGGCTGCC-3′
D1S2861-F	5′-TAGAGTCAGATGCCTGGTGC-3′
D1S2861-R	5′-CAGCCTTGAACTCCACACAT-3′

Haplotype analysis

Single nucleotide polymorphism (SNP) characterization and microsatellite marker analysis of the region adjacent to the *MPL* gene were performed on the DNA of the proband, his parents and the identified carriers.

For the identification of unique SNPs present in the mutation-bearing allele, a 708 bp DNA fragment containing the c.79 + 2T>A mutation was amplified by PCR (95 °C × 3 min, then 40 cycles of 94 °C × 30 s, 55 °C × 30 s, 72 °C × 60 s) from the DNA isolated from the proband, using two primers: a mutant allele-specific primer, MPL-mut, located in the 5′ end of intron 1, and MPL-6, located approximately 600 nt upstream of the coding sequence. The resulting PCR product was sequenced and compared to the reported *MPL* sequence (Genbank Accession # U68159.1). Following the identification of two SNPs present on the mutant allele, DNA from the parents of the proband and those who had been determined to be carriers of the c.79 + 2T>A mutation was PCR-amplified as described above for the proband, using primers MPL-6 and mutant allele-specific MPL-mut, then the DNA fragments sequenced to determine if the mutant allele carried the same two SNPs. In addition, primers flanking the SNPs, MPL-6 and MPL-2, not mutant-specific, were used to amplify a fragment of DNA from both the normal and the mutant-bearing alleles, to determine the nucleotides present on the normal allele in the same position as the mutant allele-specific SNPs. The PCR products were purified and sequenced.

For the microsatellite analysis, amplification of the D1S211, D1S443, D1S447 and D1S2861 polymorphic microsatellite markers, all consisting of CA repeats, which are adjacent to *MPL*, was performed using the respective primers listed in Table 1. The forward "F" primer of each pair was labeled on the 5′ end with 6-FAM™ (Fluorescein) dye (IDT, Coralville, IA). PCR conditions used were 94 °C × 5 min, then 40 cycles of 94 °C × 20 s, 58 °C × 30 s, 72 °C × 30 s. The amplified products were run on an ABI 3730xl Analyzer at GENEWIZ (South Plainfield, NJ) and the results analyzed using Peak Scanner v1.0 software (Applied Biosystems, Foster City, CA). For each marker analyzed, the smallest PCR product obtained from among the 30 individuals' DNA was interpreted as having the least number of CA repeats; that size PCR product was assigned an arbitrary allele number of "1," not meant to imply the actual number of repeats, but the smallest relative number for a given marker within the group of 30. A PCR product that was, for example, two bases larger, i.e., had one additional CA repeat, would be given an allele number of "2" and so on.

Results

The suspected diagnosis of CAMT in a child with hypomegakaryocytic thrombocytopenia, as described in Materials and methods,

prompted an analysis of the DNA sequence of the *MPL* gene. For this analysis, exonic regions with their flanking intronic sequences were amplified and sequenced as described [4]. The homozygous presence of a T to A transversion was detected in the invariant second base of the intron 1 donor splice site (c.79 + 2T>A) in the child's *MPL* gene sequence and the parents' DNA was determined to be heterozygous for this mutation (Fig. 1).

As no reports of the presence of the c.79 + 2T>A mutation in individuals of AJ descent appear in the literature, we performed an analysis of the frequency of this mutation in this population. For this study, primers were designed which specifically amplify the mutation-bearing allele, and the incidence of this allele was determined in 2018 DNA samples collected from a random population of individuals of AJ descent, as described in Materials and methods. Twenty-seven individuals were determined to carry a c.79 + 2T>A allele in this assay. PCR amplification of a 287 bp fragment containing the intron 1 donor splice site was performed on the DNA of these 27 individuals and the products generated were sequenced, as described in Materials and methods. All were determined to be heterozygous for this mutation. The carrier frequency for this mutation was thus determined to be approximately 1 in 75.

To examine whether the presence of the mutation in this population is due to a founder effect, a 708 bp DNA fragment containing the c.79 + 2T>A mutation and sequence 5′ of the mutation was PCR-amplified from the DNA of the affected child, using a mutation-specific primer and a primer upstream of the transcription start site, as described in Materials and methods. Sequencing of this PCR product revealed that two single nucleotide polymorphisms (SNPs), c.-400C>A and c.-298G>A, were present in a homozygous state in the upstream region of *MPL* in the child's DNA that were not present in the reported gene sequence of *MPL* (Genbank Accession #U68159.1). Using the same two primers, PCR amplification and DNA sequencing were performed on the DNA isolated from the parents of this child and from the individuals determined to be carriers for the c.79 + 2T>A mutation. All of these individuals were found to carry the SNPs, c.-400C>A and c.-298G>A, on their mutant allele. Both the normal and mutant alleles of the identified carriers were then amplified using non-mutant allele-specific primers that flanked the SNPs and sequence analysis of these PCR products confirmed the presence of the c.-400C>A and c.-298G>A SNPs in all of the carriers of the c.79 + 2T>A mutation (Table 2).

To further investigate a possible founder effect, haplotype analysis was performed using four microsatellite markers, D1S211, D1S443, D1S447 and D1S2861, all consisting of dinucleotide CA repeats, located within 700 kb of the *MPL* gene. The child was found to be homozygous for the number of repeats at each of the four loci; microsatellite markers that are "linked" to a mutation would be expected to bear the same number of repeats for each of the markers if the affected child inherited the same allele from each parent. The parents and all other carriers were found to be either homozygous or heterozygous for allele size for the markers that correspond to the allele size present in a homozygous state in the affected child (Table 2). To determine the population frequency of the haplotype consisting of the six polymorphisms associated with the c.79 + 2T>A-bearing allele, genotyping of these six loci was performed on 500 random DNA samples from individuals of AJ descent whom we had determined were not carriers of the c.79 + 2T>A mutation. The DNA of only two individuals had all of the polymorphisms observed to exist on the c.79 + 2T>A-bearing allele. Without DNA samples from parents of these two individuals, we are unable to determine whether these polymorphisms are on the same allele. We therefore calculate that the presence of all six *MPL*-associated polymorphisms present on the c.79 + 2T>A-bearing allele is a rare event and occurs in at most 0.4% of individuals of the AJ descent who do not carry the c.79 + 2T>A mutation.

Table 2
The c.79 + 2T>A allele and its associated haplotype.

Individual	c.79 + 2T>A	c.-400C>A	c.-298G>A	D1S211	D1S443	D1S447	D1S2861
Affected child	**AA**	**AA**	**AA**	**11,11**	**2,2**	**2,2**	**2,2**
Mother	**A**T	**A**C	**A**G	3,**11**	**2**,3	**2**,4	**2**,3
Father	**A**T	**A**C	**A**G	1,**11**	**2**,1	**2**,5	**2**,4
Carrier 1	**A**T	**A**C	**A**G	1,**11**	**2**,3	**2**,4	**2**,4
Carrier 2	**A**T	**A**A	**A**A	10,**11**	**2**,1	**2**,1	**2**,3
Carrier 3	**A**T	**A**A	**A**A	10,**11**	**2**,1	**2**,1	**2**,3
Carrier 4	**A**T	**A**A	**A**A	10,**11**	**2**,3	**2**,1	**2**,3
Carrier 5	**A**T	**A**A	**A**A	1,**11**	**2**,3	**2**,4	**2**,1
Carrier 6	**A**T	**A**C	**A**G	1,**11**	**2**,3	**2**,4	**2**,3
Carrier 7	**A**T	**A**A	**A**A	1,**11**	**2**,4	**2**,5	**2**,3
Carrier 8	**A**T	**A**A	**A**A	10,**11**	**2**,1	**2**,1	**2**,3
Carrier 9	**A**T	**A**C	**A**G	10,**11**	**2**,1	**2**,1	**2**,3
Carrier 10	**A**T	**A**C	**A**G	9,**11**	**2**,3	**2**,1	**2**,4
Carrier 11	**A**T	**A**C	**A**G	9,**11**	**2**,3	**2**,1	**2**,4
Carrier 12	**A**T	**A**C	**A**G	10,**11**	**2**,3	**2**,4	**2**,4
Carrier 13	**A**T	**A**A	**A**G	10,**11**	**2**,4	**2**,1	**2**,1
Carrier 14	**A**T	**A**A	**A**A	10,**11**	**2**,1	**2**,1	**2**,3
Carrier 15	**A**T	**A**C	**A**G	3,**11**	**2**,3	**2**,4	**2**,3
Carrier 16	**A**T	**A**A	**A**A	3,**11**	**2**,3	**2**,4	**2**,3
Carrier 17	**A**T	**A**C	**A**G	2,**11**	**2**,1	**2**,1	**2**,3
Carrier 18	**A**T	**A**A	**A**A	2,**11**	**2**,1	**2**,4	**2**,3
Carrier 19	**A**T	**A**C	**A**G	10,**11**	**2**,1	**2**,1	**2**,3
Carrier 20	**A**T	**A**C	**A**G	10,**11**	**2**,3	**2**,1	**2**,1
Carrier 21	**A**T	**A**A	**A**A	3,**11**	**2**,3	**2**,1	**2**,3
Carrier 22	**A**T	**A**C	**A**G	10,**11**	**2**,3	**2**,1	**2**,3
Carrier 23	**A**T	**A**C	**A**G	3,**11**	**2**,3	**2**,4	**2**,3
Carrier 24	**A**T	**A**C	**A**G	10,**11**	**2**,1	**2**,3	**2**,1
Carrier 25	**A**T	**A**A	**A**A	3,**11**	**2**,1	**2**,4	**2**,3
Carrier 26	**A**T	**A**A	**A**A	10,**11**	**2**,4	**2**,1	**2**,4
Carrier 27	**A**T	**A**C	**A**G	1,**11**	**2**,1	**2**,4	**2**,1

A designation of "1" for the CA repeat markers is a relative number and represents the least number of CA repeats seen for a given marker in this cohort of 30 individuals. Bolded nucleotides and numbers represent variants present on the mutant allele.

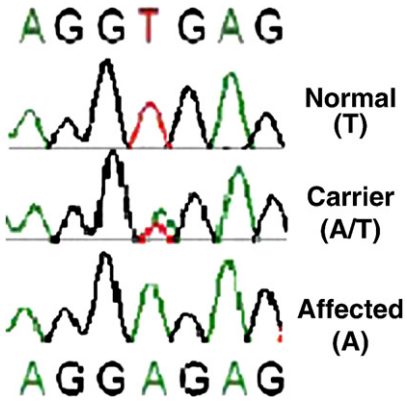

Fig. 1. Chromatograms showing sequencing of PCR products spanning the *MPL* intron 1 splice donor site. The product amplified from normal DNA contains a "T" in the 2nd position of this site, while a carrier contains a "T" and an "A." The product obtained from the DNA of an affected child is homozygous for an "A."

Discussion

Thrombocytopenia is a common clinical problem observed in neonates and can be due to placental insufficiency, a congenital infection or an autoimmune or alloimmune response [28–31]. CAMT, which is caused by the homozygous or compound heterozygous presence of mutations in the thrombopoietin receptor-encoding gene, *MPL*, is viewed as a rare cause of thrombocytopenia in a neonate. The infrequent diagnosis of CAMT has led some investigators to suggest that the incidence of this disease may be underestimated, as the thrombocytopenia can be easily misdiagnosed as idiopathic thrombocytopenic purpura, and the late onset of pancytopenia is indistinguishable from aplastic anemia [32]. The identification of mutations causing CAMT has facilitated the diagnosis of this disorder and the

recent commercial availability of clinically certified mutation analysis of *MPL* will further facilitate the diagnosis of CAMT (http://www.ncbi.nlm.nih.gov/sites/GeneTests/lab/clinical_disease_id/213279?db=genetests).

This study revealed that a child of AJ descent with thrombocytopenia and an absence of megakaryocytes in the bone marrow has a T to A transversion in the invariant second base of the intron 1 donor splice site of the *MPL* gene, a mutation termed c.79 + 2T>A. In a study performed by Germeshausen and colleagues [17], the authors describe a single patient with CAMT, homozygous for the c.79 + 2T>A mutation, who had a platelet count of 11×10^9/L at diagnosis and developed pancytopenia at 22 months, typical of the more severe CAMT, type I, phenotype [17]. This mutation is predicted to result in complete loss of function of the *MPL* gene product, the thrombopoietin receptor [17]. No information on the ethnicity of the patient was presented.

A recent report of individuals in the Israeli bone marrow donor registry identified a single Jewish individual with CAMT, in whom the homozygous causative mutation was determined to be a T to A transversion at c.1031 of the *MPL* cDNA sequence, which changes the 344th amino acid from a leucine to a glutamine [33]; the authors do not disclose whether this individual has the more severe Type I or less severe Type II phenotype. Screening for the c.79 + 2T>A mutation in a random group of 2018 AJ individuals revealed a carrier frequency of 0.0133, with a predicted incidence of CAMT in the AJ population of approximately 1 in 22,500 pregnancies. With an estimated population of approximately 3 million individuals of AJ descent living in Israel, the report of a single AJ individual with CAMT by the Israeli registry [33] is at odds with the carrier frequency of the c.79 + 2T>A mutation found in this study. Furthermore, the Israeli CAMT patient is homozygous for an unrelated mutation. The discordance between the observed carrier frequency of the c.79 + 2T>A mutation and the detection and reporting of CAMT by the Israeli bone marrow registry may reflect either the under-diagnosis of CAMT or the high incidence of fetal demise reported for couples who are carriers of mutations in *MPL* [5,14]. However, as the Israeli registry collected its data in hospitals that do not particularly serve orthodox Ashkenazi Jews, and as the carrier frequency analysis that we performed was done on samples primarily collected from the orthodox AJ population, it is possible that the lower rate of detection of individuals with CAMT by the Israeli registry reflects that this mutation is primarily present amongst the orthodox AJ population. The frequency of the c.79 + 2T>A mutation in the AJ population suggests that testing for this mutation should be considered in newborns of AJ descent presenting with thrombocytopenia.

The early identification of infants with this mutation will accelerate the search for a suitable hematopoietic stem cell donor. In AJ couples with a history of multiple miscarriages, testing for this mutation may shed light on the cause of the fetal demise. Furthermore, the identification of the c.79 + 2T>A mutation in the AJ population will enable the early diagnosis and effective clinical management of individuals determined to be homozygous for this mutation.

The homozygous presence of this mutation in the proband and its heterozygous presence in 27 identified carriers, all of whom share the same haplotype in the region adjacent to the *MPL* gene, identify the c.79 + 2T>A mutation as the first founder mutation in *MPL* in the AJ population. In light of its high frequency in this population and the significant mortality associated with CAMT disease, its inclusion in routine carrier testing should be considered.

Authorship

Dr. Berish Rubin was involved in the conception and coordination of the project, analyzed data and contributed to the writing of the manuscript. Chaim Jalas was involved in the conception of the project and DNA sequence analysis. Dr. Sylvia Anderson was involved in the coordination of the project, analyzed data and contributed to the writing of the manuscript. Tova Laufer and Kristina Martimucci performed the mutation analysis, Alex Bulanov and Xie Xie performed the haplotyping analysis and Josef Ekstein provided the blood samples from anonymous Ashkenazi Jewish individuals.

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