Published in final edited form as:

Exp Hematol. 2009 April ; 37(4): 495–503. doi:10.1016/j.exphem.2009.01.001.

Compound heterozygous c-Mpl mutations in a child with congenital amegakaryocytic thrombocytopenia: Functional characterization and a review of the literature

Norma E. Fox¹, Rose Chen¹, Ian Hitchcock¹, Jennifer Keates-Baleeiro², Haydar Frangoul², and Amy E. Geddis^{1,*}

¹ University of California, San Diego, CA

² Vanderbilt University, Nashville, TN

Abstract

Objective—To genetically and functionally characterize mutations of c-Mpl, that lead to thrombocytopenia in a child with congenital amegakaryocytic thrombocytopenia (CAMT).

Methods—We identified two c-Mpl mutations in a child with clinical features of CAMT, one a previously described mutation in the extracellular domain (R102P) and the other a novel mutation leading to truncation of the receptor after the box 1 homology domain (541Stop). Cell line models were created to examine the ability of the mutant receptors to signal in response to thrombopoietin and thrombopoietin-like agonists.

Results—Data from cell line models indicate that c-Mpl R102P does not support significant signaling in response to thrombopoietin due to impaired trafficking of the mutant receptor to the cell surface. Alternative thrombopoietic agents do not circumvent this block to signaling, likely due to the inaccessibility of the receptor. In addition, previous data indicate that c-Mpl 541Stop does not support intracellular signaling due to the loss of critical intracellular domains.

Conclusions—This case demonstrates two different mechanisms by which c-Mpl mutations can impair thrombopoietin signaling, and suggests that mutations in the extracellular domain will not be rescued by c-Mpl agonists if they interfere with normal receptor expression.

Keywords

congenital amegakaryocytic thrombocytopenia; thrombopoietin; c-Mpl; glycosylation

Introduction

CAMT is an inherited bone marrow failure syndrome caused by mutations in the receptor for thrombopoietin, c-Mpl [1,2]. Affected children typically present with thrombocytopenia at birth, with bone marrow analysis revealing severely reduced or absent megakaryocytes. Within the first decade of life, isolated thrombocytopenia progresses to pancytopenia due to trilineage bone marrow failure, and most children require treatment by stem cell transplantation.

*To whom correspondence should be addressed: Amy E. Geddis MD PhD, Assistant Professor of Pediatrics, UCSD, 9500 Gilman Drive Mailcode 0726, La Jolla, CA 92017, Ph: (858) 822-3345, Fax: (858) 822-3344, Email: E-mail: ageddis@ucsd.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Mutations have been described throughout the c-Mpl receptor, although mutations in exons 2 and 3 are the most frequent [3]. Mutations of c-Mpl have been classified as either type I, in which the receptor has lost all activity, or type II, in which the receptor retains some degree of function [1]. Clinically, type II patients have a slightly delayed onset of bone marrow failure (mean age 48 mo) compared to type I patients (22 mo) [3].

Here we describe a girl with CAMT who presented with thrombocytopenia and mild dysmegakaryopoiesis and developed pancytopenia before 2 years of age. Sequencing revealed compound heterozygosity for a previously described type II mutation in the extracellular portion of the receptor (arginine to proline substitution at amino acid 102, R102P) as well as a novel type I mutation resulting in truncation of the intracellular portion shortly after the box 1 homology domain (541Stop). Although R102P has been reported in association with type II disease [1,3], when expressed in BaF3 cells c-Mpl bearing this substitution did not support proliferation in response to thrombopoietin or a thrombopoietin mimetic, most likely because the receptor is poorly expressed at the cell surface. 541Stop results in a receptor that lacks all but the most proximal 28 amino acids of the intracellular signaling domain, including box 2 and critical distal phosphotyrosine motifs. Previous studies in BaF3 cells demonstrated that while expressed on the surface, c-Mpl truncated at this position also does not support growth in response to thrombopoietin [4]. This case demonstrates the variation that can be seen in the clinical presentation CAMT in the newborn period and the importance of genetic testing for accurate diagnosis; in addition it further illustrates the variety of mechanisms by which c-Mpl mutations can impair thrombopoietin signaling.

Methods

Patient characteristics and human subjects

The proband is a Caucasian female, 2 yr of age at the time of this investigation. All procedures described have been reviewed and approved by the Institutional Review Board of the University of California, San Diego. After getting appropriate consents from the parents of the child, we obtained EDTA-anticoagulated peripheral blood from the child, mother and father.

Plasma thrombopoietin levels and DNA preparation

Whole anti-coagulated blood was centrifuged and platelet-rich plasma and buffy coat fractions were collected. The platelet rich plasma was aliquoted into microfuge tubes and centrifuged at top speed to obtain platelet-poor plasma, which was assayed for thrombopoietin (Quantikine Human TPO ELISA, R&D, Minneapolis, MN). Genomic DNA was isolated from the buffy coat fractions (QIAamp, Qiagen, Valencia, CA). Coding regions as well as intron/exon junctions for all 12 exons of c-Mpl were amplified and products were sequenced using Big Dye terminator reactions through the DNA sequencing core facility at UCSD. Primers used for sequencing are available on request.

Materials and DNA constructs

The cDNA for human c-Mpl was cloned into pMx-Puro (the gift of Toshio Kitamura [5]); R102P and R102K mutations were introduced into the human c-Mpl cDNA using Quikchange XL-Sited Directed Mutagenesis kit (Stratagene, Cedar Creek, TX) according to the manufacturer's protocol and verified by DNA sequencing. Recombinant human thrombopoietin (rhTPO) was the gift of Zymogenetics (Seattle, WA). m-AMP4 was a gift of Amgen (Thousand Oaks, CA). Compound LGD4665 was a gift of Ligand pharmaceuticals (La Jolla, CA).

Cell lines

BaF3 cells are an IL-3 dependent murine lymphoblastic cell line that does not express c-Mpl. Cells were maintained in RPMI (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS), penicillin, streptomycin, glycine, and 2 μ l/ml murine IL-3 supernatant purified from transfected baby hamster kidney cells. WT, R102P and R102K c-Mpl were inserted into the vector pMx-puro and introduced into BaF3 cells by retroviral transduction; expressing cells were selected with puromycin and clonal cell lines were derived by single cell dilution. All clones were assayed for c-Mpl expression by western blotting, using polyclonal rabbit anti-Mpl catalog number 06-944, Upstate (now Millipore, Billerica, MA). BaF3 cells expressing murine c-Mpl truncated after box 1 (T-28) have been reported previously [4].

Assays for response to rhTPO and thrombopoietin mimetics

MTT assays—Parental BaF3 cells and BaF3 cells expressing WT or mutant c-Mpl were washed twice with PBS to remove IL-3, then transferred to RPMI containing 2% fetal bovine serum and 0.5ng/ml and 5 ng/ml of rhTPO, 3 μ M and 6 μ M LGD4665, or 50 ng/ml m-AMP4. Thrombopoietin responsiveness was defined as dose-dependent cell growth at 48 hr and measured by MTT assay as previously described [4]. Cell growth in optimal concentrations of IL-3 was simultaneously determined for comparison.

Phosphorylation of Erk and Akt—To detect activation of c-Mpl by rhTPO, BaF3 cells expressing WT or mutant c-Mpl were washed twice, starved of serum and cytokines overnight in RPMI containing 0.5% BSA, then stimulated for 15 min with 50 ng/ml rhTPO. Cells were lysed and analyzed by western blotting, using anti-phospho-p42/p44 Erk and anti-phospho-S473Akt antibodies (Cell Signaling Technologies, Beverly, MA).

Thrombopoietin uptake assay

To measure the ability of WT or mutant c-Mpl to bind thrombopoietin, we determined if cells bearing WT or mutant receptors could effectively remove rhTPO from the cell culture medium. Wells were set up containing 0 or 2×10^7 BaF3 cells expressing WT or mutant c-Mpl and incubated at 37°C in culture medium containing 0.75 ng/ml rhTPO. As a control for non-specific uptake or degradation, additional wells were prepared containing parental BaF3 cells. After 30 min, samples were placed on ice to stop uptake and then cells were removed by centrifugation and the amount of rhTPO remaining in the medium was determined using TPO ELISA (Quantikine for human TPO, R&D, Minneapolis, MN).

Total and surface expression of c-Mpl

Western blotting: BaF3 cells expressing WT or mutant c-Mpl and growing in IL-3 were lysed in NP-40 buffer (50mM tris Hcl PH 7.4, 150mM NaCl, 1 mM EDTA, 1% NP-40) containing protease and phosphatase inhibitors (Complete Protease Inhibitor (Roche, Basel, Switzerland), 2 mM Na_3VO_4 , 5 mM NaF) according to standard protocols. Protein concentrations were determined using the Protein/DC assay (Bio-Rad, Hercules, CA). For each sample, 25 μ g of total protein was denatured by boiling for 10 min in loading buffer containing 50 μ l/ml β -mercaptoethanol and separated on a 4–15% gradient polyacrylamide gel (Bio-Rad). Proteins were electrophoretically transferred to a PDVF membrane (Bio-Rad) which was then blocked for 1 h at room temperature with Tris-buffered saline (pH 8.0) containing 0.1% Tween-20 (TBST) and 5% nonfat dry milk. The membrane was incubated overnight at 4 °C in TBST with 5% milk and anti-c-Mpl antibody (1:10 000, Upstate, now Millipore, Billerica, MA). After washing, the membrane was incubated in TBST with 5% milk containing a 1:5000 dilution of secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit, Santa Cruz Biotechnologies, Santa Cruz, CA). Secondary detection was performed with ECL-plus (Amersham, Piscataway, NJ) and blots were exposed to autoradiographic film.

Determination of receptor half-life: BaF3 cells expressing WT or mutant c-Mpl and growing in IL-3 were incubated with 2 µg/ml cycloheximide (Sigma, St. Louis, MO) to block new protein synthesis. Cells were lysed at time 0, 10 min, 30 min, 60 min or 90 min thereafter, and the residual amount of c-Mpl compared to that at time 0 was determined by western blotting as above followed by densitometric analysis of scanned films using ImageJ (<http://rsb.info.nih.gov/ij/>).

Flow cytometry: Parental BaF3 cells and BaF3 cells expressing WT or mutant c-Mpl growing in IL-3 were fixed in phosphate buffered saline (PBS) containing 2% paraformaldehyde for 10 min. Cells were washed in PBS/0.5% bovine serum albumin (BSA) then incubated with a 1:100 dilution of rabbit polyclonal antibody specific for the extracellular domain of c-Mpl (gift from Amgen) in PBS/0.5% BSA for 30 min at 4°C. After washing, cells were incubated with secondary antibody conjugated to PE (goat anti-rabbit, Molecular Probes) for 30 min at 4°C, and relative fluorescence was assayed by flow cytometry using CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Background antibody staining was taken as that present in parental BaF3 cells. Relative surface expression was measured as the difference in geometric mean intensity of fluorescence compared to that in parental BaF3 cells. At least 3 clones with varied levels of total receptor expression were analyzed for each WT and R102P c-Mpl.

Results

Case presentation

The index case is a 2 yr old girl who presented at birth with petechiae, intracranial hemorrhage and severe normocytic thrombocytopenia (17,000/µl). Neither parent had abnormal blood counts. Initial bone marrow performed at 3 mo of age showed trilineage hematopoiesis with immature appearing, hypolobulated megakaryocytes that are mildly reduced in number and an increased population of hematogones (Figure 1). The morphology of the other cell lineages was normal. Thrombocytopenia did not respond to an initial transfusion of random donor platelets but maternal platelets were effective in raising the platelet count. A presumptive diagnosis of alloimmune thrombocytopenia was made based on platelet antigen testing that revealed a parental mismatch of human platelet antigen 1 (HPA1). However the persistence of transfusion requirements and the development of neutropenia and anemia starting at 14 and 19 mo of age, respectively, prompted a repeat of the marrow examination and analysis for CAMT. Repeat marrows revealed development of amegakaryocytosis and trilineage aplasia.

Mutation analysis

Plasma thrombopoietin levels were not elevated in either parent, but in the child were 2000 (normal values below 80) pg/ml, levels typical for the autosomal recessive deficiency of c-Mpl seen in CAMT. Genotyping confirmed that the child had inherited a single base pair mutation (G305>C) in exon 3 from her father resulting in the substitution of proline for arginine at amino acid 102 (R102P) in the extracellular domain of c-Mpl (Figure 2). This mutation has been previously described in several unrelated families and when homozygous is often associated with type II disease (see Table 1). In addition, the child had inherited a second single base pair mutation (C1621>T) in exon 11 from her mother, which creates a stop codon following amino acid 540 within the intracellular domain of the receptor (Figure 2). Mutations in exon 11 have not been previously described in patients with CAMT. All mutations were confirmed in both sense and antisense sequencing from the child and her parents.

Cells expressing R102P c-Mpl do not proliferate in response to rhTPO or thrombopoietin mimetics

Since the R102P c-Mpl mutation is associated with type II disease, we anticipated that it would support some level of residual proliferation in a cell line model of thrombopoietin signaling.

We generated a mutation in human WT c-Mpl to create R102P c-Mpl and introduced each of the constructs into BaF3 cells, which do not otherwise express c-Mpl and are dependent on IL-3 for survival and proliferation. We selected transfected cells using puromycin and derived clonal lines that expressed similar levels of c-Mpl, as measured by western blotting. To compare signaling by WT and mutant receptors, we washed cells of serum and IL-3, then cultured them with two doses of rhTPO for 48 h and compared cell growth in response to rhTPO by MTT assay. Although rhTPO stimulated growth as expected in cells expressing WT receptor, it failed to support detectable cell growth in more than 10 independent clonal cell lines expressing R102P c-Mpl (Figure 3a). Because the substitution R102P affects a residue within the extracellular domain and therefore may alter the ability of the receptor to bind native thrombopoietin, we hypothesized that we might be able to circumvent this defect by using a thrombopoietin mimetic that binds the receptor at an alternate site [6]. We obtained a small molecule (LGD4665) derived from a screen for compounds with thrombopoietic activity that binds to c-Mpl within the transmembrane domain and promotes signaling and growth similar to that seen with thrombopoietin. However, although LGD4665 stimulated robust growth in cells expressing WT c-Mpl, this compound did not stimulate measurable growth in cell lines expressing R102P (Figure 3a). This lack of activity could not be overcome with increased doses of LGD4665 (not shown). Similar results were obtained with a second thrombopoietic compound (m-AMP4), a peptide that binds to the extracellular domain of c-Mpl but does not have a structural resemblance to thrombopoietin (not shown).

To further evaluate the function of the mutant receptor, we starved BaF3 cells expressing WT or R102P c-Mpl of serum and IL-3 overnight, and then stimulated them with rhTPO for 15 min and asked if we could detect phosphorylation and activation of Erk and Akt. As expected, in cells expressing WT receptor rhTPO stimulation resulted in phosphorylation and activation of Erk and Akt as measured by immunoblotting. However, consistent with their lack of growth, two independent clonal cell lines expressing R102P c-Mpl did not show detectable activation of Erk or Akt in response to rhTPO (Figure 3b), confirming severely impaired c-Mpl signaling.

Substitution of arginine with proline is potentially a very disruptive amino acid change and could be envisioned to significantly affect protein folding. A second substitution, R102C, has been reported at this residue in an individual with CAMT (see Table 1). Introduction of cysteine is also likely to be quite disruptive to receptor structure and we were interested in testing a more conservative substitution at this site. In order to determine if the identity of the amino acid at position 102 must be arginine for normal receptor function or if only the introduction of a disruptive change at this site results in the loss of activity, we made the more conservative c-Mpl mutation, R102K. We found that as assessed by MTT, BaF3 cells expressing R102K c-Mpl responded to rhTPO similarly to those expressing the WT receptor (Figure 4). Therefore we presume that structural changes imposed by substitution of proline at this site are responsible for the mutant receptor's impaired responsiveness to thrombopoietin.

R102P c-Mpl does not bind thrombopoietin as efficiently as WT c-Mpl

Because rhTPO did not stimulate cells expressing R102P c-Mpl, we asked if the mutant receptor was capable of binding thrombopoietin. When thrombopoietin binds to cells expressing normal c-Mpl, it is internalized with the receptor through the process of endocytosis [7,8] and thrombopoietin is removed from the plasma. We used this phenomenon to compare the binding of rhTPO to WT or mutant c-Mpl in cultured cells by measuring the depletion of rhTPO from the medium. Equal numbers of parental BaF3 cells, BaF3 cells expressing WT receptor or 2 clones of BaF3 cells expressing c-Mpl R102P were cultured with media containing 0.75 ng/ml of rhTPO, and rhTPO levels were measured using an ELISA assay both prior to the addition of cells and following 30 min incubation at 37°C. We found that compared to BaF3 cells expressing WT receptor, which effectively removed rhTPO from the media, cells

expressing R102P c-Mpl did not bind any more rhTPO than did parental BaF3 cells (Figure 5, mean and SEM of 3 experiments). Although these measurements are relative, this result suggests that the mutant receptor is unable to efficiently bind thrombopoietin.

R102P c-Mpl is poorly glycosylated compared to WT c-Mpl and is not expressed on the cell surface

Given that R102P c-Mpl is a type II mutation creating a substitution within the extracellular domain of the receptor, we were surprised that we could not detect any proliferative response to either rhTPO or thrombopoietin mimetics that might be able to activate the intact intracellular domain. As the mutant receptor does not appear to bind thrombopoietin, we hypothesized that R102P c-Mpl might make a receptor that is either unstable or not expressed on the cell surface. Of interest, although two forms of the WT c-Mpl are routinely detected on western blotting, a higher molecular weight form that represents mature glycosylated receptor and a lower molecular weight form that represents immature receptor [9], only the smaller immature form was detected in cells expressing R102P c-Mpl (Figure 6a). Receptor glycosylation was restored in cells expressing R102K. This suggested to us that improper folding, perhaps induced by the introduction of a proline residue, was disrupting transit and glycosylation of the receptor within the golgi apparatus. To measure receptor stability, we treated BaF3 cells expressing WT and R102P c-Mpl and growing in IL-3 with cycloheximide to block new protein synthesis and measured c-Mpl half-life. We found that, comparing the immature form which is present in both cell types, the half-life of the mutant receptor was similar to that of WT c-Mpl (data not shown), and therefore the substitution of arginine to proline does not appear to markedly alter the stability of the protein. However, using an antibody to the extracellular domain of c-Mpl and flow cytometric analysis, we found that, in comparison to cells expressing WT c-Mpl in which receptor is easily detected on the cell surface, cells expressing R102P c-Mpl did not express surface receptor that was detectable above the level of parental BaF3 cells (Figure 6b). This finding was consistent in several independent clones with varying levels of total c-Mpl expression by western blotting. Consistent with our findings that BaF3 R102K c-Mpl cells grow in rhTPO and express the mature glycosylated form of the receptor by immunoblotting, this mutant is also normally expressed on the cell surface. Therefore, poor surface expression of R102P c-Mpl likely accounts for the fact that cells expressing this mutant receptor do not bind thrombopoietin and fail to grow in either rhTPO or two different thrombopoietic compounds.

541Stop is similar to the mutation T-28 previously described in murine c-Mpl

The 541Stop mutation results in a receptor that is analogous to a structural variant of murine c-Mpl generated to identify domains of the c-Mpl receptor that are critical for its ability to signal [4]. This mutant, termed T-28 in this work because it retains the most proximal 28 amino acid residues of the intracellular domain, is truncated after the box 1 homology domain and does not show any detectable signaling in response to thrombopoietin, as measured by MTT assay and by Jak2 and Stat5 phosphorylation [4]. We conclude, since 541Stop results in the truncation of human c-Mpl immediately after box 1 analogous to T-28, that this mutation also creates an inactive receptor.

Discussion

Mutations of c-Mpl have been classified as either type I mutations, in which the receptor has lost all activity, or type II mutations, in which the receptor retains some degree of function. Clinically, type II patients have a delayed onset of bone marrow failure compared to type I patients. Here we describe a girl with CAMT who had a clinical course similar to patients with type I mutations but without a clear absence of megakaryocytes at birth. Another recent report echoes our conclusion that amegakaryocytosis may not be present at birth and CAMT should

be suspected in the newborn with unexplained persistent thrombocytopenia [10]. Although we do not know the precise mechanism of thrombocytopenia in newborns with CAMT who present with thrombocytopenia that is out of proportion to the demonstrable degree of amegakaryocytosis, we speculate that this could represent a transient persistence of neonatal megakaryocytes, which are thought to be less mature and associated with lower platelet production [11].

In our case, sequencing of c-Mpl revealed two heterozygous mutations, one creating an arginine to proline substitution at amino acid 102 in the extracellular domain and the other one resulting in a stop codon at amino acid 541 in the intracellular domain. At least types of receptor are predicted: R102P c-Mpl homodimers (which have limited surface expression based on our findings) and 541Stop c-Mpl homodimers (which are not be able to signal due to truncation of key intracellular residues). It is a limitation of this work that we cannot determine if heterodimeric receptors are formed, secreted or capable of any signaling.

R102P is the most commonly occurring c-Mpl mutation reported in CAMT and is generally associated with type II disease (see Table 1), although in our cell line model, c-Mpl bearing this mutation was not expressed on the cell surface and showed no detectable ability to signal in response to rhTPO. These results are somewhat in contrast to those of Germeshausen and colleagues, who found that CD34+ cells from patients with type II disease cultured with a cocktail of stem cell factor, IL-3, IL-6 and TPO produced more megakaryocyte colonies than those from patients with type I disease [12]. It is possible that in the marrow environment or in conjunction with additional cytokines an otherwise undetectable amount of the mutant receptor does reach the cell surface and provides a minimum level of signaling, as would be predicted given the type II phenotype found in patients expressing homozygous R102P c-Mpl. Of note, because R102P c-Mpl is poorly secreted, the use of a thrombopoietin mimetic that might otherwise be anticipated to circumvent an extracellular domain mutation was unable to effectively rescue these cells in our assay. Although other mutations within the extracellular domain might be able to be stimulated by a thrombopoietin mimetic, our findings suggest that the potential utility of these new therapeutic agents in patients with CAMT will be limited.

While this manuscript was in preparation, work was published that also addressed the effect of the mutation R102P on c-Mpl signaling in cell lines [13]. In confirmation of their findings, we also demonstrated that the mutant receptor is only present as the smaller immature form and does not appear to be appropriately glycosylated, and in addition we showed that the mutant receptor is not present on the cell surface as a potential mechanism by which it fails to signal normally and that surface expression can be rescued by a more conservative mutation at this site. Whereas both groups were able to show that a thrombopoietin mimetic that binds the extracellular domain of the receptor is not able to rescue signaling from this mutant receptor, we also showed that the mutant receptor does not respond significantly to a different class of thrombopoietin mimetic that binds within the transmembrane domain. However, in contrast to their results and those of others, we did not detect significant signaling from R102P c-Mpl in response to thrombopoietin by western blot (looking at Erk and Akt) or cell proliferation assays. It is possible that these differences arise in part from the cellular models used, as we studied the receptor in the background of the growth factor-dependent lymphoblastic cell line BaF3 whereas they used K562 cells.

In contrast to R102P, 541Stop has not been previously reported and is the first c-Mpl mutation located in exon 11 (Table 1). This novel mutation is predicted to result in the truncation of the receptor shortly after the box 1 homology domain. Previous cell line studies involving the murine homolog of this mutation, which is missing all but the proximal 28 amino acids of the intracellular domain, have shown that it does not signal in response to thrombopoietin [4]. In conclusion, this case illustrates the variation in clinical phenotype that can be seen in CAMT,

describes a novel mutation in c-Mpl, and elucidates the mechanism for the lack of function in the most commonly occurring c-Mpl mutation.

Acknowledgments

The authors would like to thank Dr. Kenneth Kaushansky for critical review of the manuscript and Allison Reinhardt for administrative support. This work was supported in part with funds provided by NIH grants K08 HL44706 and R01 DK049855-15.

References

1. Ballmaier M, Germeshausen M, Schulze H, Cherkaoui K, Lang S, Gaudig A, Krukemeier S, Eilers M, Strauss G, Welte K. c-mpl mutations are the cause of congenital amegakaryocytic thrombocytopenia. *Blood* 2001;97:139–46. [PubMed: 11133753]
2. Ihara K, Ishii E, Eguchi M, Takada H, Suminoe A, Good RA, Hara T. Identification of mutations in the c-mpl gene in congenital amegakaryocytic thrombocytopenia. *Proc Natl Acad Sci U S A* 1999;96:3132–36. [PubMed: 10077649]
3. King S, Germeshausen M, Strauss G, Welte K, Ballmaier M. Congenital amegakaryocytic thrombocytopenia: a retrospective clinical analysis of 20 patients. *Br J Haematol* 2005;131:636–44. [PubMed: 16351641]
4. Drachman JG, Kaushansky K. Dissecting the thrombopoietin receptor: functional elements of the Mpl cytoplasmic domain. *Proc Natl Acad Sci U S A* 1997;94:2350–55. [PubMed: 9122198]
5. Onishi M, Mui AL, Morikawa Y, Cho L, Kinoshita S, Nolan GP, Gorman DM, Miyajima A, Kitamura T. Identification of an oncogenic form of the thrombopoietin receptor MPL using retrovirus-mediated gene transfer. *Blood* 1996;88:1399–406. [PubMed: 8695859]
6. Kuter DJ. New thrombopoietic growth factors. *Blood* 2007;109:4607–16. [PubMed: 17289815]
7. Yang C, Li YC, Kuter DJ. The physiological response of thrombopoietin (c-Mpl ligand) to thrombocytopenia in the rat. *Br J Haematol* 1999;105:478–85. [PubMed: 10233424]
8. Hitchcock IS, Chen MM, King JR, Kaushansky K. YRRL motifs in the cytoplasmic domain of the thrombopoietin receptor regulate receptor internalization and degradation. *Blood* 2008;112:2222–31. [PubMed: 18487512]
9. Moliterno AR, Spivak JL. Posttranslational processing of the thrombopoietin receptor is impaired in polycythemia vera. *Blood* 1999;94:2555–61. [PubMed: 10515857]
10. Rose MJ, Nicol KK, Skeens MA, Gross TG, Kerlin BA. Congenital amegakaryocytic thrombocytopenia: The diagnostic importance of combining pathology with molecular genetics. *Pediatr Blood Cancer* 2008;50:1263–65. [PubMed: 18240171]
11. Pastos KM, Slayton WB, Rimsza LM, Young L, Sola-Visner MC. Differential effects of recombinant thrombopoietin and bone marrow stromal-conditioned media on neonatal versus adult megakaryocytes. *Blood* 2006;108:3360–62. [PubMed: 16888093]
12. Germeshausen M, Ballmaier M, Welte K. MPL mutations in 23 patients suffering from congenital amegakaryocytic thrombocytopenia: the type of mutation predicts the course of the disease. *Hum Mutat* 2006;27:296. [PubMed: 16470591]
13. Tijssen MR, di Summa F, van den Oudenrijn S, Zwaginga JJ, van der Schoot CE, Voermans C, de Haas M. Functional analysis of single amino-acid mutations in the thrombopoietin-receptor Mpl underlying congenital amegakaryocytic thrombocytopenia. *Br J Haematol* 2008;141:808–13. [PubMed: 18422784]
14. Steinberg O, Gilad G, Dgany O, Krasnov T, Zoldan M, Laor R, Kapelushnik J, Gabriel H, Churi C, Stein J, Yaniv I, Tamary H. Congenital amegakaryocytic thrombocytopenia-3 novel c-MPL mutations and their phenotypic correlations. *J Pediatr Hematol Oncol* 2007;29:822–25. [PubMed: 18090929]
15. Savoia A, Dufour C, Locatelli F, Noris P, Ambaglio C, Rosti V, Zecca M, Ferrari S, di Bari F, Corcione A, Di Stazio M, Seri M, Balduini CL. Congenital amegakaryocytic thrombocytopenia: clinical and biological consequences of five novel mutations. *Haematologica* 2007;92:1186–93. [PubMed: 17666371]

16. van den Oudenrijn S, Bruin M, Folman CC, Peters M, Faulkner LB, de Haas M, von dem Borne AE. Mutations in the thrombopoietin receptor, Mpl, in children with congenital amegakaryocytic thrombocytopenia. *Br J Haematol* 2000;110:441–48. [PubMed: 10971406]
17. Gandhi MJ, Pendergrass TW, Cummings CC, Ihara K, Blau CA, Drachman JG. Congenital amegakaryocytic thrombocytopenia in three siblings: molecular analysis of atypical clinical presentation. *Exp Hematol* 2005;33:1215–21. [PubMed: 16219544]
18. Muraoka K, Ishii E, Ihara K, Imayoshi M, Miyazaki S, Hara T, Hamasaki Y. Successful bone marrow transplantation in a patient with c-mpl-mutated congenital amegakaryocytic thrombocytopenia from a carrier donor. *Pediatr Transplant* 2005;9:101–3. [PubMed: 15667621]
19. Passos-Coelho JL, Sebastiao M, Gameiro P, Reichert A, Vieira L, Ferreira I, Miranda N, Guimaraes A, Leal-da-Costa F, Abecasis MM. Congenital amegakaryocytic thrombocytopenia--report of a new c-mpl gene missense mutation. *Am J Hematol* 2007;82:240–41. [PubMed: 17034029]

Figure 1.

Progression of bone marrow failure in a child with CAMT. (A) Bone marrow aspiration performed at 3 months of age shows 2 megakaryocytes in a cellular particle with background hematopoiesis including erythroid and myeloid precursors without dysplasia. (B) Bone marrow aspirate 20 months later shows a hypocellular particle with no megakaryocytes and few scattered lymphocytes and histiocytes.

Figure 2.

Compound heterozygosity for mutations in exons 3 and 11 of c-Mpl. Analysis of the sequence from the proband reveals a G305>C mutation in exon 3 (confirmed to be derived from the father), which results in the amino acid substitution R102P. The second mutation, C1621>T, creates a stop codon in exon 11 and is derived from the mother.

Figure 3.

BaF3 cells expressing R102P c-Mpl do not respond to TPO or a thrombopoietin mimetic. (A) Fourteen independent clones of BaF3 cells expressing R102P c-Mpl were washed of IL-3 and tested for growth in maximal IL-3, 5 ng/ml rhTPO, and 3 μ M LGD4665. After 48 h, cells were lysed and an MTT assay was performed. Results are expressed relative to maximal growth in IL-3. Points were performed in triplicate. Error bars represent SEM. Growth of control cells expressing WT c-Mpl under the same conditions is shown for comparison. (B) BaF3 cells expressing WT c-Mpl or 2 representative clones of BaF3 cells expressing R102P c-Mpl were starved overnight and then stimulated with 50 ng/ml rhTPO for 15 min, lysed and assayed for phosphorylation of Akt and Erk by immunoblotting. Blots were reprobed for actin as a loading control. Blot shown is representative of 2 independent experiments.

Figure 4.

R102K c-Mpl is a conservative change that does not significantly impair the function of the receptor. Three independent clones of BaF3 cells expressing R102K c-Mpl were starved of IL-3 overnight and stimulated with increasing concentrations of rhTPO and growth was analyzed by MTT at 48 hr. Results are expressed as relative to maximal growth in IL-3. Growth of cells expressing WT c-Mpl (leftmost grouping) or R102P c-Mpl (rightmost grouping) under the same conditions is shown for comparison. Points were performed in triplicate. Graph shown is representative of 2 independent experiments.

Figure 5.

R102P c-Mpl does not bind TPO as efficiently as WT c-Mpl. To compare the ability of mutant and WT receptors to bind TPO, we incubated BaF3 parental cells, BaF3 cells expressing WT c-Mpl and 2 clones of BaF3 cells expressing R102P c-Mpl in medium containing fixed amounts of rhTPO and measured the ability of the cells to deplete the cytokine from the culture medium. 2×10^7 cells were washed of IL-3 and cultured with 0.75 ng/ml rhTPO at 37°C for 30 min. TPO levels were measured in duplicate and are expressed as percentage of input TPO remaining as compared to parental BaF3 cells (which should have no specific uptake). Uptake of rhTPO by cells expressing WT c-Mpl is significantly more than that of parental cells ($p=0.0012$), but there is no significant difference in rhTPO uptake between parental cells and cells expressing R102P c-Mpl. Results shown are the mean of three experiments.

Figure 6.

R102P c-Mpl is not normally glycosylated and is poorly expressed on the cell surface. (A) On western blotting, WT c-Mpl is usually seen as two bands: a more slowly migrating glycosylated protein that represents mature receptor (85 kD), and a more rapidly migrating, immature form (80 kD). Cells expressing R102P c-Mpl only produce the 80 kD, immature form of the receptor, whereas cells expressing R102K c-Mpl produce both forms of the receptor. (B) BaF3 cells expressing WT c-Mpl, R102P c-Mpl or R102K c-Mpl (as proven by western blotting) were incubated with an antibody that is specific for the extracellular domain of the receptor and analyzed by flow cytometry. Compared to the WT control, surface receptor is not detectable in cells expressing R102P c-Mpl but is restored in cells expressing R102K c-Mpl. Parental BaF3 cells serve as the negative control for nonspecific antibody binding.

Table 1

Summary of reported mutations in CAMT. A literature search of Pubmed revealed 9 reports in which specific mutations were identified from patients. Information is drawn from the respective publications. Mutations are listed in order from the extracellular to the intracellular domain along with the predicted effect on the protein and clinical phenotype.

Exon	Effect	Type I or II (predicted)	Reference
E1	Q26X	I	[14]
I1	D27fs	I	[12]
E2	R43X	I	[12,14] [15]
E2	D53fs	I	[15]
E2	Y63X	I	[14]
I2	R71fs	I	[12]
I2	Splice	II	[14]
E3	R90X	I	[12]
E3	L79EfsX84	I	[12]
E3	R102P	II	[12,16] [10], This work
E3	R102C	II	[12]
E3	F104S	II	[12]
E3	V114M	none	[17]
E3	R123X	I	[12]
E3	F126LfsX5	I	[12]
E3	D128Y	II	[15]
E3/I3	Splice	II	[17]
E4	P136H	II	[16]
E4	P136L	II	[12]
E4	W154R	II	[12,14]
E4	Q186X	I	[2,18]
E4	C193Y	II	[19]
E5	R257L	II	[12]
E5	R257C	II	[16]
E5	P275T	II	[12]
E7	V368X	I	[16]
E7	A388P	II	[14]
E8	W435C	II	[12]
E9	G443R	II	[15]
E10	W491X	I	[16]
E10	L500fs	I	[2,18]
I10/E11	Splice	II	[16]
E11	Q541X	I	This work
E12	L594W	II	[12]
E12	G614V	II	[15]
E12	P635L	II	[16]

Note: reported splicing mutations, even if they would result in creation of a non-functional truncated protein, may only partially affect splicing and therefore lead to reduced amounts of receptor rather than the more severe type I phenotype. V114M is a polymorphism and not associated with disease.

Abbreviations: single letter designations for amino acids used. E=exon, I=intron, X=nonsense mutation, fs=frame shift