Mpl Baltimore: A thrombopoietin receptor polymorphism associated with thrombocytosis

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The chronic myeloproliferative disorders (MPD) are clonal hematopoietic stem cell disorders of unknown etiology. We have reported defective thrombopoietin receptor (Mpl) protein expression in MPD patients. To determine whether the basis of abnormal Mpl protein expression was due to mutations in the Mpl gene, we sequenced Mpl cDNA from MPD patients. We found a single nucleotide substitution (G1238T) that results in a change from lysine to asparagine at amino acid 39 (K39N) in three African-American women referred for an evaluation of an MPD. We subsequently screened more than 400 patients and controls and found that the K39N substitution is a polymorphism restricted to African Americans and that ~7% of African Americans are heterozygous for K39N. African Americans with the K39N polymorphism had a significantly higher platelet count than controls without the polymorphism (P < 0.001) and reduced platelet protein Mpl expression. Expression of an Mpl cDNA containing the K39N substitution in cell lines was associated with incomplete processing and a reduction in Mpl protein, recapitulating the Mpl protein defects in cell lines that is associated with incomplete Mpl glycosylation (3, 4). The K39N substitution is a polymorphism restricted to African Americans and that is associated with incomplete Mpl glycosylation (3, 4). We have demonstrated that the majority of polycythemia vera, essential thrombocytosis, and idiopathic myelofibrosis (IMF) gene disruption or transcriptional repression. Thrombopoietin (TPO) and the TPO receptor (Mpl) are the key regulators of platelet mass and are required for both stem cell proliferation and for the terminal differentiation of platelets (2). We have demonstrated that the majority of polycythemia vera and IMF patients have a reduction in platelet Mpl protein that is associated with incomplete Mpl glycosylation (3, 4). The molecular basis of this defect is unknown, but it is not a consequence of Mpl gene disruption or transcriptional repression. To define the molecular basis for these observed Mpl protein defects, we sequenced Mpl cDNA from six MPD patients and found a missense mutation in one of these patients. We report herein that the missense mutation represents a single-nucleotide polymorphism in the Mpl gene that is restricted to African Americans. We show that this Mpl polymorphism was associated with mild thrombocytosis in African Americans heterozygous for the polymorphism and with chronic, extreme thrombocytosis in homozygous individuals.

Materials and Methods

Study Design. Patients and control subjects from a general hematology clinic were enrolled in this Institutional Review Board-approved study and gave written informed consent. The diagnosis of polycythemia vera, essential thrombocytosis, or IMF was established according to Polycythemia Vera Study Group criteria (1). Platelet counts were obtained during routine outpatient clinic visits by using an automated cell counter. Peripheral blood genomic DNA samples from racially identified healthy controls were kindly provided by Josef Prchal (Baylor College of Medicine, Houston).

Mpl Cloning and Sequencing. Platelets were harvested from peripheral blood as described in ref. 4, and RNA was extracted with phenol and chloroform (5). cDNA was generated with the SuperScript II RT kit (GIBCO/BRL) by using a gene-specific primer (5'-GGAATGTTGGCAATGTGGG-3') complementary to the distal 3' end of Mpl-P. Mpl cDNA was amplified with forward (5'-GATGGGCTAAGGCAGCCACA-3') and reverse (5'-GGAATGTTGGCAATGTGGG-3') primers under the following conditions: 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 2 min. PCR products were excised and purified from agarose gels and cloned with Topo II by using the direct PCR Topo TA cloning system (Invitrogen). Plasmids isolated from transformed clones were fully sequenced with four sequencing primers (T7, 5'-CAGCCCGGAGAAGGAGGTA-3' located in exon 4, 5'-AACGCTTTCTTCTACAC-3' located in exon 6, and SP6). DNA templates were sequenced by using the fluorescent dyeoxy terminator method cycle sequencing on an Applied Biosystems Division 3700 DNA analyzer by following the manufacturer’s protocols.

Denaturing Gradient Gel Electrophoresis. DNA was extracted from peripheral blood or buccal smears by using a commercially available kit according to the manufacturer’s instructions (Gentra, Minneapolis). Mpl exon 2 was amplified from 500 ng of peripheral blood genomic DNA by using a modified forward primer containing a 40-bp GC-rich tail (5'-CGCCCGGCACC-GCCCGGCGCCCGGCGCCCGGCCCGCGCGAATTGGAGA-3') to increase the sensitivity of mutation detection during denaturing gel electrophoresis and an unmodified reverse primer (5'-AGAGGAGTGGGATTGTGGAG-3') with the following PCR conditions: 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 2 min. Denaturing gel electrophoresis of PCR products was performed at constant voltage (80 V) for 16 h at 60°C by using a 7.5% acrylamide gel containing a linear gradient of 30–90% of the denaturants urea and formamide. Abnormally migrating bands were excised from the gel and sequenced.

Expression of K39N in 32D Cells. Retroviral expression vectors were constructed by replacement of the green fluorescent protein cDNA with the full-length Mpl cDNA (normal or K39N variant).

Abbreviations: MPD, myeloproliferative disorders; TPO, thrombopoietin.

Data deposition: The sequence reported in this paper has been deposited in the National Center for Biotechnology Information’s dbSNP database (accession no. 24779593).

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in the murine stem cell virus-based bicistronic retrovirus vector MGIN kindly provided by Linzhao Cheng (The Johns Hopkins University) (6). Retroviruses were generated by transient co-transfection into the human kidney cell line 293T of the Mpl expression vector plus a Psi-era cotransfecting packaging vector (7), then used to infect 32D cells within 1 week. Stable, polyclonal 32D cell lines expressing the wild-type or K39N Mpl were obtained by G418 selection. Mpl mRNA expression was measured with real-time RT-PCR by using the Applied Biosystems Prism 7700 sequence detection system with primers and a probe designed to bind to the exon 11 and 12 region of the Mpl gene to target full-length transcripts (forward, TCCATGACACTACAGGAGACTTG; reverse, TCCACTTCTTCACAGGTATCT-GAGACTGA; probe, 6FAM-TCTAGGCGAGTACCTTAGG-GACACTGCA-TAMRA). Mpl threshold cycle values were normalized to 18s rRNA as an internal standard by using TaqMan control reagents, and transcript levels were calibrated relative to standard curves generated with 32D-Mpl cells expressing the normal Mpl cDNA.

**Immunoblotting.** Equal amounts of protein were applied to a 10% Criterion gel (Bio-Rad), with electrophoresis, membrane transfer, and immunoblotting with antibodies against Mpl or glycoprotein IIb performed as described in ref. 4. Antiserum to β-actin was purchased from Sigma. Preparation of platelet and cell lysates was performed as described in ref. 4.

**Statistical Analysis.** Statistical significance of sets of values was analyzed with Student’s *t* test.

**Results.** We cloned and sequenced Mpl-P cDNAs from the platelet RNA of six MPD and two control subjects. In an African-American woman with a 25-year history of isolated thrombocytosis and erythrocytosis, we found a single base change (guanosine to thymidine at nucleotide 1,238 in exon 2, National Center for Biotechnology Information’s dbSNP database accession number 24779593) that predicts a lysine to asparagine substitution at amino acid 39 (K39N) (Fig. 1A). To determine the frequency of K39N in other MPD patients, we devised a PCR-based denaturing gel electrophoresis assay by using peripheral blood as the source of genomic DNA (8). The single nucleotide base change resulting in K39N produced a unique signature of the heteroduplexes compared with that of the wild-type (Fig. 1B) sequence. The K39N pattern was absent in 108 Caucasian MPD patients. However, the K39N mutation was present in two other African-American patients referred for an evaluation of anemia (one), and HIV infection (one). Both siblings had a history of acute chest syndrome. Both were genotyped and found to be homozygous for K39N. Studies of their mother and father (healthy heterozygotes, with counts of 285,000 and 313,000 platelets per microliter, respectively) were excluded from the analysis (Fig. 2A). The referring diagnoses of the seven K39N heterozygotes had an elevated platelet count (>350,000 platelets per microliter). The mean platelet count of the seven subjects with the K39N polymorphism was significantly higher than the mean platelet count of 93 individuals without the polymorphism (*P* < 0.001), even when those controls with thrombocytopenia (15 of the controls were thrombocytopenic) were excluded from the analysis (Fig. 2A). The referring diagnoses of the seven K39N heterozygous subjects were anemia (two), monoclonal gamopathy (two), von Willebrand disease (one), sickle cell anemia (one), and HIV infection (one).

Two additional patients, a sister and brother, both with sickle cell anemia, were referred to our study for chronic, extreme thrombocytosis. Both siblings had counts between 800,000 and 1,000,000 platelets per microliter chronically in the absence of infection or iron deficiency. Both siblings had a history of acute chest syndrome. Both were genotyped and found to be homozygous for K39N. Studies of their mother and father (healthy individuals with sickle cell trait) revealed both to be K39N heterozygotes, with counts of 285,000 and 313,000 platelets per microliter, respectively. Because patients with sickle cell disease have chronic elevations in platelet count because of the asplenic state, we compared the mean platelet count of these two homozygotes with the platelet counts of 29 sickle cell disease
patients that we genotyped for K39N. The mean platelet count of the two homozygotes (858,000 platelets per microliter) was significantly higher than the mean platelet count of 28 K39N-negative sickle cell patients (360,000 platelets per microliter; range 127,000–584,000) (P = <0.001) (Fig. 2B).

Expression of K39N-Mpl in Platelets and 32D Cells. We assessed Mpl protein expression in platelets from K39N-positive individuals and in murine hematopoietic cell lines engineered to express either K39N Mpl or wild-type Mpl. Platelets from a K39N homozygote expressed a markedly reduced amount of the fully processed Mpl isoform (Fig. 3A). Similarly, expression of K39N Mpl cDNA in 32D cells, an IL-3-dependent murine cell line that neither expresses Mpl nor responds to TPO, differed both qualitatively and quantitatively from the wild-type Mpl, with both a reduction in the total amount of protein and the presence of an isoform with a retarded electrophoretic mobility consistent with incomplete glycosylation (Fig. 3B) in a manner that we characterized in ref. 4. We examined mRNA expression of K39N Mpl and wild-type Mpl mRNA in 32D cells by using quantitative real-time RT-PCR (Fig. 3C). Mpl transcripts were undetectable in 32D-vector and 32D parental cell lines. Mpl gene expression relative to 18S ribosomal RNA in the 32D-K39N-Mpl cells was 1.81 relative to 18S ribosomal RNA in the 32D-vector and 32D-parental cell lines. Mpl signal was not detected in either the 32D-vector or 32D-parental cell lines, although 18S signals were detected at high levels in all four cell lines (data not shown).

Discussion

To investigate the possibility that an alteration in Mpl gene expression was responsible for polycythemia vera Mpl protein defects, we sequenced Mpl cDNA from MPD patients. During this study we identified a single base change that conferred a change in amino acid sequence, K39N, in three African-American patients referred for an evaluation of an MPD. We subsequently found that K39N was a polymorphism of the Mpl gene that is restricted to individuals of African-American descent. Studies of two geographically distinct populations of African Americans indicate that ~7% of African Americans are carriers for K39N. Examination of K39N with respect to platelet count suggests a gene dosage effect with K39N heterozygous individuals exhibiting mild thrombocytosis, whereas K39N-homozygous individuals exhibit severe thrombocytosis when compared with appropriate controls. Studies of platelets from patients homozygous for K39N and cell lines engineered to express K39N indicate that this polymorphism dramatically disrupts Mpl protein expression.

Mutations of the TPO gene and the Mpl gene have been described in families with unique clinical phenotypes. Mutations of the TPO gene that enhance translational efficiency of TPO mRNA have been demonstrated to be the cause of an autosomal dominant hereditary thrombocytopenia in three families (9–11), whereas more recently, an activating mutation in Mpl was found to be the basis of thrombocytosis in a single family with hereditary thrombocytopenia (12). Children with congenital amegakaryocytic thrombocytopenia, a disorder marked by severe congenital thrombocytopenia and eventual aplastic anemia, inherit Mpl alleles from both parents that result in a nonfunctional Mpl protein (13–17). These sporadic human mutations in TPO and Mpl generate phenotypes in a similar manner to those generated in murine models where TPO or Mpl are manipulated. Targeted deletion of either the Mpl or the TPO gene in mice results in severe thrombocytopenia and a reduction in murine bone marrow stem cells (18, 19).

K39N represents a newly identified allelic variant of Mpl associated with thrombocytosis, yet the in vivo and in vitro consequence of K39N expression is a hypofunctional protein. Impaired Mpl function in the setting of thrombocytosis is indeed counterintuitive, especially given the phenotype of marked thrombocytopenia in the Mpl knockout mice and in individuals with congenital amegakaryocytic thrombocytopenia. However,
several lines of experimental evidence indicate both a direct negative regulatory role for Mpl and the presence of platelet production pathways that are independent of Mpl function. First, deletion of the distal extracellular domain of Mpl, the site of the K39N mutation, resulted in TPO-independent growth (20). Second, induction of thrombocytosis has been demonstrated in K39N mutation, resulted in TPO-independent growth (20).

Many patients classified clinically as having essential thrombocytosis have polyclonal hematopoiesis, indicating that the etiology of essential thrombocytosis is heterogeneous and may not always be related to the transformation of a single hematopoetic stem cell (23). We have now identified an Mpl polymorphism unique to individuals of African-American descent, which we designate Mpl Baltimore. The polymorphism is autosomal and appears to conform to a pattern of autosomal dominance with incomplete penetrance in that some heterozygotes have normal platelet counts whereas others have sustained elevations of platelet counts that even satisfy MPD diagnostic criteria. The variability in the K39N phenotype that we have observed among our heterozygotes may be due to the particular genetic and/or environmental backgrounds in which K39N is expressed. Alternatively, K39N could predispose some individuals to developing clonal hematopoietic stem cell disorders. These distinctions cannot yet be addressed, as clonal markers specific for the MPDs are lacking. Larger population studies in healthy and disease states and family studies will be required to determine the relationship of K39N to the development of thrombocytosis.

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