Linkage of a Familial Platelet Disorder With a Propensity to Develop Myeloid Malignancies to Human Chromosome 21q22.1-22.2

By Carolyn Y. Ho, Brith Otterud, Robert D. Legare, Tena Varvil, Richa Saxena, David B. DeHart, Susan E. Kohler, Jon C. Aster, S. Bruce Dowton, Frederick P. Li, Mark Leppert, and D. Gary Gilliland

Linkage analysis was performed on a large pedigree with an autosomal dominant platelet disorder and a striking propensity in affected family members to develop hematologic malignancy, predominantly acute myelogenous leukemia. We report the linkage of the autosomal dominant platelet disorder to markers on chromosome 21q22. Four genetic markers completely cosegregate with the trait and yield maximum logarithm of difference scores ranging from 4.9 to 10.5 (θ = .001). Two flanking markers, D21S1265 and D21S167, define a critical region for the disease locus of 15.2 centimorgan. Further analysis of this locus may identify a gene product that affects platelet production and function and contributes to the molecular evolution of hematologic malignancy.

© 1996 by The American Society of Hematology.

From the Department of Medicine, Division of Hematology/Oncology, the Department of Pathology, Brigham and Women’s Hospital, the Dana Farber Cancer Institute and Harvard School of Public Health, Harvard Medical School, Boston, MA; Department of Genetics, Washington University School of Medicine, St Louis, MO; and the Technology Access Section of the University of Utah Genome Center, Salt Lake City, UT.

Submitted November 27, 1995; accepted February 8, 1996.

Supported in part by American Cancer Society Grant No. DHP-48 (D.G.G., R.S., and D.B.D.), the Lawrence Family Foundation (D.G.G.), a Howard Hughes Medical Institute Medical Student Fellowship (C.Y.H.), The Starr Foundation and Canto Foundation (F.P.L.) and the Technology Access Section of the University of Utah Genome Center (B.O., T.V., and M.L.). D.G.G. is the Stephen Birnbaum Scholar of the Leukemia Society of America.

Address reprint requests to D. Gary Gilliland, PhD, MD, Brigham and Women’s Hospital, Harvard Medical School, 221 Longwood Ave, LMRC 611, Boston, MA 02115.

The publication costs of this article were defrayed in part by charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.

5218
to participate in the study. Informed consent and blood samples were obtained from the remaining 52 family members. Phenotypes were assigned to these individuals before the start of genotyping, and 29 family members were considered to be affected on the basis of both (1) clinical history of a bleeding tendency and (2) thrombocytopenia and/or abnormal platelet aggregation in response to epinephrine and arachidonic acid.

Because the hallmark of a bleeding dyscrasia is a history of clinically significant bleeding, family members were asked detailed questions to assess for abnormal hemostasis, including: spontaneous, frequent epistaxis; menorrhagia; ecchymoses or petechiae; and significant bleeding associated with surgical procedures or childbirth. All affected family members had a clinical history of a significant bleeding tendency.

In addition to clinical history, laboratory studies were used to confirm affection status. Of 29 affected family members, 28 had thrombocytopenia (platelet count, <150,000/mL), whereas no clinically unaffected family member had thrombocytopenia. Serial complete blood counts since the time of the original report of this pedigree in 198515 show consistent and stable thrombocytopenia in 9 affected individuals. In 1 of 29 affected individuals with a clinical history of bleeding but with a normal platelet count (162,000/mL, patient IV-25), functional platelet abnormalities were confirmed by a prolonged Ivy bleeding time (>15 minutes) and abnormal platelet aggregation studies. Abnormal platelet aggregation, consisting of loss of the second wave of aggregation in response to epinephrine and decreased aggregation in response to arachidonic acid (Fig 2), was present in 20 of 20 affected individuals who were tested. Aggregation to ristocetin was normal (Fig 2). Platelet aggregation studies were tested on 4 unaffected family members, none of whom showed abnormal platelet aggregation.

Three large branches of the family from the originally reported pedigree13 were excluded from the linkage study because there was no evidence of segregation of the trait among individuals in these branches.

DNA collection and processing. Blood for DNA extraction was collected from 55 family members as indicated in Fig 1. Total genomic DNA was prepared according to standard methods with phenol/chloroform extraction and ethanol precipitation.16

Linkage analysis was performed using polymerase chain reaction (PCR) to amplify microsatellite repeat polymorphisms.17,18 The majority of PCR primers were obtained from the Technology Access Section of the University of Utah Genome Mapping Center (Salt Lake City, UT); the remainder were purchased from Research Genetics Inc (Huntsville, AL), based on the Genethon linkage map of chromosome 21.9 A single primer from each primer set was end-labeled by a kinase reaction consisting of 20 pmol primer, 2.0 mL 10× kinase buffer (New England Biolabs, Beverly, MA), 140 mCi of γ-<sup>32</sup>P-deoxyadenosine triphosphate (3,000 Ci/mmol; New England Nuclear, Boston, MA), and 20 U of T4 polynucleotide kinase (New England Biolabs). The kinase reaction was incubated at 37°C for 30 minutes, and the enzyme was inactivated for 2 minutes at 90°C.

PCR amplification of the repeat polymorphisms was performed in 96-well trays using an MJ Research (Watertown, MA) thermal cycler. Reaction volumes were 25 mL and consisted of 20 ng of DNA; 2.5 mL of 10× PCR buffer (100 mmol/L Tris-HCl [pH 8.4], 400 mmol/L NaCl, 15 mmol/L MgCl<sub>2</sub>; 2.5 mmol/L spermidine (Sigma Chemical Co, St Louis, MO); 200 mmol/L each of deoxyguanosine triphosphate, deoxyadenosine triphosphate, deoxythymidine triphosphate, and deoxycytidine triphosphate; 10 pmol each of forward and reverse primers; 0.3 pmol of <sup>32</sup>P-end-labeled primer; and 0.05 U of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT). The majority of markers were amplified for 30 cycles of 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C. PCR products were
creased aggregation in response to arachidonic acid. Aggregation in response to ristocetin in affected individuals was normal.

show an "aspirin-like" platelet aggregation defect, with loss of the second wave of aggregation in response to epinephrine and decreased aggregation in response to arachidonic acid. Aggregation in response to ristocetin in affected individuals was normal.

resolved on a denaturing polyacrylamide gel (5% to 7% acrylamide, 32% formamide) and were visualized by autoradiography. Allele types were assigned to each family member by direct inspection of the autoradiograph.

Linkage analysis. Two-point logarithm of difference (lod) scores were calculated using the MLMINK subroutine of the LINKAGE software package, version 5.1.26 An autosomal dominant model of disease gene inheritance was assumed. A penetrance of 90% was used in the analysis. This penetrance was considered to be valid because there was no evidence of incomplete penetrance in the family; ie, no apparently unaffected individual has given rise to an affected offspring. Disease allele frequency was assumed to be 0.001. Lod scores were first calculated assuming equal allele frequencies for the markers and were then calculated a second time with allele frequencies estimated from the unrelated members of the pedigree. Estimates of \( L_0 \) were performed with the LODSCOR subroutine of LINKAGE.

After significant linkage was obtained with the initial marker UT7582/D21S1413, seven markers in the 21q22 region were chosen from the published Cooperative Human Linkage Center (CHLC) map27 for additional genotyping. Two other polymorphic short tandem repeat markers, D21S211 (CHLC ftp db version 2.0) and D21S216,27 known to localize to this general region were also selected for genotypic analysis. To facilitate this mapping study, we attempted to place these two markers and the initial marker, UT7582, onto the CHLC framework map. This was accomplished with the CMAP and CILINK programs of LINKAGE.

RESULTS

Clinical description. A 44-year-old woman was referred to the Hematology/Oncology Clinic at the Brigham and Women's Hospital (Boston, MA) for evaluation of thrombocytopenia and refractory anemia. Clinical evaluation showed a white blood cell count of 18.7 \( \times 10^3 \)\,mL, with 7% blasts, 42% PMN, 37% lymphocytes, 5% monocytes, 7% eosinophils, and 2% basophils. Bone marrow aspirate and biopsy specimen showed a moderately hypercellular marrow (30% to 40% fat), with 5% to 10% myeloid blasts, increased numbers of early dysplastic myeloid progenitors, severe erythroid hypoplasia, and dysplastic megakaryocytes. Karyotype analysis showed del(5)(q31) and del(11)(q13) in 24 of 25 cells; 1 cell had a normal karyotype. Based on these findings, a diagnosis of MDS with possible evolution to AML was made. She subsequently developed AML and was treated with standard induction chemotherapy.

This patient was a member of a large kindred with a previously identified autosomal dominant disorder of platelet function and number (Fig 1). Affected family members have a clinical history of a bleeding diathesis (29 individuals tested positive per 29 individuals tested); thrombocytopenia (platelet count, <50,000 to 150,000/mL in 27 of 29); prolonged bleeding times (in 20 of 20); an "aspirin-like" platelet aggregation defect with an abnormal response to arachidonic acid and loss of the second wave of aggregation in response to epinephrine (in 20 of 20; Fig 2); and normal platelet size and morphology (data not shown). All affected family members with a clinically significant history of bleeding had laboratory abnormalities to support a bleeding diathesis. The platelet disorder in this family differs phenotypically from other described autosomal dominant platelet deficiencies whose findings typically include shortened platelet life span, unusually large platelets, increased number of bone marrow megakaryocytes, male predominance, morphologically abnormal platelets, neutrophil nuclear hypersegmentation, and eosinophilia.15

As previously described,15 affected family members have a striking propensity to develop hematologic malignancy in the first through sixth decades of life, predominantly AML. Of 29 affected family members, 6 have developed AML and 1 of 29 (Fig 1, patient IV-27) has developed lymphosarcoma. None of the 24 unaffected family members have developed any type of hematologic malignancy (Fig 1). Moreover, of
the 8 affected family members who have died, 6 have died from leukemia. Thus, inheritance of the gene that is responsible for the autosomal dominant platelet disorder is strongly correlated with subsequent development of myeloid malignancy. Because the incidence of AML in the general population is approximately 1 in 100,000 per year, the genetic locus that causes the platelet defect may predispose to the development of myeloid malignancy or, at least, be tightly linked to the trait.

**Linkage of familial platelet disorder (FPD) to chromosome 21.** To find linked markers for this disease locus, a general linkage study was performed. Genotypes in the disease pedigree were collected from an evenly spaced set of highly polymorphic dinucleotide, trinucleotide, and tetranucleotide repeat markers. No evidence for linkage was observed with the first 92 markers tested. Analysis of these markers excluded approximately half of the genome (data not shown).

Linkage to chromosome 21 was initially identified through marker UT7582/D21S1413, which yielded a maximum lod score of 7.73 (θ = 0.032). Linkage analysis of adjacent markers confirmed localization of the FPD locus to this region of chromosome 21. Eight additional markers yielded maximum lod scores ranging from 1.44 to 10.48, and seven of these markers gave maximum lod scores greater than 3 (Table 1). Moreover, four of nine markers tested, D21S263, D21S216, IFNAR, and D21S65, were completely linked to the disease locus and gave lod scores of 6.19, 4.89, 9.40, and 10.48, respectively (θ = .001). The haplotype that consistently segregates with the disease locus for these same four markers is 6-3-3-10 (Fig 1). The lod scores were calculated under the assumption of equal allele frequencies for all marker systems (Table 1). When the analysis was repeated with the allele frequencies estimated from the disease pedigree, the resultant lod scores did not significantly change (data not shown).

To clarify the localization of the disease gene with respect to the markers used in this linkage study, a map of all of the markers was constructed starting with the CHLC framework map of chromosome 21. Six of these markers (Mfd338/D21S1265, D21S263, IFNAR, D21S65, D21S167, and D21S168) were already included in the published genetic map. Two of the markers (UT7582/D21S1413 and D21S216) were placed on the map only as confidence intervals with odds greater than 1,000:1 (Fig 3). The third marker (D21S216) has not been typed in CEPH reference families; thus, its position could not be determined using Centre d’Etude du Polymorphisme Humain (CEPH) data. However, the localization of this marker was estimated from two-point linkage analysis performed within the disease pedigree. The results showed that D21S216 was completely linked to the marker IFNAR, with a lod score of 7.46 (0), and was closely linked to the adjacent markers D21S263 (θ = 4.09, θ = .045), and D21S65 (θ = 5.93, θ = .024). The map positions of these nine markers are summarized in Fig 3.
The localization of the FPD trait to chromosome 21 is also of interest because the FPD locus may overlap with the genetic region responsible for Down syndrome (trisomy 21). Children with Down syndrome may have hematologic abnormalities observed in Down syndrome. There are five candidate genes that map to the FPD locus, including AML1, the interferon α/β receptor (IFNAR), the cytokine receptor family 2-4 (CRF2-4), phosphoribosylglycinamidine formyltransferase (GART), and SON.

AML1 is a particularly attractive candidate gene because it has been previously implicated in leukemogenesis through its involvement in the t(8;21) translocation observed in the M2 subtype of AML, as well as in the t(3;21) translocation associated with MDS, chronic myelogenous leukemia in blast crisis, and therapy-related AML. AML1 has a 5' DNA-binding domain with significant homology to the Drosophila pair-rule segmentation gene, runt, and a 3' trans-activating domain that potentiates the transcriptional activity of the DNA-binding domain.

Several different fusion partners have been identified for the AML1 gene in translocations associated with AML. These include the ETO gene in t(8;21) and the EAP, MDS1, and EVII genes in t(3;21) translocations. A common feature for each of these translocations is expression of the DNA-binding domain and disruption of the transactivating domain by the respective fusion partners. It has been speculated that the DNA-binding domain then has dominant negative activity in precluding normal transactivation of genes by the AML1. Therefore, it is possible that an inherited missense or frameshift mutation that disrupts the AML1 transactivating domain could lead to abnormal hematopoeisis. However, the mechanism by which such a mutation would predispose to the eventual development of AML in affected individuals is unknown.

The IFNAR gene and the related CRF2-4 gene that lies in close proximity are also potential candidate genes. Although the function and pattern of expression of the CRF2-4 gene is unknown, IFN mediates a variety of effects in hematopoietic cells through interaction with its receptor, including induction of expression of IFN-regulatory factor-1, which has been implicated in pathogenesis of MDSs. SON has significant homology with the transcription factors MYC and MOS. Each of these genes has been implicated in pathogenesis of hematologic malignancy and, therefore, may be involved in pathogenesis of disease in this pedigree. Sequence analysis of each of these candidate genes is underway.

The phenotype of this pedigree is similar to other smaller pedigrees reported in the literature, including a well-characterized pedigree with an inherited platelet storage pool defect associated with a high incidence of acute myeloid leukemia. Furthermore, in sporadic cases of AML and myeloproliferative syndromes, platelets may also have aspirin-like aggregation functional abnormalities. Thus, it is possible that the mutant gene responsible for the trait in this pedigree may also be involved in other familial leukemia syndromes and in the majority of leukemias in which there is no apparent inherited predisposition.

Genetic mapping of this disorder is an important first step in our efforts to identify the underlying gene. The placement of the FPD critical region on chromosome 21q22 is of particular interest, because (1) this locus has been previously implicated in AML through the (8;21) and (3;21) chromosomal translocations involving the AML1 gene; and (2) there is a potential association between the FPD gene and the hematologic abnormalities observed in Down syndrome. There are five candidate genes that map to the FPD locus, including AML1, the interferon α/β receptor (IFNAR), the cytokine receptor family 2-4 (CRF2-4), phosphoribosylglycinamidine formyltransferase (GART), and SON.

AML1 is a particularly attractive candidate gene because it has been previously implicated in leukemogenesis through its involvement in the t(8;21) translocation observed in the M2 subtype of AML as well as in the t(3;21) translocation associated with MDS, chronic myelogenous leukemia in blast crisis, and therapy-related AML. AML1 has a 5' DNA-binding domain with significant homology to the Drosophila pair-rule segmentation gene, runt, and a 3' trans-activating domain that potentiates the transcriptional activity of the DNA-binding domain.

Several different fusion partners have been identified for the AML1 gene in translocations associated with AML. These include the ETO gene in t(8;21) and the EAP, MDS1, and EVII genes in t(3;21) translocations. A common feature for each of these translocations is expression of the DNA-binding domain and disruption of the transactivating domain by the respective fusion partners. It has been speculated that the DNA-binding domain then has dominant negative activity in precluding normal transactivation of genes by the AML1. Therefore, it is possible that an inherited missense or frameshift mutation that disrupts the AML1 transactivating domain could lead to abnormal hematopoeisis. However, the mechanism by which such a mutation would predispose to the eventual development of AML in affected individuals is unknown.

The IFNAR gene and the related CRF2-4 gene that lies in close proximity are also potential candidate genes. Although the function and pattern of expression of the CRF2-4 gene is unknown, IFN mediates a variety of effects in hematopoietic cells through interaction with its receptor, including induction of expression of IFN-regulatory factor-1, which has been implicated in pathogenesis of MDSs. SON has significant homology with the transcription factors MYC and MOS. Each of these genes has been implicated in pathogenesis of hematologic malignancy and, therefore, may be involved in pathogenesis of disease in this pedigree. Sequence analysis of each of these candidate genes is underway.

The localization of the FPD trait to chromosome 21 is also of interest because the FPD locus may overlap with the genetic region responsible for Down syndrome (trisomy 21). Children with Down syndrome may have hematologic abnormalities involving platelets and other hematopoietic cell lin-
eages, which are often present at birth. These abnormalities include thrombocytosis, thrombocytopenia, polycythemia, anemia, leukocytosis, leukemoid reactions, and transient abnormal myelopoiesis. Although these abnormalities often resolve spontaneously, these patients are at a greatly increased risk of developing leukemia, including a preponderance of acute megakaryocytic leukemia over other subtypes such as acute lymphoblastic leukemia and AML.

Therefore, it is tempting to speculate that the gene responsible for FPD is also involved in the hematologic abnormalities observed in Down syndrome. In support of this hypothesis, recent physical mapping evidence indicates that the Down syndrome chromosome region lies in close proximity to the FPD critical region. Minimal regions involved in the expression of specific features of the complex Down syndrome phenotype have been identified by analyzing patients with partial trisomy of chromosome 21. A total of 13 of 24 major features of Down syndrome has been mapped to a single region on chromosome 21q22. This region is located between markers D21S17, proximally, and D21S55, distally. However, physical mapping information of the proximal portion of this region is limited. Recent efforts to refine the physical map have used the more accurately mapped marker D21S65 as the proximal anchor of the Down syndrome chromosome region. D21S65 is tightly linked to the AML1 gene and is contained in the central portion of the FPD critical region. Therefore, the gene responsible for the platelet disorder and for the propensity for developing myeloid malignancies in the family studied in this report may also play a role in hematologic abnormalities, including AML, that are observed in Down syndrome.

The mechanism by which the inherited trait causes abnormalities in platelet number and function is unclear at this time. It is possible that the platelet disorder is an epiphenomenon that merely acts as a marker for the underlying pathophysiology. In contrast to other hematopoietic cell lines, platelet abnormalities are easily identified both clinically and through laboratory studies. As such, it is difficult to ascertain if the disease trait in this family is restricted solely to megakaryocytes or if other myeloid lineages are affected as well. It is likely that this inherited mutation acts as a first step that predisposes to the development of malignancy in affected family members. The acquisition of subsequent somatic mutations during life is necessary for the development of malignancy.

Because this family inherits a predisposition to developing AML, it may offer a unique opportunity to study the very earliest events in the pathogenesis of hematologic malignancy. Identification and characterization of the gene responsible for causing this disorder may provide further insight into the molecular basis of platelet production and function as well as of leukemogenesis.

ACKNOWLEDGMENT

We are indebted to all of the family members and their physicians and hematologists for cooperation and interest in this study. We thank David Porter for evaluation of the index patient in the Brigham and Women's Hospital Hematology/Oncology Clinic; Kerry Blanchard and Steven Perrin (Brigham and Women's Hospital) and Dora Stauffer, Lisa Baird, Mark Keating, and Richard Lifton (University of Utah, Salt Lake City, UT) for technical advice; and Diana Beardley and Nancy Upp Potter (Dana Farber Cancer Institute) for pedigree collection and evaluation. We gratefully acknowledge the long-standing support of the Lawrence Family Foundation and H. Franklin Bunn for suggesting the study and providing invaluable scientific insight and guidance.

REFERENCES

5224

31. Tighe JE, Calabi F: Alternate, out-of-frame runt/MTG8 are encoded by the der(8) chromosome in the (t(8;21) of acute myeloid leukemia M2. Blood 84:2115, 1994
33. Lutfalla G, Gardiner K, Uzé G: A new member of the cytokine receptor gene family maps on chromosome 21 at less than 35 kb from IFNAR. Genomics 16:266, 1993
Linkage of a familial platelet disorder with a propensity to develop myeloid malignancies to human chromosome 21q22.1-22.2

CY Ho, B Otterud, RD Legare, T Varvil, R Saxena, DB DeHart, SE Kohler, JC Aster, SB Dowton, FP Li, M Leppert and DG Gilliland