Molecular basis of clonal expansion of hematopoiesis in 2 patients with paroxysmal nocturnal hemoglobinuria (PNH)


Somatic mutation of PIGA in hematopoietic stem cells causes deficiency of glycosyl phosphatidylinositol–anchored proteins in paroxysmal nocturnal hemoglobinuria (PNH) that underlies the intravascular hemolysis but does not account for expansion of the PNH clone. Immune mechanisms may mediate clonal selection but appear insufficient to account for the clonal dominance necessary for PNH to become clinically apparent. Herein, we report 2 patients with PNH whose PIGA-mutant cells had a concurrent, acquired rearrangement of chromosome 12. In both cases, der(12) had a break within the 3' untranslated region of HMGA2, the architectural transcription factor gene deregulated in many benign mesenchymal tumors, that caused ectopic expression of HMGA2 in the bone marrow. These observations suggest that aberrant HMGA2 expression, in concert with mutant PIGA, accounts for clonal hematopoiesis in these 2 patients and suggest the concept of PNH as a benign tumor of the bone marrow. (Blood. 2006;108:4232-4236) © 2006 by The American Society of Hematology

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a consequence of nonmalignant clonal expansion of hematopoietic stem cells with somatic mutation of PIGA.1 Mutant PIGA2 explains the deficiency of glycosyl phosphatidylinositol–anchored proteins (GPI-APs) that underlies the intravascular hemolysis of PNH.3 However, PIGA-mutant stem cells have no intrinsic proliferative advantage,4,5 suggesting a 2-step model of pathogenesis.

Step 1 of this model, clonal selection,6,7 is envisioned as a conditional survival advantage that depends on deficiency of 1 or more GPI-APs. The close association of PNH with aplastic anemia, suggests that the selection pressure is immune mediated.6,7 But, although 60% to 70% of patients with aplastic anemia have small, subclinical populations of GPI-AP− hematopoietic cells at diagnosis,8 only 10% to 15% subsequently develop clinically apparent PNH.9 In the remainder, GPI-AP− cells persist subclinically or disappear,9 suggesting that mutant PIGA (and the consequent deficiency of GPI-APs) is necessary for clonal selection but is insufficient to account for the clonal expansion required for clinical manifestations of PNH to become apparent.

Clonal expansion, step 2 of the PNH pathogenesis model, is envisioned as a consequence of clonal evolution in which a second somatic mutation bestows on the PIGA-mutant stem cell a proliferative advantage.10 Herein, we present evidence supporting this 2-step model by showing a concurrent, acquired genetic abnormality in the PIGA-mutant cells of 2 patients that establishes a novel mechanism for the nonmalignant clonal hematopoiesis characteristic of PNH.

Patients, materials, and methods

Patients

Informed consent was obtained from patients J20 and US1 according to protocols approved by the Institutional Review Boards of Osaka University Hospital (Osaka, Japan) and the University of Utah School of Medicine (Salt Lake City, UT), respectively.

Hybrid cell lines

Monocytes derived from J20 or US1 were fused with the hypoxanthine phosphoribosyltransferase–negative mouse myeloma cell line, F3-X63-Ag8.653, as previously described.11 Lines carrying human chromosome 12 were selected by analyses of expression of both CD9 and polymorphic markers D12S77 and D12S78. The B-lymphoblastoid cell line JY2512 was used as a control in some experiments.

From the Department of Molecular Genetics, Osaka Medical Center for Cancer, Japan; the Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Japan; the Department of Immunology, Fukushima Medical University, Japan; the Department of Medicine, Duke University Medical Center, Durham, NC; the Genome Information Research Center, Osaka University, Japan; the Department of Hematology and Oncology, Osaka University School of Medicine, Japan; the Department of Medicine, Division of Hematology and Bone Marrow Transplant, University of Utah School of Medicine, Salt Lake City, UT; the Department of Medicine, Hematology/Oncology Section, George E. Whalen Veterans Administration (VA) Medical Center, Salt Lake City, UT; the Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT; the Division of Medical Genetics, Department of Pediatrics, University of Utah School of Medicine, Salt Lake City, UT; the Department of Medicine, Division of Hematology/Oncology, Baptist Medical Center, Columbia, SC; the Department of Medicine, Division of Hematology, Medical University of South Carolina, Charleston, SC; and the Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama, Japan.


An Inside Blood analysis of this article appears at the front of this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.

© 2006 by The American Society of Hematology
Determination of chromosomal breakpoints

Initial mapping of breakpoints required a combination of polymerase chain reaction (PCR), inverse PCR, and Southern blotting. For fine mapping, sequence-tagged site markers were generated by PCR using primers based on sequences of ends of bacterial artificial chromosome (BAC) clones or on data in the human genome database of the National Center for Biotechnical Information. GenBank accession numbers of BAC clones (CHORI BACPAC Resource Center) are as follows: RP11-425122 (425122), AC074030; RP11-471G7 (471G7), AC024935; RP11-150C16 (150C16), AC046129; RP11-366L20 (366L20), AC090673; RP11-474P2, AC025503; RP11-221N13, AC090023; RP11-434C1, AC007450; RP11-438119 (438119), N043819.

PCR primers used to amplify breakpoints

PCR primers used to amplify junction sequences of the breakpoints in J20 were as follows: breakpoint (BP) 1, CTTATGCTCACATGTTGCCGAC (108462-108443 in 150C16) and CCTTCACATCTGTTTAGGC (110307-110357 in 425122); BP2, TTCTCTACAGGGCCTTGCACCA (111355-111376 in 366L20) and ACTGCAACACCTCTCGACAGG (109879-109899 in 425122); BP3, TTGAAACCTTTGGCAATACGT (110477-110527 in 425122) and TATTATAACCATCTCTGACCC (97995-98016 in 150C16). Primers, GTGCCCAAGTGAGACTAAG (108443-108462 in 425122) and TATTTAACACCTATCTGACTCC (97995-98016 in 150C16) and TTGTGACTGAGCCCCATGAT (108598-108579 in 150C16), were used for positive control PCR.

Junction sequences of the breakpoints in US1 were confirmed by PCR with the following primer mixture to amplify both normal and abnormal alleles at the same time: BP3, CCAAAATGTTGGTACATAAATCAAAAA (44148-44125 in 474P2), TTGCTCTCCCACTTACATA (primer A, 111517-111498 in 366L20), and ACTCCCTGATGACATCACCTTC (primer B, 111043-111070 in 366L20); BP6, GCCGCCATGTTGCCGAC (109843-109862 in 150C16) and TGTGGACTGAGCCCCATGAT (108598-108579 in 150C16), were used for positive control PCR.

Fluorescence in situ hybridization (FISH)

BAC clones 471G7, 150C16, and 366L20 were used as probes against hybrid cell lines derived from J20 and 438119; 474P2 and 366L20 were used against bone marrow cells from US1. Signals were detected with biotinylated BAC probes and fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories, Burlingame, CA) and amplified by using biotinylated antiavidin antibody (Vector Laboratories). Chromosomes were stained with propidium iodide and biotinylated antiavidin antibody (Vector Laboratories) and FITC-conjugated avidin (Vector Laboratories, Burlingame, CA) and amplified by using biotinylated BAC probes and fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories). BAC clones 471G7, 150C16, and 366L20 were used as probes against hybrid cells. BAC clones were amplified by using biotinylated BAC probes and fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories). Chromosomes were stained with propidium iodide and biotinylated antiavidin antibody (Vector Laboratories) and FITC-conjugated avidin (Vector Laboratories). BAC clones 471G7, 150C16, and 366L20 were used as probes against hybrid cell lines derived from J20 and 438119; 474P2 and 366L20 were used against bone marrow cells from US1. Signals were detected with biotinylated BAC probes and fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories, Burlingame, CA) and amplified by using biotinylated antiavidin antibody (Vector Laboratories) and FITC-conjugated avidin (Vector Laboratories).

Results

Patients

At age 33, J20 presented with pancytopenia and a hypocellular marrow without karyotypic abnormalities.5 Five months later, an abnormal karyotype, 46, XX, t(12;12)(q13;q15), was reported in 3 of 21 metaphase cells. One year after diagnosis, blood counts were essentially normal, and marrow analysis showed normal cellularity with mild erythroid dysplasia. Cytogenetic analysis indicated expansion of the mutant clone with 10 (50%) of 20 metaphase cells having the abnormal karyotype. Laboratory evidence of hemolysis was noted, and flow cytometry showed 55% to 60% GPI-AP− erythrocytes. A mutation in exon 2 of PIGA (G715A) was shown in patient neutrophils.15 Marrow mononuclear cells were separated into GPI-AP− populations, and the abnormal karyotype was found only in GPI-AP− populations. These findings established the somatic nature of both the PIGA mutation and the chromosome 12 rearrangement and suggested that both genetic abnormalities were involved in the pathogenesis of clonal hematopoiesis.

US1 was 31 years old at presentation with complaints of fatigue and dark urine. Her white blood cell count was 4.1 × 10⁹/L (10927-10932), hemoglobin level was 38 g/L (3.8 g/dL), and platelet count was 171 000/μL. Laboratory studies indicated evidence of hemolysis was noted, and flow cytometry showed 88% GPI-AP− erythrocytes. A mutation in exon 2 of PIGA (G715A) was shown in patient neutrophils.15 Marrow mononuclear cells were separated into GPI-AP− and GPI-AP+ populations, and the abnormal karyotype was found only in GPI-AP+ cells.15 These findings established the somatic nature of both the PIGA mutation and the chromosome 12 rearrangement and suggested that both genetic abnormalities were involved in the pathogenesis of clonal hematopoiesis.

US1 was 31 years old at presentation with complaints of fatigue and dark urine. Her white blood cell count was 4.1 × 10⁹/L (10927-10932), hemoglobin level was 38 g/L (3.8 g/dL), and platelet count was 171 100/μL. Laboratory studies indicated evidence of hemolysis, and flow cytometry showed 88% GPI-AP− neutrophils. Marrow analysis revealed normal cellularity with erythroid hyperplasia without dysplasia and a karyotype of 46, XX, ins(12)(p12–13q13q12) in 20 (100%) of 20 metaphases. FISH showed the insertion split the TEL locus at 12p13. The abnormal karyotype was identified in 23% of mitogen-stimulated lymphocytes. A 14-bp deletion in the 3′ end of exon 2 of PIGA (693-706) was identified in neutrophil DNA. These findings confirmed the somatic nature of both the der(12) and mutant PIGA in US1.
Chromosomal abnormalities

Hybrid cell lines between patient monocytes and mouse myeloma cells were established. From J20, 2 lines (S1 and S2) carrying short chromosome 12 and 2 lines (L1 and L2) carrying long chromosome 12 (Figure 1B) were developed.11 From US1, hybrid cells carrying normal chromosome 12 (US1W) or der(12) (US1M) were developed (Figure 1A,C). Chromosomal abnormalities (Figure 1) were delineated by using a combination of PCR analysis based on sequence-tagged site markers, FISH (Figure 2), Southern blotting, and inverse PCR (not shown).

For J20, the abnormality was defined as insertion of an 18.5-Mbp fragment derived from one chromosome 12 (short chromosome 12) into the other (long chromosome 12) (Figure 1B). The small fragment (a) and the large fragment (b) derived from the deleted region of short chromosome 12 were inserted inversely and directly, respectively, into 12q14 of long chromosome 12, generating BP2, BP3, and BP4 (Figure 1B).

For US1, the abnormality was an intrachromosomal insertion. The large fragment (19.5 Mbp, labeled c) and the small fragment (300 kbp, labeled d) derived from a region deleted from 12q13q14 are inserted inversely and directly, respectively, into the TEL locus (Figure 1C). BP5 is generated by the deleted region. BP6, BP7, and BP8 are generated by rearranged fragments c and d. (D) Sequences of BP junctions in J20 and US1. The sequences around BP junctions 1 to 8 are shown. BAC clones containing the sequence are denoted above the lines. Arrows indicate the nucleotide numbers of the BAC clones. Arrowheads indicate one of the candidate breakpoints, and gray regions indicate ambiguous sequences shared between the 2 BAC clones at the site of the breakpoint.

Effects of chromosome 12 abnormalities

Although the molecular details are different (Figure 1), the result of the chromosome 12 rearrangements is almost identical for the 2 patients, because in both cases, HMGA2 is disrupted in the 3' UTR of exon 5 (Figure 3A). No other effects of chromosome 12 rearrangement were detected for either J20 or US1. For US1, chimeric transcripts derived from TEL and HMGA2 were not detected, and TEL transcripts appeared normal both quantitatively and qualitatively (not shown).

Real-time PCR showed that relative expression of HMGA2 in bone marrow cells of both J20 and US1 was greater than normal (Figure 3B). In addition to rearranged HMGA2, both J20 and US1 have one intact HMGA2 locus (Figure 1). To determine the allelic origin of HMGA2 expression, a polymorphic region in the 5' UTR14 was analyzed. For both patients, HMGA2 expression was derived almost exclusively from the rearranged locus (Figure 3C).

Discussion

These studies showed, in PIGA-mutant cells of 2 patients, rearrangement of chromosome 12 (Figures 1-2) that resulted in ectopic expression of HMGA2 (Figure 3). The findings identify for the first time a molecular mechanism for clonal expansion of hematopoiesis in PNH.

HMGA2 is a member of the high-mobility group of proteins (HMGA1a, HMGA1b, HMGA2) that function as architectural transcription factors.16-18 HMG members possess no intrinsic transcriptional activity. Instead, these nonhistone proteins orchestrate assembly of stereospecific transcriptional regulatory proteins into enhanceosomes.18,19 The cellular targets of HMGA2 are incompletely defined but appear to include cyclin A.19

Molecular studies established a causal role for HMGA2 in benign mesenchymal tumors.17,20 Rearrangement of 12q13-15 is observed in these neoplasms, but tumorigenesis does not depend on generation of chimeric proteins derived from fusion of HMGA2
with specific translocation partners. Rather, clonal expansion induced by HMGA2 appears to result from deregulated expression of a truncated version of the protein.\textsuperscript{21-23} For the 2 patients with PNH, ectopic expression appears to be a consequence of gain-of-function mutational events (Figure 3B) caused by disruption of the 3’ UTR (Figure 3A) shown to contain elements that negatively regulate HMGA2 transcription.\textsuperscript{24} This hypothesis is supported by experiments showing that HMGA2 transcripts from marrow of J20 and US1 are derived almost exclusively from the rearranged alleles (Figure 3C). Additional studies will be required to determine whether aberrant expression of HMGA2 underlies clonal expansion in patients with PNH without structural abnormalities of 12q13-15.

PNH manifests many of the characteristics of a benign tumor because there is limited expansion of PIGA mutant clones (the peripheral blood of patients is a relatively stable mosaic of normal and abnormal cells), PIGA-mutant cells respect tissue boundaries (there is no invasion of nonhematopoietic tissues). PIGA-mutant cells respond appropriately to signals that normally regulate hematopoiesis (function is not autonomous) and transformation into acute leukemia occurs rarely (PNH is not a premalignant condition).\textsuperscript{25} Our studies suggest the concept of PNH as a benign tumor of the bone marrow with aberrant expression of HMGA2 acting in concert with mutant PIGA (and the consequent deficiency of GPI-APs) to produce the proliferative phenotype that underlies clonal expansion. However, our studies neither establish the sequence of events that culminated in the clonal outgrowth of the

By the way, for personal use only on April 12, 2017, by the guest.
double mutant cells nor define how the aberrant expression of HMGA2 works additively or synergistically with mutant PIGA to produce the proliferative phenotype. This latter issue will be the subject of future studies.

Findings reported herein provide new insights into the cause of the nonmalignant clonal hematopoiesis of PNH. Together with observations of others,6,7 our studies support a 2-step process consisting of clonal immunoselection based on phenotype (ie, GPI-AP deficiency resulting from mutant PIGA) and clonal expansion as a consequence of a second somatic mutation that bestows the proliferative advantage. Clonal immunoselection may induce exit of PIGA-mutant stem cells from a dormant state,4 thereby favoring acquisition of the mutation that underlies clonal expansion. But the benign nature of PNH suggests that genes involved in clonal expansion of PIGA-mutant stem cells are different from those that underlie malignant clonal diseases such as acute leukemia. Characterizing the molecular basis of benign clonal hematopoiesis is important not only for understanding the pathobiology of PNH but also for developing novel strategies for treatment of bone marrow failure and for enhancing stem cell function for both transplantation and gene therapy.

Acknowledgments

We thank Andrew Zinn (The University of Texas Southwestern Medical School at Dallas) and Kiran Chada (Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey) for helpful discussions, and Kiyoh Kawata, Fumiko Ishii-Mori, and Keiko Kinoshita for technical assistance.

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Ministry of Health, Labour, and Welfare of Japan (T.K. and N.I.); the Osaka Medical Research Foundation for Incurable Diseases (N.I.); the Mochida Memorial Foundation for Medical Pharmaceutical Research (N.I.); the Japan Health Sciences Foundation (J.-I.N.); and the National Institutes of Health (grant K23 RR020043) (G.M. and C.J.P.).

Authorship

Contribution: N.I. designed research, performed research, and wrote the paper; T.I.-S. designed and performed research; Y.M., Y.E., and J.-I.N. performed research; K.K. contributed bioinformatics expertise; M.K., H.S., T.M., and Y.K. collected data; G.M. performed research; C.W. designed research; Z.C. performed research; W.B. and D.F.-L. provided essential material; and C.J.P. and T.K. designed research and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

N.I. and T.I.-S. contributed equally to this study.

Correspondence: Taroh Kinoshita, Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan; e-mail: tkinosh@biken.osaka-u.ac.jp.

References

Molecular basis of clonal expansion of hematopoiesis in 2 patients with paroxysmal nocturnal hemoglobinuria (PNH)