Acknowledgments The authors would like to thank R. Oumi and T. Tanaka of Cellular Transplantation Biology of Kanazawa University who provided some technical assistance and all of the BMF study groups who provided sera of patients to this study. This work was supported by a grant from Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Technology, Sports and Culture of Japan (KAKENHI No. 21390291) and grants from the Research Committee for Idiopathic Hematopoietic Disorders, the Ministry of Health, Labor, and Welfare, Japan.

References

1. Rosenfeld SJ, Kimball J, Vining D, Young NS (1995) Intensive immunosuppression with antithymocyte globulin and cyclosporine as treatment for severe acquired aplastic anemia. Blood 85 (11):3058–3065
2. Bacigalupo A, Broccia G, Corda G, Arcese W, Carotenuto M, Gallamini A, Locatelli F, Mori PG, Saracco P, Todeschini G, Coser P, Iacopino P, Vanlint MT, Gluckman E (1995) Antilymphocyte globulin, cyclosporin, and granulocyte colony-stimulating factor in patients with acquired severe aplastic anemia (SAA): a pilot study of the EBMT SAA Working Party. Blood 85(5):1348–1353
3. Maciejewski JP, Hibbs JR, Anderson S, Katevas P, Young NS (1994) Bone marrow and peripheral blood lymphocyte phenotype in patients with bone marrow failure. Exp Hematol 22(11):1102–1110
4. Nakao S, Yamaguchi M, Shiobara S, Yokoi T, Miyawaki T, Taniguchi T, Matsuda T (1992) Interferon-gamma gene expression in unstimulated bone marrow mononuclear cells predicts a good response to cyclosporine therapy in aplastic anemia. Blood 79 (10):2532–2535
5. Sloand E, Kim S, Maciejewski JP, Tisdale J, Follmann D, Young NS (2002) Intracellular interferon-gamma in circulating and marrow T cells detected by flow cytometry and the response to immunosuppressive therapy in patients with aplastic anemia. Blood 100(4):1185–1191
6. Takami A, Nakao S, Tatsumi Y, Wang HB, Zeng WH, Yamazaki H, Yasue S, Shiobara S, Matsuda T, Mizoguchi H (1999) High inducibility of heat shock protein 72 (hsp72) in peripheral blood mononuclear cells of aplastic anaemia patients: a reliable marker of immune-mediated aplastic anaemia responsive to cyclosporine therapy. Br J Haematol 106(2):377–384
7. Nakao S, Takamatsu H, Chuhjo T, Ueda M, Shiobara S, Matsuda T, Kaneshige T, Mizoguchi H (1994) Identification of a specific HLA class II haplotype strongly associated with susceptibility to cyclosporine-dependent aplastic anemia. Blood 84(12):4257–4261
8. Sugimori C, Chuhjo T, Feng XM, Yamazaki H, Takami A, Teramura M, Mizoguchi H, Omine M, Nakao S (2006) Minor population of CD55(-)CD59(-) blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia. Blood 107(4):1308–1314. doi:10.1182/blood-2005-06-2485
9. Sugimori C, Yamazaki H, Feng XM, Mochizuki K, Kondo Y, Takami A, Chuhjo T, Kimura A, Teramura M, Mizoguchi H, Omine M, Nakao S (2007) Roles of DRB1*1501 and DRB1*1502 in the pathogenesis of aplastic anemia. Exp Hematol 35(1):13–20. doi:10.1016/j.exphem.2006.09.002
10. Berger T, Rubner P, Schautzer F, Egg R, Ulmer H, Mayringer I, Dilitz E, Deisenhammer F, Reindl M (2003) Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. N Engl J Med 349(2):139–145
11. Ronkainen MS, Harkonen T, Perheentupa J, Knip M (2005) Characterization of the humoral immune response to glutamic acid decarboxylase in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) and/or type 1 diabetes. Eur J Endocrinol 153(6):901–906. doi:10.1530/eje.1.02026
12. Feng XM, Chuhjo T, Sugimori C, Kotani T, Lu XZ, Takami A, Takamatsu H, Yamazaki H, Nakao S (2004) Diazepam-binding inhibitor-related protein 1: a candidate autoantigen in acquired aplastic anemia patients harboring a minor population of paroxysmal nocturnal hemoglobinuria-type cells. Blood 104(8):2425–2431. doi:10.1182/blood-2004-05-1839
13. Takamatsu H, Feng XM, Chuhjo T, Lu XZ, Sugimori C, Okawa K, Yamamoto M, Iseki S, Nakao S (2007) Specific antibodies to moesin, a membrane-cytoskeleton linker protein, are frequently detected in patients with acquired aplastic anemia. Blood 109 (6):2514–2520. doi:10.1182/blood-2006-07-036715
14. Nyman TA, Rosengren A, Syyrakki S, Pellinen TP, Rautajoki K, Lahesmaa R (2001) A proteome database of human primary T helper cells. Electrophoresis 22(20):4375–4382
15. Jensen ON, Podtelejnikov A, Mann M (1996) Delayed extraction improves specificity in database searches by matrix-assisted laser desorption/ionization peptide maps. Rapid Commun Mass Spectrom 10(11):1371–1378
16. Yates JR (1998) Mass spectrometry and the age of the proteome. J Mass Spectrom 33(1):1–19
17. Camitta BM (2000) What is the definition of cure for aplastic anemia? Acta Haematol 103(1):16–18
18. Caporali R, Bugatti S, Bruschi E, Cavagna L, Montecucco C (2005) Autoantibodies to heterogeneous nuclear ribonucleoproteins. Autoimmunity 38(1):25–32. doi:10.1080/08916930400022590
19. Hassfeld W, Steiner G, Hartmuth K, Kolarz G, Scherak O, Graninger W, Thumb N, Smolen JS (1989) Demonstration of a new antinuclear antibody (anti-RA33) that is highly specific for rheumatoid arthritis. Arthritis Rheum 32(12):1515–1520
20. GA DA, Vretou E, Sekeris CE (1988) Autoantibodies to the core proteins of hnRNPs. FEBS Lett 231(1):118–124
21. KE JL, Wilson SH, Steinberg AD, Klinman DM (1988) Antibodies from patients and mice with autoimmune diseases react with recombinant hnRNP core protein A1. J Autoimmun 1(1):73–83
22. Montecucco C, Caporali R, Negri C, de Gennaro F, Cerino A, Bestagno M, Cobianchi F, Astaldi-Ricotti GC (1990) Antibodies from patients with rheumatoid arthritis and systemic lupus erythematosus recognize different epitopes of a single heterogeneous nuclear RNP core protein. Possible role of cross-reacting antikeratin antibodies. Arthritis Rheum 33(2):180–186
23. Steiner G, Hartmuth K, Skriner K, Maurerfogy I, Sinski A, Thalmann E, Hassfeld W, Barta A, Smolen JS (1992) Purification and partial sequencing of the nuclear autoantigen RA33 shows that it is indistinguishable from the A2 protein of the heterogeneous nuclear ribonucleoprotein complex. J Clin Investig 90(3):1061–1066
24. Hassfeld W, Steiner G, Studnickabenke A, Skriner K, Graninger W, Fischer I, Smolen JS (1995) Autoimmune response to the spliceosome. An immunologic link between rheumatoid arthritis, mixed connective tissue disease, and systemic lupus erythematosus. Arthritis Rheum 38(6):777–785
25. Jones DA, Yawalkar N, Suh KY, Sadat S, Rich B, Kupper TS (2004) Identification of autoantigens in psoriatic plaques using expression cloning. J Investig Dermatol 123(1):93–100. doi:10.1111/j.0022-202X.2004.22709.x
26. Bomsztyk K, VanSeuningen I, Suzuki H, Denisenko O, Ostrowski J (1997) Diverse molecular interactions of the hnRNP K protein. FEBS Lett 403(2):113–115
27. Bomsztyk K, Denisenko O, Ostrowski J (2004) HnRNP K: One protein multiple processes. Bioessays 26(6):629–638. doi:10.1002/bies.20048
28. Mandal M, Vadlamudi R, Nguyen D, Wang RA, Costa L, Bagheri-Yarmand R, Mendelsohn J, Kumar R (2001) Growth factors regulate heterogeneous nuclear ribonucleoprotein K expression and function. J Biol Chem 276(13):9699–9704

29. Li C, Hong Y, Tan YX, Zhou H, Ai JH, Li SJ, Zhang L, Xia QC, Wu JR, Wang HY, Zeng R (2004) Accurate qualitative and quantitative proteomic analysis of clinical hepatocellular carcinoma using laser capture microdissection coupled with isotope-coded affinity tag and two-dimensional liquid chromatography mass spectrometry. Mol Cell Proteomics 3(4):399–409. doi:10.1074/mcp.M300133-MCP200
30. Hatakeyama H, Kondo T, Fujii K, Nakanishi Y, Kato H, Fukuda S, Hirohashi S (2006) Protein clusters associated with carcinogenesis, histological differentiation and nodal metastasis in esophageal cancer. Proteomics 6(23):6300–6316. doi:10.1002/pmic.200600488
31. Klimek-Tomczak K, Mikula M, Dzwonek A, Paziewska A, Karczmarski J, Hennig E, Bujnicki JM, Bragoszewski P, Denisenko O, Bomsztyk K, Ostrowski J (2006) Editing of hnRNP K protein mRNA in colorectal adenocarcinoma and surrounding mucosa. Br J Cancer 94(4):586–592. doi:10.1038/sj.bjc.6602938
32. Notari M, Neviani P, Santhanam R, Blaser BW, Chang JS, Galietta A, Willis AE, Roy DC, Caligiuri MA, Marcucci G, Perrotti D (2006) A MAPK/HNRPK pathway controls BCR/ABL oncogenic potential by regulating MYC mRNA translation. Blood 107(6):2507–2516. doi:10.1182/blood-2005.09.3732
33. Valai A, Belisova A, Hayer S, Hoefler E, Steiner G (2004) The RNA binding domains of hnRNP K contain major autoepitopes targeted by patients with SLE and other autoimmune diseases. In: ICI/FOCISed. Abstract number 1148
34. Kaufman DW, Kelly JP, Levy M, Shapiro S (eds) (1991) The drug etiology of agranulocytosis and aplastic anemia: the international agranulocytosis and aplastic anemia study. Oxford University Press, New York
35. Wagatsuma M, Kimura M, Suzuki R, Takeuchi F, Matsuta K, Watanabe H (1996) Ezrin, radixin and moesin are possible autoimmune antigens in rheumatoid arthritis. Mol Immunol 33 (15):1171–1176
36. Hansen PB, Lauritzen AMF (2005) Aplastic anemia successfully treated with rituximab. Am J Hematol 80(4):292–294. doi:10.1002/ajh.20428
37. Castiglioni MG, Scatena P, Pandolfo C, Mechelli S, Bianchi M (2006) Rituximab therapy of severe aplastic anemia induced by fludarabine and cyclophosphamide in a patient affected by B-cell chronic lymphocytic leukemia. Leuk Lymphoma 47(9):1985–1986. doi:10.1080/10428190600709630
38. Fritsch R, Eselbock D, Skriner K, Jahn-Schmid B, Scheinecker C, Bohle B, Tohidast-Akrad M, Hayer S, Neumuller J, Pinol-Roma S, Smolen JS, Steiner G (2002) Characterization of autoreactive T cells to the autoantigens heterogeneous nuclear ribonucleoprotein A2 (RA33) and filaggrin in patients with rheumatoid arthritis. J Immunol 169(2):1068–1076

European Journal of Haematology 86 (226–236)

ORIGINAL ARTICLE

GPI-anchored protein-deficient T cells in patients with aplastic anemia and low-risk myelodysplastic syndrome: implications for the immunopathophysiology of bone marrow failure

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Abstract

Glycosylphosphatidylinositol-anchored protein-deficient (GPI-AP$^-$) T cells can be detected in some patients with bone marrow failure (BMF), but the link between these cells and BMF pathophysiology remains to be elucidated. To clarify the significance of GPI-AP$^-$ T cells in BMF, peripheral blood from 562 patients was examined for the presence of CD48$^-$CD59$^-$CD3$^+$ cells using high-resolution flow cytometry (FCM), and the GPI-AP$^-$ T cells were characterized with regard to their phenotype and sensitivity to inhibitory molecules, including herpesvirus entry mediator (HVEM) and a myelosuppressive cytokine, TGF-β. A multi-lineage FCM analysis detected CD48$^-$CD59$^-$CD3$^+$ T cells in 72 (12.8%) of the patients, together with GPI-AP$^-$ myeloid cells. Unexpectedly, 12 patients (10 with aplastic anemia and 2 with myelodysplastic syndrome-refractory anemia, 2.1%), who showed clinical features similar to those of other BMF patients with GPI-AP$^-$ myeloid cells, such as a good response to immunosuppressive therapy, displayed 0.01–0.3% GPI-AP$^-$ cells exclusively in T cells. The CD48$^-$CD59$^-$ T cells consisted of predominantly effector memory (EM) and terminal effector cells, while CD48$^-$CD59$^-$ T cells from non-BMF patients who had received anti-CD52 antibody only showed EM and central memory phenotypes. TGF-β and HVEM capable of inhibiting T-cell proliferation via its GPI-AP CD160 ligation suppressed the *in vitro* proliferation of GPI-AP$^+$ T cells more potently than that of GPI-AP$^-$ T cells from the same patients. The presence of GPI-AP$^-$ T cells, as well as GPI-AP$^-$ myeloid cells, may therefore reflect the immunopathophysiology of BMF in which cytokine-mediated suppression of hematopoietic stem cells via GPI-AP-type receptors takes place.

Key words aplastic anemia; myelodysplastic syndrome; paroxysmal nocturnal hemoglobinuria; GPI-anchored protein-deficient T cells

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Accepted for publication 16 December 2010

doi:10.1111/j.1600-0609.2010.01563.x

Small populations of glycosylphosphatidylinositol-anchored protein-deficient (GPI-AP$^-$) blood cells are often detectable in the peripheral blood (PB) of patients with aplastic anemia (AA) and low-risk myelodysplastic syndrome (MDS) such as refractory anemia (RA) and refractory cytopenia with multilineage dysplasia (1–6). Although such GPI-AP$^-$ blood cells often comprise $<1\%$ of granulocytes or erythrocytes, they are thought to be derived from hematopoietic stem cells (HSCs) with a *PIGA* mutation rather than committed progenitor cells because GPI-AP$^-$ granulocytes persists for many years, maintaining their individual scattergram profiles (7). Several studies have identified the presence of small populations of GPI-AP$^-$ cells as a significant factor predicting a good response to immunosuppressive therapy (IST) in patients with AA and low-risk MDS (4–6, 8–10).

Immune mechanisms are therefore thought to be involved in the increase in the GPI-AP$^-$ cells in such bone marrow failure (BMF), though the exact mechanisms responsible for the increase in the GPI-AP$^-$ cells remain unknown.

The most widely accepted mechanism for clonal expansion of GPI-AP$^-$ cells in patients with BMF is the 'escape hypothesis', which states that the relative number of *PIGA* mutant HSCs increases by avoiding immunologic attacks by T cells or NK cells (11–17). Consistent with the escape hypothesis, GPI-AP deficient cells are usually detectable in many lineages of cells, including monocytes, lymphocytes and NK cells, in addition to granulocytes and erythrocytes, in patients with classical paroxysmal nocturnal hemoglobinuria (PNH) (18–21). However, the screening of multi-lineage PB cells from patients with BMF for the presence of GPI-AP$^-$ cells using high-sensitivity flow cytometry unexpectedly revealed a few patients who showed GPI-AP$^-$ cells only in T cells (unpublished observation). The presence of GPI-AP$^-$ T cells alone appeared to contradict the escape mechanism because T-cell precursors are not the target of immune system attack in BMF. Therefore, the multi-lineage analysis was extended to a larger number of patients to determine the significance of GPI-AP$^-$ T cells in patients with BMF. The phenotypic and functional analyses of such GPI-AP$^-$ T cells provided evidence that, just like GPI-AP$^-$ myeloid cells, GPI-AP$^-$ T cells reflect the immunopathophysiology of BMF, in which the cytokine-mediated suppression of HSCs via GPI-AP-type receptors takes place.

Patients and methods

Patients and healthy volunteers

The PBs of 562 patients with various types of cytopenias were examined for the presence of GPI-AP$^-$ cells using high-sensitivity flow cytometry. Their diagnoses included classic PNH in 13, AA in 348, and MDS-RA in 201. The subgroups of MDS were defined according to the FAB classification (22). The male-to-female ratio was 1 : 1.2 (255 : 307), and the median age was 56 yr (range: 1–95 yr). PB samples from three patients (one with acute myelogenous leukemia and two with AA) who were conditioned with alemtuzumab (Campath1-H), a humanized monoclonal antibody (mAb) specific to CD52, for allogeneic stem cell transplantation as well as from 57 healthy individuals were also examined for the presence of GPI-AP$^-$ cells in all lineages of cells. All patients and healthy individuals provided their informed consent before sampling. This study protocol was approved by the ethics committee of Kanazawa University Graduate School of Medical Science.

Monoclonal antibodies (mAbs)

mAbs used for multicolor flow cytometry were anti-CD59 labeled with FITC (P282E, IgG2a; Beckman Coulter, Miami, FL, USA), anti-CD59 labeled with PE (H19, IgG2a; BD Pharmingen, San Diego, CA, USA), anti-CD55 labeled with FITC (IA10, IgG2a; BD Pharmingen), anti-CD48 labeled with FITC (J4-57, IgG1; Beckman Coulter), anti-CD48 labeled with PE (156-4H9, IgG1; eBioscience, San Diego, CA, USA), anti-CD33 labeled with APC (D3HL60.251, IgG1; Beckman Coulter), anti-CD19 labeled with APC-Cy7 (SJ25C1, IgG1; BD Pharmingen), anti-CD335 labeled with PE (BAB281, IgG1; Beckman Coulter), anti-CD3 labeled with PerCP-Cy5.5 (SK7, IgG1; BD Pharmingen), anti-CD3 labeled with APC (UCHT1, IgG1; Beckman Coulter), anti-CD11b/Mac-1 labeled with PE (ICRF44, IgG1; BD Pharmingen), anti-glycophorin A labeled with PE (JC159, IgG1; Dako, Carpenteria, CA, USA). Phenotypic analysis of GPI-AP-deficient CD3$^+$ T lymphocyte was carried out by additional staining with mAbs specific to CD45RA labeled with PE (HI100, IgG2b; BD Pharmingen), CD62L labeled with APC (DREG-56, IgG1; BD Pharmingen), CD197 labeled with PE-Cy7 (3D12, IgG2a; BD Pharmingen), CD4 labeled with APC-Cy7 (RPA-T4, IgG1; BD Pharmingen), CD8 labeled with APC-Cy7 (SK1, IgG1; BD Pharmingen).

Flow cytometry for detecting GPI-AP$^-$ cells and determining GPI-AP$^-$ T-cell phenotype

Six lineages of blood cells including granulocytes, erythrocytes, monocytes, T cells, B cells and NK cells were subjected to high-sensitivity flow cytometry for detecting small populations of GPI-AP$^-$ cells. All blood samples were analysed within 24 h to avoid false-positive results because of cell damages. The staining with the each mAb in this study was performed according to the well-established lyse-stain protocol, previously described in detail (6, 23). Briefly, 3–5 mL of heparinized blood was drawn from the patients and healthy individuals. Erythrocytes were lysed in the lysis buffer containing NH_4Cl 8.26 g/L, $KHCO_3$ 1.0 g/L, and EDTA · E4Na· 0.037 g/L to detect GPI-AP$^-$ leukocytes. After washing with saline, 50 μL of the leukocyte suspension was incubated with FITC-labeled anti-CD55 and anti-CD59 mAbs for granulocytes or FITC-labeled anti-CD48 and anti-CD59 mAbs for monocytes, T cells, B cells and NK cells in combination with mAbs specific for lineage markers including PE-labeled CD11b for granulocytes, APC-labeled CD33 for monocytes, PerCP-Cy5.5-labeled CD3 for T cells, APC-Cy7-labeled CD19 for B cells and PE-labeled CD335 for NK cells. Fresh

blood was diluted to 3% in phosphate-buffered saline (PBS), and then 50 μL was incubated with PE-labeled anti-glycophorin A and FITC-labeled anti-CD55 and anti-CD59 mAbs on ice for 30 min to detect GPI-AP$^-$ erythrocytes. Three-step gating excluded the debris and immature granulocytes that are frequently found in samples from patients with MDS. Step 1 involved the gating of granulocyte, lymphocyte or monocyte populations from the FSC–SSC scattergrams (R1). Step 2 involved the gating of the lineage marker^{bright} population on the lineage marker-SSC scattergram to exclude the lineage marker^{dim} cells that are features of either damaged cells or immature cells. Step 3 was the gating of R1 × R2 and the analysis of 10^6 cells on R1 × R2 scattergrams. The presence of ≥0.005% CD55$^-$CD59$^-$GP-A$^+$ erythrocytes, ≥0.003% CD55$^-$CD59$^-$CD11b$^+$ granulocytes, and ≥0.01% CD55$^-$CD59$^-$ CD33$^+$ monocytes, CD48$^-$CD59$^-$ CD3$^+$ T cells, CD48$^-$CD59$^-$CD19$^+$ B cells and CD48$^-$CD59$^-$ CD335$^+$ NK cells was defined as an abnormal increase (positive) based on the results obtained from 57 healthy individuals (6). When GPI-AP$^-$ cells were detected in only one lineage of cells or the percentages of GPI-AP$^-$ cells were <0.01%, then additional samples were tested, and the patients were judged to be PNH$^+$ when the examination results of the first and second samples were identical.

The phenotype of GPI-AP-deficient-CD3$^+$ T lymphocyte was determined using anti-CD45RA, anti-CD62L and anti-CCR7 mAbs and the percentages of four different T-cell subsets including naïve (CD45RA$^+$CD62L$^+$CCR7$^+$), central memory (CM) (CD45RA$^-$CD62L$^+$CCR7$^+$), effector memory (EM) (CD45RA$^-$CD62L$^-$CCR7$^-$), and terminal effector memory (TEM) (CD45RA$^+$CD62L$^-$ CCR7$^-$) cells were determined according to the methods defined by previous reports (24, 25).

Data acquisition was performed immediately after sampling using FACSCanto II, and the data were analysed using the FACSDiva software program and percentage of each population was calculated by FlowJo software 7.6.1 (Treestar, Ashland, OR, USA).

T cell culture

PB mononuclear cells (PBMCs) were isolated using density gradient centrifugation on Ficoll/Hypaque (Fresenius Kabi Norge AS, Halden, Norway). A sample of 1×10^6 PBMCs were cultured in RPMI1640 containing 10 μg/mL phytohemagglutinin (Sigma, St. Louis, MO), 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin and 100 IU/mL IL-2 for 7 d. After washing with RPMI1640, the cultured cells were subjected to cell sorting in order to analyse the *PIGA* gene as described in the following paragraphs.

Cell sorting and *PIGA* gene analysis

CD48$^-$CD59$^-$CD3$^+$ freshly isolated or cultured T cells were separated from CD3$^+$ T cells with a cell sorter (JSAN; Bay Bioscience, Kobe, Japan). More than 95% of the sorted cells were GPI-AP deficient. An analysis of the *PIGA* gene mutation was performed as described previously (26). Briefly, the coding regions of *PIGA* were amplified by nested or semi-nested PCR using 12 primer sets, and six ligation reactions were used to transform competent *Escherichia coli* JM109 cells (Nippon Gene, Tokyo, Japan). Five clones were selected randomly from each group of transfectants and subjected to sequencing with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, San Diego, CA, USA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Preparation of CD3$^+$ T cells and mAb-coated latex beads

CD3$^+$ T cells were purified from freshly isolated PBMCs by depleting non-CD3$^+$ T cells with magnetic beads using Pan T cell isolation II kit II (Miltenyi, Bergisch Gladbach, Germany); purity was judged to be over 95% by flow cytometry. Latex beads (Miltenyi) were coated with various concentration of anti-CD3 (OKT3; Miltenyi) and anti-CD28 mAbs (15E8; Miltenyi), or various concentrations of human herpesvirus entry mediator (HVEM)-Ig (mIgG1 Fc; 100-330; R&D Systems, Minneapolis, MN, USA) or mouse IgG1 (27). The mixture of latex beads were suspended in PBS and incubated for 2 h at 37°C in humid air containing 5% CO_2. The latex beads were washed once with RPMI1640 medium containing 10% FBS for 30 min at 37°C. The beads were then washed three times with PBS and thereafter were used for T-cell stimulation.

Carboxyfluorescein diacetate succinimidyl diester (CFSE) assay

CD3$^+$ T cells were washed twice with PBS and were suspended in PBS at the concentration of 5×10^6 cells/mL. One milliliter of the cell suspension was mixed with an equal volume of PBS containing 1 μM CFSE (Invitrogen, Carlsbad, CA, USA), and incubated for 10 min in a humidified atmosphere containing 5% CO_2 at 37°C with occasional mixing. Labeling was quenched by the additional of an equal volume of cold. FBS, and incubated for 5 min on ice. The cells were then centrifuged and washed three times in PBS containing 1% bovine serum albumin followed by two washes with RPMI1640 containing 5% autologous serum. The cells were plated at a density of 1×10^6 cells/mL in 96-well U-bottomed plates and were incubated in the presence of anti-CD3 mAb-coated and anti-CD28 mAb-coated beads with or

without HVEM fusion protein or TGF-β (Peprotech, Rocky Hill, NJ, USA) at various concentrations. The CFSE levels in the cultured T cells were then determined 10 d later by flow cytometry. The inhibitory effects of HVEM or TGF-β on the T-cell proliferation was assessed by comparing the mean percentage of cells that underwent cell division in the presence of the inhibitory molecules with that of the control culture.

Statistical analysis

The differences in the inhibition of the decline in the CFSE level by HVEM or TGF-β between GPI-AP$^+$ and GPI-AP$^-$ T cells of individual patients were assessed by the Student's *t*-test.

Results

GPI-AP$^-$ T cells in patients with BMF

Significant populations of GPI-AP$^-$ cells were detectable in at least one lineage of cells from 252 (44.8%) of 562 patients with BMF and CD48$^-$CD59$^-$CD3$^+$ T cells were detected in 72 (12.8%) of the patients. Clone sizes of GPI-AP$^-$ cells in different lineages of cells in patients with increased GPI-AP$^-$ cells are summarized in Table 1. The GPI-AP$^-$ cells were also detected in two or more lineages of cells including granulocytes or monocytes in 60 of the GPI-AP$^-$ T cell$^+$ patients (Fig. 1A–C). However, the remaining 12 (2.1%) patients showed GPI-AP$^-$ cells only in T cells (Fig. 1D). The similar percentages (0.01–0.3%) of GPI-AP$^-$ T cells were detectable in different samples obtained from the patients at intervals of 2–6 months (Fig. 2). Such GPI-AP$^-$ T cells >0.01% were undetectable in any of 57 healthy individuals and the other 490 patients with BMF. The clinical characteristics of the 12 patients who were provisionally referred to as 'PNH-T$^+$ patients' are summarized in Table 2. All these patients had predominant thrombocytopenia without any increase in the number of BM megakaryocytes, a common feature of BMF patients possessing small populations of GPI-AP$^-$ cells (7). Five patients (patients 1, 2, 3, 9, and 11) were red blood cell or platelet transfusion-dependent, and only patient 11 had been treated with IST (ATG) before the detection of GPI-AP$^-$ T cells. Three of the PNH-T$^+$ patients received IST (ATG + cyclosporine for patients 2 and 9, and cyclosporine alone for patient 12) after the GPI-AP$^-$ cell screening. All achieved a partial remission according to the response criteria described by Camitta (28) as described previously.

Phenotype of GPI-AP$^-$ T cells detected in patients with BMF

The functional phenotypes of GPI-AP$^-$ T cells in nine PNH-T$^+$ patients, defined by the expression of CD45RA, CD62L, and CCR7 were compared to those of GPI-AP$^-$ T cells detectable in three BM transplant recipients who were conditioned with alemtuzumab (group 1) or to those detectable in 12 patients who displayed GPI-AP$^-$ cells in all lineages of blood cells including GPI-AP$^-$ T cells that account for 0.02–41.2% of total T cells (group 2). As shown in Fig. 3A, the GPI-AP$^-$ T cells in three patients from group 1 predominantly showed EM (CD45RA$^-$CD62L$^-$CCR7$^-$, EM) and CM (CD45RA$^-$CD62L$^+$CCR7$^+$, CM) phenotypes. No naïve (CD45RA$^+$CD62L$^+$CCR7$^+$) T-cell subset was observed in this group. On the other hand, the T cells from group 2 patients mainly contained cells with the naïve phenotype with relatively small percentages of CM, EM, and TEM subsets (Fig. 3B). GPI-AP$^-$ T cells in PNH-T$^+$ (group 3) patients predominantly showed the EM phenotype with smaller percentages of naïve, CM, and TEM phenotypes (Fig. 3C), suggesting the phenotypic pattern of GPI-AP$^-$ T cells in group 3 patients to be more similar to that in group 2 patients than that in non-BMF patients treated with alemtuzumab.

Table 1 Clone size of GPI-AP$^-$ cells in different lineages of cells in patients with increased GPI-AP$^-$ cells

	PNH	AA		MDS-RA	
	Median % of GPI-AP$^-$ cells (range)	% of patients with GPI-AP$^-$ cells in all AA patients	Median % of GPI-AP$^-$ cells in AA patients with increased GPI-AP$^-$ cells (range)	% of patients with GPI-AP$^-$ cells in all RA patients	Median % of GPI-AP$^-$ cells in RA patients with increased GPI-AP$^-$ cells (range)
E	25.8 (3.8–95.6)	58.4	0.04 (0.005–48.7)	53.3	0.55 (0.005–6.4)
G	56.5 (1.2–98.1)	64.4	0.07 (0.003–37.9)	57.8	1.07 (0.003–17.4)
M	82.6 (6.1–94.2)	48.4	0.11 (0.01–82.0)	46.7	3.5 (0.01–32.2)
T	0.9 (0.01–41.2)	16.8	0.44 (0.01–6.9)	9.4	0.23 (0.01–6.6)
B	4.3 (0.8–38.0)	16.0	0.01 (0.01–13.0)	12.2	0.24 (0.01–5.1)
NK	41.7 (0.6–94.9)	15.2	0.01 (0.01–75.0)	14.4	0.55 (0.01–8.5)

E, erythrocytes; G, granulocytes; M, monocytes; T, T cells; B, B cells; NK, NK cells; PNH, paroxysmal nocturnal hemoglobinuria; AA, aplastic anemia; MDS-RA, myelodysplastic syndrome-refractory anemia; GPI-AP, glycosylphosphatidylinositol-anchored protein.

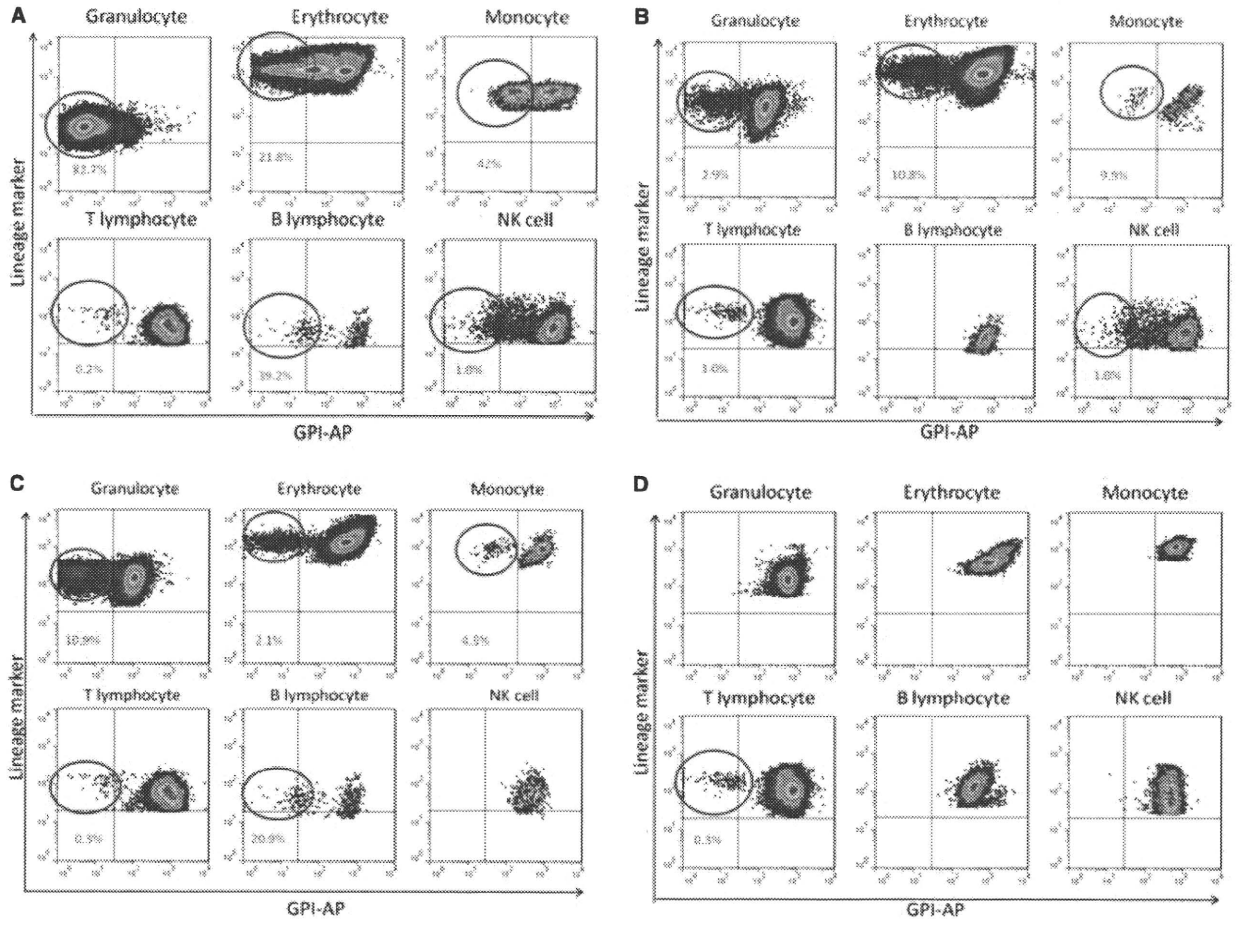

Figure 1 GPI-AP$^-$ cells in different lineages of cells. Scattergrams from four patients displaying GPI-AP$^-$ T cells are shown. (A) A patient with GPI-AP$^-$ cells in all six lineages of cells. (B) A patient with GPI-AP$^-$ cells in the granulocytes, erythrocytes, monocytes, T cells, and NK cells. (C) A patient with GPI-AP$^-$ cells in the granulocytes, erythrocytes, monocytes, T cells, and B cells. (D) A patient (patient 3) with GPI-AP$^-$ cells in T cells alone. GPI-AP, glycosylphosphatidylinositol-anchored protein.

PIGA gene analyses of GPI-AP$^-$ T cells

GPI-AP$^-$ T cells were sorted from cultured T cells from four patients (two group 1 and two group 2 patients) and were subjected to *PIGA* gene analyses. Missense mutations in exon 2 leading to a frameshift or an amino acid change were detected. These included a 16-bp deletion starting from the position of 1107 bp (frameshift), adenine insertion at the position of 1239 bp (frameshift) in group 2, the replacement of adenine (A) with guanine at 1108 bp (Arg → Gly) and 2002 bp (Gln → Arg) in a group 1 patient and the replacement of cytosine (C) with thymine (T) at 1044 bp (frameshift) and TCA insertion at 1103 bp (frameshift) in another group 1 patient.

Differences in the sensitivity to inhibitory molecules between GPI-AP$^+$ and GPI-AP$^-$ T cells

The presence of PNH-T$^+$ patients with BMF suggests that the lack of GPI-APs may confer a growth advantage to GPI-AP$^-$ T-cell precursors or memory T cells over the GPI-AP$^+$ counterparts. To test this hypothesis, T cells from three patients with BMF PNH displaying 1% and 41.2% GPI-AP$^-$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs and the effects of HVEM, a ligand of a GPI-AP CD160 which inhibits T-cell proliferation upon ligation to HVEM (27), on the proliferation of GPI-AP$^-$ and GPI-AP$^+$ T cells were investigated using the CFSE assay. Figure 4 shows the results from one of the three patients which produced similar results. CD160 expression was induced on GPI-AP$^+$ T cells by anti-CD3 and anti-CD28 mAbs in a dose-dependent fashion, while the antigen was not induced on GPI-AP$^-$ T cells (Fig. 4A). Both the GPI-AP$^-$ and GPI-AP$^+$ T cells proliferated in response to anti-CD3 and anti-CD28 mAbs (Fig. 4B,C), though GPI-AP$^-$ T cells tended to show greater proliferation than GPI-AP$^+$ T cells in keeping with previous reports (29, 30). The addition of HVEM-IgG inhibited the decline in the CFSE level of GPI-AP$^+$ T cells more

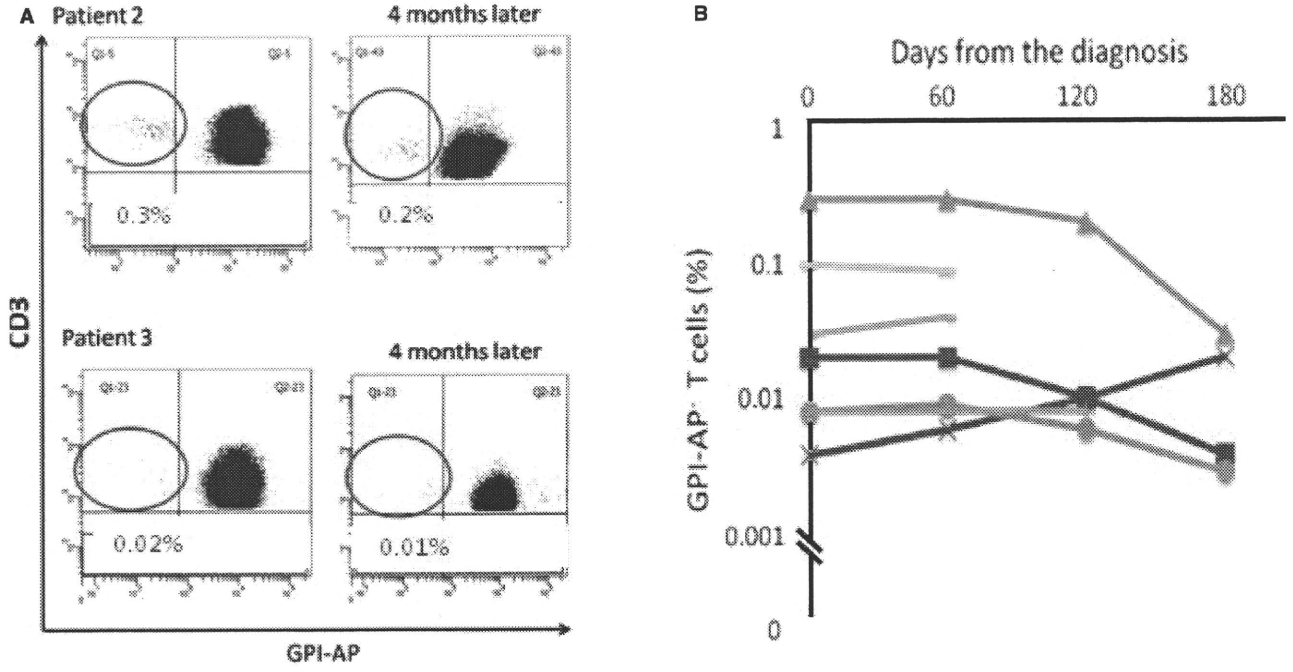

Figure 2 Changes in the percentage of GPI-AP$^-$ cells over time. (A) Scattergrams of samples obtained at 4 month internals from two PNH-T$^+$ patients are shown. (B) PB samples were obtained from the PNH-T$^+$ patients at 2-month intervals and the percentages of GPI-AP$^-$ T cells were serially determined. PNH, paroxysmal nocturnal hemoglobinuria; PB, peripheral blood; GPI-AP, glycosylphosphatidylinositol-anchored protein.

Table 2 Hematologic parameters of patients with GPI-AP$^-$ T cells alone

Patient no.	Diagnosis	Duration of illness (months)	Age at diagnosis (yr)	Gender	WBC count ($\times10^9$/L)	Neutrophil count ($\times10^9$/L)	Lymphocyte count ($\times10^9$/L)	RBC count ($\times10^{12}$/L)	Reticulocyte count ($\times10^9$/L)	Hemoglobin (g/dL)	Platelet count ($\times10^9$/L)	PNH-type T cell percentage
1	MAA	1	80	M	2.4	0.8	1.4	2.73	4.5	9.7	6.8	0.01
2	SAA	1	64	F	1.7	0.2	1.1	3.57	6.6	10.6	200	0.02
3	SAA	120	22	F	1.2	0.3	0.9	2.62	1.3	8.2	4	0.3
4	MAA	74	35	M	6.1	2.7	1.8	2.78	3.3	10.1	52	0.02
5	MAA	84	12	M	3.2	1	2	4.05	3.4	12.7	49	0.03
6	MDS-RA	4	59	M	5.7	2.9	2.7	2.06	5.6	10	53	0.02
7	SAA	12	61	F	1.2	0.4	0.6	2.52	0.8	7.6	12	0.01
8	MAA	108	83	F	2	1	0.8	3.51	3.2	9.8	64	0.03
9	MAA	49	26	F	2.6	0.9	1.2	2.22	4	7.3	0.8	0.1
10	MAA	120	59	M	2.1	0.8	1.2	1.56	2	5.9	0.9	0.03
11	MAA	1	76	M	2.7	1.1	1.5	4.03	4	12.4	8.2	0.3
12	MDS-RA	147	63	M	3.2	1.2	1.9	2.13	3.8	7.3	3	0.01

PNH, paroxysmal nocturnal hemoglobinuria; MDS-RA, myelodysplastic syndrome-refractory anemia; GPI-AP, glycosylphosphatidylinositol-anchored protein.

potently (27, 31, 32) than that of GPI-AP$^-$ T cells (Fig. 4B, 19.4 ± 8.1 vs. 0.7 ± 0.5 at 10 μg/mL, P = 0.03 and 57.5 ± 12.7 vs. 4.4 ± 4.9 at 50 μg/mL, P = 0.004). The GPI-AP$^-$ T-cell proliferation was not affected, even by a high concentration (50 μg/mL) of HVEM, which clearly inhibited GPI-AP$^+$ T-cell proliferation. Similarly, the addition of TGF-β tended to show a greater inhibition of the decline in the CFSE level of GPI-AP$^+$ T cells than that of GPI-AP$^-$ T cells (Fig. 4C, 17.4 ± 10.5 vs. 1.1 ± 0.5 at 5 ng/mL, P = 0.06 and 33.2 ± 16.2 vs. 2.1 ± 1.8 at 100 ng/mL, P = 0.05). These findings suggest that GPI-AP$^-$ CD3$^+$ T-cell precursors or memory T cells may preferentially proliferate *in vivo* in the presence of some ligands that transmit inhibitory signals for cell proliferation by their ligation to GPI-AP receptors.

Discussion

The present multi-lineage analysis of blood cells with high-sensitivity flow cytometry revealed CD48$^-$CD59$^-$ T

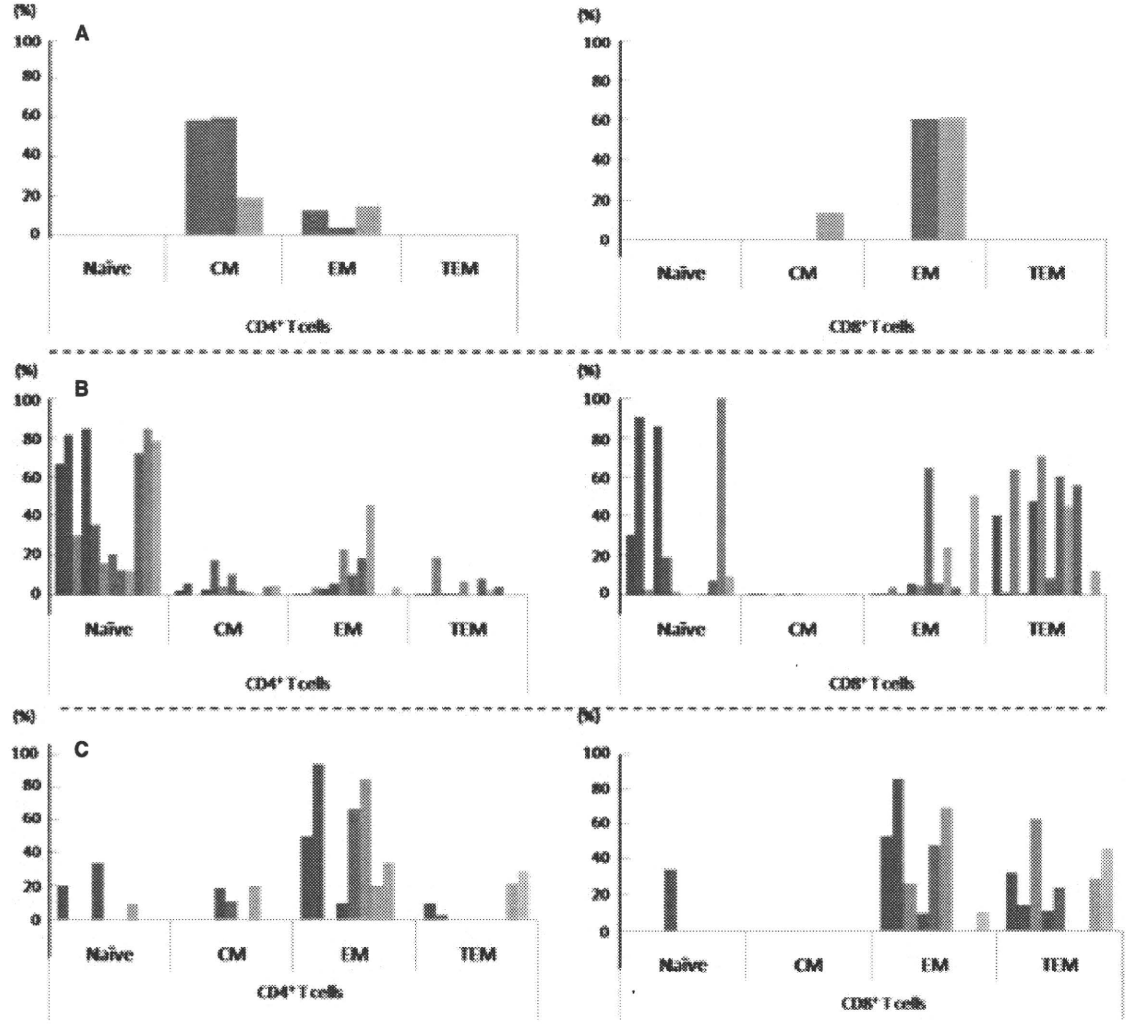

Figure 3 Phenotypic patterns of GPI-AP$^-$ T cells in different patient groups. The percentages of four different T-cell subsets defined by the expression of CD45RA, CCR7 and CD62L in CD4$^+$ and CD8$^+$ GPI-AP$^-$ T cells are shown. (A) Alemtuzumab-treated patients (n = 3); (B) bone marrow failure patients showing GPI-AP$^-$ cells in all lineages of blood cells (n = 12); (C) PNH-T$^+$ patients (n = 9). CM, central memory cells; EM, effector memory cells; TEM, terminal effector memory cells; PNH, paroxysmal nocturnal hemoglobinuria; GPI-AP, glycosylphosphatidylinositol-anchored protein.

cells in 12.8% of patients with various type of BMF. Although the percentage of GPI-AP$^-$ T cells in these patients was very low, such an increase in GPI-AP$^-$ T cells was undetectable in 57 healthy individuals and they persisted more than 2 months. The presence of GPI-AP$^-$ T cells was originally interpreted to indicate the ability of *PIGA* mutant HSC in the BMF patients to differentiate into multi-lineage blood cells (18–21). However, the GPI-AP$^-$ cells were undetectable in any other lineages of cells other than T cells in PNH-T$^+$ patients whose clinical features were similar to those of other BMF patients with GPI-AP$^-$ myeloid cells. The presence of such PNH-T$^+$ patients within the population of patients with immune-mediated BMF cannot be explained by the escape of GPI-AP$^-$ cells from T-cell attack against T-cell precursors, because T-cell precursors are not the specific target of the immune attack in patients with BMF.

The presence of PNH-T$^+$ patients can be explained by several mechanisms. One possibility is that the CD48$^-$CD59$^-$ T cells are remnants of GPI-AP$^-$ cells that used to be present in other lineages of cells. A previous study showed GPI-AP$^-$ T cells to persist in patients who underwent remission of PNH probably due to their longevity (33). The patients with long-standing disease like patients 3, 10, and 12 may have possessed small populations of GPI-AP$^-$ cells in the myeloid cells after the disease onset and lost all but the T cells with time.

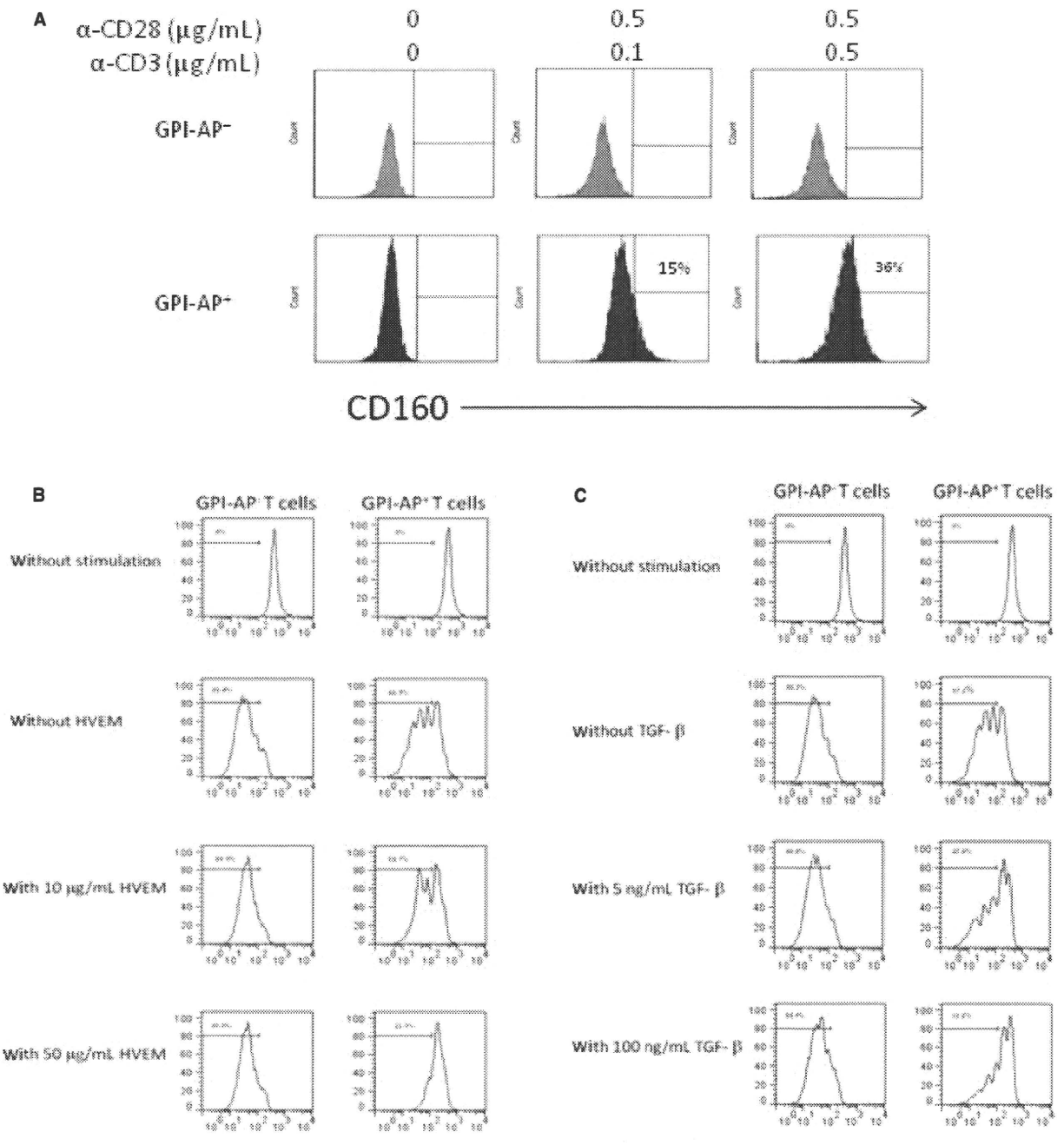

Figure 4 The effects of HVEM and TGF-β on the proliferation of GPI-AP$^+$ and GPI-AP$^-$ T cells induced by anti-CD3 and anti-CD28 mAb stimulation. PB CD3$^+$ T cells from three bone marrow failure patients were cultured in the presence of anti-CD3 and anti-CD28 mAbs for 10 d with or without HVEM and TGF-β. (A) CD160 expression by GPI-AP$^+$ T cells induced by anti-CD3 and anti-CD28 mAb stimulation compared with GPI-AP$^-$ T cells. The numbers show the percentage of CD160$^+$ cells. T-cell proliferation in the presence of different concentrations of HVEM (B) or TGF-β (C) was assessed using the carboxyfluorescein diacetate succinimidyl diester assay. The figures show representative results from one patient. The numbers denote the percentage of cells which underwent cell division. PB, peripheral blood; HVEM, herpesvirus entry mediator; GPI-AP, glycosylphosphatidylinositol-anchored protein.

However, this mechanism cannot account for PNH-T$^+$ patients in which the disease persisted for <1 yr. Another possibility is that mechanisms other than immune-mediated attack against HSCs confer proliferative advantage to GPI-AP$^-$ T-cell precursors or memory T cells. The treatment of patients with lymphoid malignancies or allogeneic stem cell transplant recipients with anti-CD52 mAb allows proliferation of GPI-AP$^-$ T cells that existed in the patients or BM donors before treatment (34, 35). Indeed, donor-derived CD48$^-$CD59$^-$

T cells were detectable in all three stem cell transplant recipients who received a conditioning regimen containing alemtuzumab in the present study. Previous studies showed auto-Abs specific to DRS-1 and moesin are frequently detected in PNH$^+$ patients (36, 37). It is thus possible that GPI-AP$^-$ T cells may be induced to proliferate by some auto-Abs specific to GPI-APs on T cells in PNH-T$^+$ patients. However, GPI-AP$^-$ T cells in alemtuzumab-treated patients showed a distinct phenotype pattern characterized by the expression of CD45RA, CCR7, and CD62L from that detectable in PNH-T$^+$ patients. There was no apparent T lymphocytopenia in PNH-T$^+$ patients which should occur in patients possessing auto-Abs specific to T cell antigens. It is therefore unlikely that CD48$^-$CD59$^-$ T cells were induced to proliferate by auto-Abs specific to GPI-APs.

The most likely explanation for the presence of PNH-T$^+$ patients is that humoral factors negatively regulating the proliferation of both HSCs and T-cell precursors via their interaction with GPI-APs are involved in the development of BMF in PNH-T$^+$ patients. Cytokine-mediated selection of *PIGA* mutant HSCs has been proposed as a mechanism for preferential proliferation of GPI-AP$^-$ cells (38), but no evidence supporting this mechanism has been shown. The present study demonstrated that GPI-AP$^-$ T cells show a decreased sensitivity to HVEM that transmit inhibitory signals through a GPI-AP receptor CD160 (27), as well as to TGF-β, a well-known inhibitor of haematopoiesis (39). Recent studies have demonstrated the presence of GPI-AP-type co-receptors for TGF-β (40). Although the T cells used in the current study were not T-cell precursors, memory T cells in the PB T cells may behave like HSCs in terms of their dormancy and activation in response to appropriate stimulation. HSCs may be rendered to express some GPI-APs capable of transmitting inhibitory signals upon activation as memory T cells express CD160 and as a result, both HSCs and T-cell precursors or memory T cells may become invulnerable to some inhibitory cytokines, such as TGF-β, because of the lack of GPI-AP type-receptors. Further analyses of T cells may therefore be useful for identifying GPI-AP type TGF-β receptors which permit the preferential proliferation of HSCs with *PIGA* mutation in patients with BMF.

Acknowledgements

The authors thank Ms. Rie Ohmi, Cellular Transplantation Biology, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science, for the excellent technical assistance. We also thank all of the institutions in Japan who contributed patients to this study.

Disclosure of conflicts of interest

All authors have no financial or personal relationships with other people or organizations that could inappropriately influence this study. The authors declare no competing financial interest.

References

1. Schubert J, Vogt HG, Zielinska-Skowronek M, Freund M, Kaltwasser JP, Hoelzer D, Schmidt RE. Development of the glycosylphosphatitylinositol-anchoring defect characteristic for paroxysmal nocturnal hemoglobinuria in patients with aplastic anemia. *Blood* 1994;**83**:2323–8.
2. Schrezenmeier H, Hertenstein B, Wagner B, Raghavachar A, Heimpel H. A pathogenetic link between aplastic anemia and paroxysmal nocturnal hemoglobinuria is suggested by a high frequency of aplastic anemia patients with a deficiency of phosphatidylinositol glycan anchored proteins. *Exp Hematol* 1995;**23**:81–7.
3. Griscelli-Bennaceur A, Gluckman E, Scrobohaci ML, Jonveaux P, Vu T, Bazarbachi A, Carosella ED, Sigaux F, Socie G. Aplastic anemia and paroxysmal nocturnal hemoglobinuria: search for a pathogenetic link. *Blood* 1995;**85**:1354–63.
4. Dunn DE, Tanawattanacharoen P, Boccuni P, Nagakura S, Green SW, Kirby MR, Kumar MS, Rosenfeld S, Young NS. Paroxysmal nocturnal hemoglobinuria cells in patients with bone marrow failure syndromes. *Ann Intern Med* 1999;**131**:401–8.
5. Wang H, Chuhjo T, Yasue S, Omine M, Nakao S. Clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria-type cells in bone marrow failure syndrome. *Blood* 2002;**100**:3897–902.
6. Sugimori C, Chuhjo T, Feng X, Yamazaki H, Takami A, Teramura M, Mizoguchi H, Omine M, Nakao S. Minor population of CD55-CD59- blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia. *Blood* 2006;**107**:1308–14.
7. Sugimori C, Mochizuki K, Qi Z, Sugimori N, Ishiyama K, Kondo Y, Yamazaki H, Takami A, Okumura H, Nakao S. Origin and fate of blood cells deficient in glycosylphosphatidylinositol-anchored protein among patients with bone marrow failure. *Br J Haematol* 2009;**147**:102–12.
8. Maciejewski JP, Follmann D, Nakamura R, Saunthararajah Y, Rivera CE, Simonis T, Brown KE, Barrett JA, Young NS. Increased frequency of HLA-DR2 in patients with paroxysmal nocturnal hemoglobinuria and the PNH/aplastic anemia syndrome. *Blood* 2001;**98**:3513–9.
9. Ishiyama K, Chuhjo T, Wang H, Yachie A, Omine M, Nakao S. Polyclonal hematopoiesis maintained in patients with bone marrow failure harboring a minor population of paroxysmal nocturnal hemoglobinuria-type cells. *Blood* 2003;**102**:1211–6.

10. Ishikawa T, Tohyama K, Nakao S, *et al.* A prospective study of cyclosporine A treatment of patients with low-risk myelodysplastic syndrome: presence of CD55 (-)CD59(-) blood cells predicts platelet response. *Int J Hematol* 2007;**86**:150–7.
11. Nakao S, Takamatsu H, Yachie A, Itoh T, Yamaguchi M, Ueda M, Shiobara S, Matsuda T. Establishment of a CD4+ T cell clone recognizing autologous hematopoietic progenitor cells from a patient with immune-mediated aplastic anemia. *Exp Hematol* 1995;**23**:433–8.
12. Karadimitris A, Manavalan JS, Thaler HT, Notaro R, Araten DJ, Nafa K, Roberts IA, Weksler ME, Luzzatto L. Abnormal T-cell repertoire is consistent with immune process underlying the pathogenesis of paroxysmal nocturnal hemoglobinuria. *Blood* 2000;**96**:2613–20.
13. Young NS, Maciejewski JP. Genetic and environmental effects in paroxysmal nocturnal hemoglobinuria: this little PIG-A goes "Why? Why? Why?" *J Clin Invest* 2000;**106**:637–41.
14. Murakami Y, Kosaka H, Maeda Y, Nishimura J, Inoue N, Ohishi K, Okabe M, Takeda J, Kinoshita T. Inefficient response of T lymphocytes to glycosylphosphatidylinositol anchor-negative cells: implications for paroxysmal nocturnal hemoglobinuria. *Blood* 2002;**100**:4116–22.
15. Gargiulo L, Lastraioli S, Cerruti G, Serra M, Loiacono F, Zupo S, Luzzatto L, Notaro R. Highly homologous T-cell receptor beta sequences support a common target for autoreactive T cells in most patients with paroxysmal nocturnal hemoglobinuria. *Blood* 2007;**109**:5036–42.
16. Rotoli B, Luzzatto L. Paroxysmal nocturnal haemoglobinuria. *Baillieres Clin Haematol* 1989;**2**:113–38.
17. Young NS. The problem of clonality in aplastic anemia: Dr Dameshek's riddle, restated. *Blood* 1992;**79**:1385–92.
18. Nicholson-Weller A, Spicer DB, Austen KF. Deficiency of the complement regulatory protein, "decay-accelerating factor," on membranes of granulocytes, monocytes, and platelets in paroxysmal nocturnal hemoglobinuria. *N Engl J Med* 1985;**312**:1091–7.
19. van der Schoot CE, Huizinga TW, van 't Veer-Korthof ET, Wijmans R, Pinkster J, von dem Borne AE. Deficiency of glycosyl-phosphatidylinositol-linked membrane glycoproteins of leukocytes in paroxysmal nocturnal hemoglobinuria, description of a new diagnostic cytofluorometric assay. *Blood* 1990;**76**:1853–9.
20. Schubert J, Alvarado M, Uciechowski P, Zielinska-Skowronek M, Freund M, Vogt H, Schmidt RE. Diagnosis of paroxysmal nocturnal haemoglobinuria using immunophenotyping of peripheral blood cells. *Br J Haematol* 1991;**79**:487–92.
21. Hall SE, Rosse WF. The use of monoclonal antibodies and flow cytometry in the diagnosis of paroxysmal nocturnal hemoglobinuria. *Blood* 1996;**87**:5332–40.
22. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Proposals for the classification of the acute leukaemias. French–American–British (FAB) co-operative group. *Br J Haematol* 1976;**33**:451–8.
23. Araten DJ, Nafa K, Pakdeesuwan K, Luzzatto L. Clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals. *Proc Natl Acad Sci USA* 1999;**96**:5209–14.
24. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999; **401**:708–12.
25. Chalasani G, Dai Z, Konieczny BT, Baddoura FK, Lakkis FG. Recall and propagation of allospecific memory T cells independent of secondary lymphoid organs. *Proc Natl Acad Sci USA* 2002;**99**:6175–80.
26. Mochizuki K, Sugimori C, Qi Z, Lu X, Takami A, Ishiyama K, Kondo Y, Yamazaki H, Okumura H, Nakao S. Expansion of donor-derived hematopoietic stem cells with PIGA mutation associated with late graft failure after allogeneic stem cell transplantation. *Blood* 2008;**112**:2160–2.
27. Cai G, Anumanthan A, Brown JA, Greenfield EA, Zhu B, Freeman GJ. CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. *Nat Immunol* 2008;**9**:176–85.
28. Camitta BM. What is the definition of cure for aplastic anemia? *Acta Haematol* 2000;**103**:16–8.
29. Chen G, Kirby M, Zeng W, Young NS, Maciejewski JP. Superior growth of glycophosphatidy linositol-anchored protein-deficient progenitor cells *in vitro* is due to the higher apoptotic rate of progenitors with normal phenotype *in vivo*. *Exp Hematol* 2002;**30**:774–82.
30. Ismail MM, Tooze JA, Flynn JA, Gordon-Smith EC, Gibson FM, Rutherford TR, Elebute MO. Differential apoptosis and Fas expression on GPI-negative and GPI-positive stem cells: a mechanism for the evolution of paroxysmal nocturnal haemoglobinuria. *Br J Haematol* 2003;**123**:545–51.
31. Classen S, Zander T, Eggle D, *et al.* Human resting CD4+ T cells are constitutively inhibited by TGF beta under steady-state conditions. *J Immunol* 2007;**178**:6931–40.
32. Das L, Levine AD. TGF-beta inhibits IL-2 production and promotes cell cycle arrest in TCR-activated effector/memory T cells in the presence of sustained TCR signal transduction. *J Immunol* 2008;**180**:1490–8.
33. Nakakuma H, Nagakura S, Kawaguchi T, Iwamoto N, Hidaka M, Horikawa K, Kagimoto T, Tsuruzaki R, Takatsuki K. Persistence of affected T lymphocytes in long-term clinical remission in paroxysmal nocturnal hemoglobinuria. *Blood* 1994;**84**:3925–8.
34. Rawstron AC, Rollinson SJ, Richards S, Short MA, English A, Morgan GJ, Hale G, Hillmen P. The PNH phenotype cells that emerge in most patients after CAMPATH-1H therapy are present prior to treatment. *Br J Haematol* 1999;**107**:148–53.
35. Garland RJ, Groves SJ, Diamanti P, *et al.* Early emergence of PNH-like T cells after allogeneic stem cell transplants utilising CAMPATH-1H for T cell depletion. *Bone Marrow Transplant* 2005;**36**:237–44.

36. Feng X, Chuhjo T, Sugimori C, Kotani T, Lu X, Takami A, Takamatsu H, Yamazaki H, Nakao S. Diazepam-binding inhibitor-related protein 1: a candidate autoantigen in acquired aplastic anemia patients harboring a minor population of paroxysmal nocturnal hemoglobinuria-type cells. *Blood* 2004;**104**:2425–31.
37. Takamatsu H, Feng X, Chuhjo T, Lu X, Sugimori C, Okawa K, Yamamoto M, Iseki S, Nakao S. Specific antibodies to moesin, a membrane-cytoskeleton linker protein, are frequently detected in patients with acquired aplastic anemia. *Blood* 2007;**109**:2514–20.
38. Parker CJ. The pathophysiology of paroxysmal nocturnal hemoglobinuria. *Exp Hematol* 2007;**35**:523–33.
39. Yamazaki S, Iwama A, Takayanagi S, Eto K, Ema H, Nakauchi H. TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. *Blood* 2009;**113**:1250–6.
40. Finnson KW, Tam BY, Liu K, Marcoux A, Lepage P, Roy S, Bizet AA, Philip A. Identification of CD109 as part of the TGF-beta receptor system in human keratinocytes. *FASEB J* 2006;**20**:1525–7.

Mutations in the ribosomal protein genes in Japanese patients with Diamond-Blackfan anemia

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Acknowledgments: the authors are grateful to all physicians of the institutions listed in the Appendix for their contribution to the present study.

Funding: this work was supported in part by a grant from the Ministry of Health, Labour and Welfare of Japan.

Manuscript received on December 3, 2009. Revised version arrived on January 2, 2010. Manuscript accepted on January 7, 2010.

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ABSTRACT

Background

Diamond-Blackfan anemia is a rare, clinically heterogeneous, congenital red cell aplasia: 40% of patients have congenital abnormalities. Recent studies have shown that in western countries, the disease is associated with heterozygous mutations in the ribosomal protein (RP) genes in about 50% of patients. There have been no studies to determine the incidence of these mutations in Asian patients with Diamond-Blackfan anemia.

Design and Methods

We screened 49 Japanese patients with Diamond-Blackfan anemia (45 probands) for mutations in the six known genes associated with Diamond-Blackfan anemia: *RPS19*, *RPS24*, *RPS17*, *RPL5*, *RPL11*, and *RPL35A*. *RPS14* was also examined due to its implied involvement in 5q- syndrome.

Results

Mutations in *RPS19*, *RPL5*, *RPL11* and *RPS17* were identified in five, four, two and one of the probands, respectively. In total, 12 (27%) of the Japanese Diamond-Blackfan anemia patients had mutations in ribosomal protein genes. No mutations were detected in *RPS14*, *RPS24* or *RPL35A*. All patients with *RPS19* and *RPL5* mutations had physical abnormalities. Remarkably, cleft palate was seen in two patients with *RPL5* mutations, and thumb anomalies were seen in six patients with an *RPS19* or *RPL5* mutation. In contrast, a small-for-date phenotype was seen in five patients without an *RPL5* mutation.

Conclusions

We observed a slightly lower frequency of mutations in the ribosomal protein genes in patients with Diamond-Blackfan anemia compared to the frequency reported in western countries. Genotype-phenotype data suggest an association between anomalies and *RPS19* mutations, and a negative association between small-for-date phenotype and *RPL5* mutations.

Key words: protein genes, Diamond-Blackfan anemia, *RPL5* mutation.

Citation: Konno Y, Toki T, Tandai S, Xu G, Wang RN, Terui K, Ohga S, Hara T, Hama A, Kojima S, Hasegawa D, Kosaka Y, Yanagisawa R, Koike K, Kanai R, Imai T, Hongo T, Park M-J, Sugita K, and Ito E. Mutations in the ribosomal protein genes in Japanese patients with Diamond-Blackfan anemia. Haematologica 2010;95(8):1293-1299. doi:10.3324/haematol.2009.020826

Introduction

Diamond-Blackfan anemia (DBA, MIM#105650) is a congenital, inherited bone marrow failure syndrome, characterized by normochromic macrocytic anemia, reticulocytopenia, and absence or insufficiency of erythroid precursors in normocellular bone marrow.[1] DBA was first reported by Josephs in 1936 and defined as a distinct clinical entity 2 years later by Diamond and Blackfan. Recent investigations have shown that the cellular defect in DBA fibroblasts is primarily caused by reduced proliferation and a prolonged cell cycle corresponding to the bone marrow characteristics of DBA.[2] DBA is a rare disease with a frequency of two to seven cases per million live births and has no ethnic or gender predilection.[1]

Approximately 90% of affected patients typically present in infancy or early childhood, although patients with a 'non-classical', mild phenotype are diagnosed later in life.[3,4] Macrocytic anemia is a prominent feature of DBA, but the disease is also characterized by growth retardation and congenital anomalies, including craniofacial, upper limb/hand, cardiac, and genitourinary malformations that are present in approximately half of the patients.[3-5] In addition, DBA patients have a predisposition to malignancies including acute myeloid leukemia, myelodysplastic syndrome, and osteogenic sarcoma.[3] The diagnosis of DBA is often difficult because incomplete phenotypes and wide variability of clinical expression are present.[4-6] The central hematopoietic defect is enhanced sensitivity of hematopoietic progenitors to apoptosis along with evidence of stress erythropoiesis, including elevations in fetal hemoglobin and mean red cell volume.[2] The majority of patients have an increase in erythrocyte adenosine deaminase activity.[7]

Proteins are universally synthesized in ribosomes. This macromolecular ribonucleoprotein machinery consists of two subunits: one small and one large. The mammalian ribosome comprises four RNA and 80 ribosomal proteins.[8] The first genetic anomaly identified in DBA involves the *RPS19* gene, which is mutated in approximately 25% of DBA patients. This gene is located at chromosome 19q13.2 and encodes a protein belonging to the small subunit of the ribosome.[9,10] Haploinsufficiency of the *RPS19* gene product has been demonstrated in a subset of cases[11] and appears to be sufficient to cause DBA. The RPS19 protein plays an important role in 18S rRNA maturation and small ribosomal subunit synthesis in human cells.[12,13] Deficiency of RPS19 leads to increased apoptosis in hematopoietic cell lines and bone marrow cells. Suppression of *RPS19* inhibits cell proliferation and early erythroid differentiation but not late erythroid maturation in RPS19-deficient DBA cell lines.[14]

Mutations in two other genes, *RPS24* and *RPS17*, encoding proteins of the small ribosomal subunits have been found in approximately 2% of patients.[15,16] Furthermore, mutations in genes encoding large ribosomal subunit-associated proteins, *RPL5*, *RPL11* and *RPL35A*, have been reported in 9% to 21.4%, 6.5% to 7.1%, and 3.3% of patients, respectively.[17-19] To date, approximately 50% of DBA patients in western countries have been found to have a single heterozygous mutation in a gene encoding a ribosomal protein.[1,3] These findings imply that DBA is a disorder of ribosome biogenesis and/or function. However, there have been no studies of the incidences of these mutations in Asian DBA patients.

In this study, we screened 49 Japanese DBA patients (45 probands) for mutations of the six known DBA genes and *RPS14*, which has been implicated in the 5q- syndrome, a subtype of myelodysplastic syndrome characterized by a defect in erythroid differentiation.[20]

Design and Methods

Patients

Forty-nine patients were studied in order to define the frequency and type of mutations of ribosomal protein genes associated with DBA in Japan. Eight patients were from families with more than one affected member, whereas 41 were from families with only one affected patient. The diagnosis of DBA was based on the criteria of normochromic, often macrocytic anemia; reticulocytopenia; a low number or lack of erythroid precursors in bone marrow; and, in some patients, congenital malformations, without known causes of single cytopenia including acquired or congenital infection, transient erythroblastopenia of childhood, metabolic disorders, malignancies, or autoimmune diseases. All clinical samples were obtained with informed consent from 28 pediatric and/or hematology departments throughout Japan. Additional information was obtained by a standardized questionnaire including information on birth history, age of onset or diagnosis, family history, physical examination (especially regarding malformations), hematologic data, response to therapeutic procedures, and prognosis. This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine.

Ribosomal protein gene analysis

DNA was extracted from peripheral blood using a standard proteinase K, phenol and chloroform protocol.[21] A polymerase chain reaction (PCR) was used to amplify fragments from genomic DNA using primer sets designed to amplify the coding exons and exon/intron boundaries of the *RPS19*, *RPS17*, *RPS24*, *RPS14*, *RPL5*, *RPL11* and *RPL35A*. PCR products were directly sequenced in the forward and/or reverse direction using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Tokyo, Japan) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). *RPS19* was analyzed by determining the genomic DNA sequence of the non-coding first exon, with flanking regions, and the 450-base pair (bp) sequence upstream of the first exon (5'UTR) for each DNA sample, as previously described.[5]

To clarify the sequence of heterozygous insertion/deletion sequence variations, the respective PCR products were cloned into a TA pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and their sequences were confirmed.

Genotype-phenotype correlations and statistical analysis

Physical abnormalities in the Japanese DBA patients were evaluated from a viewpoint of correlations with genotype. Although growth retardation can be modified by several factors such as steroid therapy, chronic anemia, and iron overload, the retardation was considered pathognomonic for DBA if it was marked, being below -3 standard deviations (SD). Response to treatment is usually seen within 1 month of treatment in DBA, but a prediction of response has not been reported previously.[1,3] We, therefore, also examined the correlation between genotype and response to the first round of steroid therapy. Associations between two groups of variables were assessed with Fisher's exact test. All tests were two-sided and P levels less than 0.05 were considered statistically significant. Data were analyzed with SPSS 11.0J software (SPSS Inc., Chicago, IL, USA).

Results

Patients' characteristics

Overall, 49 patients (45 probands) were available for analysis. The male to female ratio was 1:1.2. Forty-one index cases were classified as sporadic without unexplained anemia in first-degree relatives, while the remaining eight patients were from four families. All patients were Japanese except two cases: case 10 was Chinese and case 23 was a Brazilian of Japanese extraction. Case 15 had a Filipina mother and a Japanese father.

Genetics

RPS19

Five different mutations were detected in five probands out of 45 families (11%) (Table 1). The median age at presentation of the index cases with *RPS19* mutations was 1 month (range, 0 to 2 months). There appears to be a lower percentage of *RPS19* mutations in Japanese DBA patients than in patients from western countries. All mutations were in the coding region of the gene. Missense mutations resulting in amino acid substitutions were noted in four index cases. The three mutations, p.R62Q in case 30, p.R62W in case 44 and p.0 in case 43, have been reported in seven, ten and two families, respectively,[5,10,11,22-26] whereas one mutation, p.G95V in case 25, was novel, and could not be found in the Single Polymorphism Database (dbSNP at *www.ncbi.nlm.nih.gov/SNP*). Furthermore, the mutation was not observed in DNA from 50 normal individuals. An insertion of one nucleotide was found in one case (case 28), resulting in a novel frameshift mutation.

RPL5 and RPL11

The human *RPL5* gene consists of eight exons and is located on chromosome 1. Four novel mutations were found among the 45 probands (9%) (Table 1). The median age at presentation of the index cases with *RPL5* mutations was 10 months. A deletion of two nucleotides was found in case 10, and an insertion of one nucleotide was found in case 65, each affecting the reading frame. Two cases (cases 41 and 55) had point mutations that resulted in a loss of the translation initiation codon.

The human *RPL11* gene, which consists of six exons, is also located on chromosome 1. All exons and exon/intron boundaries were PCR-amplified and sequenced in DBA patients who were negative for mutations in *RPS19* and *RPL5*. Two mutations (4%) were found, and they were diagnosed at 18 and 20 months old, respectively (Table 1). A deletion of two nucleotides was found in case 9, and a deletion of one nucleotide was found in case 23, in each patient leading to a shift in the reading frame and the introduction of a premature stop codon.

RPS17

The *RPS17* gene is located on chromosome 15, and consists of five exons. *RPS17* mutations are rare and have been reported in only two patients with DBA. A novel one-nucleotide deletion in *RPS17* was identified in one patient (2%), resulting in the introduction of a premature stop codon (Table 1). The patient with the *RPS17* mutation (case 56) was born to healthy non-consanguineous parents and diagnosed as having DBA at the age of 1 month. He responded to the initial steroid treatment, and had a course of steroid-dependent therapy. No physical anomalies were seen in this patient.

RPL35A, RPS24 and RPS14

Mutations in *RPS24* and *RPL35A* are rare and have been reported in only eight and six patients with DBA, respectively. DBA patients were screened for *RPS24* and *RPL35A*, in addition to *RPS14*, which is implicated in the 5q- syndrome. No mutations were detected in *RPS24*, *RPL35A* or *RPS14* in Japanese DBA patients.

In total, sequence changes were found in four out of seven screened ribosomal protein genes (Table 2). Mutations in *RPS19*, *RPS17*, *RPL5*, and *RPL11* were detected in 11%, 2%, 9%, and 4% of the probands, respectively. The frequency of ribosomal protein gene mutations in Japanese DBA patients was 27%.

Genotype-phenotype correlations: congenital anomalies

The patients' characteristics are summarized in Table 3.

Table 1. Mutations identified in *RPS19*, *RPL5*, *RPL11*, and *RPS17* in Japanese DBA patients.

Patients (gender)	Inheritance	Age at diagnosis	Mutation	Predicted amino acid change
Mutations in the *RPS19* gene				
43 proband (F)	Sporadic	0 D	Exon2:g.3G>A	p.0
28 proband (M)	Sporadic	6 D	Exon3:g.130_131insA	E44fsX50
44 proband (F)	Sporadic	1 M	Exon4:g.184C>T	R62W
30 proband (F)	Familial	1 M	Exon4:g.185G>A	R62Q
30 father (M)	Familial	0 M	Exon4:g.185G>A	R62Q
25 proband (M)	Sporadic	2 M	Exon4:g.284G>T	G95V
Mutations in the *RPL5* gene				
10 proband (F)	Sporadic	0 M	Exon5:g.473_474delAA	K158fsX183
41 proband (F)	Sporadic	1 Y	Exon1:g.3G>T	p.0
55 proband (F)	Sporadic	3 Y	Exon1:g.3G>A	p.0
65 proband (F)	Sporadic	4 M	Exon2:g.37_38insT	F13fsX14
Mutations in the *RPL11* gene				
9 proband (F)	Sporadic	1 Y 10 M	Exon2:g.58_59delCT	L20fsX53
23 proband (M)	sporadic	1 Y 6 M	Exon5:g.460delA	R154fsX189
Mutations in the *RPS17* gene				
56 proband (F)	Sporadic	1 M	Exon2:g.26delT	V9fsX17

Anomalies associated with DBA were found in 27 patients (55%). Sixteen had two or more malformations (33%). All six patients with an *RPS19* mutation had physical anomalies, and three of them had multiple anomalies. In contrast, clinical data from European and American DBA patients showed that the frequency of malformations was 31% in patients with *RPS19* mutations, which is not significantly different from that of the entire DBA population.[26] *RPS19* mutations are characterized by a wide variability of phenotypic expression.[26] A mutation is frequently associated with various degrees of anemia, different responses to treatment, and dissimilar malformations. Even various family members having the same mutation in *RPS19* present with different clinical expressions. Cases 30, 44 and 43 harbored the same *RPS19* mutations reported in multicase families (p.R62Q, p.R62W, p.0).[6,10,11,22-27] Comparable to previous observations, no consistent clinical features were found in patients from different families displaying mutations in *RPS19*. For example, the father of case 30 harboring the same mutation had no finger anomalies, although case 30 had syndactyly and thumb polydactyly.

Consistent with reports that patients with *RPL5* and *RPL11* mutations are at high risk of developing malformations,[17,18] all four patients with *RPL5* mutations had physical anomalies. Furthermore, three of them had multiple physical anomalies, particularly case 41, who had very severe congenital heart disease (Table 3). One of two patients with *RPL11* mutations had physical anomalies. In contrast, of the 36 patients with no mutations, physical anomalies were seen in 16 (44%).

Nine patients had craniofacial anomalies. Of these, two had *RPL5* mutations, while the remaining patients had no mutations. Gazda *et al.* suggested an association between *RPL5/RPL11* mutation and cleft lip and/or palate.[17] Data in the Diamond-Blackfan Anemia Registry (DBAR) of North America also suggest that the DBA phenotype associated with cleft lip/palate is caused by non-*RPS19* mutations.[4] In our cohort, the frequency of cleft palate was significantly different between *RPL5*-mutated and *RPL5* non-mutated groups ($P<0.05$): cleft palate was seen in three patients, two of whom had *RPL5* mutations while the other patient belonged to the *RPL5* non-mutated group.

Thumb anomalies were seen in six patients, four of whom had *RPS19* mutations while two had *RPL5* mutations. There was a statistically significant difference in the frequency of thumb anomalies between *RPS19*-mutated and *RPS19* non-mutated groups ($P<0.05$). Flat thenar was seen in one patient with an *RPL5* mutation. In contrast to previous reports on patients with *RPL11* mutations, thumb anomalies were not found in our patients with these mutations.

A small-for-date phenotype was seen in seven patients (14%): one had an *RPS19* mutation, one had an *RPL11* mutation, and the four others had no mutations. None of the patients with *RPL5* mutations was born small-for-date.

Genotype-phenotype correlations: therapeutic response

Corticosteroids and transfusions are the mainstays of DBA treatment.[1,3] Of 45 patients evaluable for first treatment response, 73% responded to steroid therapy, 8% did not respond and 16% were never treated with steroids. The proportions of patients who responded to the first steroid treatment were 5/5 (*RPS19*), 2/3 (*RPL5*), 1/2 (*RPL11*), 1/1 (*RPS17*), and 22/27 (no mutation). There were no significant differences in the response rates among these patients.

Sixty-nine percent of patients received red blood cell transfusions. Of 48 patients available for therapy in follow-up, 8 patients (17%) were transfusion-dependent, 18 patients (37%) were steroid-dependent, and 18 patients (37%) were transfusion-independent with no other treatment. Three patients received bone marrow transplants and were alive and well (Table 3). A malignancy was detected in one case (case 50, proband), who developed a myelodysplastic syndrome 1 year after the diagnosis of DBA.

Table 2. **Summary of sequence changes in seven ribosomal protein genes identified in Japanese DBA patients.**

Gene symbol	N. of tested DNA samples from unrelated probands	N. of probands with mutations	N. of subjects with mutations	Mutation types
RPS19	45	5 (11%)	6	missense, loss of 1st methionine, small insertion
RPL5	45	4 (9%)	4	loss of 1st methionine, small deletion, small insertion
RPL11	34	2 (4%)	2	small deletion
RPS17	45	1 (2%)	1	small deletion

Discussion

This is the first report of an investigation of DBA patients in Japan. Twelve types of mutations were detected in four ribosomal protein genes. These mutations occurred in 27% of Japanese DBA patients. Mutations in *RPS19*, which have been found in 25% of patients in western countries,[26] were detected in only five of 45 probands (11%) in Japan, and two of these mutations were unique. Novel mutations in *RPL5* (four probands; 9%), *RPL11* (two probands; 4%) and *RPS17* (one proband; 2%) were identified. The frequencies of mutations in *RPL5*, *RPL11* and *RPS17* were very similar to those in western countries.[16-19] These results may suggest that a lower incidence of mutations in ribosomal protein genes in Japanese patients with DBA is due to a lower incidence of *RPS19* mutations, although we might have missed large deletions or re-arrangements in this study.

Physical abnormalities and growth retardation were detected in 55% of the Japanese DBA patients, consistent with previous reports from western countries.[4-6] Recent studies suggest that patients with *RPL5* mutation are more likely to have physical malformations including craniofacial, thumb, and heart anomalies.[17,18] Remarkably, patients with *RPL5* mutations tend to have cleft lip and/or palate or cleft soft palate, isolated or in combination with other physical abnormalities.[17,18] We found that three of four patients with *RPL5* mutations had multiple physical malformations, and two had cleft palate, whereas only one patient without an *RPL5* mutation had cleft palate. In the general population, 0.1% to 0.2% of children are born with cleft lip and/or palate.[28] Our data, and those from previous findings, suggest that *PRL5* mutations are associ-

Table 3. **Characteristics of Japanese DBA patients.**

Patient	Malformation status	Response to first steroid therapy	Present therapy
Patients with mutation of *RPS19*			
25 proband	Thumb polydactyly, growth retardation (-2.0SD), etc.	ND	ND
28 proband	Thumb polydactyly, CHD, etc.	Response	Steroid-dependent
30 proband	Thumb polydactyly, syndactyly, growth retardation (-3.4SD)	Response	Steroid-dependent
30 father	Growth retardation (-3.6SD)	NA	CR
43 proband	Thumb polydactyly	Response	Steroid-dependent
44 proband	SFD	Response	CR
Patients with mutation of *RPL5*			
10 proband	Flat thenar, cleft palate, CHD, etc.	Poor	Transfusion-dependent
41 proband	Craniofacial abnormalities, cleft palate, CHD, etc.	ND	Transfusion-dependent
55 proband	Thumb polydactyly	Response	Steroid-dependent
65 proband	Growth retardation (-3.0SD)	Response	Steroid
Patients with mutation of *RPL11*			
9 proband	CHD, SFD, etc.	Response	CR
23 proband	None	Poor	Steroid-dependent
Patient with mutation of *RPS17*			
56 proband	None	Response	Steroid-dependent
Patients without mutation of seven RP genes			
1 proband	Growth retardation (-4.0SD)	Response	CR
1 daughter	None	Response	CR
3 proband	Growth retardation (-3.6SD)	Response	Steroid-dependent
4 proband	Craniofacial abnormalities, SFD, short stature, webbed neck	Response	Steroid-dependent
5 proband	None	Response	CR
6 proband	Cleft palate, SFD, etc.	Poor	BMT
7 proband	Craniofacial abnormalities, SFD, growth retardation, etc.	Response	CR
8 proband	Growth retardation, webbed neck	Response	Steroid-depndent
13 proband	None	NA	CyA, BMT
14 proband	None	Response	CR
15 proband	None	Response	Transfusion-dependent
20 proband	Craniofacial abnormalities, CHD, etc.	Rsponse	Transfusion-dependent
21 proband	None	Response	Steroid-dependent
22 proband	None	Response	CR
24 proband	Growth retardation (-4.0SD)	Response	Steroid-dependent
26 proband	Growth retardation (-4.1SD), craniofacial abnormalities, etc.	Response	Transfusion-dependent
33 proband	None	Response	BMT
36 proband	Hypospadias, cryptorchidism	Response	Steroid-dependent
36 cousin	None	Response	Steroid-dependent
37 proband	Hypospadias, cryptorchidism	ND	CR
42 proband	None	Response	CR
45 proband	Craniofacial abnormalities, growth retardation, etc.	Poor	Transfusion-dependent
48 proband	Fetal hydrops	ND	CR
49 proband	None	Response	Steroid-dependent
50 proband	None	Response	Steroid-dependent, CBT (due to MDS)
50 sister	None	Response	Steroid-dependent
51proband	None	Poor	CR
54 proband	None	ND	Transfusion-dependent
59 proband	None	ND	Transfusion
60 proband	SFD	ND	Transfusion
61 proband	None	Response	Cyclosporine
62 proband	CHD, SFD, growth retardation (-3.1SD)	Response	Steroid-dependent
63 proband	Craniofacial abnormalities, growth retardation (-7.5SD)	Response	Steroid-dependet
64 proband	None	Response	Steroid-depndent
66 proband	None	NA	Transfusion-dependent
67 proband	None	NA	NA

*ND: not done; NA: not available; SFD: small-for-date; CHD: congenital heart disease; MDS: myelodysplastic syndrome; BMT: bone marrow transplantation; CBT: cord blood stem cell transplantation; CR: complete remission. * RPS19, RPS24, RPS17, RPS14, RPL5, RPL11, RPL35A.*

ated with multiple physical abnormalities, especially cleft lip and/or palate.

Cmejla *et al.* reported that 87.5% of *RPL5*-mutated patients were born small-for-date, whereas only 42.9% of *RPS19*-mutated patients were born small-for-date.[18] However, in our series, the small-for-date phenotype was seen in seven patients, and all of them were *RPL5*-non-mutated patients. Our data suggest that *RPL5* mutations in Japanese DBA patients have no relevance to the small-for-date phenotype, which may be a unique characteristic of Japanese DBA.

According to recent studies, the frequency of malformation, particularly thumb anomalies, in *RPS19*-mutated patients, was relatively low compared to that in *RPL5*- or *RPL11*-mutated patients.[22-24,29] In Italian DBA patients, the risk of malformation was 7-fold higher in *RPL5*-mutated patients than in *RPS19*-mutated patients.[29] In contrast, all of the Japanese DBA patients with *RPS19* mutations had one or more malformations. The frequency of thumb anomalies was significantly higher in patients with *RPS19* mutations, as well as in patients with *RPL5* mutations, compared to in the other groups of patients.

Although steroid therapy is one of the established treatments for DBA, the mechanism of action is unknown and reliable prediction of response to initial steroid therapy is not available.[1,3] *RPS19* mutation status has not been predictive of response in any series.[3] In our cohort, responsiveness to first steroid therapy in Japanese DBA patients was as good as that reported in western populations.[1,3] In this study, no significant differences in response to initial steroid therapy were found between *RPS19*-mutated and *RPS19*-non-mutated groups, or between the groups with *RPS19* mutations and other ribosomal protein gene mutations.

In summary, we found that heterozygous mutations in *RPS19*, *RPL5*, *RPL11* or *RPS17* were present in 27% of Japanese DBA patients. No mutations were detected in *RPS14*, *RPS24* or *RPL35A*. We observed a slightly lower frequency of mutations in ribosomal protein genes in our cohort of Japanese DBA patients than the frequencies reported previously from western countries, although the data from both populations are based on relatively low numbers of patients and values showing significant differences between populations are lacking. Our data suggest an association between *RPL5* mutation and malformations, especially cleft palate, and between *RPS19* mutation and malformations, particularly thumb anomalies. This study also suggests that no association exists between *RPL5* mutations and the small-for-date phenotype or between *RPS19* mutations and non-responsiveness to initial steroid therapy in Japanese DBA patients.

Authorship and Disclosures

EI was the principal investigator and takes primary responsibility for the paper. YK, TT, ST, GX, RNW, KT, and SO performed the laboratory work for this study. SO, TH, AH, SK, DH, YK, RY, KK, RK, TI, TH, MHP, and KS enrolled the patients. EI and YK wrote the paper.

The authors reported no potential conflicts of interest.

List of hospitals and people who cooperated in collecting clinical samples from the DBA patients

Iwate prefectural Chubu Hospital (N. Onodera); Iwata City Hospital (M. Shirai); Osaka City General Hospital (J. Hara) ; Kagoshima City Hospital (K. Kawakami); Kagoshima University (Y. Okamoto); Kyoto University (K. Watanabe); Kyoto Prefectural Yosanoumi Hospital (H. Ogawa); Saitama Children's Medical Center (K. Koh); Shiga Medical Center for Children (T. Kitoh); Shizuoka Children's Hospital (K. Sakaguchi); Tokyo University (K. Ida); National Hospital Organization Saitama Hospital (I. Kamimaki); Dokkyo University (H. Kurosawa); Nakadori General Hospital (A. Watanabe); East Medical Center Moriyama Municipal Hospital, City of Nagoya (M. Yazaki); Nara Medical University (Y. Takeshita); Japanese Red Cross Narita Hospital (S. Igarashi); Hiroshima Red Cross Hospital & Atomic-bomb Survivors Hospital (N. Fujita); Fukushima Medical University (A. Kikuta); Yamagata University (T. Mitsui); Wakayama Medical University (M.Yoshiyama).

References

1. Alter BP, Young NS. The bone marrow failure syndromes. In: Nathan DG, Orkin HS, editors. Hematology of Infancy and Childhood. Volume 1. Saunders; Philadelphia, PA: 1998. pp. 237-335.
2. Badhai J, Fröjmark AS, J Davey E, Schuster J, Dahl N. Ribosomal protein S19 and S24 insufficiency cause distinct cell cycle defects in Diamond-Blackfan anemia. Biochim Biophys Acta. 2009;1792(10): 1036-42.
3. Vlachos A, Ball S, Dahl N, Alter BP, Sheth S, Ramenghi U, et al. Diagnosing and treating Diamond Blackfan anaemia: results of an international clinical consensus conference. Br J Haematol. 2008;142(6):859-76.
4. Lipton JM, Atsidaftos E, Zyskind I, Vlachos A. Improving clinical care and elucidating the pathophysiology of Diamond Blackfan anemia: an update from the Diamond Blackfan Anemia Registry. Pediatr Blood Cancer. 2006;46(5):558-64.
5. Willig TN, Niemeyer CM, Leblanc T, Tiemann C, Robert A, Budde J, et al. Identification of new prognosis factors from the clinical and epidemiologic analysis of a registry of 229 Diamond-Blackfan anemia patients. DBA group of Societe d'Hematologie et d'Immunologie Pediatrique (SHIP), Gesellshaft fur Padiatrische Onkologie und Hamatologie (GPOH), and the European Society for Pediatric Hematology and Immunology (ESPHI). Pediatr Res. 1999;46(5):553-61.
6. Campagnoli MF, Garelli E, Quarello P, Carando A, Varotto S, Nobili B, et al. Molecular basis of Diamond-Blackfan anemia: new findings from the Italian registry and a review of the literature. Haematologica. 2004;89(4):480-9.
7. Glader BE, Backer K, Diamond LK. Elevated erythrocyte adenosine deaminase activity in congenital hypoplastic anemia. N Engl J Med. 1983;309(24):1486-90.
8. Lecompte O, Ripp R, Thierry JC, Moras D, Poch O. Comparative analysis of ribosomal proteins in complete genomes: an example of reductive evolution at the domain scale. Nucleic Acids Res. 2002; 30(24):5382-90.
9. Gustavsson P, Willing TN, van Haeringen A, Tchernia G, Dianzani I, Donnér M, et al. Diamond-Blackfan anaemia: genetic homogeneity for a gene on chromosome 19q13 restricted to 1.8 Mb. Nat Genet. 1997;16(4):368-71.
10. Draptchinskaia N, Gustavsson P, Andersson B, Pettersson M, Willig TN, Dianzani I, et al. The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. Nat Genet. 1999;21(2): 169-75.
11. Gazda HT, Zhong R, Long L, Niewiadomska E, Lipton JM, Ploszynska A, et al. RNA and protein evidence for haplo-insufficiency in Diamond-Blackfan anaemia patients with RPS19 mutations. Br J Haematol. 2004;127(1):105-13.
12. Choesmel V, Bacqueville D, Rouquette J, Noaillac-Depeyre J, Fribourg S, Crétien A, et al. Impaired ribosome biogenesis in Diamond-Blackfan anemia. Blood. 2007; 109(3):1275-83.
13. Flygare J, Aspesi A, Bailey JC, Miyake K, Caffrey JM, Karlsson S, et al. Human

RPS19, the gene mutated in Diamond-Blackfan anemia, encodes a ribosomal protein required for the maturation of 40S ribosomal subunits. Blood. 2007;109(3): 980-6.
14. Miyake K, Utsugisawa T, Flygare J, Kiefer T, Hamaguchi I, Richter J, et al. Ribosomal protein S19 deficiency leads to reduced proliferation and increased apoptosis but does not affect terminal erythroid differentiation in a cell line model of Diamond-Blackfan anemia. Stem Cells. 2008;26(2): 323-9.
15. Gazda HT, Grabowska A, Merida-Long LB, Latawiec E, Schneider HE, Lipton JM, et al. Ribosomal protein S24 gene is mutated in Diamond-Blackfan anemia. Am J Hum Genet. 2006;79(6):1110-8.
16. Cmejla R, Cmejlova J, Handrkova H, Petrak J, Pospisilova D. Ribosomal protein S17 gene (RPS17) is mutated in Diamond-Blackfan anemia. Hum Mutat. 2007;28(12): 1178-82.
17. Gazda HT, Sheen MR, Vlachos A, Choesmel V, O'Donohue MF, Schneider H, et al. Ribosomal protein L5 and L11 mutations are associated with cleft palate and abnormal thumbs in Diamond-Blackfan anemia patients. Am J Hum Genet. 2008;83(6):769-80.
18. Cmejla R, Cmejlova J, Handrkova H, Petrak J, Petrtylova K, Mihal V, et al. Identification of mutations in the ribosomal protein L5 (RPL5) and ribosomal protein L11 (RPL11) genes in Czech patients with Diamond-Blackfan anemia. Hum Mutat. 2009;30(3): 321-7.
19. Farrar JE, Nater M, Caywood E, McDevitt MA, Kowalski J, Takemoto CM, et al. Abnormalities of the large ribosomal subunit protein, Rpl35a, in Diamond-Blackfan anemia. Blood. 2008;112(5):1582-92.
20. Ebert BL, Pretz J, Bosco J, Chang CY, Tamayo P, Galili N, et al. Identification of RPS14 as a 5q- syndrome gene by RNA interference screen. Nature. 2008;451 (7176):335-9.
21. Xu G, Nagano M, Kanezaki R, Toki T, Hayashi Y, Taketani T, et al. Frequent mutations in the GATA-1 gene in the transient myeloproliferative disorder of Down syndrome. Blood. 2003;102(8):2960-8.
22. Willig TN, Draptchinskaia N, Dianzani I, Ball S, Niemeyer C, Ramenghi U, et al. Mutations in ribosomal protein S19 gene and Diamond Blackfan anemia: wide variations in phenotypic expression. Blood. 1999;94(12):4294-306.
23. Ramenghi U, Campagnoli MF, Garelli E, Carando A, Brusco A, Bagnara GP, et al. Diamond-Blackfan anemia: report of seven further mutations in the RPS19 gene and evidence of mutation heterogeneity in the Italian population. Blood Cells Mol Dis. 2000;26(5):417-22.
24. Cmejla R, Blafkova J, Stopka T, Zavadil J, Pospisilova D, Mihal V, et al. Ribosomal protein S19 gene mutations in patients with Diamond-Blackfan anemia and identification of ribosomal protein S19 pseudogenes. Blood Cells Mol Dis. 2000;26(2):124-32.
25. Proust A, Da Costa L, Rince P, Landois A, Tamary H, Zaizov R, et al. Ten novel Diamond-Blackfan anemia mutations and three polymorphisms within the rps19 gene. Hematol J. 2003;4(2):132-6.
26. Campagnoli MF, Ramenghi U, Armiraglio M, Quarello P, Garelli E, Carando A, et al. RPS19 mutations in patients with Diamond-Blackfan anemia. Hum Mutat. 2008;29(7):911-20.
27. Gazda H, Lipton JM, Willig TN, Ball S, Niemeyer CM, Tchernia G, et al. Evidence for linkage of familial Diamond-Blackfan anemia to chromosome 8p23.3-p22 and for non-19q non-8p disease. Blood. 2001;97(7): 2145-50.
28. Lidral AC, Murray JC. Genetic approaches to identify disease genes for birth defects with cleft lip/palate as a model. Birth Defects Res A Clin Mol Teratol. 2004; 70(12):893-901.
29. Quarello P, Garelli E, Carando A, Brusco A, Calabrese R, Dufour C, et al. Diamond-Blackfan anemia: genotype-phenotype correlation in Italian patients with RPL5 and RPL11 mutations. Haematologica. 2010; 95(2):206-13.