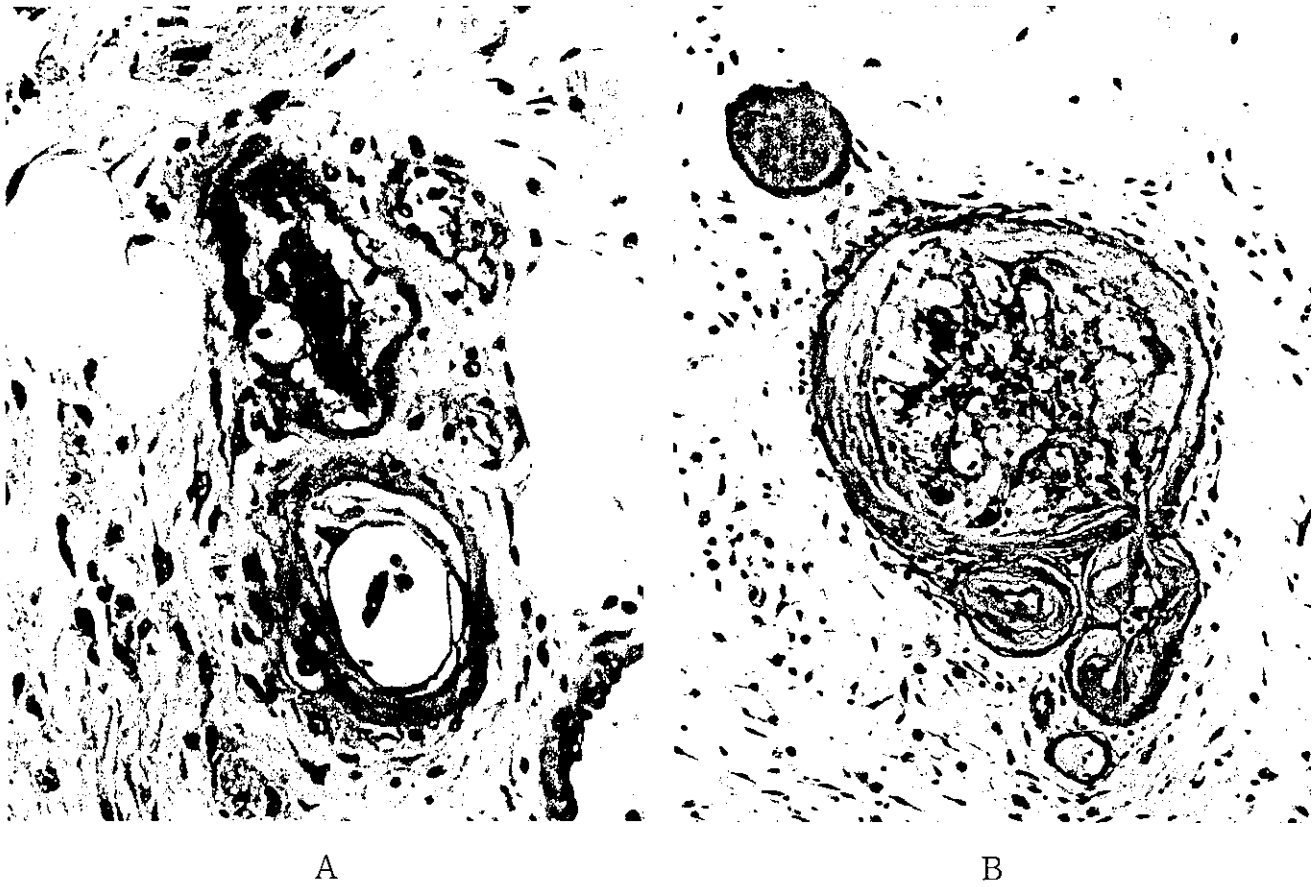
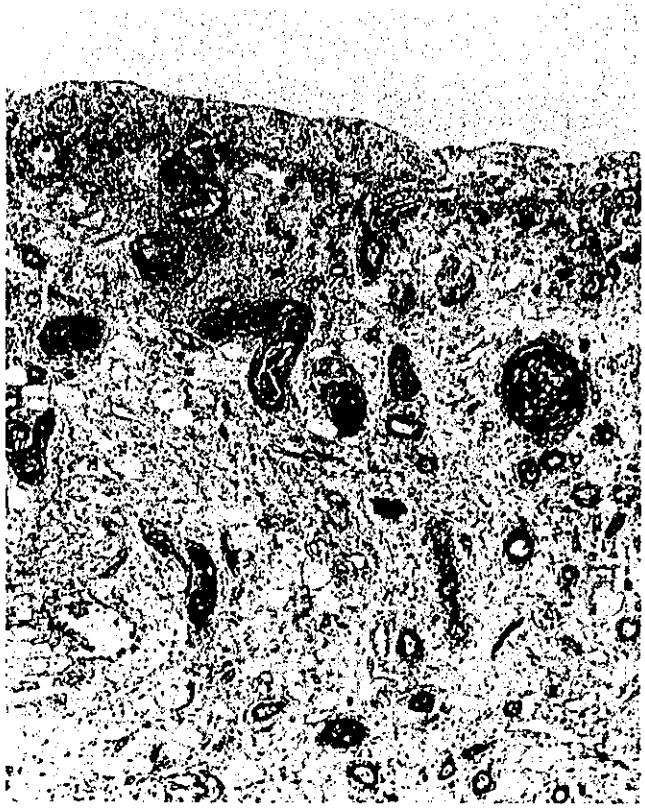


A B



C

Figure 2. Subintimal mucoid lesions of arterioles of the ileum at autopsy (A and B), and a low-magnification view (C) (HE stain). The mucosa overlying the involved arterioles shows marked atrophy (C).

episode of TMA, which showed ischemic colitis, was partially responsive to antithymocyte globulin. A reduction in methylprednisolone administered for skin GVHD was not accompanied by the exacerbation of bloody diarrhea.

In conclusion, we have reported a case of ischemic colitis as a manifestation of BMT-TMA and reviewed the characteristics of the clinical course and the pathological findings. Early detection, an accurate diagnosis and an optimal treatment strategy for this complication are needed to improve the treatment-related mortality in stem cell transplantation.

References

1) Symmers W. Thrombotic microangiopathic haemolytic anemia. Br Med J **2**: 897–903, 1952.
2) Moake JL. Thrombotic microangiopathies. N Engl J Med **347**: 589–600, 2002.
3) Moschcowitz E. An acute febrile pleiochromic anemia with hyaline thrombosis of the terminal arterioles and capillaries. An undescribed disease. Thromb Haemost **40**: 4–8, 1978.
4) Singer K, Bornstein FP, Wile SA. Thrombotic thrombocytopenic purpura. Blood **2**: 542–554, 1947.
5) Gasser C, Gautier E, Steck A, Siebenmann RE, Oechslin R. Hemolytic-uremic syndrome. Bilateral kidneys in necrosis by acute aquired hemolytic anemia. Schweiz Med Wochenschr **85**: 905–909, 1955 (in German).
6) Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. J Biol Chem **276**: 41059–41063, 2001.
7) Kokame K, Matsumoto M, Soejima K, et al. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. Proc Natl Acad Sci USA **99**: 11902–11907, 2002.
8) Furlan M, Robles R, Galbusera M, et al. von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. N Engl J Med **339**: 1578–1584, 1998.
9) Furlan M, Robles R, Lamie B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. Blood **87**: 4223–4234, 1996.
10) Tsai H. Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. Blood **87**: 4235–4244, 1996.
11) Pettitt AR, Clark RE. Thrombotic microangiopathy following bone marrow transplantation. Bone Marrow Transplant **14**: 495–504, 1994.
12) Kanamori H, Maruta A, Sasaki S, et al. Diagnostic value of hemostatic parameters in bone marrow transplant-associated thrombotic microangiopathy. Bone Marrow Transplant **21**: 705–709, 1998.
13) van der Plas RM, Schiphorst ME, Huizinga EG, et al. von Willebrand factor proteolysis is deficient in classic, but not in bone marrow transplantation-associated, thrombotic thrombocytopenic purpura. Blood **93**: 3798–3802, 1999.
14) Zeigler ZR, Shadduck RK, Nemunaitis J, Andrews DF, Rosenfeld CS. Bone marrow transplant-associated thrombotic microangiopathy: a case series. Bone Marrow Transplant **15**: 247–253, 1995.
15) Hirabayashi N. Thrombotic microangiopathy (TMA) (1). TMA in the target organs of graft-versus-host disease. Rinsho Ketsueki **41**: 491–495, 2000 (in Japanese).
16) Selby DM, Rudzki JR, Bayever ES, Chandra RS. Vasculopathy of small muscular arteries in pediatric patients after bone marrow transplantation. Hum Pathol **30**: 734–740, 1999.
17) Iwata H, Kami M, Hori A, Hamaki T, Takeuchi K, Mutou Y. An autopsy-based retrospective study of secondary thrombotic thrombocytopenic purpura. Haematologica **86**: 669–670, 2001.
18) Berkowitz LR, Dalldorf FG, Blatt PM. Thrombotic thrombocytopenic purpura: a pathology review. JAMA **241**: 1709–1710, 1979.
19) Dignan CR, Greenson JK. Can ischemic colitis be differentiated from C difficile colitis in biopsy specimens? Am J Surg Pathol **21**: 706–710, 1997.
20) Sale GE, Shulman HM, McDonald GB, Thomas ED. Gastrointestinal graft-versus-host disease in man. A clinicopathologic study of the rectal biopsy. Am J Surg Pathol **3**: 291–299, 1979.
21) Sarode R, McFarland JG, Flomenberg N, et al. Therapeutic plasma exchange does not appear to be effective in the management of thrombotic thrombocytopenic purpura/hemolytic uremic syndrome following bone marrow transplantation. Bone Marrow Transplant **16**: 271–275, 1995.
22) Sarode R, Gottschall JL, Aster RH, McFarland JG. Thrombotic thrombocytopenic purpura: early and late responders. Am J Hematol **54**: 102–107, 1997.
23) Mori T, Okamoto S, Watanabe R, Ikeda Y. Successful treatment of bone marrow transplant—associated thrombotic microangiopathy with antithrombin-III. Am J Med **112**: 81–82, 2002.

American Journal of Hematology 72:27–30 (2003)

Rapid Diagnosis of Invasive Pulmonary Aspergillosis by Quantitative Polymerase Chain Reaction Using Bronchial Lavage Fluid

Masahito Kawazu, Yoshinobu Kanda, Susumu Goyama, Masataka Takeshita, Yasuhito Nannya, Miyuki Niino, Yukiko Komeno, Tetsuya Nakamoto, Mineo Kurokawa, Shiho Tsujino, Seishi Ogawa, Katsunori Aoki, Shigeru Chiba, Toru Motokura, Nobuya Ohishi, and Hisamaru Hirai*

Department of Hematology & Oncology, University of Tokyo, Tokyo, Japan

Polymerase chain reaction (PCR) is a sensitive method for detection of *Aspergillus* DNA in bronchoalveolar lavage fluid, but it has not yet been able to distinguish infection from contamination. We have established a technique to quantify *Aspergillus* DNA using a real-time PCR method to resolve this problem, and we report herein a successful application of real-time PCR to diagnose invasive pulmonary aspergillosis by comparing the amount of *Aspergillus* DNA in bronchial lavage fluid from an affected area to that from an unaffected area. This novel tool will provide rapid, sensitive, and specific diagnosis of pulmonary aspergillosis. Am. J. Hematol. 72:27–30, 2003. © 2002 Wiley-Liss, Inc.

Key words: real-time polymerase chain reaction; aspergillosis; bone marrow transplantation; bronchial lavage fluid

INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is a major complication among immunocompromised patients, especially who undergo hematopoietic stem cell transplantation. Because IPA, once overtly developed, may become refractory to antifungal treatments, strategies for an early detection of IPA have been eagerly investigated [1]. Chest computed tomography (CT) scan has been reported to be useful for early diagnosis of IPA [2], and actually antifungal therapy is apt to be started empirically on the basis of the clinical course and radiographic findings. However, because the toxicity of amphotericin B, the most reliable agent against aspergillosis, is not negligible, antifungal therapy is better started on the basis of a definite diagnosis established by pathological or microbiological examinations of the lung specimen. Transbronchial lung biopsy, however, can hardly be performed, because thrombocytopenia and/or coagulopathy are common among immunosuppressed patients. In contrast, bronchoalveolar or bronchial lavage fluid (BLF) can be safely obtained and shown to be useful in diagnosing IPA [3]. Although culture of BLF is specific and it allows determination of species, culture assay is time-consuming and not sensitive enough [4].

Some molecular and/or serological methods have been investigated for early detection of *Aspergillus* in blood and/or BLF samples. One of them is a sandwich enzyme-linked immunosorbent assay (ELISA) to detect galactomannan antigen (GM) [5], but its sensitivity is not clinically sufficient [6]. Polymerase chain reaction (PCR) to amplify *Aspergillus* DNA has been expected to be a sensitive method [7–9]. However, since conidia are ubiquitous in the environment, a high frequency of false-positive results has been problematic; in addition, it was difficult to distinguish true aspergillosis from upper-airway colonization when BLF samples were used [10]. To resolve these problems, we have established a technique to quantify *Aspergillus* DNA using a real-time

*Correspondence to: Hisamaru Hirai, M.D., Department of Hematology & Oncology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: hhirai-tky@umin.ac.jp

Received for publication 15 February 2002; Accepted 15 October 2002

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ajh.10259

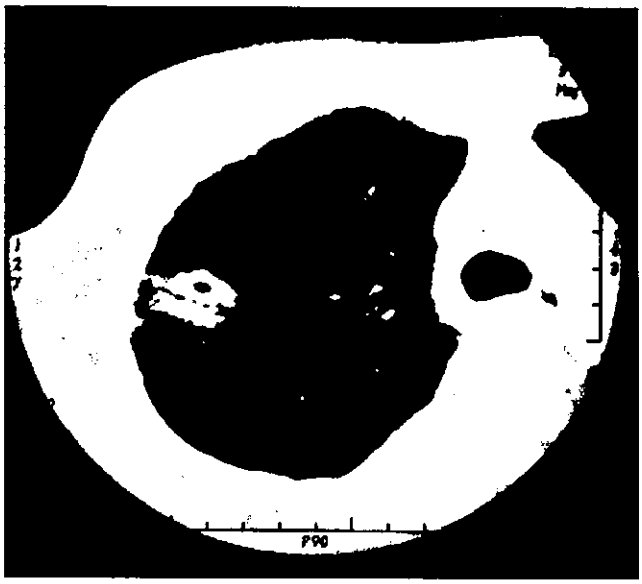


Fig. 1. Chest computed tomography scan on day 61. A nodular lesion with cavitation was seen in the right upper lobe (S_3). The ground-glass like lesion at the periphery was supposed to be bronchiolitis obliterans organizing pneumonia.

PCR method [11]. Here we report a successful and rapid diagnosis of IPA using quantitative PCR (Q-PCR) comparing BLF from an affected lesion and that from a normal area.

CASE REPORT

A 38-year-old woman with acute myeloblastic leukemia (M2) in second complete remission underwent bone marrow transplantation from an human leukocyte antigen-matched unrelated male donor after a conditioning regimen consisting of total body irradiation (2 Gy twice daily on days −9, −8, and −7) and cyclophosphamide (60 mg/kg/day on days −4 and −3). Engraftment defined by neutrophil count >500/mm^3 was achieved on day 18, and bone marrow aspiration on days 28 and 60 revealed sustained complete remission. Regimen-related toxicity was mild except for development of congestive heart failure, which was resolved by an administration of diuretics and catecholamines.

Cyclosporine A (3 mg/kg/day, from day −1) and methotrexate (12 mg/m^2/day on day 1, and then 7 mg/m^2/day on days 3, 6, and 11) were administered for prophylaxis of graft-versus-host disease (GVHD). However, cyclosporine A was discontinued when congestive heart failure developed and was substituted by 1 mg/kg/day of methyl-prednisolone (mPSL). Subsequently, cutaneous acute GVHD of stage 3 developed on day 33, which was improved by the resumption of cyclosporine A, and then we began to gradually decrease the dose of mPSL. A routine chest X ray on day 53 showed nodular lesion with a cavitation in the right upper field of lung without any symptoms. Chest CT scan on day 61 revealed nodular lesion with air-crescent sign in the right upper lobe (S_3 segment) and diffuse ground-glass opacity at the periphery of the left lower lobe and the right upper, middle, and lower lobes (Fig. 1). The ground-glass-like lesion was suspected to be bronchiolitis obliterans organizing pneumonia (BOOP), whereas the nodular lesion was suspected to be fungal infection. In order to confirm the diagnosis of fungal infection, bronchoscopy was performed on day 62. BLF and brush samples obtained from the right B_3 and B_5 bronchi were subjected to culture, cytology, flow cytometry, GM detection by ELISA (*Platelia aspergillus*, Fuji Revio, Japan), and Q-PCR for *Aspergillus* DNA.

We could not make a definite diagnosis of BOOP, since biopsy was not performed due to the low platelet count. However, the CD4/CD8 ratio of leukocytes in the BLF sample obtained from the B_5 bronchus was 0.2, which was compatible with the diagnosis of BOOP [12]. The results of microbiological examinations are summarized in Table I. The yields of *Aspergillus* DNA estimated by Q-PCR in the B_3 brush sample, B_3 BLF, and B_5 BLF were 30,000, 6,000, and 100 copies/mL, respectively. A few days later, two colonies of *Aspergillus fumigatus* were detected in the culture of the B_3 brush sample. Several days after bronchoscopy was performed, she also complained of a subcutaneous nodular lesion of the right femur, which was histologically diagnosed as subcutaneous aspergillosis. Therefore, we made the diagnosis of definite systemic aspergillosis. Weekly obtained blood samples were persistently negative for β-D-glucan, GM antigen, and *Aspergillus* DNA throughout the clinical course (Table II).

We started AMPH-B just after obtaining the results of Q-PCR and subsequently changed to liposomal AMPH-B and oral itraconazole because of the development of renal toxicity. Aspergillosis gradually improved as the dose of mPSL was decreased.

TABLE I. Results of Microbiological Examinations for Bronchial Lavage Fluid Samples*

	B_5	B_3	
	BLF	BLF	Brush
Culture	No growth	No growth	2 colonies of *A. fumigatus*
β-D-Glucan (pg/mL)	<0.2	<2.0	<2.0
Galactomannan ODI	0.3	0.8	1.1
Aspergillus DNA (copies/mL)	100	6,000	30,000

*Cutoff values for β-D-glucan, galactomannan, and quantitative PCR were 11.0 pg/mL, 1.5, and 40 copies/mL, respectively. BLF, bronchial lavage fluid; ODI, optical density index.

TABLE II. Surveillance of *Aspergillus* Infection Using Blood Samples*

	Days after BMT								
	Day 3	Day 10	Day 17	Day 24	Day 31	Day 38	Day 45	Day 53	Day 59
β-D-Glucan (pg/mg)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	2.8	<2.0	<2.0
Galactomannan ODI	0.3	0.4	0.5	0.6	0.6	0.5	0.9	0.4	0.6
Aspergillus DNA (copies/mL)	0	0	0	10.5	0	0	31	0	33.5

*Cut-off values for β-D-glucan, galactomannan, and quantitative PCR were 11.0 pg/mL, 1.5, and 40 copies/mL, respectively. ODI, optical density index.

DISCUSSION

Varieties of PCR methods, including conventional PCR [7], competitive PCR, [8] and nested PCR [9], have been applied in the detection of *Aspergillus* DNA in clinical specimens, and they have demonstrated their high sensitivities [6]. However, false-positive results are frequent, and up to 25% of BLF from healthy volunteers showed positive results [13]. This low specificity may be due to environmental contamination or airway colonization of *Aspergillus,* and it has been difficult to differentiate false-positive results from true infection by PCR [10]. We applied Q-PCR to resolve this problem. In the present patient, 6,000 copies/mL of *Aspergillus* DNA was detected in the BLF from an affected bronchus (B_3), whereas the amount of *Aspergillus* DNA was estimated as only 100 copies/mL in the BLF obtained from right B_5 area, which was supposed to be free from infection. The low amount of *Aspergillus* DNA in the B_5 BLF could be explained by upper-airway colonization, contaminated by the insertion of bronchoscope. Actually, we have found that induced sputa from normal volunteers were sometimes positive for *Aspergillus* DNA at lower levels (unpublished data). By comparing the amount of *Aspergillus* DNA from the affected area with that from an unaffected area, we could distinguish true positive samples. Although culture is the gold standard of microbiological examination and also allows identification of species and tests for susceptibility [14], it is time-consuming and less sensitive. The result of real-time PCR is available within 24 hr. GM detection by ELISA in BLF has also been suggested to be useful for the diagnosis of IPA [6]. In this case, however, GM was not detected in the B_3 BLF, in which 6,000 copies/mL of *Aspergillus* DNA were detected, suggesting that Q-PCR is more sensitive than GM detection by ELISA.

Because blood samples are easily available without invasive procedures, the detection of GM by ELISA [15] or *Aspergillus* DNA by PCR [16,17] has been investigated using blood samples. However, both tests became positive only at an advanced stage [18,19], and they cannot predict IPA at an early stage. In this case, we could not detect *Aspergillus* infection using blood samples, despite both tests being performed weekly. Therefore, the sensitivity of these tests using blood samples was not clinically sufficient.

In conclusion, quantification of *Aspergillus* DNA in BLF by real-time PCR could differentiate true infection from colonization or contamination by comparing the amount of *Aspergillus* DNA in BLFs from an affected with that from an unaffected area. This method may shorten the time to diagnosis and improve the sensitivity compared with conventional culture.

REFERENCES

1. Denning DW. Therapeutic outcome in invasive aspergillosis. Clin Infect Dis 1996;23:608–615.
2. Caillot D, Couailier JF, Bernard A, Casasnovas O, Denning DW, Mannone L, Lopez J, Couillault G, Piard F, Vagner O, Guy H. Increasing volume and changing characteristics of invasive pulmonary aspergillosis on sequential thoracic computed tomography scans in patients with neutropenia. J Clin Oncol 2001;19:253–259.
3. Kahn FW, Jones JM, England DM. The role of bronchoalveolar lavage in the diagnosis of invasive pulmonary aspergillosis. Am J Clin Pathol 1986;86:518–521.
4. Levy H, Horak DA, Tegtmeier BR, Yokota SB, Forman SJ. The value of bronchoalveolar lavage and bronchial washing in the diagnosis of invasive pulmonary aspergillosis. Respir Med 1992;86:243–248.
5. Stynen D, Goris A, Sarfati J, Latge JP. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. J Clin Microbiol 1995;33:497–500.
6. Becker MJ, de Marie S, Willemse D, Verbrugh HA, Bakker-Woudenberg IA. Quantitative galactomannan detection is superior to PCR in diagnosing and monitoring invasive pulmonary aspergillosis in an experimental rat model. J Clin Microbiol 2000;38:1434–1438.
7. Tang CM, Holden DW, Aufauvre-Brown A, Cohen J. The detection of *Aspergillus* spp. by the polymerase chain reaction and its evaluation in bronchoalveolar lavage fluid. Am Rev Respir Dis 1993;148:1313–1317.
8. Bretagne S, Costa JM, Marmorat-Khuong A, Poron F, Cordonnier C, Vidaud M, Fleury-Feith J. Detection of *Aspergillus* species DNA in bronchoalveolar lavage samples by competitive PCR. J Clin Microbiol 1995;33:1164–1168.
9. Buchheidt D, Baust C, Sklandny H, Ritter J, Suedhoff T, Baldus M, Seifarth W, Leib-Moesch C, Hehlmann R. Detection of *Aspergillus* species in blood and bronchoalveolar lavage samples from immunocompromised patients by means of 2-step polymerase chain reaction: clinical results. Clin Infect Dis 2001;33:428–435.
10. Hayette MP, Vaira D, Susin F, Boland P, Christiaens G, Melin P, De

Mol P. Detection of *Aspergillus* species DNA by PCR in bronchoalveolar lavage fluid. J Clin Microbiol 2001;39:2338–2340.
11. Kami M, Fukui T, Ogawa S, Kazuyama Y, Machida U, Tanaka Y, Kanda Y, Kashima T, Yamazaki Y, Hamaki T, Mori S, Akiyama H, Mutou Y, Sakamaki H, Osumi K, Kimura S, Hirai H. Use of real-time PCR on blood samples for diagnosis of invasive aspergillosis. Clin Infect Dis 2001;33:1504–1512.
12. Izumi T, Kitaichi M, Nishimura K, Nagai S. Bronchiolitis obliterans organizing pneumonia. Clinical features and differential diagnosis. Chest 1992;102:715–719.
13. Latge JP. *Aspergillus fumigatus* and Aspergillosis. Clin Microbiol Rev 1999;12:310–350.
14. Denning DW. Invasive aspergillosis. Clin Infect Dis 1998;26:781–805.
15. Maertens J, Verhaegen J, Lagrou K, Eldere JV, Boogaerts M. Screening for circulating galactomannan as a noninvasive diagnostic tool for invasive aspergillosis in prolonged neutropenic patients and stem cell transplantation recipients: a prospective validation. Blood 2001;97: 1604–1610.
16. Einsele H, Hebart H, Roller G, Loffler J, Rothenhofer I, Muller CA, Bowden RA, van Burik J, Engelhard D, Kanz L, Schumacher U. Detection and identification of fungal pathogens in blood by using molecular probes. J Clin Microbiol 1997;35:1353–1360.
17. Yamakami Y, Hashimoto A, Tokimatsu I, Nasu M. PCR detection of DNA specific for *Aspergillus* species in serum of patients with invasive aspergillosis. J Clin Microbiol 1996;34:2464–2468.
18. Kami M, Tanaka Y, Ogawa S, Kanda Y, Honda H, Chiba S, Mitani K, Yazaki Y, Hirai H. The limitation of the circulating *Aspergillus* antigen detection methods for BMT recipients. Bone Marrow Transplant 1998;22:832–833.
19. Bretagne S, Costa JM, Bart-Delabesse E, Dhedin N, Rieux C, Cordonnier C. Comparison of serum galactomannan antigen detection and competitive polymerase chain reaction for diagnosing invasive aspergillosis. Clin Infect Dis 1998;26:1407–1412.

American Journal of Hematology 72:220–222 (2003)

Reduced-Intensity Bone Marrow Transplantation From an Alternative Unrelated Donor for Myelodysplastic Syndrome of First-Donor Origin

Yukiko Komeno, Yoshinobu Kanda, Koji Kandabashi, Masahito Kawazu, Susumu Goyama, Masataka Takeshita, Yasuhito Nannya, Miyuki Niino, Tetsuya Nakamoto, Mineo Kurokawa, Shiho Tsujino, Seishi Ogawa, Katsunori Aoki, Shigeru Chiba, Toru Motokura, and Hisamaru Hirai*

Department of Cell Therapy & Transplantation Medicine, University of Tokyo Hospital, Tokyo, Japan

A male patient had a relapse of myelodysplastic syndrome (MDS) 2 years after BMT from a female matched unrelated donor. Conventional cytogenetics, FISH, and short-tandem repeat chimerism analysis proved a relapse of donor origin. He underwent reduced-intensity BMT after a conditioning with fludarabine and busulfan, since he had impaired renal, liver, and pulmonary functions. Chimerism analysis on day 28 after the second BMT showed mixed chimerism of the first and the second donors, which later turned to full second-donor chimerism on day 60. He developed grade II acute GVHD of the skin and cytomegalovirus reactivation, but both were improved with methylprednisolone and ganciclovir, respectively. He remains in complete remission 6 months after the second BMT. Reduced-intensity second BMT from an alternative donor appeared to be a tolerable treatment option for donor-derived leukemia/MDS after the first conventional transplantation. Am. J. Hematol. 72:220–222, 2003. © 2003 Wiley-Liss, Inc.

Key words: reduced-intensity stem-cell transplantation; donor-derived leukemia; second transplantation

INTRODUCTION

Several cases of donor-cell leukemia have been reported after allogeneic bone marrow transplantation (BMT) [1–4]. Since donor-cell leukemia is a rare event, treatment of such patients has not been established. We experienced a patient who developed donor-derived myelodysplastic syndrome (MDS) with unfavorable karyotypic abnormality after the first unrelated BMT, who subsequently underwent a successful reduced-intensity BMT from an alternative unrelated donor. Here we report the clinical course as well as the kinetics of chimerism and immunological reconstitution after the second BMT.

CASE REPORT

A 43-year old man was diagnosed with MDS (RA) in June 1996. Chromosome analysis revealed a cytogenetic abnormality with 47, XY, +8, del(20)(q11). He was observed without treatment until leukocytopenia and anemia progressed in September 1998. He became transfusion-dependent and underwent allogeneic BMT from a female matched unrelated donor in June 1999. The conditioning regimen consisted of cyclophosphamide (60 mg/kg for 2 days) and fractionated total body irradiation (2 Gy twice daily for 3 days). Cyclosporin A and a short course of methotrexate were administered for GVHD prophylaxis. Neutrophil engraftment was confirmed on day 21. The clinical course after the first BMT was generally uneventful. He developed only grade I acute GVHD restricted to the skin. Cytomegalovirus antigenemia became positive on day 38, which was treated with

*Correspondence to: Hisamaru Hirai, M.D., Department of Cell Therapy & Transplantation Medicine, University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: hhirai-tky@umin.ac.jp

Received for publication 12 July 2002; Accepted 15 November 2002

Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/ajh.10286

ganciclovir. Manifestation of chronic GVHD was observed in xerophthalmia, mild elevation of liver enzymes, and dry skin. Donor-recipient chimerism was serially analyzed with XY chromosome FISH, and persistent full-donor chimerism was confirmed.

In June 2001, however, 20% of blasts appeared in peripheral blood, and a bone marrow aspirate showed a relapse of MDS as RAEB-t. The leukemic cells were positive for CD3, CD33, CD34, and HLA-DR. Conventional cytogenetics of the seven dividing cells revealed 47, XX, +11 in 6 cells, and 46, XX in one cell. XY FISH analysis of both 502 bone marrow cells and 590 peripheral blood cells showed 100% XX signals. Short tandem repeat chimerism analysis confirmed that the blood cells were completely of donor origin [5]. In addition, these analyses were also performed with CD34-positive sorted cells, and the results described above were reproduced. Therefore, MDS of donor-cell origin was diagnosed. A second BMT from an alternative unrelated donor was planned at this time, because the prognosis of AML/MDS with trisomy 11 has been shown to be poor [6]. Complete remission was achieved with a combination of daunorubicin, cytarabine, and 6-MP, which was followed by two courses of consolidation chemotherapy. Conventional cytogenetics revealed normal female karyotype.

Since he had renal (creatinine clearance 30 mL/min), hepatic (alkaline phosphatase 300–400 U/L with a normal upper limit of 200 U/L), and respiratory dysfunction (FEV 60%), we chose a reduced-intensity conditioning regimen with busulfan 4 mg/kg for 2 days and fludarabine 18 mg/m^2 for 6 days. Prophylaxis against GVHD consisted of cyclosporine A (3 mg/kg) and methotrexate (5 mg/m^2) on days 1, 3, 6, and 11. The doses of fludarabine and methotrexate were adjusted considering the renal dysfunction. On January 25, 2002, a second BMT from an alternative female matched unrelated donor was performed. The number of all nucleated cells infused was 1.7×10^8/kg.

Regimen-related toxicity was generally mild, and the only manifestation was stomatitis of grade II by the Bearman's criteria [7]. Neutrophil engraftment was achieved on day 27. He developed grade II acute GVHD of the skin on day 41, which was improved with methylprednisolone at a dose of 1 mg/kg. It was tapered and finally discontinued on day 69. On day 52, cytomegalovirus antigenemia became positive, and pre-emptive therapy with ganciclovir at a maintenance dose was started [8,9]. CMV antigenemia became negative on day 87 without any sign of CMV diseases. He remains in complete remission 6 months after the second BMT.

Short tandem repeat chimerism analysis of bone marrow cells on day 28 showed mixed chimerism; 31% of the first-donor cells and 69% of the second-donor cells. This turned to full second-donor chimerism on day 60 (Fig. 1). Conventional cytogenetics of bone marrow cells

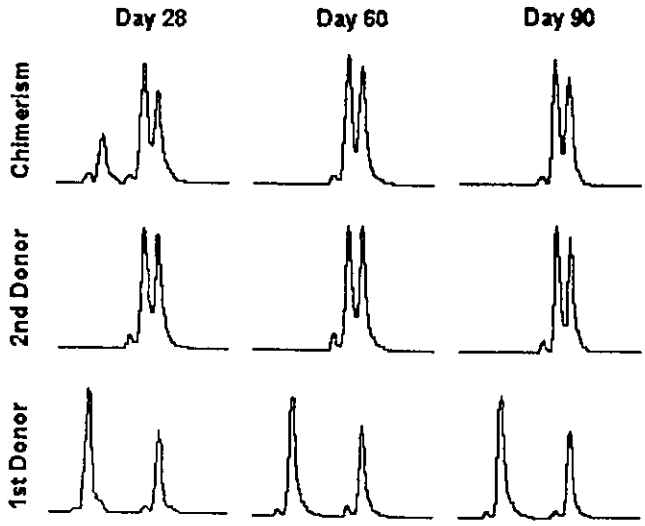


Fig. 1. Results of chimerism analysis of bone marrow cells on days 28, 60, and 90 after the second transplantation. Bone marrow cells on day 28 showed mixed chimerism; 31% of the first-donor cells and 69% of the second-donor cells. This turned to full second-donor chimerism on day 60.

persistently showed normal female karyotype on days 28, 60, and 90. On the other hand, chimerism of peripheral blood CD3-positive cells showed full second-donor chimerism on day 28 and thereafter.

Indices of immunological recovery are shown in Table I. Each subclass of immunoglobulin continued to decrease from day −10 to day 90, probably due to the use of methotrexate after BMT and the omission of intravenous administration of immunoglobulin. Flow cytometry analyses showed no CD19 positive cells during the period. The number of CD4 positive cells on day 90 was lower than that after conventional transplantation [10] but showed an increasing tendency.

DISCUSSION

Hematological malignancy of donor-cell origin after allogeneic stem-cell transplantation is a rare event [11]. The optimal treatment of donor-cell malignancy may depend on the character of the secondary malignancy and general condition of the patient. Since the patient was classified into the high-risk group by the international prognostic scoring index [12] and he had an unfavorable abnormal karyotype of trisomy 11 [6], we considered that the only curative treatment was the second hematopoietic stem-cell transplantation.

The risk of transplant-related mortality associated with second BMT, however, has been reported to be extremely high [13]. Moreover, this patient had several organ dysfunctions. Therefore, we planned a reduced-intensity BMT. We searched an alternative unrelated donor, because the graft-versus-leukemia effect could not

TABLE I. Immune Recovery After the Second BMT

	Day −10	Day 28	Day 60	Day 90
IgG	982 mg/dL	898 mg/dL	700 mg/dL	453 mg/dL
IgA	26 mg/dL	45 mg/dL	22 mg/dL	22 mg/dL
IgM	135 mg/dL	95 mg/dL	30 mg/dL	24 mg/dL
CD4	362 cells/μL	N.T.[a]	117 cells/μL	282 cells/μL
CD8	213 cells/μL	N.T.	249 cells/μL	454 cells/μL

[a]N.T., not tested.

be expected by BMT from the first donor. A very similar experience has been reported by Hambach et al. [3]. They used a very low-intensity conditioning with fludarabine 90 mg/m^2 in total and TBI of only 2 Gy [14]. However, we chose Slavin-type conditioning consisting of fludarabine and busulfan, because this regimen has been safely applied for the second allogeneic transplantation and is considered to have a higher activity against myeloid malignancies [15]. Anti-thymocyte globulin (ATG) was omitted because he was considered to be already severely immunosuppressed by the first BMT and subsequent chemotherapies. Regimen-related toxicity was mild and well tolerated. Full second-donor chimerism was achieved early after the second BMT. He remained in complete remission after the second BMT, although the duration of observation is still too short.

The recovery of CD4-positive cells after the second BMT was delayed compared to that after conventional transplantation. Actually, the patient experienced rising CMV antigenemia with a maximum number of positive cells greater than 1,000 cells per 2 slides, which suggested that the host-immunity was severely suppressed [16,17]. This could not be attributable to the use of reduced-intensity regimen, because we did not use ATG [18]. Taken altogether, the second allogeneic transplantation itself may be the main reason for the delay in the recovery of cellular immunity.

In conclusion, reduced-intensity BMT from an alternative donor for donor-derived leukemia/MDS appeared to be well tolerated. Although the impact of graft-versus-leukemia effect originated from the second donor remains unclear, this treatment could be considered as a treatment option.

REFERENCES

1. Cooley LD, Sears DA, Udden MM, et al. Donor cell leukemia: report of a case occurring 11 years after allogeneic bone marrow transplantation and review of the literature. Am J Hematol 2000;63:46–53.
2. Gopcsa L, Barta A, Banyai A, et al. Acute myeloid leukaemia of donor cell origin developing 5 years after allogeneic bone marrow transplantation for chronic myeloid leukaemia. Bone Marrow Transplant 2002; 29:449–452.
3. Hambach L, Eder M, Dammann E, et al. Donor cell-derived acute myeloid leukemia developing 14 months after matched unrelated bone marrow transplantation for chronic myeloid leukemia. Bone Marrow Transplant 2001;28:705–707.
4. Lawler M, Locasciulli A, Longoni D, et al. Leukaemic transformation of donor cells in a patient receiving a second allogeneic bone marrow transplant for severe aplastic anaemia. Bone Marrow Transplant 2002; 29:453–456.
5. Thiede C, Florek M, Bornhauser M, et al. Rapid quantification of mixed chimerism using multiplex amplification of short tandem repeat markers and fluorescence detection. Bone Marrow Transplant 1999; 23:1055–1060.
6. Heinonen K, Mrozek K, Lawrence D, et al. Clinical characteristics of patients with de novo acute myeloid leukaemia and isolated trisomy 11: a Cancer and Leukemia Group B study. Br J Haematol 1998;101: 513–520.
7. Bearman SI, Appelbaum FR, Buckner CD, et al. Regimen-related toxicity in patients undergoing bone marrow transplantation. J Clin Oncol 1988;6:1562–1568.
8. Kanda Y, Mineishi S, Saito T, et al. Pre-emptive therapy against cytomegalovirus (CMV) disease guided by CMV antigenemia assay after allogeneic hematopoietic stem cell transplantation: a single-center experience in Japan. Bone Marrow Transplant 2001;27:437–444.
9. Kanda Y, Mineishi S, Saito T, et al. Response-oriented preemptive therapy against cytomegalovirus disease with low-dose ganciclovir: a prospective evaluation. Transplantation 2002;73:568–572.
10. Saito T, Kanda Y, Kami M, et al. Therapeutic potential of a reduced-intensity preparative regimen for allogeneic transplantation with cladribine, busulfan, and antithymocyte globulin against advanced/refractory acute leukemia/lymphoma. Clin Cancer Res 2002;8:1014–1020.
11. Boyd CN, Ramberg RC, Thomas ED. The incidence of recurrence of leukemia in donor cells after allogeneic bone marrow transplantation. Leuk Res 1982;6:833–837.
12. Greenberg P, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 1997; 89:2079–2088.
13. Wolff SN. Second hematopoietic stem cell transplantation for the treatment of graft failure, graft rejection or relapse after allogeneic transplantation. Bone Marrow Transplant 2002;29:545–552.
14. Sandmaier BM, McSweeney P, Yu C, Storb R. Non-myeloablative transplants: preclinical and clinical results. Semin Oncol 2000;27:78–81.
15. Slavin S, Nagler A, Naparstek E, et al. Non-myeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. Blood 1998;91: 756–763.
16. Nichols WG, Corey L, Gooley T, et al. Rising pp65 antigenemia during preemptive anticytomegalovirus therapy after allogeneic hematopoietic stem cell transplantation: risk factors, correlation with DNA load, and outcomes. Blood 2001;97:867–874.
17. Kanda Y, Mineishi S, Nakai K, et al. Frequent detection of rising cytomegalovirus antigenemia after allogeneic stem cell transplantation following a regimen containing antithymocyte globulin. Blood 2001; 97:3676–3677.
18. Nakai K, Kanda Y, Mineishi S, et al. Suspected delayed immune recovery against cytomegalovirus after reduced-intensity stem cell transplantation using anti-thymocyte globulin. Bone Marrow Transplant 2002;29:237–241.

International Journal of
HEMATOLOGY

Molecular Cytogenetic Analyses of HIG, a Novel Human Cell Line Carrying t(1;3)(p36.3;q25.3) Established from a Patient with Chronic Myelogenous Leukemia in Blastic Crisis

Noriko Hosoya,[a] Seishi Ogawa,[a] Tohru Motokura,[a] Akira Hangaishi,[a] Lili Wang,[a] Ying Qiao,[a] Yasuhito Nannya,[a] Mieko Kogi,[b] Hisamaru Hirai[a]

[a]*Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo;* [b]*Hematologic Research Center, BML Tokyo Assay Laboratories Co, Ltd, Tokyo, Japan*

Received June 9, 2003; received in revised form August 11, 2003; accepted August 13, 2003

Abstract

Chromosomal abnormalities involving 1p36, 3q21, and/or 3q26 have been reported in a subset of myeloid neoplasms having characteristic dysmegakaryopoiesis, and the overexpression of *EVI1* on 3q26 or of *MEL1* on 1p36 has been implicated in their pathogenesis. We describe molecular cytogenetic analyses of a novel human cell line, HIG, established from a unique case in which a novel translocation t(1;3)(p36;q26) appeared as the sole additional chromosomal abnormality at the time of blastic transformation of chronic myelogenous leukemia. The patient displayed clinical features resembling those of the 3q21q26 syndrome. The HIG cell line retained der(1)t(1;3)(p36;q26) but lost t(9;22)(q34;q11). To identify the relevant gene that would be deregulated by this translocation, we molecularly cloned the translocation's breakpoints. They were distant from the breakpoint cluster regions of the 3q21q26 syndrome or t(1;3)(p36;q21), and neither the *EVI1* nor the *MEL1* transcript was detected in the HIG cell line. None of the genes located within 150 kilobase pairs of the breakpoints were aberrantly expressed, suggesting that in this case other gene(s) more distant from the breakpoints are deregulated by possible remote effects. Further analyses of the deregulated genes in the HIG cell line should provide important insight into the mechanisms involved in these types of leukemias. *Int J Hematol.* 2003;78:432-438.

Key words: Chromosomal translocation; Leukemia; t(1;3)(p36;q26); 3q21q26 Syndrome

1. Introduction

Chromosomal abnormalities involving chromosomes 3q21 and 3q26, such as inv(3)(q21q26), ins(3;3)(q21;q21q26), and t(3;3)(q21;q26), are observed in approximately 2% of cases of acute myelogenous leukemia and myelodysplastic syndrome and also are found in blastic transformation of chronic myelogenous leukemia (CML) [1]. There is a well-known association between these abnormalities and their clinical features, known as acute leukemia syndrome [2] or, more specifically, 3q21q26 syndrome [1,3], which shows normal or elevated peripheral platelet counts at the initial diagnosis, hyperplasia of bone marrow with dysplasia of megakaryocytes, poor response to chemotherapy, and a dismal prognosis [4].

With regard to the molecular mechanism, the *EVI1* gene at 3q26 has been implicated in the pathogenesis of this syndrome [5-7]. The chromosomal breakpoints on 3q26 are clustered within the 5′ region of the *EVI1* gene in t(3;3)(q21;q26) and within the 3′ region in inv(3)(q21q26), whereas the breakpoints at 3q21 in both t(3;3)(q21;q26) and inv(3)(q21q26) are clustered within the 50-kilobase pair (kbp) region near the ribophorin I (*RPN1*) gene [3,6,8]. Thus, it has been postulated that in the 3q21q26 syndrome the 5′ enhancer element of *RPN1* translocated to the *EVI1* loci is responsible for transcriptional activation of the *EVI1* gene. Intriguingly, a novel homologue of the *EVI1* gene has recently been identified near the 1p36 breakpoint of t(1;3)

Correspondence and reprint requests: Seishi Ogawa, MD, PhD, Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; 81-3-5800-8741; fax: 81-3-5804-6261 (e-mail: sogawa-tky@umin.ac.jp).

(p36;q21) [9], another well-known translocation that resembles 3q21q26 syndrome [10,11]. In this translocation, the *MEL1* gene near the 1p36 breakpoint is also supposed to be expressed under the control of the enhancer of the *RPN1* gene 100 to 160 kbp apart from the 3q21 breakpoints [9,12]. There exists a remarkable correlation between the clinical features and the underlying genetic aberrations in the 3q21q26 syndrome and t(1;3)(p36;q21) cases.

We describe a case of translocation involving both 1p36 and 3q26, ie, t(1;3)(p36;q26). This translocation has rarely been found in leukemias. We found it in a patient with CML in blastic crisis who had clinical features similar to those of the 3q21q26 syndrome and t(1;3)(p36;q21), such as an increased platelet count and severe dysmegakaryopoiesis. In this study, we established a cell line from the patient and performed molecular cytogenetic analyses of this translocation. The breakpoints thus identified were, unexpectedly, quite distinct from the *EVI1* and *MEL1* loci, implying the existence of another gene target responsible for this acute leukemia syndrome.

2. Materials and Methods

2.1. Case History

The HIG cell line was established from the bone marrow leukemic cells of a 59-year-old Japanese man with CML in blastic crisis. He received the diagnosis of CML in the chronic phase in November 1984. Cytogenetic examination showed a karyotype of 46,XY,t(9;22)(q34;q11) in all metaphases examined. The disease had been kept under good control until April 1989, when novel translocation t(1;3)(p36;q26) appeared as a sole additional chromosomal abnormality in 18 of 30 examined bone marrow metaphases (Figure 1A). During the following month, the white blood cell count rapidly increased to 85.9 × 10^9/L with 53% blasts, and the peripheral platelet count also increased to 634 × 10^9/L. The bone marrow was markedly hypercellular with 31% blasts and numerous micromegakaryocytes, showing the blastic transformation of CML. In May 1989, all 30 marrow metaphases had the karyotype 46,XY,t(1;3)(p36:q26), t(9;22)(q34:q11). The patient died of sepsis during chemotherapy one month after blastic transformation.

2.2. Establishment and Characterization of the Cell Line

Mononuclear cells were separated by Ficoll-Conray gradient centrifugation from a bone marrow sample obtained on May 10, 1989. Leukemic cells were cultured at 37°C in a 5% carbon dioxide atmosphere in RPMI 1640 medium with 20% fetal calf serum. Cytogenetic analyses of the patient's bone marrow samples and the HIG cell line were performed with the conventional Giemsa banding technique. The HIG cell line was also subjected to multiplex-fluorescence in situ hybridization (FISH) analysis [13] using the Sky Paint kit (Applied Spectral Imaging, Migdal Ha'Emek, Israel). Ten metaphases were analyzed with a Zeiss microscope (Axioplan 2; Zeiss, Oberkochen, Germany) equipped with appropriate filters (4',6-diamidino-2-phenylindole, Texas Red, Spectra Orange, fluorescein isothiocyanate, Cy5, and Cy5.5) and MetaSystems Isis imaging software programs (MetaSystems, Altlussheim, Germany).

2.3. Breakpoint Mapping Using Gene Walking and FISH Analyses

Breakpoint mapping on chromosome 3q was performed by iterated FISH experiments using a series of probes already mapped to or newly isolated from 3q. The yeast artificial chromosome (YAC) clones mapped to 3q were purchased from CEPH (Paris, France). Between the two adjacent YAC clones 938F10 and 855E4, a contig of P1-derived artificial chromosome (PAC) and bacterial artificial chromosome (BAC) clones was constructed by polymerase chain reaction (PCR)-based screening of "Down-to-the-Well" Human PAC/BAC DNA pools (Genome Systems, St. Louis, MO, USA) according to the manufacturer's instructions. The obtained contig clones were used as FISH probes. To identify chromosomes 1 and 3 on FISH analysis, we used two alphoid probes, D1Z7 on chromosome 1 and D3Z1 on chromosome 3 (American Type Culture Collection, Manassas, VA, USA). FISH analyses on metaphase chromosomes of HIG cells were performed essentially as previously described [14].

2.4. Southern and Northern Blot Analyses

High molecular weight DNA and polyA+ RNA were prepared from cell lines or normal controls as previously described [15]. Ten micrograms of DNA digested with appropriate restriction enzymes or 5 μg polyA+ RNA were electrophoresed, transferred onto nylon membranes, and hybridized to DNA probes labeled by the random hexamer method. Probe A within RP11-290K4 was obtained by PCR using 100 ng DNA isolated from the RP11-290K4 clone and the following 2 primers: the 20 mer 5' sense primer (5'-CTT TATATGTACTCTGGGCC-3') initiated at nucleotide 40,436 and the 20 mer 3' antisense primer (5'-TGTGTTTAT TTATTCAATCA-3') initiated at nucleotide 40,685 of RP11-290K4 (Genbank accession no. AC079943). PCR amplifications were performed in a thermal cycler with the following profile: 94°C for 2 minutes followed by 35 cycles of 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute and a final extension at 72°C for 10 minutes. The probes for the *EVI1*, *SHOX2*, and *MEL1* genes have been described in previous reports [9,15,16]. The expressed sequence tag (EST) clones were purchased from Invitrogen (Tokyo, Japan) or from the Department of ATCC, Summit Pharmaceuticals International Corporation (Tokyo, Japan). The EST clones' full-length inserts were obtained by digesting the clones with appropriate restriction enzymes, and the inserts were used as probes.

2.5. Long-Distance Inverse PCR

Genomic DNA (0.4 μg) from HIG cells was digested with *Sac*I and self-ligated overnight at 15°C in a volume of 500 μL. A portion (1/40) of the self-ligated DNA was directed to inverse PCR reactions. The conditions for the PCR reactions

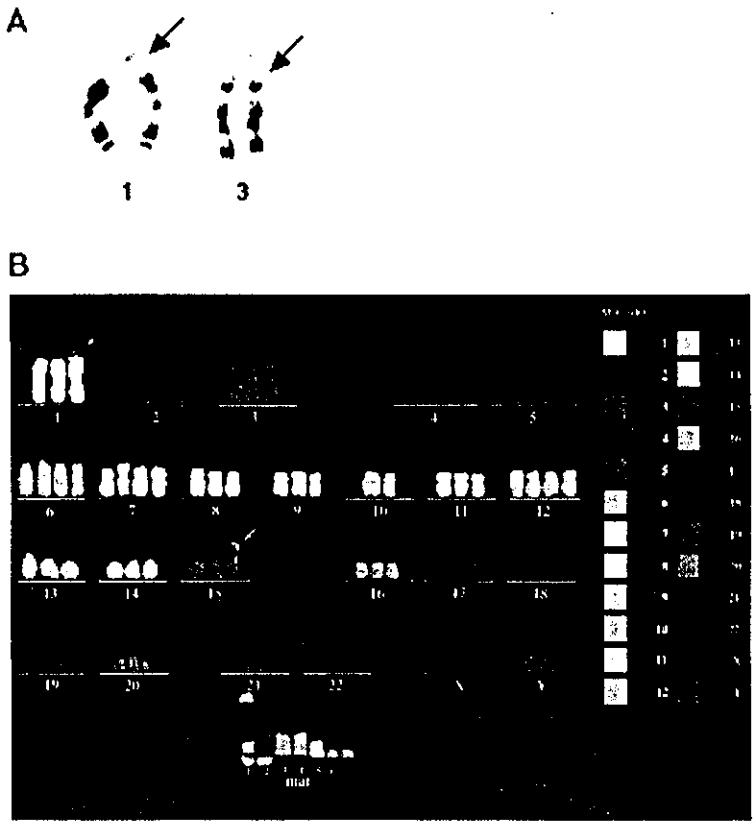


Figure 1. Karyotypes of the original leukemic cells and the HIG cell line. A, Partial karyotype of a bone marrow cell showing t(1;3)(p36;q26). The arrows indicate the derivative chromosomes 1 and 3. B, Multiplex-fluorescence in situ hybridization analysis showing a representative karyotype of HIG cells. The corresponding Giemsa-band karyotyping data together with these data indicate that the karyotype may be as follows: 80,XXYY,-1,der(1)t(1;3)(p36:q26),-3,-5,-5,-8,-9,-10,-10,-11,-13,-14,-15,add(15)(p13),-16,-17,-18,-20,-21,-21,-22,+7mar.

have been described in previous reports [17]. Primers were designed within the breakpoint-containing region at 3q25.3 so that the amplification products would include short stretches from these regions in addition to the translocated region at 1p36.3. Primers A5 and B1 were used in the first PCR reactions, and primers A1 through A4 were each used with B2 in the second reactions. The primer sequences are as follows: A1, 5′-TGGCCAACATGGTGAAACCCT-3′; A2, 5′-TGGGCAGATCACCTGAGTTTG-3′; A3, 5′-AAGCTC CAGTGCCAAAGGAAG-3′; A4, 5′-TGGAGCCAAACA TTGCAAAGA-3′; A5, 5′-GGAGGATATGACTTGTTG GCC-3′; B1, 5′-CTTGGTGCTCAGCCTAACATT-3′; and B2, 5′-GACGGTGAAAGTGGTGTCTGA-3′.

2.6. Reverse Transcriptase–PCR

Five micrograms of the total RNA sample were transcribed to complementary DNA (cDNA) with 2 units of Moloney murine leukemia virus reverse transcriptase (Stratagene, La Jolla, CA, USA) and a random hexamer. One tenth of the synthesized cDNA was directed to PCR analysis. The primers used for amplifications of the major *BCR/ABL*, *EVI1*, *MEL1*, *GAPDH*, and *SHOX2* transcripts were those described in previous reports [9,16,18,19]. Amplifications of the two EST (IMAGE nos. 1845365 and 6104862) transcripts were performed with primers C-F (5′-GCTTGGAAGAAG AATGAAGGA-3′) and C-R (5′-CAATCTGGCCTTATG

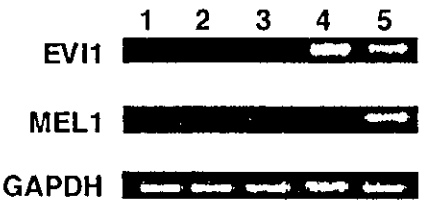


Figure 2. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of the *EVI1* and *MEL1* transcripts. Lanes 1 through 5 represent the origins of the complementary DNA templates prepared from cell lines U937, KCL22, HIG, and MOLM1 and human uterus, respectively. RT-PCR analyses were performed with RT-PCR primers for *EVI1* (upper panel), *MEL1* (middle panel), and *GAPDH* as a control (lower panel). Neither the *EVI1* nor the *MEL1* transcript was detected in the HIG cell line (lane 3), although they were detected in the positive control(s). The *EVI1* transcript was detected in MOLM1 (lane 4) and human uterus (lane 5), and that of *MEL1* was detected only in human uterus (lane 5).

TTGTAA-3') and carried out under the conditions described above.

3. Results

3.1. HIG, a Novel Cell Line Carrying der(1)t(1;3)(p36;q26)

After a 3-month culture, the leukemic cells became continuously proliferating in liquid suspension with a doubling time of approximately 30 hours. The cells were heterogeneous in size and had a tendency to aggregate. They were positive for CD15, CD25, CD71, and HLA-DR, weakly positive for CD4, CD5, CD22, CD56, CD64, and CD65, and negative for all other lineage-specific markers and did not show differentiation into any specific cell lineages, even in the presence of dimethyl sulfoxide, phorbol myristate acetate, or thrombopoietin (data not shown). Although the patient's original leukemic cells carried both t(1;3)(p36;q26) and t(9;22)(q34;q11), the established cell line, named HIG, had lost t(9;22)(q34;q11) and der(3)t(1;3)(p36;q26) during in vitro culture, retained only der(1)t(1;3)(p36;q26), and showed a near-tetraploid karyotype (Figure 1B). Reverse transcriptase–PCR (RT-PCR) results were also negative for the major *BCR/ABL* gene fusion product. The expression of the *EVI1* and *MEL1* transcripts was also examined, but they were not detected in the HIG cell line, either by Northern blot hybridization or by RT-PCR (Figure 2).

3.2. Identification of a Breakpoint-Containing PAC Clone at 3q25.3

Initially, we mapped the breakpoint of t(1;3)(p36;q26) on 3q by repeatedly performing FISH analyses using as probes a series of YAC clones already mapped to 3q. We defined the breakpoint between two adjacent YAC clones, 938F10 and 855E4 (Figure 3A). In metaphase FISH analyses, the former produced a signal only on the 3 normal chromosome 3s, whereas the latter also did so on the der(1)t(1;3) chromosome in addition to the 3 normal chromosome 3s (data not shown). Then, we further narrowed the location of the breakpoint by making a PAC/BAC contig that covered these YAC

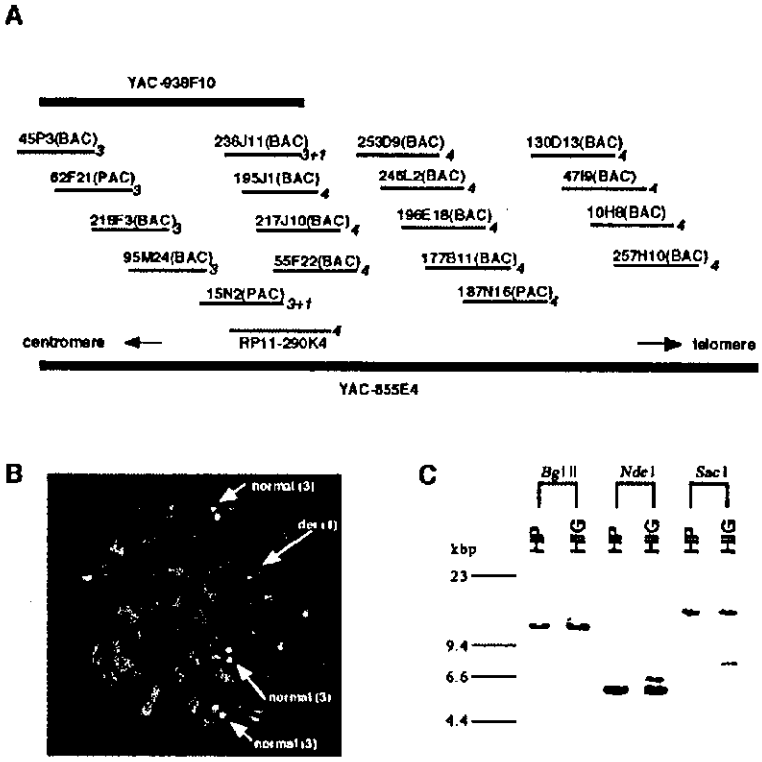


Figure 3. Identification of the breakpoints on 3q. A, The P1-derived artificial chromosome (PAC)/bacterial artificial chromosome (BAC) contig between 2 adjacent yeast artificial chromosome (YAC) clones 938F10 and 855E4. Directions of telomere and centromere are indicated. The number of signals that each clone created on the fluorescence in situ hybridization (FISH) analysis in the HIG cells is indicated to the right of the clone. "*3+1*" indicates that a weaker signal appeared on the derivative chromosome in addition to 3 authentic signals on the normal chromosome 3s, showing that the breakpoint exists on the indicated clones. B, FISH analysis in a metaphase chromosome array of the HIG cell line, revealing that signals of the 15N2 probe (green) are hybridized on the long arm of the 3 normal chromosome 3s and weakly on der(1)t(1;3)(p36;q26). The D1Z7 probe (red) is hybridized to the centromeres of the chromosome 1s. C, Identification of the rearrangement at 3q25.3 in the HIG cell line by Southern blot analysis. The filter was hybridized with probe A as described in "Materials and Methods." Sources of DNA and restriction enzymes used are indicated at the top of each blot. HP indicates human placenta.

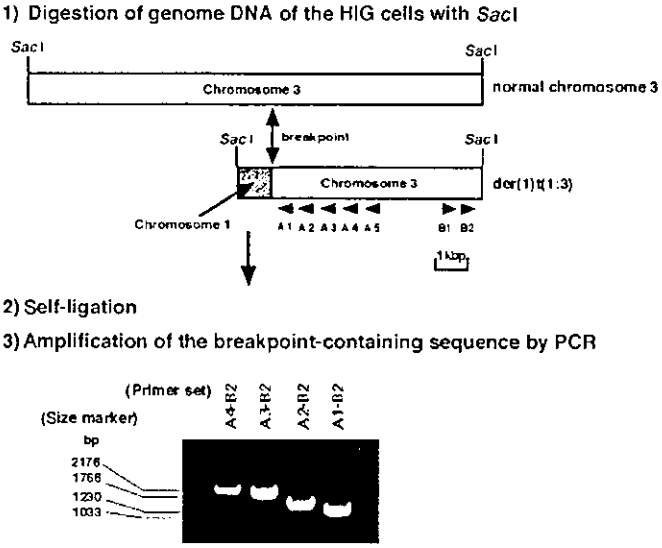


Figure 4. The strategy and results of long-distance inverse polymerase chain reaction (PCR) analysis. Positions and directions of the primers are illustrated. The primer sets used in the second PCR reactions are indicated at the top of each lane.

clones, and using this contig's component clones as FISH probes (Figure 3A), we identified the 3q breakpoint on the PAC-15N2 clone, which showed a weaker signal on the der(1)t(1;3)(p36;q26) chromosome (Figure 3B). To further pinpoint the location of the 3q breakpoint, the sequence of the 15N2 clone was determined by random sequencing of its shotgun library (Genbank accession no. AB107101). The partial sequence of 15N2 was 91,914 bp in length with an unclosed gap at sequence position 79,605. Clone 15N2 later was found to partly overlap BAC clone RP11-290K4 (Genbank accession no. AC079943) mapped to 3q25.3 (rather than to 3q26), which is likely located more than 10 megabase pairs (Mbp) centromeric to the *EVI1* gene at 3q26 according to mapping information from the Ensembl Genome Data Resources (http://www.ensembl.org). An array of genomic probes based on the genomic sequences was prepared within 15N2 by genomic PCR. After a series of Southern blot analyses using these probes, the gene rearrangement specific to the HIG cell line was identified in *Bgl*II, *Nde*I, and *Sac*I digests with probe A (Figure 3C).

3.3. Identification of Genomic Breakpoint Sequences at 3q25.3 and 1p36.3

To identify the exact breakpoint sequences in the HIG cell line, we employed the long-distance inverse PCR method (Figure 4) in which the breakpoint-containing *Sac*I fragment was self-ligated, PCR-amplified, and then directly sequenced (Figures 4 and 5A). By using the Basic Local Alignment Search Tool (BLAST), we were able to successfully identify breakpoint sequences at nucleotide 37,464 within RP11-290K4 on 3q25.3 and at nucleotide 175,231 within RP11-206L10 on 1p36.3 (Genbank accession no. AL669831) (Figure 5A). RP11-671C15 was also identified as an overlapping clone that was centromeric to RP11-206L10, and this clone was subjected to shotgun sequencing. Although 3400 shotgun clones were sequenced, 13 subcontigs remained unassembled (Genbank accession no. AB107102) because of a heavy load of highly homologous sequences. These BAC clones had been mapped in the subtelomeric region at 1p, which is likely to be located more than 1 Mbp telomeric to the *MEL1* gene according to the mapping information of Ensembl Genome Data Resources.

3.4. Analyses of the Transcribed Units near the Breakpoints

To identify the genes relevant to this translocation, we have extensively searched for structural genes near the breakpoints at 1p36.3 and 3q25.3 whose expressions might be altered in the HIG cell line. We identified 8 genes possibly encoded in the 1p36.3 region containing RP11-206L10 and RP11-671C15. We found an EST clone (IMAGE no. 346493) spanning the breakpoint and thus considered it a plausible candidate for the target gene (Figure 5B). On the other hand, we found a homeobox gene, *SHOX2* (also called *SHOT* or *OG12X*) [16,20,21], 121 kbp telomeric to the breakpoint on 3q25.3 (Figure 5B). We additionally found 3 EST clones (IMAGE nos. 1845365, 6104862, and 4668287) mapped between the 3q25.3 breakpoint and the *SHOX2* gene, one of which (IMAGE no. 1845365) encoded an exon only 611 bp distal to the breakpoint (Figure 5B), suggesting that it may be the nearest transcriptome to the 3q25.3 breakpoint. Nucleotide sequencing of the full inserts of the two clones, EST 1845365 and EST 6104862, indicated that the clones represented different splicing variants of the same gene. We examined the expression levels of all these transcriptomes in the HIG cell line as well as in other human hematopoietic cell lines, including THP1, U937, KCL22, and P30OHK, by Northern blot analyses and/or RT-PCR analyses, but none of the transcriptomes were aberrantly expressed in the HIG cell line (data not shown), showing that the potential transcriptomes at or near the breakpoints at 1p36.3 and 3q25.3 were not deregulated by this t(1;3)(p36.3;q25.3) translocation.

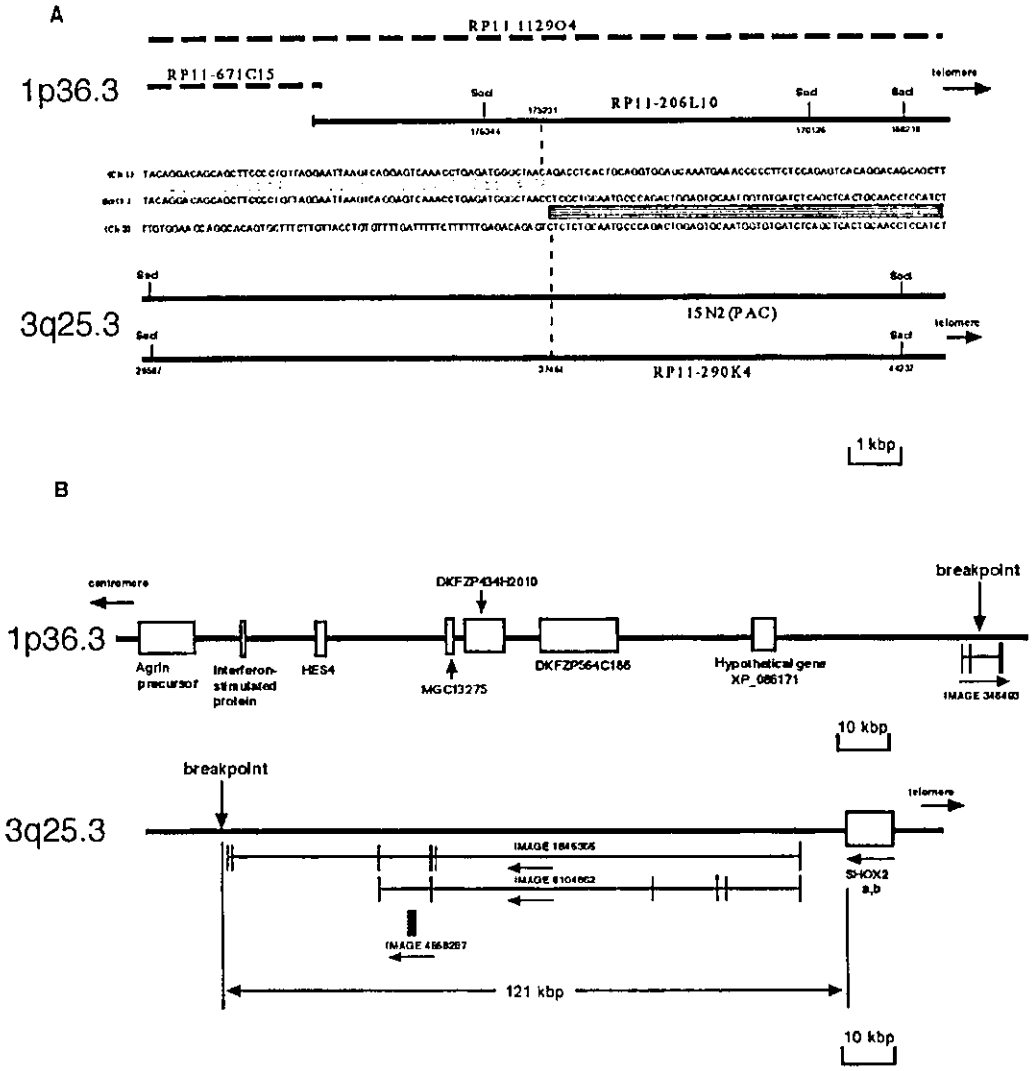


Figure 5. Physical maps of the chromosomal breakpoints at 1p36.3 and 3q25.3 in the HIG cell line. A, Genomic sequences surrounding the breakpoints of der(1)t(1;3)(p36.3;q25.3). The breakpoint sequences are depicted in parallel with the corresponding germline sequences. *Sac*I sites are shown by vertical lines. B, Transcriptomic units near the breakpoints at 1p36.3 and 3q25.3 in the HIG cell line. The breakpoints are shown by vertical arrows. The positions of the expressed sequence tag clones and the genes located near the breakpoints are presented. Their 5'-to-3' orientations are indicated by horizontal arrows.

4. Discussion

We have established a novel human leukemic cell line HIG from a unique case in which t(1;3)(p36;q26) emerged as the sole chromosomal abnormality in addition to t(9;22)(q34;q11) at the time of the blastic transformation of CML. Noted were a marked elevation of the peripheral platelet count and severe dysmegakaryocytosis, as are frequently observed in the 3q21q26 syndrome and t(1;3)(p36;q21)-positive leukemias. No other additional chromosomal abnormalities were detected at the time of cytogenetic evolution, and the established cell line still retains der(1)t(1;3)(p36;q26), although t(9;22)(q34;q11) was lost, suggesting that the appearance of der(1)t(1;3)(p36;q26) may have been a critical event responsible for the dysmegakaryopoiesis as well as the transformation of CML to the acute phase in this case. Thus, this cell line will be useful for investigating the leukemogenic mechanism of this translocation.

Together with their similar clinical features, the 3q26 and 1p36 breakpoints in our case were also cytogenetically indistinguishable from those found in 3q21q26 syndrome and t(1;3)(p36;q21)-positive leukemias in which the *EVI1* and *MEL1* genes have been implicated [5-7,9]. However, the molecularly determined breakpoints in the current case were found to be quite distant from the *EVI1* or *MEL1* loci (>10 Mbp from *EVI1* and >1 Mbp from *MEL1*). This finding is consistent with the fact that neither the *EVI1* nor the *MEL1* transcript was detected in the HIG cell line. Thus, leukemias determined to have the 1p36 or 3q26 breakpoint by the conventional Giemsa-band karyotyping technique may be more

heterogeneous in terms of molecular breakpoints and target genes and may even show a common clinical phenotype. It is possible that a yet unknown gene other than *EVI1* or *MEL1* exists in 1p36.3 or 3q25.3 and is relevant to progression of leukemias with dysmegakaryopoiesis and thrombocytosis.

With regard to the target genes for the current case, we searched extensively for transcriptional units located within regions of as long as 150 kbp at or near the breakpoints at 1p36.3 and 3q25.3 and identified several genes and potential transcriptomes as possible targets for this translocation. Unfortunately, however, we failed to show any alteration of these genes' expression in the HIG cell line, and we could not assess their expression in the original leukemic cells because the samples were not available. These findings suggest that the deregulation of the genes close to the breakpoints was not responsible for leukemogenesis in this patient, but it is likely that some unidentified genes more distant from the breakpoints are the target and are deregulated by possible remote effects. Supporting this hypothesis are observations of the ability of translocations to affect gene expression over distances of as far as 260 kbp, as has been demonstrated for the *MYC* gene [22-24]. We speculate that the chromosomal rearrangements in these cases may introduce an element that affects chromatin structure so as to deregulate the expression of the target genes. Further investigation to identify such critical genes in the current case is of great interest and is now in progress.

Acknowledgments

This work was supported in part by grants-in-aid for scientific research (KAKENHI 13307029) sponsored by the Japan Society for the Promotion of Science. We thank Dr. Akiko Yoneyama, Hiromitsu Yokota (University of Tokyo), and Dr. Ieharu Yamasaki (Inoue Memorial Hospital) for their assistance in the characterization of the cell line.

References

1. Mitelman F, Mertens F, Johansson B. A breakpoint map of recurrent chromosomal rearrangements in human neoplasia. *Nat Genet.* 1997;15:417-474.
2. Koeffler HP. Syndromes of acute nonlymphocytic leukemia. *Ann Int Med.* 1987;107:748-758.
3. Suzukawa K, Parganas E, Gajjar A, et al. Identification of a breakpoint cluster region 3' of the ribophorin I gene at 3q21 associated with the transcriptional activation of the EVI1 gene in acute myelogenous leukemias with inv(3)(q21q26). *Blood.* 1994;84:2681-2688.
4. Testoni N, Borsaru G, Martinelli G, et al. 3q21 and 3q26 cytogenetic abnormalities in acute myeloblastic leukemia: biological and clinical features. *Haematologica.* 1999;84:690-694.
5. Fichelson S, Dreyfus F, Berger R, et al. Evi-1 expression in leukemic patients with rearrangements of the 3q25-q28 chromosomal region. *Leukemia.* 1992;6:93-99.
6. Morishita K, Parganas E, Willman CL, et al. Activation of EVI1 gene expression in human acute myelogenous leukemias by translocations spanning 300-400 kilobases on chromosome band 3q26. *Proc Natl Acad Sci U S A.* 1992;89:3937-3941.
7. Levy ER, Parganas E, Morishita K, et al. DNA rearrangements proximal to the EVI1 locus associated with the 3q21q26 syndrome. *Blood.* 1994;83:1348-1354.
8. Suzukawa K, Taki T, Abe T, et al. Identification of translocational breakpoints within the intron region before the last coding exon (exon 12) of the EVI1 gene in two cases of CML-BC with inv(3)(q21q26). *Genomics.* 1997;42:356-360.
9. Mochizuki N, Shimizu S, Nagasawa T, et al. A novel gene, MEL1, mapped to 1p36.3 is highly homologous to the MDS1/EVI1 gene and is transcriptionally activated in t(1;3)(p36;q21)-positive leukemia cells. *Blood.* 2000;96:3209-3214.
10. Bloomfield CD, Garson OM, Volin L, Knuutila S, de la Chapelle A. t(1;3)(p36;q21) in acute nonlymphocytic leukemia: a new cytogenetic-clinicopathologic association. *Blood.* 1985;66:1409-1413.
11. Welborn JL, Lewis JP, Jenks II, Walling P. Diagnostic and prognostic significance of t(1;3)(p36;q21) in the disorders of hematopoiesis. *Cancer Genet Cytogenet.* 1987;28:277-285.
12. Shimizu S, Suzukawa K, Kodera T, et al. Identification of breakpoint cluster regions at 1p36.3 and 3q21 in hematologic malignancies with t(1;3)(p36;q21). *Genes Chromosomes Cancer.* 2000;27: 229-238.
13. Eils R, Uhrig S, Saracoglu K, et al. An optimized, fully automated system for fast and accurate identification of chromosomal rearrangements by multiplex-FISH (M-FISH). *Cytogenet Cell Genet.* 1998;82: 160-171.
14. Qiao Y, Ogawa S, Hangaishi A, et al. Identification of a novel fusion gene, TTL, fused to ETV6 in acute lymphoblastic leukemia with t(12;13)(p13;q14), and its implication in leukemogenesis. *Leukemia.* 2003;17:1112-1120.
15. Ogawa S, Kurokawa M, Tanaka T, et al. Structurally altered Evi-1 protein generated in the 3q21q26 syndrome. *Oncogene.* 1996;13: 183-191.
16. Blaschke RJ, Monaghan AP, Schiller S, et al. SHOT, a SHOX-related homeobox gene, is implicated in craniofacial, brain, heart, and limb development. *Proc Natl Acad Sci U S A.* 1998;95:2406-2411.
17. Willis TG, Jadayel DM, Coijnet LJA, et al. Rapid molecular cloning of rearrangements of the IGHJ locus using long-distance inverse polymerase chain reaction. *Blood.* 1997;90:2456-2464.
18. Kawasaki ES, Clark SS, Coyne MY, et al. Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia-specific mRNA sequences amplified in vitro. *Proc Natl Acad Sci U S A.* 1988;85:5698-5702.
19. Zoccola D, Legros L, Cassuto P, Fuzibet JG, Nucifora G, Raynaud SD. A discriminating screening is necessary to ascertain EVI1 expression by RT-PCR in malignant cells from the myeloid lineage without 3q26 rearrangement. *Leukemia.* 2003;17:643-645.
20. De Baere E, Speleman F, Van Roy N, De Paepe A, Messiaen L. Assignment of SHOX2 (alias OG12X and SHOT) to human chromosome bands 3q25→q26.1 by in situ hybridization. *Cytogenet Cell Genet.* 1998;82:228-229.
21. Semina EV, Reiter RS, Murray JC. A new human homeobox gene OGI2X is a member of the most conserved homeobox gene family and is expressed during heart development in mouse. *Hum Mol Genet.* 1998;7:415-422.
22. Cory S, Graham M, Webb E, Corcoran L, Adams JM. Variant (6;15) translocations in murine plasmacytomas involve a chromosome 15 locus at least 72 kb from the c-myc oncogene. *EMBO J.* 1985;4: 675-681.
23. Graham M, Adams JM. Chromosome 8 breakpoint far 3' of the c-myc oncogene in a Burkitt's lymphoma 2;8 variant translocation is equivalent to the murine pvt-1 locus. *EMBO J.* 1986;5:2845-2851.
24. Henglein B, Synovzik H, Groitl P, Bornkamm GW, Hartl P, Lipp M. Three breakpoints of variant t(2;8) translocations in Burkitt's lymphoma cells fall within a region 140 kilobases distal from c-myc. *Mol Cell Biol.* 1989;9:2105-2113.

Bone Marrow Transplantation (2003) 32, 1175–1179

www.nature.com/bmt

Post-transplant complications

Male predominance among Japanese adult patients with late-onset hemorrhagic cystitis after hematopoietic stem cell transplantation

Y Asano, Y Kanda, N Ogawa, M Sakata-Yanagimoto, M Nakagawa, M Kawazu, S Goyama, K Kandabashi, K Izutsu, Y Imai, A Hangaishi, M Kurokawa, S Tsujino, S Ogawa, K Aoki, S Chiba, T Motokura and H Hirai

Department of Cell Therapy & Transplantation Medicine, University of Tokyo Hospital, Tokyo, Japan

Summary:

Late-onset hemorrhagic cystitis (LHC) after hematopoietic stem cell transplantation (HSCT) is mainly caused by viral infections. We retrospectively analyzed the records of 141 Japanese adult patients who underwent a first allogeneic HSCT from 1995 to 2002. In all, 19 patients developed LHC a median of 51 days after HSCT. Adenovirus (AdV) was detected in the urine of 10 LHC patients, of whom eight had AdV type 11. Five of the six available serum samples from these patients were also positive for AdV type 11, but the detection of AdV in serum was not associated with a worse outcome. Male sex and the development of grade II–IV acute graft-versus-host disease were identified as independent significant risk factors for LHC. Male predominance was detected in LHC after HSCT, as has been previously shown in children with AdV-induced acute HC. The detection of AdV DNA in serum did not predict a poor outcome.
Bone Marrow Transplantation (2003) **32,** 1175–1179. doi:10.1038/sj.bmt.1704274
Published online 24 November 2003
Keywords: hemorrhagic cystitis; adenovirus; hematopoietic stem cell transplantation

Hemorrhagic cystitis (HC) is a common complication after hematopoietic stem cell transplantation (HSCT), with a reported incidence of 7–70%.[1] In the HSCT setting, it is well known that late-onset HC (LHC) is mainly attributed to viral infection, whereas early-onset HC is mainly due to the toxicity of cyclophosphamide. Adenovirus (AdV) type 11 is the prominent pathogen for LHC in Japan,[2,3] while BK virus (BKV)-associated LHC is frequently seen all over the world.[4,5]

Several risk factors associated with LHC, including old age, allogeneic HSCT, graft-versus-host-disease (GVHD), and the use of busulfan in a conditioning regimen, have been reported in several studies.[1,5,6] However, most of these risks have not been observed consistently. Furthermore, the incidence and risk factors may differ among populations, considering that LHC is mainly caused by viral infections. In this study, we retrospectively analyzed the etiology and risk factors of LHC in Japanese adult HSCT recipients.

Correspondence: Dr H Hirai, Department of Hematology & Oncology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: hhirai-tky@umin.ac.jp
Received 24 January 2003; accepted 09 May 2003; published online 24 November 2003

Patients and methods

Study population

We analyzed the records of 141 consecutive adult patients (>15 years old) who underwent conventional allogeneic HSCT for the first time at the University of Tokyo Hospital between June 1995 and May 2002. The median age was 35.0 years (range 16–59). Patients who received reduced-intensity conditioning were not included in the study. In all, 79 and 62 patients received graft from relatives and unrelated donors, respectively. Unrelated HSCT was performed exclusively using bone marrow, whereas 14 relatives donated a peripheral blood stem cell graft. In total, 47 patients received an HLA-mismatched graft. The underlying disease was acute myeloblastic leukemia (AML) in 32, acute lymphoblastic leukemia (ALL) in 36, chronic myelogenous leukemia (CML) in 42, myelodysplastic syndrome (MDS) in 15, non-Hodgkin's lymphoma (NHL) in eight, aplastic anemia (AA) in seven, and adult T-cell leukemia/lymphoma (ATL) in one. Acute leukemia in first remission, CML in first chronic phase, MDS without leukemic transformation, NHL in complete response, and AA were defined as low-risk disease.

Transplantation procedure

The preparative regimen was mainly either cyclophosphamide at 120 mg/kg with busulfan at 16 mg/kg or cyclophosphamide at the same dose with total body irradiation at 1200 cGy. Mesna was administered at 400–800 mg three times a day with hyperhydration to prevent cyclophosphamide-induced HC. Prophylaxis against GVHD consisted of cyclosporine or tacrolimus combined with short-term methotrexate.

As herpes simplex virus prophylaxis, acyclovir was given 1000 mg/day orally or 750 mg/day intravenously from days −7 to 35 after HSCT. Pre-emptive therapy against

cytomegalovirus (CMV) was performed by monitoring CMV antigenemia weekly after engraftment.

Definition of LHC and samples for virus detection

To exclude regimen-related HC, only patients who developed macroscopic hematuria or sustained microhematuria for more than 14 days with clinical signs of cystitis *de novo* at least 10 days after HSCT and who did not have a tendency for generalized bleeding or bacteriuria were considered to have LHC. The severity of LHC was graded according to previously reported criteria;[1] mild: sustained microscopic hematuria, moderate: gross hematuria and dysuria with or without clots, and severe: passage of clots with bladder pain requiring irrigation or the presence of complications including urinary tract obstruction and renal dysfunction requiring surgical intervention or chemical coagulation. Urine samples from all of the 19 patients with LHC were subjected to the following analyses to detect viral infections. Available frozen serum samples from six patients were also analyzed.

Urine viral culture

A volume of 2 ml of urine was centrifuged overnight at 20 000*g* and the sediment was cocultured with Hep-2 cells for up to 4 weeks. When a cytopathic sign of viral infection was observed, the viral species were identified using monoclonal antibodies against AdV.

PCR of urine samples

After 2 ml of a urine sample was centrifuged at 15 000*g* for 1 h at 4°C, DNA was extracted from the sediment using a GIAmp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). On the other hand, 200 μl of serum sample was directly applied to the kit to extract DNA. Next, 5 μg of purified DNA was subjected to a PCR assay using a GeneAmp Kit and GeneAmp PCR System 9600 (Perkin-Elmer, NJ, USA) with the following primers; AD185S (5-tccagcaacttcatgtccatgg-3) and AD 185A (5-tcgatgacgccgcggt-3) to screen for AdV infection, AD11BKOS (5-aatacaactggtgaggaacacgtaacag-3), AD11-BXUA (5-ccgcatcaaaaaactccatgtcgatatcat-3), AD11BXIS (5-tggaagtttcagatgaagaaagtaaaccga-3), and AD11BTIA (5-caaaggacccgtagcatggtttc-3) for AdV type 11-specific nested PCR, and BKV-1 (5-gcaagtgccaaaactactaat-3) and BKV-2 (5-tgcatgaaggttaagcatgc-3) to detect BKV infection. The size of the final products was confirmed by 3% agarose gel electrophoresis. The sensitivities of the PCR assay to screen AdV infection, Adv type 11-specific PCR, and BKV PCR were 200, 50, and 200 copies/ml, respectively.

Statistical analysis

The cumulative incidence of LHC and the impact of risk factors on LHC were evaluated using Gray's method, considering death without LHC as a competing risk.[7] Possible pretransplant factors analyzed in this study included age, sex, donor, graft source, risk of primary disease, and the use of busulfan in the preparative regimen. To evaluate the influence of the development of grade II–IV acute GVHD, proportional hazard modeling was used, treating the development of acute GVHD as a time-dependent covariate. Factors associated with at least borderline significance ($P<0.10$) in the univariate analyses were subjected to a multivariate analysis using backward stepwise proportional-hazard modeling. *P*-values of less than 0.05 were considered statistically significant.

Results

The incidence of LHC

In all, 19 patients developed LHC with a median onset of 51 days (range 11–380) after HSCT, with a 2-year cumulative incidence of LHC of 14.2%. All but one of the patients with LHC were male. Eight, nine, and two patients had severe, moderate, and mild LHC, respectively. The median duration of symptoms was 35 days (range 3–151). While LHC was mainly treated by supportive modalities including hydration and/or blood transfusions, seven patients required bladder irrigation and two received antiviral agents including cidofovir and vidarabine.

Clinical outcome of LHC patients

Nine of the 19 LHC patients died at a median of 57 days (range 29–364) after the onset of LHC. The cause of death was thrombotic microangiopathy in three, acute GVHD in two, invasive aspergillosis in two, and interstitial pneumonitis in two. Two of the nine patients developed AdV pneumonia, which was not the major cause of death. The 3-year actuarial survival rate among LHC patients was only 27%.

Analysis of urine and serum samples

AdV was detected in the urine samples of 10 LHC patients, of whom eight had AdV type 11 (Table 1). Serum samples were available in six of these eight patients and five of them were also positive for AdV type 11. The outcome of the patients with positive serum AdV DNA was similar to that of the other LHC patients (the median duration of symptoms was 45 days and three died due to unrelated causes). BKV was detected in the urine of 10 LHC patients, but six of them also had AdV type 11 infection.

Risk factors for LHC

Among the possible confounding factors, only the male sex and the development of grade II–IV acute GVHD were identified as significant risk factors for LHC by a univariate analysis (Table 2, Figure 1). These two factors were confirmed to be independently significant by proportional hazard modeling, with relative risks of 7.52 (95% CI; 1.00–56.4, $P=0.050$) and 4.37 (95% CI; 1.65–11.6, $P=0.0031$), respectively.

Table 1 Characteristics of patients who developed late-onset hemorrhagic cystitis

	Sex/age	*Diagnosis*	*aGVHD grade*	*Lymphocytes at the onset (cells/mm³)*	*Onset of LHC (days)*	*Severity of LHC*	*Treatment for LHC*	*Duration of symptom (days)*	*Virus in urine sample (detection method)*	*Virus in serum sample (PCR)*	*Survival (months)*	*Cause of death*
1	M/22	AA	II	1961	43	Severe	Irrigation	38	(—)	N.E.	76.0+	
2	M/24	ALL	I	224	79	Severe	Irrigation	55	BKV	N.E.	54.2+	
3	M/37	AML	—	495	380	Moderate	Hydration	45	AdV type 11 (C)	AdV type 11	24.4	IP
4	M/39	CML	IV	0	59	Severe	—	35	(—)	N.E.	3.1	TMA
5	M/26	CML	IV	264	72	Moderate	Hydration	12	(—)	N.E.	33.9+	
6	M/45	AML	III	90	19	Severe	Irrigation	41	AdV type 11 (C)	AdV type 11	2.0	TMA
7	M/31	CML	IV	306	28	Mild	Hydration	30	(—)	N.E.	3.4	TMA
8	M/50	AML	III	1860	231	Severe	Hydration	32	AdV type 11 (C)	N.E.	34.9+	
9	M/55	ALL	III	640	173	Severe	Surgical Intervention	57	BKV	N.E.	7.6	aGVHD
10	M/27	AML	III	244	34	Moderate	Hydration	74	AdV type 2, 11 (C) BKV	N.E.	21.7+	
11	M/57	CML	II	1012	101	Mild	Hydration	14	AdV common (C) BKV	N.E.	23.6+	
12	M/32	MDS	III	49	63	Moderate	Hydration	14	BKV	N.E.	8.3	IA
13	M/43	AML	I	0	11	Moderate	Hydration	3	(—)	N.E.	17.6+	
14	M/26	ALL	I	1632	44	Severe	Irrigation	33	AdV type 11, 34 (C)	AdV type 11	18.5+	
15	F/25	CML	III	144	26	Moderate	Irrigation	105	BKV	N.E.	11.7+	
16	M/53	AML	III	360	27	Severe	Hydration	151	AdV type 11 (P) BKV	AdV type 11	5.8	IP
17	M/42	MDS	I	0	247	Severe	Vidaravin	29	AdV type 11 (P) BKV	(—)	9.1	IA
18	M/35	MDS	I	731	51	Severe	Instillation of alum	45	AdV type 11 (C) BKV	AdV type 11	8.2+	
19	M/44	ATL	IV	176	30	Moderate	Hydration	33	AdV common (P) BKV	N.E	2.1	aGVHD

N.E. = not examined, AdV = adenovirus, BKV = BK virus, C = culture, P = PCR, CMV = cytomegalovirus, IP = interstitial pneumonitis, TMA = thrombotic microangiopathy, aGVHD = acute graft-versus-host-disease; IA = invasive aspergillosis.

Table 2 Influence of confounding factors on the incidence of late-onset hemorrhagic cystitis after hematopoietic stem cell transplantation

Univariate analysis

Factors	*Variables*	*Incidence (%)*	*P-value*
Pretransplant factors			
Sex	Male (101)	18.7	0.020
	Female (40)	2.5	
Age	< 35 (69)	11.7	0.53
	≥ 35 (72)	16.8	
Risk of primary disease	High (63)	15.4	0.76
	Low (78)	13.3	
Donor	Related (79)	10.6	0.17
	Unrelated (62)	18.8	
Graft source	Bone marrow (127)	14.7	0.52
	Peripheral blood (14)	10.0	
Preparative regimen	Containing busulfan (34)	15.2	0.83
	Not containing busulfan (107)	13.9	
Post transplant factor			
Acute GVHD	Grade 0–I (80)	6.8	0.0029
	Grade II–IV (61)	24.1	

Multivariate analysis

Factors	*Relative risk*	*95% CI*	*P-value*
Sex	7.52 (male *vs* female)	1.00–56.4	0.050
Acute GVHD	4.37 (grade II–IV *vs* 0–I)	1.65–11.6	0.0031

Numbers in parentheses show the number of patients in each group. GVHD = graft-versus-host disease, CI = confidence interval.

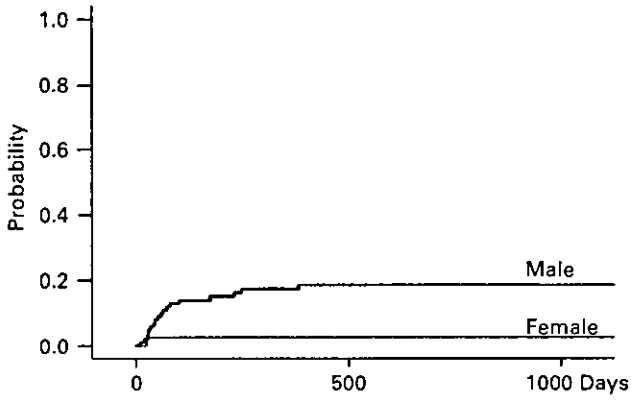


Figure 1 Cumulative incidence of late-onset hemorrhagic cystitis after hematopoietic stem cell transplantation in male and female patients.

Discussion

In this population, the cumulative incidence of LHC was 14.3% and approximately half of the LHC patients had AdV infection. Male sex and the development of acute GVHD were identified as independent risk factors for LHC. While patients who developed LHC showed poor survival, the severe background clinical status, rather than LHC itself, appeared to be responsible for the poor outcome. The incidence of LHC in our series was almost the same as that of Japanese adult HSCT recipients reported by Miyamura *et al*[2] and Akiyama *et al*.[3] In contrast, Kondo *et al*[8] reported that a lower incidence of LHC in Japanese pediatric HSCT recipients.

The development of grade II–IV acute GVHD was identified as an independent risk factor for LHC. Among the 13 LHC patients who developed acute GVHD, only one did so after the onset of LHC. This patient had received steroid pulse therapy against engraftment syndrome before he developed LHC. The median dose of corticosteroid and the median absolute lymphocyte count at the onset of LHC were 75 mg/day and 264/mm^3. These findings suggest that immunosuppression due to the development of acute GVHD and/or the use of steroid might be responsible for the development of LHC.

AdV was isolated by means of viral culture and/or PCR in 53% of the urine specimens of LHC patients, and most of the isolates were classified as AdV type 11. The incidence of AdV-associated LHC in our group of patients agreed with that in the previous reports from Japan, but was much higher than that in reports from the West.[4,5] The relationship between BKV infection and the development of LHC has been reported to be unclear.[4] BKV was frequently detected in the urine of non-LHC patients, whereas AdV was almost exclusively detected in LHC patients in a previous Japanese study.[3] In this study, four of the eight patients with AdV type 11 LHC were coinfected with BKV. However, neither the severity of LHC (three severe and one moderate in both groups) nor the outcome (two died and two are alive in both groups) was different between BKV-positive and -negative groups. We used a qualitative