Histological analysis of the bone marrow compartment of mice engrafted with myelodysplastic syndrome-originated cells

Successful engraftment of MDS-originated cells from six patients prompted us to dissect out the phenotypes of MDS-originated cells in the murine bone marrow environment. Although MDS is normally a disease of normal- to hyper-cellularity, the total number of bone marrow cells recovered from injected tibiae of the mice engrafted with MDS-originated cells was significantly lower than that of the mice engrafted with normal cells (Table 1, MDS-originated cell engrafted tibiae: $2.48\pm1.81\times10^6$, n=11, normal cell engrafted tibiae: $5.59\pm3.14\times10^6$, n=13, P=0.004). We then analyzed histological sections of mice engrafted with bone marrow cells from patients 11, 13, and 14. Consistent with the above finding, the marrow of the animals engrafted with MDS-originated cells from patient 11 appeared distinctly hypocellular compared to the marrow of normal cell-engrafted animals (Figure 2A). Human cells expressing human CD45, CD15, CD31, CD61, or glycophorin A (GlyA) were scattered throughout the marrow compartment in the injected tibiae of mice engrafted with normal cells or cells from patient 11. Neither human hematopoietic cells nor mesenchymal cells were observed in the contralateral tibiae of the same mice (Figure 2B and *data not shown*), consistent with our previous findings that intramedullary injected cells, especially when a limited number of cells were used, had a tendency to stay in the injected tibia.[16,18]

The cytology and bone histology of the mice engrafted with cells from patients 13 and 14 revealed that most of the marrow compartment was filled with large human CD45$^+$ leukemic blasts with prominent nucleoli (Figures 1D and 2A, and *data not shown*), leaving little space for normal murine hematopoiesis. Consistently, the number of non-human cells in the tibiae of mice engrafted with MDS-originated cells was significantly lower than that of the mice engrafted with normal cells (MDS-originated cell-engrafted tibia: $1.98\pm1.8\times10^6$, n=11, normal cell-engrafted tibia: $4.59\pm2.7\times10^6$, n=13, P=0.006). Although all experimental animals were irradiated equally, to exclude a possibility of heterogeneous response to sublethal irradiation as a cause of this decreased bone marrow cellularity in tibiae engrafted with MDS-originated cells, the number of non-human cells was also compared with that in the contralateral tibia of the same mice. The number of non-human cells in the injected tibia was significantly lower in the mice engrafted with MDS-originated cells (injected tibia: $3.08\pm1.35\times10^6$, contralateral tibia: $5.12\pm0.84\times10^6$, n=4, P=0.02), while there was no difference in non-human cell cellurality in mice engrafted with normal cells (injected tibia: $5.4\pm0.8\times10^6$, contralateral tibia: $5.2\pm1.2\times10^6$, n=3, P=0.4). The cell surface phenotypes of cells from patients 13 and 14 in murine bone marrow were primarily CD34$^+$, CD31$^+$, or CD61$^+$. Unlike the mice engrafted with normal cells or bone marrow cells from patient 11, the cells derived from patients 13 and 14 in the bone marrow rarely expressed CD15 or GlyA, two lineage markers used in this histological study.

Interestingly, proliferating primitive CD34$^+$ cells were prominently clustered along the endosteum, contrasting with the bone marrow of animals transplanted with nor-

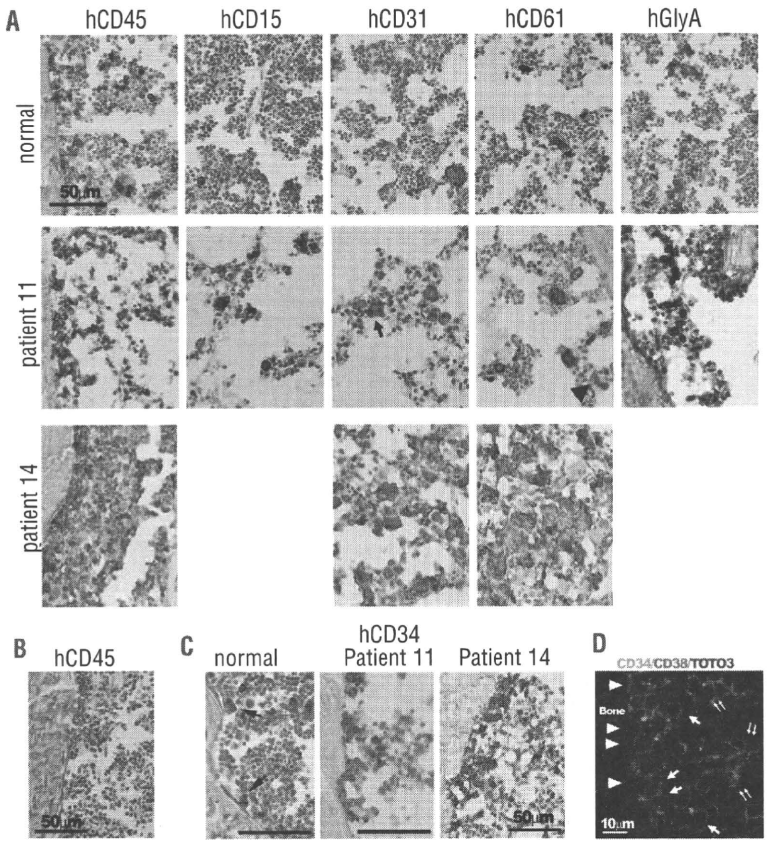

Figure 2. **Histological analysis of human MDS-originated cells in murine bone marrow (BM).** (A) **Immunohistochemical staining of bone sections of the mice engrafted with BM cells from normal individuals and patients 11 and 14. Human cells were recognized by specific staining for human antigens. Cells that reacted with antibodies specific to human CD45, CD15, CD31, CD61, and glycophorin A (GlyA) were detected throughout the murine BM compartment in the bone samples of normal individuals and patient 11. Relatively small megakaryocytes with separated nuclei (an arrow) were often observed in the CD31 stained sections of the mice engrafted with cells from patient 11. Note, murine megakaryocytes are negative for human CD61 (an arrowhead), confirming the specificity of the antigen-antibody reaction. In bone samples of patient 14, human CD45-expressing cells occupied most of the marrow compartment. Cells expressing megakaryocytic markers, CD31 and CD61, were noticeable.** (B) **A human CD45-stained bone section of contra-lateral tibia of the mice intramedullary injected with MDS-originated bone marrow CD34$^+$ cells from patient 11 and MSC. Neither obvious hypocellularity nor human CD45$^+$ cells were detected.** (C) **In bone samples of patient 11, MDS-originated CD34$^+$ cells proliferated along the surface of the endosteum, while individual CD34$^+$ cells (arrows) attached to the endosteum in normal cell-engrafted mice. Invasion of CD34$^+$ cells was prominent in bones engrafted with cells from patient 14.** (D) **The cells expressing only CD34 (arrowheads) attached to the endosteum, but the cells expressing both CD34 and CD38 did not (arrows). The cells expressing only CD38 (double arrows) were located distant from the bone.**

mal CD34⁺ cells in which individual CD34⁺ cells attached to the endosteum (patient 11, Figure 2C). The endosteal surface of primary recipient mice for patient 14 was covered with CD34⁺ cells, indicating an invasion of the putative hematopoietic stem cell niche by MDS-originated CD34⁺ cells (Figure 2C). Consistent with a previous report,[15] many of the MDS-originated CD34⁺ cells adhering to the endosteal surface lacked CD38 expression. The immunophenotypes of human cells gradually changed from CD34⁺CD38⁻ to CD34⁺CD38⁺, and eventually to CD34⁻CD38⁺ as cells were located further away from the endosteum (Figure 2D).

Sequential engraftment of myelodysplastic syndrome-originated cells

Serial transplants were conducted using bone marrow cells recovered from mice engrafted with bone marrow CD34⁺ cells from patients 11, 13, and 14. MDS-originated cells from patients 13 and 14, two patients whose cells demonstrated limited differentiation ability in murine bone marrow, but not from patient 11, successfully engrafted in secondary recipient mice (Figure 3A). In addition, it was possible to maintain the MDS-originated bone marrow cells from patient 14 for more than 2 years *in vivo* through passaging until the 8th recipient. The immunophenotypes of the engrafted cells were basically maintained throughout the experiments despite a gradual decline in the percentages of human cells over the period of the serial transplants. Interestingly, the frequency of CD34-expressing cells increased in the later transplant animals (*Online Supplementary Table S4* and Figure 3B), even though the frequency of CD34⁺ cells (%) in the endosteal area (within the distance of 5 cells) declined (53.79 ± 3.23, 40.56 ± 1.95, 38.85±0.87, and 29.88±6.74, for the 3rd, 5th, 6th, and 7th transplants, respectively), indicating a widespread distribution of primitive CD34⁺ cells and, thus, the selection or overgrowth of blastic cells against lineage differentiation. As expected, CD34⁺ cells, but not CD34⁻ cells, were able to sustain neoplastic cell growth into the next generation (*Online Supplementary Table S4*). Importantly, fluorescent activated cells sorting (FACS) analysis of human cells recovered from the engrafted mice demonstrated the maintenance of approximately the same proportion of CD34⁺CD38⁻ cells, a subpopulation of cells that includes leukemic stem cells,[13,15] until the 7th engraftment even though the overall human cell chimerism declined (Figure 3B).

Human cells were localized in the endosteal region in the

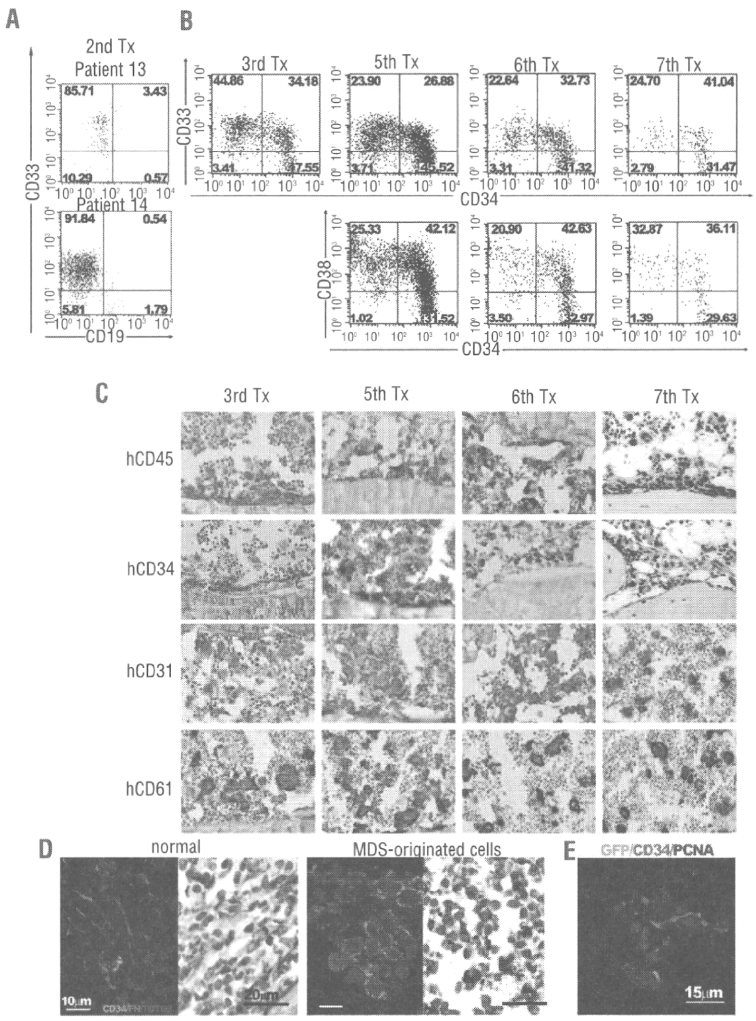

Figure 3. **Serial transplantation of MDS-originated cells and the bone marrow microenvironment** (A) **Flow cytometric profiles of bone marrow (BM) cells recovered from secondary hosts. Tx indicates transplantation.** (B) **Flow cytometric profiles of BM cells recovered from mice that had undergone serial transplants with BM cells from patient 14.** (C) **Immunohistochemical staining of bones obtained from mice serially transplanted with cells of patient 14 demonstrated a lineage cell staining pattern similar to that of the primary recipient mice, while CD45 and CD34-expressing cells were more confined to the endosteal region.** (D) **Immunofluorescent and immunohisotochemical staining for fibronectin (FN) of bones of normal cell- and MDS-originated cell-engrafted mice. Murine and human cells in normal cell-transplanted mice were tightly enveloped by fibronectin while the fibronectin network of MDS-originated cell-engrafted mice was disrupted. A light field photograph confirmed the well-structured fibronectin network in the BM of normal cell-engrafted mice, but only fibronectin fibrils were detected in the MDS-originated cell-engrafted mice. Stained sections of the mice engrafted with cells from patient 11 are shown. The same staining patterns were confirmed in bone sections of mice engrafted with cells from patients 13 and 14.** (E) **MDS-originated CD34⁺ cells expressing a proliferating marker, PCNA, interacted with human MSC marked with green fluorescent protein.**

bone marrow of serially transplanted mice, in contrast to the situation in the primary recipient mice in which human CD45$^+$ cells were ubiquitously located (Figures 2A and 3C). Even so, the MDS-originated CD34$^+$ cells occupied the murine endosteal surface, in striking resemblance to the situation in the bones of the primary recipients, thus indicating the persistence of leukemic stem cells, at least until the 6th transplant. Although the FACS analysis indicated the maintenance of a CD34$^+$CD38$^-$ subpopulation that included leukemic stem cells until the 7th serial transplant (Figure 3B), the 8th transplant did not result in obvious human cell engraftment (*Online Supplementary Table S4*). Consistent with this, in histological studies of the bone marrow of the recipient of the 7th transplantation, MDS-originated CD34$^+$ cells attached to the bone surface had disappeared (Figure 3C). These results are also consistent with our previous finding that CD34$^+$CD38$^-$ fractions are heterogeneous and stem cells reside at the endosteal surface.[22]

The microenvironment of mice engrafted with myelodysplastic syndrome-originated cells

The proliferation and survival of neoplastic cells are influenced by the host microenvironment. In this study, decreased bone marrow cellularity was a distinctive feature of all the MDS-originated cell engrafted samples analyzed (Table 1), thus indicating the suppression of murine hematopoiesis when neoplastic cells were present. To shed light on how neoplastic cells gained competitive advantages over normal host cells, the marrow compartments of animals engrafted with MDS-originated cells and normal bone marrow cells were examined. In the mice engrafted with normal bone marrow cells, the fibronectin network was well-structured throughout the marrow cavity (Figure 3D). In contrast, fibronectin network formation was irregular or disrupted in the mice engrafted with MDS-originated cells. Clusters of human CD34$^+$ cells were proliferating around the disrupted fibronectin fibrils in the central medulla of the bone marrow compartment. These observations, combined with an earlier histological finding that contralateral tibiae of the mice engrafted with MDS-originated cells exhibited normal cellularity (Figure 2B), suggest that the disruption of fibronectin network favors survival and proliferation of MDS-originated cells, while making it difficult to sustain normal hematopoiesis. In addition, CD34$^+$ cells with a proliferation marker, PCNA$^+$, were associated with co-transplanted MSC (Figure 3E) in MDS-originated cell engrafted bone marrow, which suggests the involvement of transplanted MSC in the engraftment, survival and proliferation of MDS-originated cells in the murine microenvironment.

Discussion

The recent development of mouse models of human acute leukemia are helping us to understand the phenotypes and physiology of leukemic stem cells. Although the engraftment of clonal MDS cells has been reported,[23,24] little is known about the behavior of MDS stem cells *in vivo* because of the difficulties of propagation of MDS cells up to a level that allows detailed investigation of MDS biology. In this study, in order to establish a reliable murine model for human MDS, bone marrow CD34$^+$ cells from patients with MDS and AML-MRC were co-injected with human MSC into the bone marrow of mice, a method proven to help engraftment and differentiation of human hematopoietic cells in the murine bone marrow microenvironment.[16,18,25] Successful engraftment of MDS-originated cells was observed in three out of six MDS cases (3 out of 8 mice engrafted with patients' cells) and three out of eight AML-MRC cases (9 out of 12 mice engrafted with patients' cells). Much to our surprise, the mice engrafted with MDS-originated cells uniformly exhibited significant decreases in the number of non-human bone marrow cells in the injected tibiae in comparison to the number of such cells in mice engrafted with normal cells, even when the percentage of human cells was less than 1% of total mononuclear cells. This is consistent with the fact that suppression of normal hematopoiesis can occur even when the tumor burden is relatively low. We found that most of the endosteal surface was covered with MDS-originated CD34$^+$ cells, a phenomenon also seen in murine models of acute leukemia,[14,15] which could be the underlying basis for the selective outgrowth of MDS-originated clones in patients over time.

The MDS-originated cells recovered from the human cell-engrafted mice maintained many characteristics of the original patients' cells, such as the cell surface phenotype and cytogenetic abnormalities. Unlike the previous two studies in which clonal abnormalities were detected in a fraction of cells,[23,24] we confirmed the presence of abnormalities in all cells examined by either FISH or chromosomal analysis, probably because purified bone marrow CD34$^+$ cells were used, rather than bone marrow mononuclear cells or T-cell-depleted blood or bone marrow cells. This makes our method more attractive for studying the behavior of MDS-originated cells *in vivo* because the majority, if not all, of the engrafted cells can be assumed to originate from clonal MDS cells. The majority of cells capable of engrafting in murine hosts were derived from patients carrying one or more genetic abnormalities (5/6; *Online Supplementary Table S1*). It is also noteworthy that four of those patients harbored an abnormality in chromosome 7, monosomy 7 or a partial deletion of chromosome 7, which is commonly found in Asian MDS cases and an indicator of aggressive disease with a poor prognosis.[26,27] On the other hand, five of eight cases that did not engraft or engrafted with normal cells were genetically normal. Taken together, the engraftment ability of MDS-originated cells positively correlated with the cytogenetic abnormalities of the patients.

In the current study, we attempted to explore the importance of MSC in engraftment of MDS-originated cells by comparison to non-bone marrow-derived stromal cells and the non-stromal component of bone marrow cells as auxiliary cells of transplantation. The presence of non-bone marrow-derived stromal cells (dermal fibroblasts) or non-stromal cells (cells of the CD34$^-$ fraction) did not help engraftment of MDS-originated cells. In contrast, the presence of MSC consistently improved engraftment of MDS-originated bone marrow CD34$^+$ cells. The pro-engraftment effect observed in our study could, therefore, be uniquely attributed to the presence of bone marrow-derived MSC. An investigation of how MSC help engraftment of human hematopoietic cells was beyond the scope of this study, but our observation of a physical interaction between MDS-originated CD34$^+$ cells and MSC (Figure 3E) suggests that MSC create a favorable environment for human MDS-originated cells to survive in the murine microenvironment, possibly through the physical interaction itself and

production of human cytokines, as indicated by our previous study.[18] It has been speculated that microenvironmental changes are involved in the pathogenesis of MDS.[28-30] However, since there was no significant difference in the engraftment of MDS-originated cells between normal (allogeneic-) and patient-derived (autologous-) MSC transplanted groups tested in this study, humanization of the microenvironment appeared more important, at least in our present study. One interesting finding in the serial transplantation study was that AML-MRC cells that had already engrafted in mice no longer required auxiliary cells or an intramedullary route of administration in subsequent transplants, perhaps because the cells with an ability to overcome hurdles to homing and engraftment in a murine host were selected during the serial transplants.

Local regulatory signals from the surrounding microenvironment to stem/progenitor cells play restrictive roles not only for normal cell development but also for tumorigenesis and metastasis.[31] It has also been reported that leukemic cells disrupt the behavior of normal hematopoietic progenitor cells by creating an abnormal microenvironment.[32] The microenvironment consists of heterogeneous types of cells and extracellular matrix proteins. Fibronectin is one of the major components of microenvironment structure. It was shown that mice lacking the enzyme needed to produce galactocerebrosides, a class of glycolipids in the nervous system, had an altered fibronectin network in the marrow microenvironment, which resulted in defective intramedullary lymphopoiesis and a hypocellular bone marrow.[33] Several studies found that interactions between leukemic cells and fibronectin prevented the apoptosis of leukemic cells from patients with AML, acute lymphocytic leukemia, and B-cell chronic lymphocytic leukemia, as well as leukemic cell lines *in vitro*.[34-37] In this study, mice engrafted with MDS-originated cells had an overall disruption of the fibronectin network in the bone marrow compartment and a striking decrease in the number of non-human bone marrow cells, indicating the importance of the three-dimensional structure of the fibronectin network in normal hematopoiesis. The reason for this fibronectin disruption and decrease in bone marrow cellularity are currently undetermined, but matrix metalloproteinases may play a role in creating a microenvironment that favors neoplastic cell growth over normal hematopoietic development. This idea is consistent with the results from previous studies demonstrating the increased matrix metalloproteinase production in cells obtained from MDS patients[38,39] and degradation of fibronectin by matrix metalloproteinases in a number of pathological processes.[40-43]

In summary, this study describes the establishment of a xenograft model of human MDS, using cells obtained from patients with low-risk MDS, high-risk MDS, and AML-MRC. Our murine xenograft MDS model consisted of human cell progeny, the majority of which, if not all, originated from clonal MDS cells and which demonstrated the defective cytological features of the original patients' cells. The efficiency of engraftment in the AML-MRC group was lower than that of *de novo* AML study.[15,44] This is probably because our AML-MRC group included patients with low percentages of blasts, formally categorized as having refractory anemia with excess blasts in transformation according to the French-American-British criteria. The study shows that the transplantability of neoplastic MDS-originated cells is partly determined by genetic abnormality and clinical aggressiveness of disease, which reflects the pathogenesis and progression of MDS towards leukemia.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Steensma DP, Tefferi A. The myelodysplastic syndrome(s): a perspective and review highlighting current controversies. Leuk Res. 2003;27(2):95-120.
2. Nimer SD. Myelodysplastic syndromes. Blood. 2008;111 (10):4841-51.
3. Heaney ML, Golde DW. Myelodysplasia. N Engl J Med. 1999;340(21):1649-60.
4. Haase D, Fonatsch C, Freund M, Wormann B, Bodenstein H, Bartels H, et al. Cytogenetic findings in 179 patients with myelodysplastic syndromes. Ann Hematol. 1995;70(4):171-87.
5. Parlier V, van Melle G, Beris P, Schmidt PM, Tobler A, Haller E, et al. Hematologic, clinical, and cytogenetic analysis in 109 patients with primary myelodysplastic syndrome. Prognostic significance of morphology and chromosome findings. Cancer Genet Cytogenet. 1994;78(2):219-31.
6. Raskind WH, Tirumali N, Jacobson R, Singer J, Fialkow PJ. Evidence for a multistep pathogenesis of a myelodysplastic syndrome. Blood. 1984;63(6):1318-23.
7. Tsukamoto N, Morita K, Maehara T, Okamoto K, Karasawa M, Omine M, et al. Clonality in myelodysplastic syndromes: demonstration of pluripotent stem cell origin using X-linked restriction fragment length polymorphisms. Br J Haematol. 1993;83(4):589-94.
8. Nilsson L, Astrand-Grundstrom I, Arvidsson I, Jacobsson B, Hellstrom-Lindberg E, Hast R, et al. Isolation and characterization of hematopoietic progenitor/stem cells in 5q-deleted myelodysplastic syndromes: evidence for involvement at the hematopoietic stem cell level. Blood. 2000;96(6):2012-21.
9. Van Etten RA, Shannon KM. Focus on myeloproliferative diseases and myelodysplastic syndromes. Cancer Cell. 2004;6 (6):547-52.
10. Uckun FM. Severe combined immunodeficient mouse models of human leukemia. Blood. 1996;88(4):1135-46.
11. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001;414(6859):105-11.
12. Lapidot T. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994;367(6464): 645-8.
13. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730-7.
14. Ninomiya M, Abe A, Katsumi A, Xu J, Ito M, Arai F, Suda T, et al. Homing, proliferation and survival sites of human leukemia cells in vivo in immunodeficient mice. Leukemia. 2007;21(1):136-42.
15. Ishikawa F, Yoshida S, Saito Y, Hijikata A, Kitamura H, Tanaka S, et al. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. Nat Biotechnol. 2007;25(11):1315-21.
16. Yahata T, Ando K, Sato T, Miyatake H, Nakamura Y, Muguruma Y, et al. A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. Blood. 2003;101(8):2905-13.
17. Muguruma Y, Reyes M, Nakamura Y, Sato T, Matsuzawa H, Miyatake H, et al. In vivo and in vitro differentiation of myocytes

from human bone marrow-derived multipotent progenitor cells. Exp Hematol. 2003;31(12):1323-30.
18. Muguruma Y, Yahata T, Miyatake H, Sato T, Uno T, Itoh J, et al. Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. Blood. 2006;107(5):1878-87.
19. Hiramatsu H, Nishikomori R, Heike T, Ito M, Kobayashi K, Katamura K, et al. Complete reconstitution of human lymphocytes from cord blood CD34+ cells using the NOD/SCID/gammacnull mice model. Blood. 2003;102(3):873-80.
20. Ishikawa F, Saito Y, Yoshida S, Harada M, Shultz LD. The differentiative and regenerative properties of human hematopoietic stem/progenitor cells in NOD-SCID/IL2rgamma(null) mice. Curr Top Microbiol Immunol. 2008;324:87-94.
21. Giassi LJ, Pearson T, Shultz LD, Laning J, Biber K, Kraus M, et al. Expanded CD34+ human umbilical cord blood cells generate multiple lymphohematopoietic lineages in NOD-scid IL2rgamma(null) mice. Exp Biol Med (Maywood). 2008;233(8):997-1012.
22. Yahata T, Muguruma Y, Yumino S, Sheng Y, Uno T, Matsuzawa H, et al. Quiescent human hematopoietic stem cells in the bone marrow niches organize the hierarchical structure of hematopoiesis. Stem Cells. 2008;26 (12):3228-36.
23. Thanopoulou E, Cashman J, Kakagianne T, Eaves A, Zoumbos N, Eaves C. Engraftment of NOD/SCID-beta2 microglobulin null mice with multilineage neoplastic cells from patients with myelodysplastic syndrome. Blood. 2004;103(11): 4285-93.
24. Kerbauy DM, Lesnikov V, Torok-Storb B, Bryant E, Deeg HJ. Engraftment of distinct clonal MDS-derived hematopoietic precursors in NOD/SCID-beta2-microglobulin-deficient mice after intramedullary transplantation of hematopoietic and stromal cells. Blood. 2004;104(7):2202-3.
25. Nakamura Y, Yahata T, Muguruma Y, Uno T, Sato T, Matsuzawa H, et al. Angiopoietin-1 supports induction of hematopoietic activity in human CD34- bone marrow cells. Exp Hematol. 2007;35 (12):1872-83.
26. Haase D, Germing U, Schanz J, Pfeilstocker M, Nosslinger T, Hildebrandt B, et al. New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients. Blood. 2007;110(13):4385-95.
27. Pozdnyakova O, Miron PM, Tang G, Walter O, Raza A, Woda B, et al. Cytogenetic abnormalities in a series of 1,029 patients with primary myelodysplastic syndromes: a report from the US with a focus on some undefined single chromosomal abnormalities. Cancer. 2008;113 (12):3331-40.
28. Borojevic R, Roela RA, Rodarte RS, Thiago LS, Pasini FS, Conti FM, et al. Bone marrow stroma in childhood myelodysplastic syndrome: composition, ability to sustain hematopoiesis in vitro, and altered gene expression. Leuk Res. 2004;28(8):831-44.
29. Flores-Figueroa E, Arana-Trejo RM, Gutirrez-Espindola G, Perez-Cabrera A, Mayani H. Mesenchymal stem cells in myelodysplastic syndromes: phenotypic and cytogenetic characterization. Leuk Res. 2005;29(2):215-24.
30. Raaijmakers MH, Mukherjee S, Guo S, Zhang S, Kobayashi T, Schoonmaker JA, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. Nature. 2010;464(7290):852-7.
31. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer. 2004;4(11):839-49.
32. Colmone A, Amorim M, Pontier AL, Wang S, Jablonski E, Sipkins DA. Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. Science. 2008;322(5909):1861-5.
33. Katayama Y, Frenette PS. Galactocerebrosides are required postnatally for stromal-dependent bone marrow lymphopoiesis. Immunity. 2003;18(6):789-800.
34. Bendall LJ, Makrynikola V, Hutchinson A, Bianchi AC, Bradstock KF, Gottlieb DJ. Stem cell factor enhances the adhesion of AML cells to fibronectin and augments fibronectin-mediated anti-apoptotic and proliferative signals. Leukemia. 1998;12 (9):1375-82.
35. de la Fuente MT, Casanova B, Garcia-Gila M, Silva A, Garcia-Pardo A. Fibronectin interaction with alpha4beta1 integrin prevents apoptosis in B cell chronic lymphocytic leukemia: correlation with Bcl-2 and Bax. Leukemia. 1999;13(2):266-74.
36. Mudry RE, Fortney JE, York T, Hall BM, Gibson LF. Stromal cells regulate survival of B-lineage leukemic cells during chemotherapy. Blood. 2000;96(5):1926-32.
37. Matsunaga T, Takemoto N, Sato T, Takimoto R, Tanaka I, Fujimi A, et al. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nat Med. 2003;9 (9):1158-65.
38. Ries C, Loher F, Zang C, Ismair MG, Petrides PE. Matrix metalloproteinase production by bone marrow mononuclear cells from normal individuals and patients with acute and chronic myeloid leukemia or myelodysplastic syndromes. Clin Cancer Res. 1999;5(5):1115-24.
39. Travaglino E, Benatti C, Malcovati L, Della Porta MG, Galli A, Bonetti E, et al. Biological and clinical relevance of matrix metalloproteinases 2 and 9 in acute myeloid leukaemias and myelodysplastic syndromes. Eur J Haematol. 2008;80 (3):216-26.
40. Nakahara H, Howard L, Thompson EW, Sato H, Seiki M, Yeh Y, et al. Transmembrane/cytoplasmic domain-mediated membrane type 1-matrix metalloprotease docking to invadopodia is required for cell invasion. Proc Natl Acad Sci USA. 1997;94(15):7959-64.
41. Matsumura S, Iwanaga S, Mochizuki S, Okamoto H, Ogawa S, Okada Y. Targeted deletion or pharmacological inhibition of MMP-2 prevents cardiac rupture after myocardial infarction in mice. J Clin Invest. 2005;115(3):599-609.
42. Clark ES, Whigham AS, Yarbrough WG, Weaver AM. Cortactin is an essential regulator of matrix metalloproteinase secretion and extracellular matrix degradation in invadopodia. Cancer Res. 2007;67(9):4227-35.
43. Hsu CC, Lai SC. Matrix metalloproteinase-2, -9 and -13 are involved in fibronectin degradation of rat lung granulomatous fibrosis caused by Angiostrongylus cantonensis. Int J Exp Pathol. 2007;88(6):437-43.
44. Saito Y, Uchida N, Tanaka S, Suzuki N, Tomizawa-Murasawa M, Sone A, et al. Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. Nat Biotechnol. 2010;28 (3):275-80.

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Letter to the Editor

"Triage" of lymphoid malignancies in the peripheral blood using the Extended Immunofluorescent Application of the CELL-DYN Sapphire automated hematology analyzer

Hiromichi Matsushita[1,*], Kazumi Gondo[2], Yumiko Tanaka[2] and Hayato Miyachi[1]

[1] Department of Laboratory Medicine, Tokai University School of Medicine, Isehara, Kanagawa, Japan
[2] Clinical Laboratory, Tokai University Hospital, Isehara, Kanagawa, Japan

Keywords: CELL-DYN Sapphire; leukemic B-lymphoid malignancy; monoclonality; surface immunoglobulin light chain.

Automated hematology analyzers are useful for differentiation of white blood cells. However, they often fail to detect infiltration of lymphoid malignancies in the peripheral blood, particularly when the tumor cells appear infrequently or are morphologically similar to normal lymphocytes. Microscopic observations by experts may lead to the identification of the suspicious cells, but such examinations often cannot confirm whether they are neoplastic. Following the identification of suspicious cells, an immunophenotypic analysis of these cells is needed to determine whether there is monoclonal proliferation, but this requires staff that are proficient in the techniques and time for sample preparation and analysis. The development of a simple and rapid discriminating "triage" assay system for lymphoid lineage cells is therefore needed in hospital laboratories.

There have been several automated hematology analyzers that have provided additional functions for the evaluation of abnormal leukocyte fractions detected by common light and laser technology. For example, the Advia Leukocyte Hematology Analyzer (Siemens) has a peroxidase channel to evaluate blasts and atypical lymphocytes as large unstained cells (LUCs) (1). XE-2100 and 5000 (Sysmex) instruments can also detect blasts through the immature information (IMI) channel, which were reported to correspond to $CD34^+$ and $CD34^-$ hematopoietic stem cells and progenitors (2, 3). However, these technologies are dependent on the biochemical features of LUCs and blasts, rather than the direct detection of their immunophenotypes. Therefore, they cannot be utilized to distinguish mature lymphoid malignancies from normal lymphocytes.

The CELL-DYN Sapphire automated hematology analyzer has a blue laser lamp and detectors. Therefore, it is able to evaluate the immunophenotypes of blood cells in addition to the analysis of complete blood cell counts and leukocyte differential counts. The systems for the analysis of T-cell subsets (CD4/CD8 ratio) and the immunological detection of platelets using anti-CD61 antibody have already been validated. It takes only 7 min to perform T-cell subset analysis and 5 min to perform platelet detection for automatic measurements after blood collection (4–6). Recently, the flow cytometric function using arbitrary antibodies and the ability to download FCS (flow cytometry standard) files have together been implemented as Extended Immunofluorescent Applications, which thus makes it possible to analyze various kinds of surface antigens on blood cells (7).

We have developed a simple and rapid assay system for monoclonal B-cells in the peripheral blood using the CELL-DYN Sapphire. The performance of a system using antibodies for CD19, CD20, CD3, and κ and λ types of surface immunoglobulin light chains (DAKO), including assay reproducibility and linearity, was comparable with the conventional method using the FACScalibur (BD) (8). To evaluate its applicability for clinical samples, we examined the peripheral blood of normal individuals and from those with different types of leukemic mature B-lymphoid malignancies, including chronic lymphocytic leukemia (CLL) (12 cases, morphologically abnormal cells: 50%–95%), follicular lymphoma (FL) (3 cases, 4%–28%), mantle cell lymphoma (MCL) (1 case, 8%), and diffuse large B cell lymphoma (1 case, 61%), in addition to T-large granular lymphocytic leukemia (T-LGL) (1 case, 95%), using the antibody combinations of anti-CD19/anti-CD3, anti-CD20/anti-CD3 and anti-κ/anti-λ (Figure 1).

As expected, CD19 and CD20-positive cells were counted less frequently than CD3-positive cells in normal individuals without laterality of surface immunoglobulin light chains, thus suggesting that there were no monoclonal B-cell subsets. In contrast, blood specimens from the cases with leukemic mature B-lymphoid malignancies, such as CLL, MCL and FL, often showed that there was dominant expression of CD19 and CD20 in the lymphoid cells gated by optical scattering with 0° axial light loss and 7° intermediate angle scat-

*Corresponding author: Hiromichi Matsushita, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
Phone: +81-463-93-1121, Fax: +81-463-93-8607,
E-mail: hmatsu@is.icc.u-tokai.ac.jp
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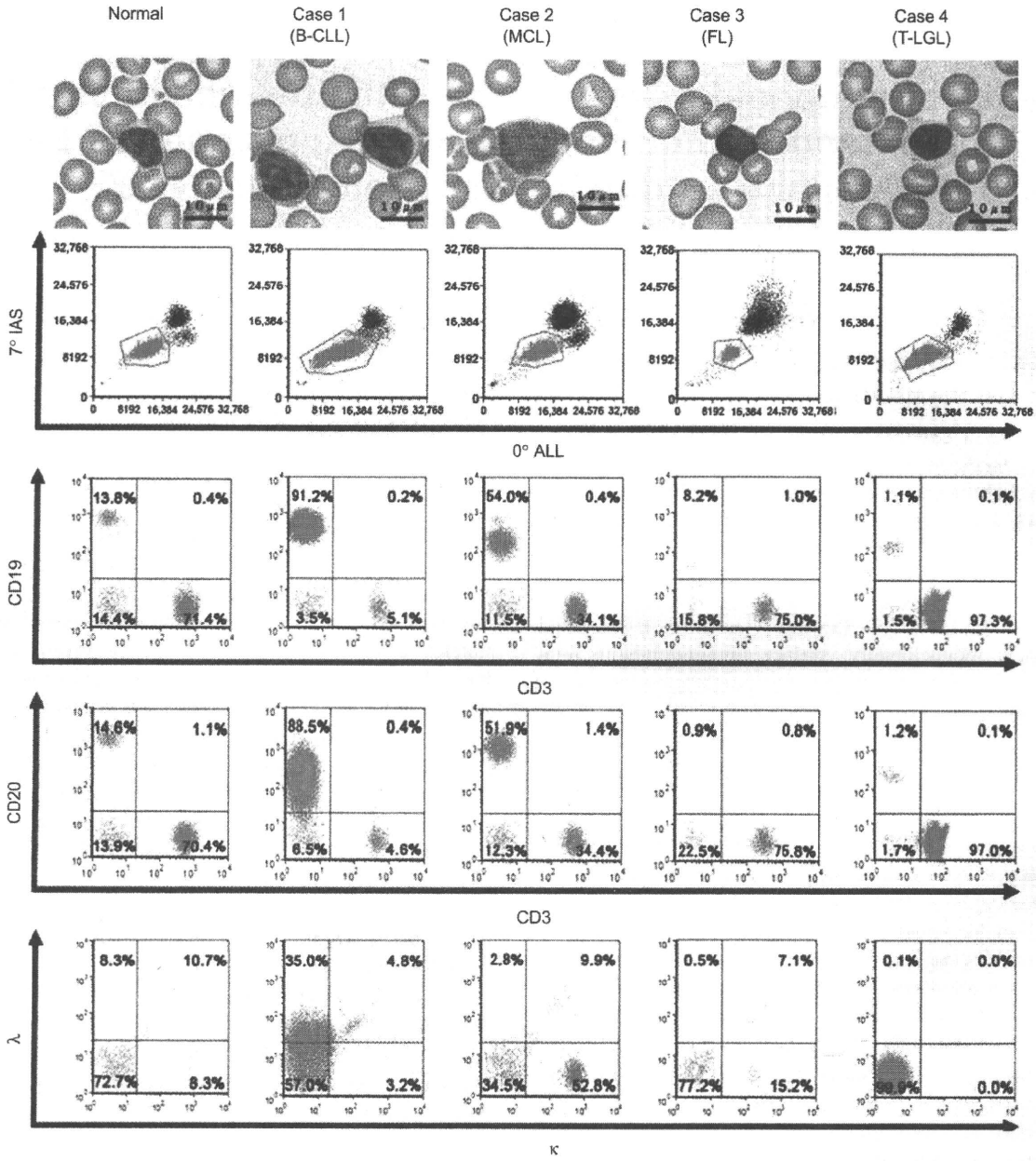

Figure 1 Cellular morphologies and scattergrams in the Extended Immunofluorescent Applications of the CELL-DYN Sapphire automated hematology analyzer for various B-lymphoid malignancies.
For the immunophenotypic analyses, the anti-CD19 and anti-CD3 antibodies were applied directly to the blood samples, whereas the anti-κ and anti-λ antibodies were added to the samples washed with phosphate-buffered saline. Representative cases were depicted. Case 1: Chronic lymphocytic leukemia (CLL). The leukocyte count was 27,100/μL with 87.0% CLL cells. Their nuclei were round with rather coarse chromatin formation, and their cytoplasm was narrow. They had dominant expression of the λ type of surface immunoglobulin light chain, although its signal intensity was low. Case 2: Mantle cell lymphoma (MCL). The leukocyte count was 12,400/μL with 8.0% MCL cells. The shape of their nucleus was round, with irregular contour of the nuclear membrane. They had dominant expression of the κ type surface immunoglobulin light chain. Case 3: Follicular lymphoma (FL). The leukocyte count was 1100/μL with 4% FL cells. They had a scant cytoplasm and a round nucleus with slightly fine chromatin formation. The κ type surface immunoglobulin light chain was dominantly expressed. Case 4: T-large granular lymphocytic leukemia (T-LGL). The leukocyte count was 72,800/μL with 95% LGL cells. The cells had a cytoplasm with a few large azurophilic granules. They expressed neither B-cell markers nor surface immunoglobulin light chains. The settings used were: 0° ALL, 0° axial light loss; 7° IAS, 7° intermediate angle scatter.

ter, although their fluorescence intensities were dependent on the type of disease (Case 1, 2 and 3). The unbalanced expression of surface immunoglobulin light chains was detected in all cases analyzed, with mature B-cell malignancies. As the malignant cells were enriched in the lymphocyte population using optical scattering, this system could detect monoclonal

proliferation of B-lymphoid cells in the peripheral blood, as low as only 4% lymphoid cells (Case 3). In addition, T-cell lymphoma cells could be detected by demonstrating that almost all of the lymphoid cells expressed CD3, but not B-cell markers (Case 4).

These data indicate that the Extended Immunofluorescent Applications of the CELL-DYN Sapphire automated hematology analyzer using simple panels of CD3, CD19, CD20 and immunoglobulin light chains can easily discriminate the lineage of various lymphoid malignancies, even if their frequencies in the peripheral blood are low. This simple and rapid assay system would be helpful not only for the efficient and economical detection of morphologically-indistinguishable lymphoid malignancies in the peripheral blood, but would also be useful for rapid monitoring of the disease. However, the Extended Immunofluorescent Applications using arbitrary monoclonal antibodies requires another computer system with a software program for flow cytometry data analysis, such as the FCS Express version 3.0 programs. In addition, the antibodies against the immunoglobulin light chains indicated a non-specific binding pattern, shown as a diagonal line of dots from the bottom left to the top right on the scattergrams, unless the blood samples were pre-washed with phosphate-buffered saline prior to the antibody-reactions (data not shown). It therefore takes more time and effort to perform this assay, compared to the CD61 immuno-platelet measurement and T-cell subset detection, which can be analyzed using only the CELL-DYN Sapphire main unit without pre-washing the blood samples (6). By improving these issues, we are expecting this ''triage'' system to positively contribute to the appropriate management of patients with lymphoid malignancies in the peripheral blood.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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References

1. Bononi A, Lanza F, Ferrari L, Gusella M, Gilli G, Abbasciano V, et al. Predictive value of hematological and phenotypical parameters on postchemotherapy leukocyte recovery. Cytometry Part B (Clinical Cytometry) 2009;76B:328–33.
2. Letestu R, Marzac C, Audat F, Belhocine R, Tondeur S, Baccini V, Use of hematopoietic progenitor cell count on the Sysmex XE-2100 for peripheral blood stem cell harvest monitoring. Leuk Lymphoma 2007;48:89–96.
3. Wang FS, Rowan RM, Creer M, Hay A, Dorfner M, Peesapati S, et al. Detecting human CD34+ and CD34-hematopoietic stem and progenitor cells using a Sysmex automated hematology analyzer. Lab Hematol 2004;10:200–5.
4. Cid J, Nascimento JD, Vicent A, Aguinaco R, Escoda L, Ugarriza A, et al. Evaluation of low platelet counts by optical, impedance, and CD61-immunoplatelet methods: estimation of possible inappropriate platelet transfusion. Transfusion 2010;50:795–800.
5. Grimaldi E, Del Vecchio L, Scopacasa F, Lo Pardo C, Capone F, Pariante S, et al. Evaluation of the platelet counting by Abbott CELL-DYN SAPPHIRE haematology analyser compared with flow cytometry. Int J Lab Hematol 2009;31:151–60.
6. Johannessen B, Roemer B, Flatmoen L, Just T, Aarsand AK, Scott CS. Implementation of monoclonal antibody fluorescence on the Abbott CELL-DYN Sapphire haematology analyzer: evaluation of lymphoid, myeloid and platelet markers. Clin Lab Haematol 2006;28:84–96.
7. Molero T, Lemes A, De La Iglesia S, Scott CS. Monoclonal antibody fluorescence for routine lymphocyte subpopulation analysis with the Abbott CELL-DYN Sapphire haematology analyser. Int J Lab Hematol 2007;29:446–53.
8. Gondo K, Tanaka Y, Hirota H, Tanaka Y, Matsushita H, Miyachi H. Screening test for lymphoid malignancies in the peripheral blood using the extended immunofluorescent application of automated hematology analyzer CELL-DYN Sapphire. J Jpn Soc Lab Hematol 2011;12:44–51 [Japanese].

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Short communication

FLT3-internal tandem duplication in a pediatric patient with t(8;21) acute myeloid leukemia

Machiko Kawamura[a,*], Hidefumi Kaku[a], Tateki Ito[b], Nobuaki Funata[b], Tomohiko Taki[c], Akira Shimada[d], Yasuhide Hayashi[d]

[a]*Department of Pediatrics, Tokyo Metropolitan Cancer and Infectious Disease Center Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan*

[b]*Department of Pathology, Tokyo Metropolitan Cancer and Infectious Disease Center Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan*

[c]*Department of Molecular Diagnostics and Therapeutics, Kyoto Prefectural University of Medicine Graduate School of Medical Science, 465 Kajii-cho Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan*

[d]*Department of Hematology/Oncology, Gunma Children's Medical Center, 779 Shimohakoda, Hokkitsu, Shibukawa, Gunma 377-8577, Japan*

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Abstract Patients diagnosed with t(8;21)-acute myeloid leukemia (AML) are currently considered to have good prognoses, but about half of these patients relapse. *FLT3*-internal tandem duplication (ITD) is generally thought to be strongly associated with poor prognosis in AML, but is rarely reported in patients with t(8;21)-AML. Expression of the neural cell-adhesion molecule (CD56) is also associated with a significantly shorter complete remission duration and survival in patients with t(8;21)-AML. Patients with t(8;21)-AML expressing CD56 have been reported to exhibit a higher incidence of granulocytic sarcoma (GS), and t(8;21)-AML with GS results in a less favorable prognosis than AML with this translocation alone. Here, we report on a 15-year-old girl with t(8;21)-AML having both CD56 expression and *FLT3*-ITD. This patient underwent unrelated donor bone marrow transplantation and achieved complete remission, but thereafter presented with obstructive jaundice caused by GS compression of the common bile duct without bone marrow invasion at relapse. Autopsy revealed multiple nodules of the stomach membrane and invasion into the head of the pancreas. For earlier detection of relapse, we suggest that it would be useful to examine existence of GS in CD56-positive t(8;21)-AML patients at diagnosis and hematologic remission. Even though t(8;21)-AML is less likely to co-occur with *FLT3*-ITD in pediatric patients, this report suggests that prognostic factors, including *FLT3* and *KIT* genes and the surface marker CD56, should be analyzed in these patients.

1. Introduction

Even though patients with t(8;21)-acute myeloid leukemia (AML) are thought to have a good prognosis, it is reported that 50% relapse and have poor prognosis [1,2]. High-presenting leukocyte count, neural cell-adhesion molecule (CD56) expression, and extramedullary disease has been reported to be associated with a poor prognosis in t(8;21)-AML [1,3]. *FLT3*-internal tandem duplication (ITD) is also considered to be strongly associated with a poor prognosis in adult and pediatric AML patients [4], but is rarely reported in patients with t(8;21)-AML [5,6]. Patients with t(8;21)-AML expressing CD56 have been reported to have a high incidence of granulocytic sarcoma (GS). However, the correlation of *FLT3*-ITD, CD56 expression, and GS with the prognosis of t(8;21)-AML remains unclear. We report on a patient with t(8;21)-AML having CD56 expression and *FLT3*-ITD, who also showed obstructive jaundice caused by GS compressing the bile ducts at relapse and had a poor prognosis.

2. Materials and methods

2.1. Case report

A 15-year-old girl presenting with a persistent fever was admitted to our hospital with anemia and general fatigue. Upon admission, the patient's face was pale and she

* Corresponding author. Tel.: +81(3)3823-2101; fax: +81(3)3824-1552.

E-mail address: m.kawamura@cick.jp (M. Kawamura).

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demonstrated no signs of cervical lymphadenopathy or hepatosplenomegaly. Blood tests revealed a white blood cell (WBC) count of 12,700/μL containing 54.5% blasts, a hemoglobin concentration of 5.7g/dL, and a platelet count of 2.2×10^4/μL. Bone marrow examination revealed 82.6% blasts that were positive for peroxidase staining (100%), as well as both nonspecific (21%) and specific (53%) esterase staining. A diagnosis of AML-M2 was made according to the morphologic and immunophenotypic criteria of French–American–British (FAB) classification. Immunophenotypic analysis of the CD45 dim cells showed the presence of CD13 (74.6%), CD33 (48%), CD34 (96.5%), CD38 (99.8%), HLA-DR (94.6%), CD56 (27.3%), and CD19 (51.2%). Cytogenetic analysis of the bone marrow cells demonstrated the 46, XX, t(8;21)(q22;q22), del(9)(q13q22) in 20 bone marrow cells. The *AML1/RUNX1-MTG8/ETO/RUNX1T1* fusion transcript was also detected at a concentration of 34×10^6 copies/μg RNA by real-time quantitative reverse-transcriptase polymerase chain reaction (RQ-RT-PCR).

The patient was treated in accordance with the low-risk treatment protocol of the Japanese Childhood AML Cooperative Study Group Protocol AML99 [7] due to the presence of t(8;21) and a WBC of less than 5×10^4/μl. Treatment consisted of etoposide, cytosine arabinoside, and mitoxantrone for induction therapy. After induction therapy, she achieved complete remission, and cytogenetic analysis revealed normal results in 20 bone marrow cells. After five courses of treatment, she remained in complete remission, although the *RUNX1-RUNX1T1* transcript was still detected at a concentration of 1.8×10^3 copies/μg RNA. Two months later, bone marrow analysis showed relapse and chemotherapy was continued. Finally, she could not achieve a second remission before allogeneic transplantation, and *RUNX1-RUNX1T1* transcript was 6.3×10^6 copies/μg RNA from bone marrow. Seven months after diagnosis, she underwent an unrelated-donor bone marrow transplant with the following conditioning regimen and acute graft-versus-host disease (GVHD) prophylaxis: busulfan and cyclophosphamide administration, as well as total lymph node irradiation followed by FK506 and short-term methotrexate administration. On day 15, she developed grade IIa GVHD with diffuse pruritic erythroderma, which improved after treatment with methylprednisolone. On day 25, her skin condition worsened and she reported severe abdominal pain and diarrhea. Lower GI endoscopy was performed and the biopsy revealed GVHD. The *RUNX1-RUNX1T1* fusion transcript of bone marrow cells was not detected. The volume of bloody diarrhea was almost 4 liters/day at its peak. She received steroid pulse therapy, octreotide acetate, and low-dose antithymocyte globulin, with her condition gradually improving after 2 months. During hematologic remission, ultrasound resonance of the abdomen did not show GS, although gastro-intestinal endoscopy was not performed at that time. On day 102, diarrhea symptoms returned and steroid pulse therapy and mycophenolate mofetil treatment were initiated. On day 238, she presented with jaundice and the *RUNX1-RUNX1T1* transcript was elevated up to 4.3×10^6 copies/μg RNA without bone marrow involvement. Computed tomography scanning disclosed thickening of the common bile duct wall and obstruction. Ultrasound resonance of the abdomen also showed obstructive findings (Fig. 1). She died on the day of the scheduled endoscopic retrograde biliary drainage, most likely due to septic shock caused by biliary tract infection, 2 years

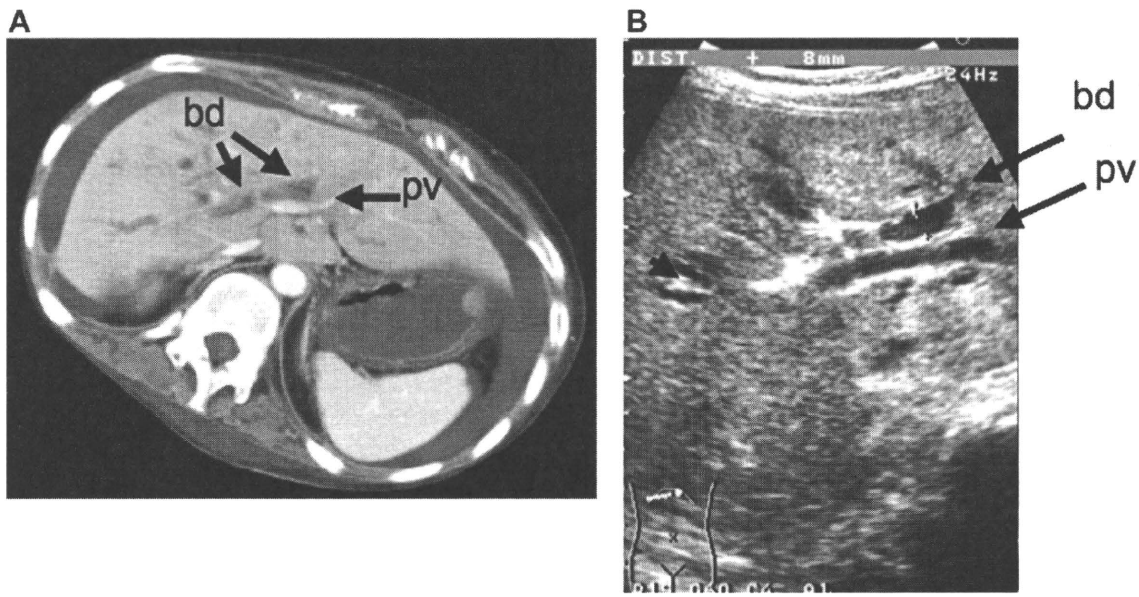

Fig. 1. (A) Enhanced computed tomography of the abdomen showing dilatation of the intrahepatic bile duct (bd) and obstruction of portal vein (pv). (B) Ultrasonography of abdomen showing dilatated intrahepatic bile duct (bd) and its accompanying hepatic portal vein (pv) with "parallel channel sign" of biliary obstruction.

after AML was diagnosed. The autopsy revealed multiple GS of the common bile duct, the head of the pancreas, and six nodes in the stomach mucosa that were formed by GS. The junction of the bile duct, pancreatic duct, and duodenum was also compressed by leukemic cells invading the tissues and causing obstructive jaundice (Fig. 2).

2.2. Analysis of FLT3 and KIT genes

RNA extracted from bone marrow cells at diagnosis was reverse-transcribed to cDNA, and alterations in the *FLT3* and *KIT* genes were examined according to previous reports [7]. Mutations of *FLT3*-D835/I836 were examined by restriction fragment length polymorphism PCR, and *FLT3*-ITD was analyzed by RT-PCR. Mutational analysis of exons 8–11 and exons 17–18 of the *KIT* gene was performed with RT-PCR followed by direct sequencing. In this patient, a 48-base pair (bp) *FLT3*-ITD was identified, while *KIT* or *FLT3* mutations did not show at diagnosis and at the first relapse.

This study was approved by the institute's ethics committee, and written informed consent was obtained from our patient's parents.

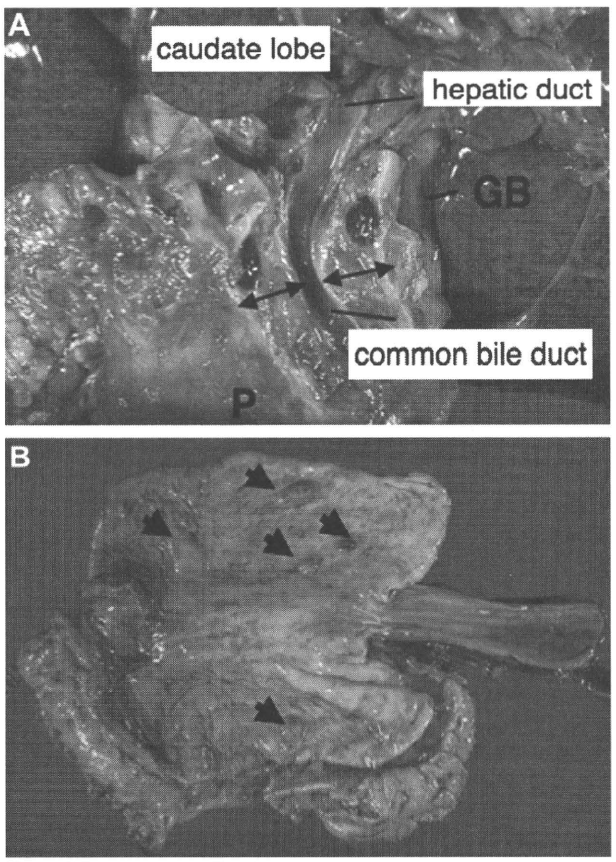

Fig. 2. (A) Granulocytic sarcoma invasion into the junction of gall bladder, duodenum, and common bile duct. P, pancreas; GB, gall bladder. (B) Multiple granulocytic sarcomas in stomach (indicated by arrows).

3. Results and discussion

t(8;21)-AML is a heterogeneous disease in terms of its clinical and biologic features, as well as its clinical outcome. *KIT* mutations are identified in 12–47% of patients with t(8;21) and are strongly associated with a poor prognosis in pediatric t(8;21)-AML [7,8]. In contrast, *FLT3*-ITD of AML is present in approximately 15% of pediatric and 25% of adult patients [4,5,9]. *FLT3*-ITD was demonstrated in only 2 (4.6%) of 46 patients with t(8;21)-AML entered in the study of the Japanese Childhood AML Cooperative Study Group [7] similar to the previous study [1]. This case of 1 of 2 patients with t(8;21)-AML carrying *FLT3*-ITD is thought to be strongly associated with a poor prognosis in AML, but is rarely reported in patients with t(8;21)-AML [1,7].

It has been shown recently that in pediatric AML, patients with ITD longer than 48 bp had a worse relapse-free survival rate (19 vs. 51%, $P = 0.035$), while the presence of more than one ITD was not clinically significant. Physical characteristics, including the length of *FLT3*-ITD, may influence *FLT3* activation state by altering its structure and may impact response to therapy [10]. In this patient, the length of the *FLT3*-ITD was 48 bp, while the position of the ITD within this sequence has been reported to be associated with poor prognosis. Prognostic factors other than *FLT3*-ITD were also thought to influence the prognosis of this patient.

The analysis of immunosurface markers, such as CD56 expression, on leukemic blasts may also determine the risk of developing extramedullary relapse. The expression of CD56 may also be correlated to the predisposition of t(8;21)-AML to develop GS [11]. CD56 expression in t(8;21)-AML was associated with significantly shorter complete remission duration and survival [3]. Although some studies suggest that the presence of GS adversely affects the prognosis of patients with t(8;21)-AML [1], other reports have indicated that GS does not influence the outcome [12]. Extramedullary leukemia does not appear to be a sufficient explanation for the adverse treatment outcome of t(8;21)-AML with CD56 expression and, thus, additional prognostic factors must be investigated. The high relapse rate of t(8;21)-AML patients with leukemia cells expressing CD56 could possibly be explained by an association between CD56 expression and drug resistance [13]. Clonal evolution of leukemic blasts with CD56 expression has been observed in AML patients who were originally CD56 negative after relapse [14,15]. CD56 expression has also been shown to be related to relapse and metastasis [16]. In this study, we could not examine CD56 expression in leukemic cells of GS. The association of GS, CD56 expression, and *FLT3*-ITD in t(8;21)-AML, all of which are considered to be poor prognosis factors, currently remains unknown. It is possible that CD56 positivity of leukemic blasts might be related to GS, while CD56 expression and *FLT3*-ITD remain independent.

While GS tends to occur most commonly in the skin, lymph nodes, spine, and small intestine, it has also been

Table 1
AML presenting with obstructive jaundice and granulocytic sarcoma

Patient no.	Age/sex	FAB/karyotype	CD56 expression	Onset of jaundice	AML	Site of GS	Outcome	Reference
1	73/M	NA	NA	Concurrent	Diagnosis	Head of pancreas	D	[25]
2	51/M	NA	NA	Precedent	1CR	Gallbladder wall, bile duct, pretropancreatic lymph node	D	[20]
3	48/F	NA	(−)	Precedent	2CR	Gallbladder wall	D	[21]
4	37/F	NA	NA	Concurrent	3CR	Head of pancreas	D	[26]
5	31/M	NA	(−)	Precedent	Diagnosis	Head of pancreas	CR (NA)	[27]
6	61/F	NA/trisomy 8,13	NA	Concurrent	Diagnosis	Head of pancreas	D (10 mo)	[27]
7	75/F	NA	NA	Precedent	Diagnosis	Bile duct	D (1 mo)	[28]
8	84/M	M0	NA	Precedent	Diagnosis	Gallbladder wall	D	[29]
9	36/M	NA/46XY	NA	Concurrent	Diagnosis	Bile duct	CR (12 mo)	[18]
10	51/F	M2	NA	NA	Relapse	Head of pancreas	NA	[20]
11	47/M	M2	NA	Concurrent	1CR	Common bile duct	CR (NA)	[30]
12	20/F	M2/t(8;21)	NA	Precedent	Diagnosis	Bile duct, ovary	CR (3 mo)	[31]
13	64/M	M2/t(8;21)	NA	Precedent	1CR	Head of pancreas	D (4 mo)	[32]
14	55/M	M2/trisomy 21	NA	Concurrent	Diagnosis	Common bile duct	CR (8 mo)	[33]
15	60/M	M4	NA	Concurrent	Diagnosis	Liver, colon	NA	[34]
16	49/M	M2/t(8;21)	(+)	Precedent	1CR	Paraspinal	NA	[22]
17	36/F	M4/46XX	NA	Precedent	Diagnosis	Head of pancreas	CR (7 mo)	[19]
18	17/M	M2/t(8;21)	(+)	Precedent	2CR	Bile duct	D	This patient

Abbreviations: NA, not available; D, dead; CR, complete remission.

described in a variety of other organs [17]. The association of obstructive jaundice with AML has been reported infrequently. While jaundice caused by GS compression of the bile ducts as a primary manifestation of AML is well known in some cases [18–20], it appears to be very uncommon as the first manifestation of relapse [21]. Our patient exhibited periods of nausea, vomiting, and abdominal pain before the development of jaundice. It appears likely that we need to pay more attention to these symptoms and further examine the possibility of GS in the abdomen at relapse in AML rather than intestinal GVHD. The increase in hyperbilirubinemia and liver transferase, however, occurred very rapidly. Table 1 shows AML patients with obstructive jaundice that have been reported to date. In some of these patients, GS may have occurred before bone marrow invasion. One report (patient no. 16) was the patient with t(8;21)-AML with CD56 expression and paraspinal GS without bone marrow involvement, who relapsed 8 months after successful induction chemotherapy [22]. We are unable to identify the status of both *FLT3* gene and CD56 expression in patients with t(8;21)-AML with obstructive jaundice and GS in the literature.

Our patient was considered to have a good prognosis due to her clinical characteristics of t(8;21)-AML, including a low WBC count and absence of extramedullary myeloid tumor symptoms at diagnosis. However, her disease was multidrug-resistant and she relapsed despite severe GVHD after an unrelated bone marrow transplant. We hypothesized that AML clones in this patient could not survive in the bone marrow due to a graft-versus-leukemia (GVL) effect, but those that escaped from a GVL effect had managed to survive and grow in the common bile duct. Different GVL effects both inside and outside of the bone marrow may depend on the quantity and/or activity of effector cells in those areas [23].

In this case report especially, when the minimal residual disease was detected without bone marrow recurrence in morphology after allogeneic transplantation, one should infer not only hematologic recurrence, but also the existence of GS, presenting as a soft-tissue mass, and examine any site of the body. Therefore, for earlier detection of relapse, we suggest that it would be useful to examine the existence of GS in CD56-positive t(8;21)-AML patients at diagnosis and hematologic remission.

Finally, recent reports have demonstrated that both the mutations of *KIT*, *FLT3*, *JAK2*, and *RAS* genes, and the secondary chromosome aberrations of del(9q) related to a loss of *TLE1* and *TLE4* genes occur in addition to t(8;21)(q22;q22) [8]. Although non-Caucasian patients with t(8;21) having del (9q) exhibited longer survival compared with patients with t(8;21) alone and with other cytogenetic abnormalities, there were no differences in the long-term survival observed among Caucasian patients [24]. Additional chromosome aberrations of del(9q) remain as unknown prognostic factors in Japanese patients. We were unable to analyze the *RAS* and *JAK2* genes in this patient. Even though pediatric patients with t(8;21)-AML are unlikely to have *FLT3*-ITD, this report suggests that molecular prognostic factors, including *FLT3* and *KIT* genes, as well as surface marker CD56, should be analyzed.

Acknowledgments

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References

[1] Rubnitz JE, Raimondi SC, Halbert AR, Tong X, Srivastava DK, Razzouk BI, Pui CH, Downing JR, et al. Characteristics and outcome of t(8;21)-positive childhood acute myeloid leukemia: a single institution's experience. Leukemia 2002;16:2072–7.

[2] Schlenk RF, Benner A, Krauter J, Buchner T, Sauerland C, Ehninger G, et al. Individual patient data-based meta-analysis of patients aged 16–60 years with core binding factor acute myeloid leukemia: a survey of the German Acute Myeloid Leukemia Intergroup. J Clin Oncol 2004;22:3741–50.

[3] Baer MR, Stewart CC, Lawrence D, Arthur DC, Byrd JC, Davey FR, et al. Expression of the neural cell adhesion molecule CD56 is associated with short remission duration and survival in acute myeloid leukemia with t(8;21)(q22;q22). Blood 1997;90:1643–8.

[4] Yokota S, Kiyoi H, Nakao M, Iwai T, Misawa S, Okuda T, et al. Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. Leukemia 1997;11:1605–9.

[5] Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Platzbecker U, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood 2002;99:4326–35.

[6] Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood 2001;98:1752–9.

[7] Shimada A, Taki T, Tabuchi K, Tawa A, Horibe K, Tsuchida M, et al. KIT mutations, and not FLT3 internal tandem duplication, are strongly associated with a poor prognosis in pediatric acute myeloid leukemia with t(8;21): a study of the Japanese Childhood AML Cooperative Study Group. Blood 2006;107:1806–9.

[8] Mrozek K, Marcucci G, Paschka P. Advances in molecular genetics and treatment of core-binding factor acute myeloid leukemia. Curr Opin Oncol 2008;20:711–8.

[9] Zwaan CM, Meshinchi S, Radich JP, Veerman AJ, Huismans DR, Munske L, et al. FLT3 internal tandem duplication in 234 children with acute myeloid leukemia: prognostic significance and relation to cellular drug resistance. Blood 2003;102:2387–94.

[10] Meshinchi S, Stirewalt DL, Alonzo TA, Boggon TJ, Gerbing RB, Rocnik JL, et al. Structural and numerical variation of FLT3/ITD in pediatric AML. Blood 2008;111:4930–3.

[11] Byrd JC, Weiss RB. Recurrent granulocytic sarcoma. An unusual variation of acute myelogenous leukemia associated with 8;21 chromosomal translocation and blast expression of the neural cell adhesion molecule. Cancer 1994;73:2107–12.

[12] Schwyzer R, Sherman GG, Cohn RJ, Poole JE, Willem P. Granulocytic sarcoma in children with acute myeloblastic leukemia and t (8;21). Med Pediatr Oncol 1998;31:144–9.

[13] Pearson L, Leith CP, Duncan MH, Chen IM, McConnell T, Trinkaus K, et al. Multidrug resistance-1 (MDR1) expression and functional dye/drug efflux is highly correlated with the t(8;21) chromosomal translocation in pediatric acute myeloid leukemia. Leukemia 1996;10:1274–82.

[14] Daniels JT, Davis BJ, Houde-McGrail L, Byrd JC. Clonal selection of CD56+ t(8;21) AML blasts: further suggestion of the adverse clinical significance of this biological marker? Br J Haematol 1999;107: 381–3.

[15] Itoh S, Sugawara T, Enomoto S, Ono Y, Numaoka H, Utsugisawa T, et al. Clonal evolution of blasts in an elderly patient with CD56(+) relapsed acute promyelocytic leukemia. Am J Hematol 2002;69: 59–63.

[16] Yang DH, Lee JJ, Mun YC, Shin HJ, Kim YK, Cho SH, et al. Predictable prognostic factor of CD56 expression in patients with acute myeloid leukemia with t(8:21) after high dose cytarabine or allogeneic hematopoietic stem cell transplantation. Am J Hematol 2007;82:1–5.

[17] Byrd JC, Edenfield WJ, Shields DJ, Dawson NA. Extramedullary myeloid cell tumors in acute nonlymphocytic leukemia: a clinical review. J Clin Oncol 1995;13:1800–16.

[18] Goor Y, Goor O, Michalewitcz R, Cabili S. Acute myeloid leukemia presenting as obstructive jaundice. J Clin Gastroenterol 2002;34:485–6.

[19] King DJ, Ewen SW, Sewell HF, Dawson AA. Obstructive jaundice. An unusual presentation of granulocytic sarcoma. Cancer 1987;60:114–7.

[20] Lillicrap DP, Ginsburg AD, Corbett WE. Relapse of acute myelogenous leukemia presenting with extrahepatic obstruction of the biliary tract. Can Med Assoc J 1982;127:1000–1.

[21] Hurley R, Weisdorf DJ, Jessurun J, Vercellotti GM, Miller WJ. Relapse of acute leukemia presenting as acute cholecystitis following bone marrow transplantation. Bone Marrow Transplant 1992;10:387–9.

[22] Krishnan K, Ross CW, Adams PT, Pereira A, Roth MS. Neural cell-adhesion molecule (CD 56)-positive, t(8;21) acute myeloid leukemia (AML, M-2) and granulocytic sarcoma. Ann Hematol 1994;69:321–3.

[23] Dermime S, Mavroudis D, Jiang YZ, Hensel N, Molldrem J, Barrett AJ. Immune escape from a graft-versus-leukemia effect may play a role in the relapse of myeloid leukemias following allogeneic bone marrow transplantation. Bone Marrow Transplant 1997;19:989–99.

[24] Marcucci G, Mrozek K, Ruppert AS, Maharry K, Kolitz JE, Moore JO, et al. Prognostic factors and outcome of core binding factor acute myeloid leukemia patients with t(8;21) differ from those of patients with inv(16): a Cancer and Leukemia Group B study. J Clin Oncol 2005;23:5705–17.

[25] Rikitake O, Kodama T, Hisano S, Sakata T, Niho Y, Matsukuma, Yamaguchi A. [Obstructive jaundice caused by tumor-forming acute myeloblastic leukemia—a case study]. Nippon Rinsho 1980;38: 4692–6.

[26] Marcos HB, Semelka RC, Woosley JT. Abdominal granulocytic sarcomas: demonstration by MRI. Magn Reson Imaging 1997;15:873–6.

[27] Ravandi-Kashani F, Cortes J, Giles FJ. Myelodysplasia presenting as granulocytic sarcoma of mediastinum causing superior vena cava syndrome. Leuk Lymphoma 2000;36:631–7.

[28] Ascani S, Piccaluga PP, Pileri SA. Granulocytic sarcoma of main biliary ducts. Br J Haematol 2003;121:534.

[29] Matsueda K, Yamamoto H, Doi I. An autopsy case of granulocytic sarcoma of the porta hepatis causing obstructive jaundice. J Gastroenterol 1998;33:428–33.

[30] Scully RE, Mark EJ, McNeely WF, McNeely BU. Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Case 32-1988. Obstructive jaundice in a man with treated colon cancer and leukemia. N Engl J Med 1988;319:356–64.

[31] Scully RE, Mark EJ, McNeely WF, McNeely BU. Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Case 22-1990. A 19-year-old woman with a mass in the shoulder, jaundice, and hepatosplenomegaly. N Engl J Med 1990; 322:1585–94.

[32] Servin-Abad L, Caldera H, Cardenas R, Casillas J. Granulocytic sarcoma of the pancreas. A report of one case and review of the literature. Acta Haematol 2003;110:188–92.

[33] Mano Y, Yokoyama K, Chen CK, Tsukada Y, Ikeda Y, Okamoto S. [Acute myeloid leukemia presenting with obstructive jaundice and granulocytic sarcoma of the common bile duct]. Rinketsu 2004;45:1039–43.

[34] Sevinc A, Buyukberber S, Camci C, Koruk M, Savas MC, Turk HM, et al. Granulocytic sarcoma of the colon and leukemic infiltration of the liver in a patient presenting with hematochezia and jaundice. Digestion 2004;69:262–5.

2 Ferrajoli A, Lee BN, Schlette EJ, O'Brien SM, Gao H, Wen S *et al.* Lenalidomide induces complete and partial remissions in patients with relapsed and refractory chronic lymphocytic leukemia. *Blood* 2008; **111**: 5291–5297.
3 Aue G, Njuguna N, Tian X, Soto S, Hughes T, Vire B *et al.* Lenalidomide-induced upregulation of CD80 on tumor cells correlates with T-cell activation, the rapid onset of a cytokine release syndrome and leukemic cell clearance in chronic lymphocytic leukemia. *Haematologica* 2009; **94**: 1266–1273.
4 Andritsos LA, Johnson AJ, Lozanski G, Blum W, Kefauver C, Awan F *et al.* Higher doses of lenalidomide are associated with unacceptable toxicity including life-threatening tumor flare in patients with chronic lymphocytic leukemia. *J Clin Oncol* 2008; **26**: 2519–2525.
5 Moutouh-de Parseval LA, Weiss L, DeLap RJ, Knight RD, Zeldis JB. Tumor lysis syndrome/tumor flare reaction in lenalidomide-treated chronic lymphocytic leukemia. *J Clin Oncol* 2007; **25**: 5047 (letter).
6 Lapalombella R, Andritsos L, Liu Q, May SE, Browning R, Pham LV *et al.* Lenalidomide treatment promotes CD154 expression on CLL cells and enhances production of antibodies by normal B cells through a PI3-kinase dependent pathway. *Blood* 2009; **115**: 2619–2629.
7 Egle A, Steurer M, Melchardt T, Stoll M, Greil R. The REVLIRIT CLL5 AGMT Study – a phase I/II trial combining Fludarabine/Rituximab with escalating doses of lenalidomide followed by Rituximab/Lenalidomide in untreated CLL: results of a planned interim analysis. *Blood* 2009; **114**: 3453 (abstract).
8 Ferrajoli A, Badoux XC, O'Brien S, Wierda WG, Faderl S, Estrov Z *et al.* Combination therapy with Lenalidomide and Rituximab in patients with relapsed chronic lymphocytic leukemia (CLL). *Blood* 2009; **114**: 206 (abstract).

High frequencies of simultaneous *FLT3*-ITD, *WT1* and *KIT* mutations in hematological malignancies with *NUP98*-fusion genes

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Acute myeloid leukemia (AML) is heterogeneous in clinical features and molecular pathogenesis. Cooperating alterations of several genes, including oncogenes or tumor suppressor genes, lead to AML development.[1] AML leukemogenesis is thought to require at least two different types of genetic change: class I mutations, which confer a proliferative or survival advantage; and class II mutations, which block myeloid differentiation and provide self-renewability.[1] In hematological malignancies with 11p15 translocations, the nucleoporin (*NUP*) *98* gene is reportedly fused to various partner genes, often including homeobox genes, such as *HOXA9, A11, A13, C11, C13, D11, D13* and *PMX1*.[2] With respect to the oncogenic mechanism of NUP98-HOX fusion proteins, a previous study using a murine bone marrow transplantation assay revealed that NUP98-HOXA9, -HOXD13 and -PMX1 fusion proteins induce myelodysplastic syndrome (MDS) or myeloproliferative neoplasm (MPN), which progress to AML.[2] This latency period indicates that additional genetic events might be required for leukemic transformation. Therefore, we examined somatic mutations of the *FLT3, KIT, WT1, RUNX1, CEBPA, NPM1, NRAS, KRAS* and *MLL* genes, which are prevalent in AML, in leukemia patients with *NUP98* fusion genes. This study was approved by local ethical committee.

Sixteen patients with chromosomal 11p15 translocations included nine with *NUP98-HOXA9*, two with *NUP98-HOXA13*, two with *NUP98-HOXA11* and one each with *NUP98-HOXC11*, *NUP98-HOXD11*, *NUP98-HOXD13* or *NUP98-NSD3* (Table 1). The partner gene fused to *NUP98* could not be detected in one patient with t(4;11)(q21;p15); however, fluorescent *in situ* hybridization analysis using a probe containing *NUP98* showed split signals (data not shown). No patients had any additional chromosomal abnormality except for chromosomal 11p15 translocations (Supplementary data). Two patients with t(7;11)(p15;p15) had double *NUP98* fusion transcripts: patient (PN) 13 had simultaneous *NUP98-HOXA9* and *NUP98-HOXA13* fusions, and PN14 had simultaneous *NUP98-HOXA9* and *NUP98-HOXA11* fusions. In all, 15 of the 16 patients with *NUP98*-related hematological malignancies were diagnosed as having myeloid malignancies, and the other patient (PN16) were initially diagnosed as having T-cell non-Hodgkin's lymphoma with t(4;11)(q21;p15), and transformed into acute myelomonocytic leukemia with the same t(4;11) (lineage switch). Patients with myeloid malignancies consisted of 10 patients with AML, 2 patients with MDS and 3 patients with MPN.

We examined the internal tandem duplications (ITDs) and tyrosine kinase domain (TKD) mutations of the *FLT3* gene in 16 patients, and detected ITDs in nine (56.3%) patients, and TKD mutations in none (Table 1, Figure 1a). The incidence of *FLT3*-ITD in our study was much higher than that in an AML cohort reported previously (12–35%).[1] A high frequency of *FLT3*-ITD was previously reported in 30–35% of AML patients with either normal karyotype or with t(15;17)(q21;q11) resulting in *PML-RARA*, and in 70% of AML patients with t(6;9)(p23;q34) resulting in *DEK-CAN/NUP214*.[1] Interestingly, both *NUP98* and *NUP214* encode a part of the nucleoporin complex. The general activation effects on reporters of the DEK-CAN/NUP214 fusion protein are specific for myeloid cells.[3] Moreover, in murine bone marrow transplantation assays, NUP98-related fusion proteins such as NUP98-HOXA9, -HOXD13 and -PMX1 induced MDS or MPN, which progressed to AML.[2] These results demonstrate that the nucleoporin-related proteins share a common ability for myeloid differentiation. Furthermore, the very tight correlation between *nucleoporin*-related fusion genes and *FLT3*-ITD suggest that *FLT3*-ITD may contribute to the myeloid leukemogenesis involved in *nucleoporin*-related fusions.

We further examined mutations of the *KIT, WT1, AML1, CEBPA, NPM1, NRAS, KRAS* and *MLL* genes,[4] which are prevalent in AML. *KIT, NRAS* and *KRAS* mutations were found in four (25.0%), three (18.8%) and two (12.5%) patients, respectively (Table 1, Figure 1b). *WT1* aberrations were found in eight patients (50.0%; Table 1, Figure 1c). No mutations were found in the other four genes (*RUNX1, CEBPA, NPM1* and *MLL*). The mutations in *KIT* were all missense mutations including Val399Ile, Met541Leu and Asp816Val, and all mutations of *NRAS* and *KRAS* were Gly13Asp. All of *KIT, NRAS* and *KRAS* mutations were heterozygous. The aberrations in *WT1* comprised a frameshift insertion of exon 7 in four patients, missense mutation of exon 9 in one, deletion of exon 5 in one and deletion of the whole cording region in two. Frameshift and

Table 1 Clinical features and additional mutations of patients with *NUP98*-related leukemias

PN	*Age*	*Sex*	*Disease*	*WBC at diagnosis*	*Karyotype*	*Fusion partner gene of NUP98*	*CR*	*Relapse*	*Therapy*	*Prognosis*	*FLT3*	*KIT*	*WT1*	*NRAS*	*KRAS*
PN1	14	M	AML-M1	12500	t(11;12)	*HOXC11*	yes	yes	Chemo+SCT	Death	ITD	Val399Ile	del	WT	WT
PN2	12	F	AML-M2	133100	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	WT	WT	WT	Gly13Asp	WT
PN3	13	M	AML-M2	460000	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	ITD	Met541Leu	ins4bpfsX	WT	WT
PN4	13	F	AML-M2	147000	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Alive	WT	WT	WT	WT	WT
PN5	15	M	AML-M2	22700	t(7;11)	*HOXA9*	yes	no	Chemo+SCT	Alive	WT	WT	WT	WT	Gly13Asp
PN6	57	M	AML-M2	252000	t(7;11)	*HOXA13*	yes	yes	Chemo	Death	ITD	WT	WT	WT	WT
PN7	38	M	AML-M2	6400	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	ITD	Asp816Val	ins4bpfsX	WT	WT
PN8	15	M	AML-M4	187900	t(2;11)	*HOXD11*	yes	no	Chemo+SCT	Alive	WT	WT	ins4bpfsX	WT	Gly13Asp
PN9	56	M	AML-M4	204500	t(7;11)	*HOXA9*	yes	yes	Chemo	Lost to follow-up	ITD	WT	WT	WT	WT
PN10	62	M	AML-M4	6500	t(2;11)	*HOXD13*	yes	no	Chemo	Alive	ITD	WT	WT	WT	WT
PN11	60	M	RA	6250	t(8;11)	*NSD3*	no	ND	Chemo	Death	ITD	Met541Leu	ins4bpfsX	WT	WT
PN12	69	F	RAEB	2500	t(7;11)	*HOXA9*	no	ND	Chemo	Death	WT	WT	WT	WT	WT
PN13	45	M	CMML	29800	t(7;11)	*HOXA9/HOXA13*	yes	yes	Chemo	Death	ITD	WT	Arg250Trp	WT	WT
PN14	58	F	CML(Ph-)	11200	t(7;11)	*HOXA9/HOXA11*	yes	no	Chemo	Alive	ITD	WT	del	WT	WT
PN15	3	F	JMML	39400	t(7;11)	*HOXA11*	yes	no	Chemo+SCT	Alive	WT	WT	del exon5	Gly13Asp	WT
PN16	51	F	T-NHL	2600	t(4;11)	undetermined	yes	yes	Chemo+SCT	Death	WT	WT	WT	Gly13Asp	WT

Abbreviations: AML, acute myeloid leukemia; Chemo, chemotherapy; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; CR, complete remission; del, deletion; F, female; JMML, Juvenile myelomonocystic leukemia; M, male; ND, not determined; Ph-, Philadelphia chromosome; PN, patient number; RA, refractory anemia; RAEB-t, refractory anemia with excess of blasts in transformation; SCT, stem cell transplantation; T-NHL, T-cell non-Hodgkin's lymphoma; WBC, white blood cell; WT, wild type.
t(11;12), t(11;12)(p15;q13); t(2;11), t(2;11)(q31;p15); t(4;11), t(4;11)(q21;p15); t(7;11), t(7;11)(p15;p15); t(8;11), t(8;11; p11; p15).

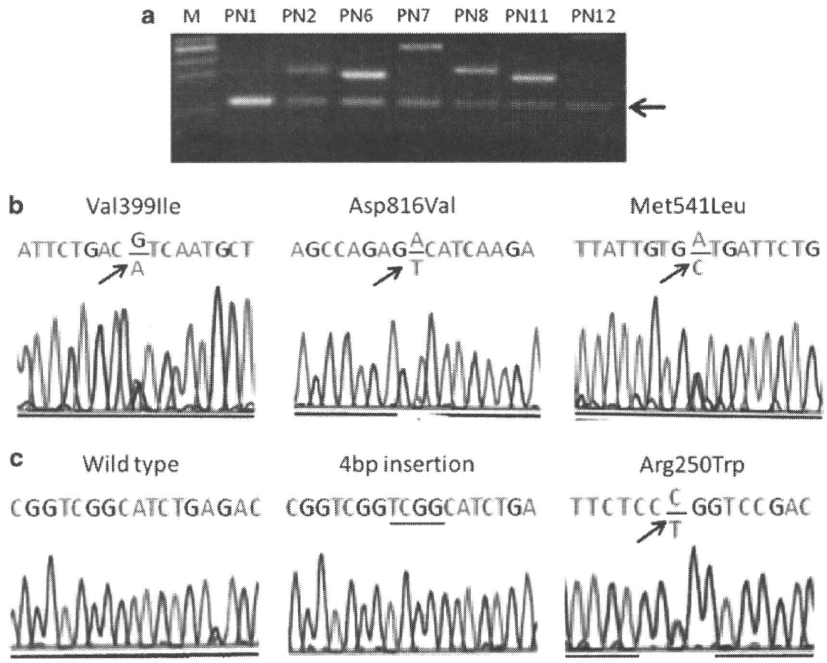

Figure 1 *FLT3*-ITD, *KIT* and *RAS* mutations, and *WT1* aberrations. (**a**) Identification of *FLT3*-ITD by reverse transcription PCR. M, size marker; arrow indicates wild-type allele. (**b**) *KIT* mutations. All figures show the sequence of PCR products. (**c**) *WT1* aberrations. Left panel shows wild type of *WT1* exon 7. Middle panel shows 4-bp insertion in exon 7 of *WT1*. Right panel shows *WT1* missense mutation. Left and middle panels show the sequence of each plasmid subclone, and right panel shows that of PCR products.

missense mutation of *WT1* are heterozygous, whereas deletion was homozygous. *FLT3*-ITD, *KIT* and *RAS* mutations reportedly confer cellular proliferative abilities.[1] In our study, 14 patients (88%) had at least one mutation involved in cellular proliferation (*FLT3*, *KIT* or *RAS*). Recently, Chou *et al.*[5] reported that the *NUP98-HOXA9* fusion is strongly associated with *KRAS* and *WT1* mutations. *Nras* and *Kras* mutations were frequently found in AML developed in transgenic mice expressing *NUP98-HOXD13*.[6] These results indicate that *NUP98*-related leukemias have a high frequency of mutations involved in growth advantage.

Interestingly, five of the six patients with *WT1* aberrations had *FLT3*-ITD, and three of the five patients with both *FLT3*-ITD and *WT1* aberrations had a *KIT* mutation, although the simultaneous *FLT3*-ITD and *KIT* mutations are reportedly very rare[1]. These results suggest that the *NUP98*-related leukemias share a distinct molecular subgroup in leukemias. In addition, all four patients with *KIT* mutations had both *FLT3*-ITD ($P=0.04$) and *WT1* aberrations ($P=0.03$), whereas all five patients with *RAS* mutations did not have *FLT3*-ITD. In all, 14 (88%) of the 16 patients had either *FLT3*-ITD or *RAS* mutations, but they were mutually exclusive as described in previous papers.[1] These

suggest the distinct molecular basis between *NUP98*-related leukemias having *FLT3*-ITD and those having *RAS* mutations.

The relationships between clinical features and gene mutations were described in Table 1. In our study, male patients were more likely than female patients to have *FLT3*-ITD ($P=0.01$) and patients with *FLT3*-ITD have leukocytosis ($P=0.08$) more than those without *FLT3*-ITD. Patients with *RAS* mutations were significantly younger than those without the mutations (median age of 15 vs 56 years; $P=0.04$). In total, 9 (64.3%) of the 14 patients who achieved complete remission relapsed, and 9 (60.0%) of the 15 patients whose data were available died, although they were treated by different protocols (Table 1). All three patients who had both *FLT3*-ITD and *KIT* mutations, and five (83.3%) of the six patients who had both *FLT3*-ITD and *WT1* aberrations, died. Many studies have shown that *FLT3*-ITD is related to a poor prognosis in AML patients,[1] and that *KIT* mutations are associated with a worse outcome in CBF-leukemia patients.[1] *WT1* mutations are also reported to be a poor prognostic factor in adult AML patients with normal karyotypes.[7] These results suggest that simultaneous occurrence of *FLT3*-ITD, *KIT* mutations and *WT1* aberrations in *NUP98*-related leukemia may be associated with poor prognosis.

FLT3-ITD, *KIT* and *RAS* mutations lead to constitutive activation of downstream pathway, resulting in acquirement of a proliferative advantage.[1] In a mouse model, *FLT3*-ITD alone does not induce AML, and *RAS* mutations can induce myeloid leukemia with distinct leukemogenic strengths and phenotypes.[1] *NUP98*-related fusions alone require long periods of time to induce AML, although these fusions induce MDS or MPN by impaired myeloid differentiation.[2] Cooperation between BCR-ABL (which enhances proliferation) and NUP98-fusion (which inhibits differentiation) lead to CML blast crisis.[2] Moreover, the *WT1* mutations were clustered within the DNA binding domain, and were subsequently considered to impair the ability of DNA to bind to target genes associated with apoptosis, cell cycle or cellular proliferation.[8] These results suggest that a high frequency of cell proliferation gene mutations may contribute to leukemogenesis in *NUP98*-related leukemia, and that simultaneous occurrence of *FLT3-ITD* and *WT1* aberrations may have an important role in the clinical outcome of *NUP98*-related leukemia.

Conflict of interest

The authors declare no conflict of interest.

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T Taketani[1,2], T Taki[3], T Nakamura[4], Y Kobayashi[5], E Ito[6], S Fukuda[2], S Yamaguchi[2] and Y Hayashi[7]
[1]*Division of Blood Transfusion, Shimane University Hospital, Shimane, Japan;*
[2]*Department of Pediatrics, Shimane University School of Medicine, Shimane, Japan;*
[3]*Department of Molecular Diagnostics and Therapeutics, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, Japan;*
[4]*Department of Carcinogenesis, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan;*
[5]*Hematology Division, National Cancer Center Hospital, Tokyo, Japan;*
[6]*Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki, Japan and*
[7]*Department of Hematology/Oncology, Gunma Children's Medical Center, Gunma, Japan*
E-mail: hayashiy-tky@umin.ac.jp

References

1 Renneville A, Roumier C, Biggio V, Nibourel O, Boissel N, Fenaux P *et al.* Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia* 2008; **22**: 915–931.
2 Moore MA, Chung KY, Plasilova M, Schuringa JJ, Shieh JH, Zhou P *et al.* NUP98 dysregulation in myeloid leukemogenesis. *Ann N Y Acad Sci* 2007; **1106**: 114–142.
3 Ageberg M, Drott K, Olofsson T, Gullberg U, Lindmark A. Identification of a novel and myeloid specific role of the leukemia-associated fusion protein DEK-NUP214 leading to increased protein synthesis. *Genes Chromosomes Cancer* 2008; **47**: 276–287.
4 Schlenk RF, Döhner K, Krauter J, Fröhling S, Corbacioglu A, Bullinger L *et al.* Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med* 2008; **358**: 1909–1918.
5 Chou WC, Chen CY, Hou HA, Lin LI, Tang JL, Yao M *et al.* Acute myeloid leukemia bearing t(7;11)(p15;p15) is a distinct cytogenetic entity with poor outcome and a distinct mutation profile: comparative analysis of 493 adult patients. *Leukemia* 2009; **23**: 1303–1310.
6 Slape C, Liu LY, Beachy S, Aplan PD. Leukemic transformation in mice expressing a NUP98-HOXD13 transgene is accompanied by spontaneous mutations in Nras, Kras, and Cbl. *Blood* 2008; **112**: 2017–2019.
7 Virappane P, Gale R, Hills R, Kakkas I, Summers K, Stevens J *et al.* Mutation of the wilms' tumor 1 gene is a poor prognostic factor associated with chemotherapy resistance in normal karyotype acute myeloid leukemia: The United Kingdom Medical Research Council Adult Leukaemia Working Party. *J Clin Oncol* 2008; **26**: 5429–5435.
8 Ariyaratana S, Loeb DM. The role of the Wilms tumour gene (WT1) in normal and malignant haematopoiesis. *Expert Rev Mol Med* 2007; **9**: 1–17.

bjh research paper

Prospective study of a therapeutic regimen with all-*trans* retinoic acid and anthracyclines in combination of cytarabine in children with acute promyelocytic leukaemia: the Japanese childhood acute myeloid leukaemia cooperative study

Masue Imaizumi,[1] Akio Tawa,[2] Ryoji Hanada,[3] Masahiro Tsuchida,[4] Ken Tabuchi,[5] Hisato Kigasawa,[5] Ryoji Kobayashi,[6] Akira Morimoto,[7] Hideki Nakayama,[8] Kazuko Hamamoto,[9] Kazuko Kudo,[10] Hiromasa Yabe,[11] Keizo Horibe,[12] Shigeru Tsuchiya[13] and Ichiro Tsukimoto[14]

[1]Department of Haematology and Oncology, Miyagi Children's Hospital, Sendai, [2]Department of Paediatrics, National Hospital Organization Osaka National Hospital, Osaka, [3]Department of Haematology/Oncology, Saitama Children's Medical Centre, Saitama, [4]Department of Paediatrics, Ibaraki Children's Hospital, Mito, [5]Division of Haematology, Kanagawa Children's Medical Centre, Yokohama, [6]Department of Paediatrics, Sapporo Hokuyu Hospital, Sapporo, [7]Department of Paediatrics, Jichi Medical University School of Medicine, Tochigi, [8]Department of Paediatrics, Fukuoka-Higashi Medical Centre, Koga, [9]Department of Paediatrics, Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital, Hiroshima, [10]Division of Haematology and Oncology, Shizuoka Children's Hospital, Shizuoka, [11]Specialized Clinical Science, Paediatrics, Tokai University School of Medicine, Isehara, [12]Clinical Research Centre, National Hospital Organization Nagoya Medical Centre, Nagoya, [13]Department of Paediatrics, Tohoku University School of Medicine, Sendai, and [14]Children's Medical Centre, Saiseikai Yokohamashi, Tobu Hospital, Yokohama, Japan

Summary

In childhood acute promyelocytic leukaemia (APL), the efficacy of therapy combining cytarabine with all-*trans* retinoic acid (ATRA) and anthracyclines remains unclear in terms of long-term prognosis. Between August 1997 and March 2004, 58 children with APL (median age: 11 years) were enrolled into an acute myeloid leukaemia (AML) study (AML99-M3) and followed up for a median time of 86 months. The regimen included ATRA and anthracyclines combined with cytarabine in both induction and consolidation. In induction, two patients died of haemorrhage and four patients developed retinoic acid syndrome. Of 58 patients, 56 (96·6%) achieved complete remission, two of whom relapsed in the bone marrow after 15 and 19 months respectively. Sepsis was a major complication, with an incidence of 5·6–10·9% in the consolidation blocks, from which all but one of patients recovered. Consequently, 7-year overall and event-free survival rates were 93·1% and 91·4% respectively, and cumulative incidence of relapse plateaued at 3·6% after 2 years. Follow-up survey of 54 patients revealed no patients with late cardiotoxicity or secondary malignancy, except one with asymptomatic prolongation of QTc interval. This study suggests that the combination of cytarabine with ATRA and anthracycline-based therapy may have useful implications in the perspective of long-term prognosis and late adverse effects for childhood APL.

Keywords: childhood acute promyelocytic leukaemia, all-*trans* retinoic acid, anthracyclines, cytarabine, long-term prognosis.

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Correspondence: Dr Masue Imaizumi, Department of Haematology and Oncology, Miyagi-Children's Hospital, Sendai, 989-3126, Japan. E-mail: imaizumi@miyagi-children.or.jp

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The prognosis of patients with acute promyelocytic leukaemia (APL), a distinct subtype of acute myeloid leukaemia (AML) (Grignani *et al*, 1994), has been improved dramatically by the introduction of differentiation induction therapy with all-*trans* retinoic acid (ATRA) (Fenaux *et al*,1999; Tallman *et al*, 2002; Sanz *et al*, 2004). However, recent clinical trials with ATRA and anthracycline-based chemotherapy found that recurrent disease posed a major problem, especially for high-risk patients. (Sanz *et al*, 2000, 2009).

Childhood APL, which consists of only 7–10% of all patients, is often associated with risk factors such as hyperleucocytosis (Guglielmi *et al*,1998; Mann *et al*, 2001); however, few studies of paediatric patients have specifically examined its long-term prognosis. In those studies, the complete remission (CR) and overall survival (OS) rates have been improved to >80%, but event-free survival (EFS) remains at around 70–80% because of increased cumulative incidence of relapse (CIR). (de Botton *et al*, 2004; Ortega *et al*, 2005; Testi *et al*, 2005) In addition to frequent relapse in the bone marrow, extramedullary (EM) relapse involving mostly the central nervous system (CNS) occurs at incidence of 1–5%. (Ko *et al*, 1999; de Botton *et al*, 2006; Chow & Feusner, 2009) The therapeutic effectiveness of cytarabine added to anthracycline-based consolidation therapy has been reported for high-risk adult patients(Adès *et al*, 2006, 2008), but the efficacy of cytarabine in addition to the combination of ATRA and anthracyclines in consolidation remains unknown for paediatric patients.

More recently, there has been increasing concern regarding long-term adverse effects, including cardiotoxicity and secondary malignancy, for children with leukaemia. The cumulative dosage of anthracyclines may be related to the risk of late cardiotoxicity as well as therapy-related myelodysplastic syndrome (t-MDS)/AML for childhood malignancies (Nysom *et al*,1998; Le Deley *et al*, 2003). Although such effects of anthracyclines are yet undetermined for APL, the cumulative dosage of anthracyclines may be an important perspective of the long-term prognosis of children with APL.

This report describes the outcome of a prospective study for childhood APL, AML99-M3, in which patients received therapy with cytarabine in addition to ATRA and anthracyclines. The improved outcome of this study suggests that the combination of cytarabine, ATRA and anthracyclines may have useful implications in the perspective of long-term prognosis and late adverse effects for childhood APL.

Patients and methods

Patients

Between August 1997 and March 2004, 58 children with *de novo* APL (31 males and 27 females; median age of 11 years [range:11 months – 16 years] were enrolled in the AML99-M3 study of the Japanese Childhood AML Cooperative Study Group, and a follow-up survey was performed in May 2010 (Table I). Three patients with APL were not recruited to this study: two had already started another chemotherapeutic regimen for AML when APL was diagnosed; the other died of intracranial haemorrhage (ICH) at diagnosis. The relevant institutional review board approved the protocol. Written informed consent was obtained from the parents of all patients. APL was diagnosed according to the French–American–British (FAB) criteria (Bennett *et al*, 1982); the involvement of t(15;17) translocation was examined cytogenetically. APL patients with t(15;17) translocation or *PML-RARA* chimaeric gene confirmed through examinations with fluorescence *in situ* hybridization (FISH) or reverse transcription–polymerase chain reaction (RT-PCR) were registered to this study.

Treatment protocol

In remission induction therapy, ATRA was initiated (45 mg/m^2, until CR) and then daunorubicin (45 mg/m^2 per d, days 6–8) and cytarabine (200 mg/m^2, days 6–12) were added (Fig 1). For patients with a white blood cell (WBC) count $>10 \times 10^9$/l at diagnosis or after the initiation of ATRA therapy, chemotherapy was started before day 6. Consolidation

Table I. Characteristics of patients with APL (N = 58).

Characteristics	Median	Range	No. (%)
Age, years	11	0·9–16	
<5			9 (16)
5 to 10			14 (24)
>10			35 (60)
Sex			
Male			31 (53)
Female			27 (47)
WBC, $\times10^9$/l	4·3	0·7–171	
<10			36 (62)
≥10			22 (38)
Haemoglobin, g/l	91	37–131	
<10			44 (76)
≥10			14 (24)
Platelets, $\times10^9$/l	2·3	5–233	
<40			48 (83)
≥40			10 (17)
FAB subtype			
Typical			53 (91)
Variant			5 (9)
Cytogenetics			
t(15;17)			47 (81)
t(15;17) + others			9 (15)
Normal			1 (2)
Unknown			1 (2)
PML-RARA			
Examined			47 (81)
Long isoform (bcr1)			21
Short isoform (bcr3)			8
bcr not determined			18
Not examined			11 (19)

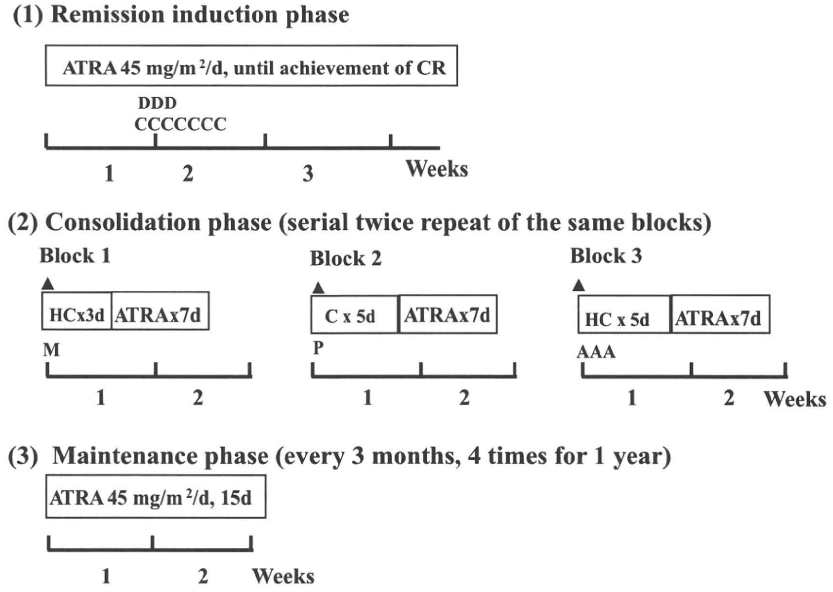

Fig 1. Scheme of AML99-M3 protocol. In remission induction, oral administration of ATRA (45 mg/m^2 per d) was combined with daunorubicin (D) (45 mg/m^2) and cytarabine (C) (200 mg/m^2). In consolidation, administration of ATRA (45 mg/m^2 per d for 7 d) was combined with mitoxantrone (M) (10 mg/m^2 per d, day 1) and high-dose cytarabine (HC) (3 g/m^2 × 2/d, days 1–3) in Block 1, with pirarubicin (P) (45 mg/m^2 per d, day 1) and cytaratibine (C) (200 mg/m^2 per d, days 1–5) in Block 2, and with aclarubicin (A) (30 mg/m^2 per d, days 1–3) and high-dose cytarabine (HC) (3 g/m^2 per d, days 1–5) in Block 3. For CNS prophylaxis, intrathecal injection (▲) of methotrexate, cyatarbine and hydrocortisone at day 1 of every consolidation block in age-adjusted doses as described in Methods. In maintenance therapy, ATRA (45 mg/m^2 per d, days 1–15) was administrated every 3 months for 1 year.

therapy consisted of six courses of block treatment (Blocks 1, 2 and 3), in which each block was performed every month and the same block was repeated serially twice. In respective blocks, chemotherapy with cytarabine and each of the different anthracycline agents was administrated respectively and then ATRA (45 mg/m^2 per d, 7 d) was administrated consecutively. Block 1 consisted of mitoxantrone (10 mg/m^2 per d × day 1) and cytarabine (3 g/m^2 × 2/d × days 1–3), Block 2 of pirarubicin (45 mg/m^2 per d × day 1) and cytarabine (200 mg/m^2 per d × days 1–5), and Block 3 of aclarubicin (30 mg/m^2 per d × days 1–3) and cytarabine (3 g/m^2 per d × days 1–5). On the first day of each consolidation block, patients received intrathecal (IT) therapy with methotrexate (3 mg for <3 months; 6 mg for 3 months to <1 year; 7·5 mg for 1 year; 10 mg for 2 years; 12·5 mg for 3 years or older), cytarabine (6 mg for <3 months; 12 mg for 3 months to <1 year; 15 mg for 1 year; 20 mg for 2 years; 25 mg for 3 years or older) and hydrocortisone (10 mg for <3 months; 10 mg for 3 months to <1 year; 15 mg for 1 year; 20 mg for 2 years; 25 mg for 3 years or older). In maintenance therapy, ATRA alone (45 mg/m^2 per d) for 15 consecutive days was given every 3 months, for a total of four times during 1 year.

Adverse effects

Retinoic acid (RA) syndrome was diagnosed based on clinical signs, including fever, respiratory distress, pulmonary infiltration, pleural and pericardial effusion and renal failure. (Ko *et al*, 1999) When RA syndrome was diagnosed or strongly suspected, ATRA therapy was stopped and the patients received administration of dexamethasone (8 mg/m^2 per d, i.v. in two doses) unless they clinically improved. Disseminated intravascular coagulopathy (DIC), bacterial infections, and other adverse effects were summarized in each phase of treatment. Long-term adverse effects, including cardiotoxicity and secondary malignancy, were surveyed through followe-up analysis. For evaluation of the potential risk of cardiotoxicity, cumulative doses of anthracyclines were converted to equivalent doses of daunorubicin using ratios in 1:3–1:5 for idarubicin/mitoxantrone, 1:1·6 for pirarubicin, and 1:0·2 for aclarubicin (Warrell, 1986; Lenk *et al*, 1990; Sakata-Yanagimoto *et al*, 2004).

Minimal residual disease (MRD) monitoring

For MDR monitoring, the *PML-RARA* chimaeric mRNA in marrow samples was detected using RT-PCR as described (Suzuki *et al*, 2001). Serial evaluation of MRD monitoring was performed every 3 months for 17 patients whose bone marrow samples were sent to the reference laboratory.

Statistical analysis

The OS and EFS were calculated from the beginning date of induction therapy to the date of events; failure to achieve CR, relapse or death of any cause. The OS and EFS were analyzed

using the Kaplan–Meier method. Statistical analyses used the Statistical Package for the Social Sciences (SPSS) software, version 16 (SPSS Japan Inc., Tokyo, Japan), estimated by the log-rank test and considered to be significant when a P value is <0·05. For patients who achieved CR, cumulative incidence functions of relapse as well as death without relapse were calculated using the competing risk method with the cmprsk software package (http://biowww.dfci.harvard.edu/~gray), ver.2.1-5 on R ver.2.10.1.

Results

Patient characteristics

The median follow-up period of 58 patients was 86 months (range: 16 d–12·1 years) (Table I). The median age of patients was 11 years (range: 11 months–16 years); 35 (60%) patients were over 10 years old; 31 patients were male and 27 were female. The WBC counts at diagnosis were 0·9–171 $\times$ 10^9/l (median: 4·3 $\times$ 10^9/l) and 22 patients (38%) had WBC counts >10 $\times$ 10^9/l. The proportion of these high-risk patients was comparable to that (35–48%) reported by other studies for childhood APL (de Botton *et al*, 2004; Ortega *et al* 2005; Testi *et al*, 2005). Haemoglobin levels were 37–131 g/l (median: 91 g/l). Platelet counts were 5–233 $\times$ 10^9/l (median: 23 $\times$ 10^9/l) and 48 patients (83%) had a platelet count <40 $\times$ 10^9/l at diagnosis. Haematological examination identified FAB:M3 morphology in 53 patients and five others exhibited the microgranular FAB: M3v morphology. No patient showed leukaemic infiltration in the cerebrospinal fluid obtained by lumbar puncture performed for CNS prophylaxis at the beginning of consolidation therapy.

Cytogenetic examination revealed that 47 patients had t(15;17) translocation abnormality alone, nine had t(15;17) with additional chromosomal abnormalities, one with normal karyotype, and one with no result. In the latter two patients, the involvement of *PML-RARA* chimaeric gene was confirmed using RT-PCR. Examinations for *PML-RARA* were performed in 47 patients. RT-PCR detected *PML-RARA* in 29 patients, 21 of whom showed the long type (bcr1) isoform; eight showed the short type (bcr3) isoform. Eighteen patients had *PML-RARA* detected by FISH analysis without differentiation of the isoform types. No patient had ATRA-insensitive fusion genes, such as the *ZBTB16-RARA* caused by the t(11;17) chromosomal translocation.

Clinical course and statistical analysis

In induction therapy, two patients (3·4%) died from ICH and pulmonary bleeding after 16 and 24 d respectively. CR was achieved in 56 patients (96·6%), two of whom exhibited relapse at bone marrow; one relapsed at 15 months and died of ICH, the other relapsed at 19 months and remains in second CR after treatment with marrow transplantation. For patients who achieved CR, the period of ATRA administration in induction was a median of 29 d (range 14–60 d), during which 13 patients temporarily discontinued the administration of ATRA for a median 4 d (range 1–31 d). Overall, four patients died: two of DIC with haemorrhage during induction, one of sepsis and meningitis in remission, and one of ICH after relapse. Consequently, the OS and EFS rates at 7 years were respectively, 93·1% (95% confidence interval [CI], 86·5–99·7%) and 91·4% (95% CI, 84·0–98·4%) (Fig 2A). No significant difference was found in the OS and EFS rates between patients with or without haematological risk factors, such as WBC count >10 $\times$ 10^9/l or platelet count <40 $\times$ 10^9/l. (Sanz *et al*, 2000) (Figs 2B, C) The CIR was 3·6% (95%CI: 0–8·5%) at 7 years, while the cumulative incidence of death without relapse, one of the competing events, was 1·8% (95%CI: 0–5·3%) at 7 years (Fig 2D).

Adverse effects and events

Table II presents the incidence of adverse effects and the duration of neutropenia. In induction therapy, DIC was observed in 10 patients (17%) and four of these patients (7%) showed haemorrhagic complications including retinal haemorrhage in two patients and ICH and/or pulmonary haemorrhages in the other two who died. RA syndrome, which occurred in 7% of cases, was resolved with cessation of ATRA and administration of dexamethasone, the incidence of which was comparable to those (7–19%) reported by other studies of childhood APL (de Botton *et al*, 2004; Ortega *et al* 2005; Testi *et al*, 2005).

Bacterial infection was the major adverse effect in induction and consolidation, and sepsis with documented microbes was determined at a higher incidence during consolidation than induction. Although one patient died in remission of pseudomonas sepsis and meningitis after Block 2 consolidation, all other patients recovered from sepsis with treatment. A proportional relationship was apparent between the periods of neutropenia (<0·1 $\times$ 10^9/l) and the incidence of whole infections at any sites, including gingivitis, stomatitis, bronchopneumonia, enteritis, or cellulites during neutropenia, and herpes zoster only in maintenance. Other complications included impaired consciousness or convulsion associated with pseudotumour cerebri and aclarubicin-related dysuria in consolidation Block 3. Severe headache/nausea associated with ATRA therapy was experienced at an incidence of 8–22% throughout treatment.

Table III shows the characteristics of five patients with early death or relapse, two of whom exhibited at least one of the following: WBC count >10 $\times$ 10^9/l, M3v morphology, *PML-RARA* bcr3 isoform. The proportion of these patients was not significantly different from that of the whole population of 58 patients. Because of adverse effects, Block 3 consolidation was omitted or reduced in dosage at the physician's discretion in five patients, including two with WBC count >10 $\times$ 10^9/l, of whom all remained in remission for 4·9–8·9 years.

