develop organ failure *in utero*, which may lead to premature delivery.

In summary, the present study found that EGA, higher WBC count, and liver dysfunction were significantly associated with early death in DS neonates with TL. A simple risk stratification system based on EGA and the WBC count was developed to identify patients with a poor prognosis. Although validation by an independent data set is warranted, this risk classification could be useful for identifying high-risk patients who need intervention, including low-dose chemotherapy. We are now planning a prospective study that will assess early intervention with low-dose chemotherapeutic agents in high-risk patients with DS and TL.

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Authorship

Contribution: HM designed and performed research, analyzed data and prepared manuscript, and NW, KM, TN, YH, JM, CS and MH performed research and analyzed data, KK and SK helped in interpretation of the data and in writing of the manuscript.

References

Abildgaard, L., Ellebaek, E., Gustafsson, G., Abrahamsson, J., Hovi, L., Jonmundsson, G., Zeller, B. & Hasle, H. (2006) Optimal treatment intensity in children with Down syndrome and myeloid leukaemia: data from 56 children treated on NOPHO-AML protocols and a review of the literature. *Annals of Hematology*, **85,** 275–280.

Al-Kasim, F., Doyle, J.J., Massey, G.V., Weinstein, H.J. & Zipursky, A. (2002) Incidence and treatment of potentially lethal diseases in transient leukemia of Down syndrome: Pediatric Oncology Group Study. *Journal of Pediatric Hematology/oncology*, **24,** 9–13.

Bunin, N.J. & Pui, C.H. (1985) Differing complications of hyperleukocytosis in children with acute lymphoblastic or acute nonlymphoblastic leukemia. *Journal of Clinical Oncology*, **3,** 1590–1595.

Creutzig, U., Reinhardt, D., Diekamp, S., Dworzak, M., Stary, J. & Zimmermann, M. (2005) AML patients with Down syndrome have a high cure rate with AML-BFM therapy with reduced dose intensity. *Leukemia*, **19,** 1355–1360.

Dormann, S., Kruger, M., Hentschel, R., Rasenack, R., Strahm, B., Kontny, U. & Niemeyer, C. (2004) Life-threatening complications of transient abnormal myelopoiesis in neonates with Down syndrome. *European Journal of Pediatrics*, **163,** 374–377.

Doyle, J.J. (1994) Down syndrome children with neonatal transient leukemia. Survival and risk of subsequent myelodysplasia and leukemia [abstract]. *Blood*, **84,** 316a.

Gamis, A.S., Woods, W.G., Alonzo, T.A., Buxton, A., Lange, B., Barnard, D.R., Gold, S. & Smith, F.O. (2003) Increased age at diagnosis has a significantly negative effect on outcome in children with Down syndrome and acute myeloid leukemia: a report from the Children's Cancer Group Study 2891. *Journal of Clinical Oncology*, **21,** 3415–3422.

Hayashi, Y., Eguchi, M., Sugita, K., Nakazawa, S., Sato, T., Kojima, S., Bessho, F., Konishi, S., Inaba, T., Hanada, R. & Yamamoto, K. (1988) Cytogenetic findings and clinical features in acute leukemia and transient myeloproliferative disorder in Down's syndrome. *Blood*, **72,** 15–23.

Homans, A.C., Verissimo, A.M. & Vlacha, V. (1993) Transient abnormal myelopoiesis of infancy associated with trisomy 21. *The American Journal of Pediatric Hematology/Oncology*, **15,** 392–399.

Hoskote, A., Chessells, J. & Pierce, C. (2002) Transient abnormal myelopoiesis (TAM) causing multiple organ failure. *Intensive Care Medicine*, **28,** 758–762.

Kojima, S., Kato, K., Matsuyama, T., Yoshikawa, T. & Horibe, K. (1993) Favorable treatment outcome in children with acute myeloid leukemia and Down syndrome. *Blood*, **81,** 3164.

Kojima, S., Sako, M., Kato, K., Hosoi, G., Sato, T., Ohara, A., Koike, K., Okimoto, Y., Nishimura, S., Akiyama, Y., Yoshikawa, T., Ishii, E., Okamura, J., Yazaki, M., Hayashi, Y., Eguchi, M., Tsukimoto, I. & Ueda, K. (2000) An effective chemotherapeutic regimen for acute myeloid leukemia and myelodysplastic syndrome in children with Down's syndrome. *Leukemia*, **14,** 786–791.

Kudo, K., Kojima, S., Tabuchi, K., Yabe, H., Tawa, A., Imaizumi, M., Hanada, R., Hamamoto, K., Kobayashi, R., Morimoto, A., Nakayama, H., Tsuchida, M., Horibe, K., Kigasawa, H. & Tsukimoto, I. (2007) Prospective study of a pirarubicin, intermediate-dose cytarabine, and etoposide regimen in children with Down syndrome and acute myeloid leukemia: the Japanese Childhood AML Cooperative Study Group. *Journal of Clinical Oncology*, **25,** 5442–5447.

Massey, G.V., Zipursky, A., Chang, M.N., Doyle, J.J., Nasim, S., Taub, J.W., Ravindranath, Y., Dahl, G. & Weinstein, H.J. (2006) A prospective study of the natural history of transient leukemia (TL) in neonates with Down syndrome (DS): Children's Oncology Group (COG) study POG-9481. *Blood*, **107,** 4606–4613.

Miyauchi, J., Ito, Y., Kawano, T., Tsunematsu, Y. & Shimizu, K. (1992) Unusual diffuse liver fibrosis accompanying transient myeloprolif-

erative disorder in Down's syndrome: a report of four autopsy cases and proposal of a hypothesis. *Blood*, **80**, 1521–1527.

Schunk, G.J. & Lehman, W.L. (1954) Mongolism and congenital leukemia. *Journal of the American Medical Association*, **155**, 250–251.

Schwab, M., Niemeyer, C. & Schwarzer, U. (1998) Down syndrome, transient myeloproliferative disorder, and infantile liver fibrosis. *Medical and Pediatric Oncology*, **31**, 159–165.

Supplementary material

The following supplementary material is available for this article online:

Table SI. Detailed information about congenital heart disease in the study group.

The material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2141.2008.07231.x

(This link will take you to the article abstract).

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A conditioning regimen of busulfan, fludarabine, and melphalan for allogeneic stem cell transplantation in children with juvenile myelomonocytic leukemia

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Abstract: A pilot study was undertaken using a myeloablative conditioning with fludarabine, busulfan, and melphalan to improve the outcome of HSCT in 10 children, aged six months to six yr, with JMML. All patients were conditioned with oral busulfan (560 mg/m^2), fludarabine (120 mg/m^2), and melphalan (180–210 mg/m^2) prior to HSCT, and received stem cells from bone marrow in seven cases, and from cord blood in three cases. Engraftment was documented in eight patients, whereas graft failure occurred in two, one of whom had received HLA-mismatched cord blood and other had received bone marrow from HLA-mismatched mother. Three patients, including two in who graft failure had occurred, relapsed. Five patients developed acute GVHD and two developed chronic GVHD. Seven patients are alive and in remission 27–69 months after transplantation. Thus, our study showed that HSCT following conditioning with fludarabine, busulfan, and melphalan was well tolerated and appeared to be effective for JMML.

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Key words: juvenile myelomonocytic leukemia – hematopoietic stem cell transplantation – fludarabine-containing regimen

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Abbreviations: AML, acute myelogenous leukemia; BMT, bone marrow transplantation; CBT, cord blood transplantation; CR, complete remission; Cs-A, cyclosporin A; EBMT, European Blood and Marrow Transplantation; EWOG, European Working Group on Childhood; FISH, fluorescence *in situ* hybridization; FLAG, fludarabine, cytarabine, and granulocytes; G-CSF, granulocytes colony stimulating factor; GVHD, graft-vs.-host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; JMML, juvenile myelomonocytic leukemia; MDS, myelodysplastic syndrome; MTX, methotrexate; RRT, regimen-related toxicity; STR, short tandem repeats; TBI, total body irradiation.

JMML is a rare hematologic malignancy of early childhood, which runs an aggressive clinical course (1). Allogeneic HSCT is presently the only curative treatment available for JMML (2). Some studies have reported that a graft-vs.-leukemia effect seems to play an essential role in HSCT for JMML because the development of chronic GVHD protects the patients against the risk of disease relapse (2, 3). Until recently, most studies published on the results of HSCT in patients with JMML had been performed on a limited number of patients conditioned with heterogeneous regimens. The EWOG-MDS/EBMT group reported the outcome of JMML in 100 children, who were given HSCT after a homogenous preparative regimen consisting of three alkylating agents: busulfan, cyclophosphamide, and melphalan, without TBI (4). These results, when compared, were favorable to those published previously, curing approximately 50% of patients with JMML. In addition, the outcome of HSCT recipients who received transplantation from unrelated donor was comparable to that of children who had received transplants from HLA-identical siblings. Although allogeneic HSCT for JMML has been shown to improve outcome, leukemia recurrence has been represented as the main cause of treatment failure after HSCT in JMML patients (5, 6). In this report, we describe the outcome of 10 children with JMML, who were given unmanipulated HSCT after a uniform preparative regimen comprising oral busulfan, fludarabine, and melphalan.

Patients and methods

Patients

Ten consecutive patients with primary JMML underwent HSCT after being given conditioning with fludarabine, busulfan, and melphalan between June 2001 and November 2005. The patients in this study were diagnosed as suffering from JMML according to previously published criteria (7, 8). The patient characteristics at the time of diagnosis and transplantation are listed in Tables 1 and 2, respectively. Karyotypic abnormality was detected in one patient (no. 7), who was diagnosed with monosomy-7, and chemotherapy designed for AML was employed prior to transplantation in this patient. The remaining patients were treated with various regimens. Low-intensity chemotherapy was based on the use of mercaptopurine or low-dose cytosine arabinoside. Splenectomy before HSCT was performed in one patient,

Table 1. Patient data at diagnosis

No.	Sex	Age (yr)	WBC count ($\times 10^9$/L)	Platelets ($\times 10^9$/L)	HbF (%)	Cytogenetics	Mutations
1	M	0.1	97.0	23.0	60.0	46,XY	N.T
2	F	3.1	19.0	26.0	55.0	46,XX	PTPN11
3	M	5.4	9.6	13.0	43.9	46,XY	NRAS
4	F	0.3	37.2	1.0	19.5	46,XX	N.T
5	M	1.6	56.6	63.0	62.3	46,XY	N.T
6	M	2.4	14.3	33.0	43.3	46,XY	N.T
7	F	0.6	23.8	102.0	9.2	46,XX, -7,+der(?)t(?;7)(?;q11)	N.T
8	M	1.0	53.1	1.0	5.2	46,XY	N.T
9	F	0.9	61.0	12.0	50.4	46,XX	NRAS
10	M	3.7	56.6	10.0	26.4	46,XY	PTPN11

F, female; M, male; WBC, white blood cell; HbF, fetal hemoglobin.

Table 2. Patient characteristics at transplantation

No.	Age (yr)	WBC count ($\times 10^9$/L)	BM blast (%)	Spleen size (cm)	Previous therapy	Interval to HSCT (months)
1	0.5	9.7	3.6	5	6MP	5
2	3.7	1.0	60.9	12	6MP, VP, MIT, low-dose CA, splenic irradiation 600 cGy	6
3	6.8	6.2	5.4	11.5	6MP	16
4	2.2	15.0	9.0	Splenectomized	6MP, low-dose CA, splenectomy	22
5	1.9	10.1	10.0	6	6MP	4
6	2.8	4.7	4.0	3	6MP	4
7	2.2	2.6	5.0	15	VP + CA (AML protocol)	19
8	1.6	8.9	1.4	Not palpable	6MP, PSL	7
9	1.2	1.0	6.0	6	6MP, PSL, VP	4
10	3.9	5.0	2.6	15	6MP, low-dose CA, splenic irradiation 600 cGy	3

F, female; M, male; WBC, white blood cell; BM, bone marrow; HSCT, hematopoietic stem cell transplantation; 6MP, mercaptopurine; VP, etoposide; MIT, mitoxantrone; CA, cytosine arabinoside; AML, acute myelogenous leukemia; PSL, prednisolone.

while two patients underwent splenic irradiation (6 Gy) to palliate symptomatic splenomegaly. Only one patient (no. 2) had >20% blasts in her bone marrow at the time of transplantation, and one patient (no. 3) was older than four yr at diagnosis.

Donor choice

Serologic typing for HLA-A and -B antigens, and a low-resolution generic DRB1 oligotyping were available for all donor–recipient pairs. High or middle-resolution DNA typing for all loci in unrelated donor–recipient pairs and mismatched family donor–recipient pairs confirmed the previous serological typing.

The patient–donor characteristics are shown in Table 3. Of the 10 patients who received allogeneic HSCT, two patients received allogeneic BMT from fully matched unrelated donor (n = 2), one patient received allogeneic BMT from an antigen-mismatched unrelated donor (n = 1), one patient received fully matched unrelated cord blood (n = 1), two patients received antigen-mismatched unrelated cord blood (n = 2), three patients received matched bone marrow from their siblings (n = 3), and one received BMT from antigen-mismatched family donor (n = 1).

Preparative regimen and transplantation

All 10 patients were conditioned with busulfan 140 mg/m^2 p.o. in divided doses daily for four days (total dose 560 mg/m^2), fludarabine 30 mg/m^2 once daily i.v. for four days (total dose 120 mg/m^2) and melphalan 90–100 mg/m^2 once daily i.v. for two days or 70 mg/m^2 once daily i.v. for three days (total dose 180–210 mg/m^2). Grafts for BMT were non-T-cell-depleted marrow cells, whereas those for CBT were cord blood stem cells. Basically, GVHD prophylaxis for a matched sibling allograft was MTX alone for patients younger than 10 yr old, and Cs-A was added to short-term MTX for patients older than 10 yr old. But they varied and a single administration of MTX, Cs-A + short-term MTX or tacrolimus + short-term MTX was employed in three patients. Four patients given allograft from an alternative donor received tacrolimus and short-term MTX. The combination of Cs-A and short-term MTX was used in three patients who received unrelated CBT. Detailed characteristics regarding transplantation and its outcome are shown in Table 3. This study was carried out according to the guidelines of the Declaration of Helsinki and according to good clinical practice, after informed consent.

Analysis of chimerism

Engraftment of the donor marrow was assayed using STR analysis or FISH with XY chromosome-specific probes.

Results

Engraftment and GVHD occurrence

At a median of 28 days (range, 13–55 days), eight patients had neutrophil engraftment (>0.5 × 10^9/L) and at a median of 49 days (range, 24–138 days), eight patients had an unsupported platelet count of >50 × 10^9/L. Transplant outcomes are detailed in Table 3. Two patients failed to engraft (nos. 4 and 10), of which one had received 3-antigen mismatched cord blood, while the other had received 2-antigen mismatched bone marrow from his mother. This patient possessed an anti-HLA antibody against HLA-DR9, which was also present in the mother. The patient later received a second successful HSCT from an unrelated donor with mismatched antigen, who did not possess HLA-DR9. Eight patients had 97.6–100% donor cells,

Table 3. Transplantation characteristics and outcome

No.	Conditioning Bu (mg/m^2)	Flu (mg/m^2)	L-PAM (mg/m^2)	Donor-stem cell source	HLA matching (DNA)	Cell dose (×10^8/kg)	GVHD prophylaxis	Engraftment donor cell (%; day)	Acute GVHD	Chronic GVHD	Outcome, months (interval after HSCT)
1	560	120	210	MUD-CB	6/6	0.96	MTX + Cs-A	100% (+45)	I	Absent	Alive (69)
2	560	120	200	MUD-BM	6/6	2.06	MTX + FK	97.6% (+29)	0	Lim	Alive (39)
3	560	120	210	MSD-BM	6/6	3.66	MTX	99.2% (+22)	II	Ext	Alive (34)
4–1	560	120	210	MMUD-CB	3/6	0.80	MTX + Cs-A	No	NA	NA	Relapse (day69)
4–2	TBI (10 Gy) + CY (120 mg/kg)			MMUD-BM	4/6	Unknown	MTX + FK	Yes	I	Absent	Relapse (seven after second BMT), died (30)
5–1	560	120	210	MSD-BM	6/6	4.30	MTX + FK	100% (+29)	I	Absent	Relapse (day120)
5–2	TBI (12 Gy) + CY (120 mg/kg) + VP			MUD-BM	6/6	Unknown	MTX + FK	NA	NA	NA	Died (10), IP
6	560	120	200	MUD-BM	6/6	5.20	MTX + FK	100% (+28)	I	Absent	Alive (30)
7	560	120	180	MMUD-BM	5/6	5.00	MTX + FK	99% (+27)	0	Absent	Alive (29)
8	560	120	210	MSD-BM	6/6	4.20	MTX + Cs-A	100% (+28)	0	Absent	Alive (27)
9	560	120	210	MMUD-CB	5/6	1.00	MTX + Cs-A	100% (+113)	IV	Absent	Alive (27)
10–1	560	120	180	MMFD-BM	4/6	4.73	MTX + FK	No	NA	NA	Relapse (day47)
10–2	TBI (12 Gy) + CY (120 mg/kg)			MMUD-BM	5/6	5.34	MTX + FK	Yes	0	NA	Relapse (two after second BMT), died (8)

Bu, busulfan; Flu, fludarabine; L-PAM, melphalan; TBI, total body irradiation; CY, cyclophosphamide; VP, etoposide; GVHD, graft-vs.-host disease; MUD, matched unrelated donor; MSD, matched sibling donor; MMUD, mismatched unrelated donor; MMFD, mismatched family donor; CB, cord blood; BM, bone marrow; MTX, short-term methotrexate; Cs-A, cyclosporin A; FK, tacrolimus; Lim, limited type; Ext, extensive type; NA, not available; HSCT, hematopoietic stem cell transplantation; IP, interstitial pneumonia.

22–113 days after HSCT. Acute GVHD developed in five of eight evaluable patients, grade I in three patients, grade II in one and grade IV in one. Chronic GVHD was observed in two out of eight evaluable patients surviving beyond 100 days after HSCT, with a limited form in one patient and extensive form in the other.

Toxicity and survival

RRT including moderate mucositis, hepatic veno-occlusive disease, cardiac toxicity (of grade II, according to Bearman's grading system) and hemorrhagic cystitis was observed in one patient each; however, none of these complications were fatal. Relapse occurred in three of 10 patients at 69, 120, and 47 days after HSCT, respectively (nos. 4, 5, and 10). These patients received a second BMT from an unrelated donor using a preparative regimen consisting of TBI (10–12 Gy) and cyclophosphamide (120 mg/kg). One patient (no. 5) died of interstitial pneumonitis at the time of second transplantation whereas two other patients (nos. 4 and 10) relapsed again at seven and two months after the second transplantation, respectively, and eventually died. The remaining seven patients are still alive and are in complete remission after HSCT, with a median observation time of 30 months (range, 27–69).

Discussion

The purpose of this study was to improve the outcome of HSCT in JMML using a fludarabine-containing regimen without using TBI. Fludarabine is a nucleoside analogue that has been successfully employed for the treatment of low-grade lymphoid malignancies (9). However, several investigators have reported that it has also been active in cases with acute myeloid leukemia and myelodysplastic syndrome (10). The combination chemotherapy of FLAG (G-CSF) seemed to produce good results in children with relapsed, poor-prognosis acute monocytic leukemia (11). The use of fludarabine may be effective in suppressing the aggressive growth of malignant clone of monocytes in JMML. The second point, which favors the use of fludarabine, is its strong cytotoxic activity against lymphocytes, which consistently prolongs immunosuppression, facilitating the engraftment of hematopoietic stem cells both from HLA-identical siblings and unrelated donors (12). But graft failure was seen in two who had received HLA-mismatched HSCT. To overcome graft failure, particularly in mismatched transplant, it may be necessary to use low-dose TBI or more immunosuppressive agents. Conventional CBT utilized a TBI or a busulfan-based myeloablative conditioning regimen, which carries a high risk of morbidity and mortality (13). On the other hand, Bradley et al. (14) reported that reduced intensity CBT may result in graft failure in specific high-risk chemo-naive patients (chronic myelogenous leukemia, hemophagocytic lymphohistiocytosis, and myelodysplastic syndrome). In our study, two of three patients were successfully transplanted with unrelated umbilical cord blood cells using fludarabine-containing regimen. Although there are very few data in the literature reporting specific results and prognostic factors of CBT in JMML, our experience suggests that a conditioning with fludarabine, busulfan, and melphalan may possibly decrease the mortality rate and the risk of graft failure even in the case of CBT.

Fludarabine in combination with melphalan, cyclophosphamide, or other agents can replace TBI or can be used together with low-dose TBI regimens (15, 16). Occurrence of long-term complications such as growth retardation, infertility (17) and appearance of a second malignancy are the major concerns following TBI therapy in children (18). Therefore, we decided to avoid radiotherapy for treatment of JMML, a condition that occurs during early childhood. In our study, no patient experienced life-threatening regimen-related grade III/IV toxicities, such as severe viral infection, idiopathic pneumonitis, thrombotic microangiopathy, and veno-occlusive disease of the liver. Grade IV acute GVHD developed in one patient (no. 9). In this case, her hepatosplenomegaly progressed, and Cs-A was discontinued on day +17. Following resumed Cs-A therapy, her acute GVHD improved and she has maintained CR. Thus, the preparative regimen consisting of busulfan, fludarabine, and melphalan seems safe, because no patient died of transplantation-related causes. Further long-term follow-up is necessary to evaluate growth retardation, infertility, and second malignancy.

Koyama et al. (19) presented a case using a reduced intensity regimen consisting of fludarabine (30 mg/m^2 for four days) and melphalan (70 mg/m^2 for two days) after AML-type chemotherapy. JMML patients who respond to chemotherapy might be considered as candidates for a non-myeloablative preparative, reduced intensity preparative regimen. Disease recurrence remains the major cause of treatment failure for JMML, and it is believed that both intensive myeloablative conditioning and a graft-vs.-leukemia effect are needed to eradicate the disease (2, 3). Thus, further studies in the future are necessary to compare the results between transplants

conditioned with myeloablative regimens and those conditioned with reduced intensity, non-myeloablative regimens.

A high relapse rate has been the major cause of failure of HSCT in JMML. In patients with JMML, relapse occurs early, generally within the first year after the allograft (20). In this study, only two out of 10 patients bear high-risk features (age more than four yr, blast count at HSCT > 20%) as defined by the EWOG-MDS/EBMT study. The development of chronic GVHD might be associated with better survival, although the association was not significant, possibly because of the small sample size of the study. Seven of 10 patients survived in complete remission for more than two yr after HSCT even though only two patients developed chronic GVHD. Among three patients who relapsed, two failed to engraft, and one had no signs of chronic GVHD. Despite the use of a TBI conditioning regimen for second BMT, two patients without chronic GVHD relapsed again within the first year after BMT. Yoshimi et al. (21) reported that none of the six patients who developed chronic GVHD after second HSCT relapsed. It is reasonable to speculate that chronic GVHD led to a stronger graft-vs.-leukemia effect and resulted in a favorable outcome for allogeneic HSCT in JMML.

Although the number of patients was too small for statistical analysis, this study indicates that a preparative regimen consisting of fludarabine, busulfan, and melphalan can be used satisfactorily in conditioning patients with JMML who receive transplantation, either in form of cord blood or bone marrow not only from HLA-matched siblings but also from alternative donors. Further large studies are needed to confirm any advantage of this choice.

References

1. Niemeyer CM, Aricò M, Basso G, et al. Chronic myelomonocytic leukemia in childhood: A report of 110 cases. Blood 1997: 89: 3534–3543.
2. Manabe A, Okamura J, Yumura-Yagi K, et al. Allogeneic hematopoietic stem cell transplantation for 27 children with juvenile myelomonocytic leukemia diagnosed based on the criteria of the International JMML Working Group. Leukemia 2002: 16: 645–649.
3. Smith FO, King R, Nelson G, et al. Unrelated donor bone marrow transplantation for children with juvenile myelomonocytic leukemia. Br J Haematol 2002: 116: 716–724.
4. Locatelli F, Nöllke P, Zecca M, et al. Hematopoietic stem cell transplantation (HSCT) in children with juvenile myelomonocytic leukemia (JMML): Results of the EWG-MDS/EBMT trial. Blood 2005: 105: 410–419.
5. Matthes-Martin S, Mann G, Peters C, et al. Allogeneic bone marrow transplantation for juvenile myelomonocytic leukemia: A single center experience and review of the literature. Bone Marrow Transplant 2000: 26: 377–382.
6. Korthof ET, Snijder PP, De Graaff AA, et al. Allogeneic bone marrow transplantation for juvenile myelomonocytic leukemia: A single center experience of 23 patients. Bone Marrow Transplant 2005: 35: 455–461.
7. Niemeyer CM, Fenu S, Hasle H, Mann G, Stary J, Van Wering E. Differentiating juvenile myelomonocytic leukemia from infectious disease. Blood 1998: 91: 365–367.
8. Hasle H, Niemeyer CM, Chessells JM, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative disease. Leukemia 2003: 17: 277–282.
9. Keating M, O'Brien S, Lerner S, et al. Long-term follow-up of patients with chronic lymphocytic leukemia (CLL) receiving fludarabine regimens as initial therapy. Blood 1998: 92: 1165–1171.
10. Virchis A, Koh M, Rankin P, et al. Fludarabine, cytosine arabinoside, granulocyte-colony stimulating factor with or without idarubicin in the treatment of high risk acute leukemia and myelodysplastic syndrome. Br J Haematol 2004: 124: 26–32.
11. Fleischhack G, Hasan C, Graf N, Mann G, Bode U. Ida-FLAG (idarubicin, fludarabine, cytarabine, G-CSF) an effective remission-induction therapy for poor-prognosis AML of childhood prior to allogeneic or autologus bone marrow transplantation: Experiences of a phase II trial. Br J Haematol 1998: 102: 647–655.
12. Giralt S, Thall PF, Khouri I, et al. Melphalan and purine analog-containing preparative regimens: Reduced-intensity conditioning for patients with hematologic malignancies undergoing allogeneic progenitor cell transplantation. Blood 2001: 97: 631–637.
13. Gluckman E, Rocha V, Boyer-Chammard A, et al. Outcome of cord-blood transplantation from related and unrelated donors. Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. N Engl J Med 1997: 337: 373–381.
14. Bradley MB, Satwani P, Baldinger L, et al. Reduced intensity allogeneic umbilical cord blood transplantation in children and adolescent recipients with malignant and non-malignant diseases. Bone Marrow Transplant 2007: 40: 621–631.
15. Slavin S, Nagler A, Naparstek E, et al. Nonmyeloablative stem cell transplantation and cell therapy as alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. Blood 1998: 91: 756–763.
16. Niederwieser D, Maris M, Shizuru JA, et al. Low-dose total body irradiation (TBI) and fludarabine followed by hematopoietic cell transplantation (HCT) from HLA-matched or mismatched unrelated donors and post grafting immunosuppression with cyclosporine and mycophenolate mofetil (MMF) can induce durable complete chimerism and sustained remissions in patients with hematological disease. Blood 2003: 101: 1620–1629.
17. Georgiani G, Bozzola M, Locatelli F, et al. Role of busulfan and total body irradiation on growth of pre-pubertal children receiving bone marrow transplantation and results of treatment with recombinant human growth hormone. Blood 1995: 86: 825–831.
18. Socie G, Curtis RE, Deeg HJ, et al. New malignant diseases after allogeneic marrow transplantation for childhood acute leukemia. J Clin Oncol 2000: 18: 348–357.
19. Koyama M, Nakano T, Takeshita Y, et al. Successful treatment of JMML with related bone marrow transplantation after reduced-intensity conditioning. Bone Marrow Transplant 2005: 36: 453–454.

20. Locatelli F, Niemeyer C, Angelucci E, et al. Allogeneic bone marrow transplantation for chronic myelomonocytic leukemia in childhood: A report from the European Working Group on Myelodysplastic Syndrome in Childhood. J Clin Oncol 1997: 15: 566–573.

21. Yoshimi A, Mohamed M, Bierings M, et al. Second allogeneic hematopoietic stem cell transplantation (HSCT) results in outcome similar to that of first HSCT for patients with juvenile myelomonocytic leukemia. Leukemia 2007: 21: 556–560.

bjh research paper

Acute megakaryoblastic leukaemia (AMKL) in children: a comparison of AMKL with and without Down syndrome

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Summary

To characterize childhood acute megakaryoblastic leukaemia (AMKL), we reviewed 45 children with AMKL diagnosed between 1986 and 2005 at Nagoya University Hospital and Japanese Red Cross Nagoya First Hospital. Twenty-four patients (53%) had AMKL associated with Down syndrome (DS-AMKL) and 21 (47%) had non-DS-AMKL. The median age of the DS-AMKL patients was 21 months (range, 8–38 months) and that of non-DS-AMKL patients was 15 months (range, 2–185 months). The morphology of blast cells was categorized into three groups according to the stage of megakaryocyte maturation. The blast cells were more immature in DS-AMKL than in non-DS-AMKL in terms of morphology and immunophenotyping. Cytogenetic abnormalities of leukaemic cells were classified into seven categories: normal karyotype including constitutional trisomy 21 in DS-AMKL; numerical abnormalities only; t(1;22)(p13;q13); 3q21q26 abnormalities; t(16;21)(p11;q22); −5/del(5q) and/or −7/del(7q); and other structural changes. The outcome of children with either DS-AMKL or non-DS-AMKL is excellent. The 10-year overall survival estimate was 79% [95% confidence interval (CI): 54–90] for DS-AMKL and 76% (95% CI: 58–91) for non-DS-AMKL ($P = 0{\cdot}81$) with a median follow-up of 78 months (range, 20–243 months). Our study shows the diverse heterogeneity of childhood AMKL and the need for subclassification according to cytogenetic and morphological features.

Keywords: acute megakaryoblastic leukaemia, Down syndrome, children, cytogenetics, morphology.

Acute megakaryoblastic leukaemia (AMKL), M7 according to the French-American-British (FAB) classification (Bennett *et al*, 1985), is a subtype of acute myeloid leukaemia (AML) (Bennett *et al*, 1985). Until now, only a few series of AMKL have been reported in a consecutive cohort of children (Ribeiro *et al*, 1993; Athale *et al*, 2001; Paredes-Aguilera *et al*, 2003; Reinhardt *et al*, 2005). Large collaborative studies have shown that AMKL occurs in 4·1% to 15·3% of patients with childhood AML (Ravindranath *et al*, 1992; Gamis *et al*, 2003; Creutzig *et al*, 2005; Zeller *et al*, 2005; Rao *et al*, 2006). These previous studies revealed a diverse heterogeneity of the disease including morphology and cytogenetic studies. In children, two major subgroups have been described: AMKL in patients with Down syndrome (DS-AMKL) and AMKL in patients without Down syndrome (non-DS-AMKL). AMKL is the most frequent type of AML in children with DS (Zipursky *et al*, 1987). Somatic mutations of *GATA1* are found in almost all children with DS-AMKL (Wechsler *et al*, 2002; Hirose *et al*, 2003; Rainis *et al*, 2003). It is well known that patients with DS-AMKL have an excellent outcome with less intensive chemotherapy. In general, the remission rate is approximately 90% with event-free survival (EFS) of 70% to 80% (Gamis, 2005). The favourable outcome in patients with DS-AMKL has been explained by the increased sensitivity of leukaemic cells to anticancer drugs (Taub *et al*, 1999, 2000). On the other hand, patients with non-DS-AMKL appear to be more heterogeneous and several cytogenetic groups have been identified (Lu *et al*, 1993; Dastugue *et al*, 2002; Duchayne *et al*, 2003). Among these groups, the occurrence of t(1;22)(p13;q13) is restricted to infants with AMKL (Carroll *et al*, 1991; Lion *et al*, 1992; Bernstein *et al*, 2000). The outcome of children with non-DS-AMKL is generally poor (Ribeiro *et al*, 1993; Athale

et al, 2001), but a recent study reported long-term survivors after intensive chemotherapy (Reinhardt *et al*, 2005).

We reviewed 45 children with AMKL (24 DS-AMKL, 21 non-DS-AMKL) diagnosed between 1986 and 2005 at Nagoya University Hospital and Japanese Red Cross Nagoya First Hospital. The main purpose of this study was to compare clinical and biological characteristics of patients with DS-AMKL and non-DS-AMKL.

Patients and methods

Patients and diagnostic criteria of AMKL

Forty-five children with newly diagnosed AMKL (24 DS-AMKL, 21 non-DS-AMKL) at Nagoya University Hospital and Japanese Red Cross Nagoya First Hospital between 1986 and 2005 were retrospectively reviewed. Acute leukaemia was diagnosed by the presence of at least 20% blasts in the bone marrow (BM) according to the World Health Organization classification (Harris *et al*, 1999). In patients with poor quality BM aspiration smears, the presence of more than 20% blasts in the BM core biopsy or 20% or more circulating blasts were used to support the diagnosis of acute leukaemia. The diagnosis of AMKL was established on the basis of FAB classification (Bennett *et al*, 1985) by studies of cell morphology and cytochemistry and was confirmed by immunophenotyping.

For immunophenotyping of leukaemic blasts, mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation from the BM. In patients who did not have an adequate BM aspirate, the immunological studies were performed on peripheral blood (PB) mononuclear cells. The cells were analysed by flow cytometry with a panel of monoclonal antibodies. For the assessment of megakaryocytic lineage, at least 10% of the blast cells needed to be positive for one or more of the platelet-associated antigens (CD36, CD41, CD42 or CD61) (San Miguel *et al*, 1988). For the other immunological markers, the samples were defined as positive if more than 20% of the cells were stained. In the absence of immunophenotyping, the diagnosis was confirmed by electron microscopic identification of platelet peroxidase (PPO) activity or CD41 expression in the histopathological examination in malignant cells.

Cytogenetic studies were performed on BM or PB samples taken at the time of diagnosis; samples were processed and analysed by standard methods.

Analysis of GATA1 mutation

After obtaining informed consent from the parents of the children for the purpose of sample banking and molecular analysis, BM or PB samples were obtained from all of the patients with AMKL at the time of diagnosis. High-molecular weight DNA was extracted from the samples using standard methods. For screening of *GATA1* mutations, we amplified the genomic DNA that corresponded to exon 2 of *GATA1* by using polymerase chain reaction that employed one primer pair as previously reported (Hirose *et al*, 2003). Amplified products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced on a DNA sequencer (310; Applied Biosystems, Foster City, CA, USA) using a BigDye terminator cycle sequencing kit (Applied Biosystems).

Treatment

In patients with DS-AMKL, six patients received chemotherapy consisting of cytosine arabinoside (AraC) and dounorubicin (Kojima *et al*, 1990, 1993) and 16 patients received chemotherapy consisting of AraC, etoposide, and dounorubicin or pirarubicin (Kojima *et al*, 2000). One patient with mosaic DS received intensive chemotherapy according to non-DS-AML protocol containing high-dose AraC (AML99) (Shimada *et al*, 2006) and one patient received chemotherapy according to an acute lymphoblastic leukaemia oriented protocol (Kojima *et al*, 1990). One patient who failed to achieve complete remission (CR) by induction therapy underwent bone marrow transplantation (BMT) from a human leucocyte antigen (HLA)-matched unrelated donor.

In the patients with non-DS-AMKL, 18 patients were treated in one of two national co-operative studies for AML (12 on ANLL91, six on AML99). Two patients received less intensive chemotherapy according to DS-AMKL protocol (Kojima *et al*, 1993). Twelve patients received BMT (autologous, seven; allogeneic, five) in the first CR. Three patients who failed to achieve CR after induction therapy received allogeneic stem cell transplant (SCT: BM, two; cord blood, one). Two patients who relapsed after allogeneic BMT in the first CR (one from a matched sibling, the other from a matched unrelated donor) received a second allogeneic BMT (one from a matched unrelated donor, the other from a haploidentical related donor). The indication for SCT varied over the study period and was defined by individual protocol. In the ANLL91 study, allogeneic BMT was indicated in the first CR for patients who had a matched sibling donor; patients without a matched sibling donor were eligible for autologous BMT. AML99 study did not indicate SCT for patients with AMKL in the first CR without poor prognostic chromosome abnormalities such as monosomy 7 or t(16;21). Conditioning regimens included busulfan and melphalan; fludarabine was added in the unrelated donor settings. Prophylaxis against graft-versus-host disease consisted of ciclosporin and short-term methotrexate therapy in BMT from a matched related donor and tacrolimus and short-term methotrexate in SCT from an unrelated donor or a mismatched related donor.

Statistical analysis

Non-parametric Mann–Whitney test was used to analyse statistical differences in the distribution of continuous variables. χ^2 test or Fisher's exact test was used for differences in frequencies. Survival distributions were estimated by using

the method of Kaplan and Meier (1958) and were compared using the log-rank test. All estimates of outcomes are reported with 95% confidence intervals (CI). The duration of overall survival (OS) was defined as the period between the date of diagnosis and the date of death from any cause or the date of the last follow-up examination. The duration of EFS was defined as the period between the date of diagnosis and the date of an adverse event (relapse, death from any cause) or the most recent follow-up examination. Early death or remission induction failure was recorded as an event at zero time, with an EFS value of zero. SAS release 6.12 software (SAS Institute, Cary, NC, USA) was used to perform the statistical analysis. *P* values <0·05 were considered significant.

Results

Patients

During the period of study, 194 children with AML were diagnosed in two hospitals; 45 (23·2%) of these had AMKL. Twenty-four patients (53·3%) had DS-AMKL and 21 (46·7%) had non-DS-AMKL. Among them, 11 patients with DS-AMKL had been previously reported (Kojima *et al*, 1990, 1993, 2000). Twenty-seven children with DS-AML/myelodysplastic syndrome (MDS) were diagnosed during the same period of study. Among them, one patient was diagnosed as AML (M0) and two were diagnosed as MDS. One of the patients with DS-AMKL was trisomy 21 mosaicism. In the patients with DS-AMKL, eight patients (33·3%) had a history of transient myeloproliferative disorder and 12 (50·0%) had prior MDS. Congenital heart anomalies were present in 11 patients (45·8%) with DS-AMKL. None of the patients with AMKL had secondary leukaemia or mediastinal germ cell tumour. The median age of DS-AMKL patient was 21 months (range, 8–38 months) and that of non-DS-AMKL was 15 months (range, 2–185 months). Patients younger than 4 years at diagnosis accounted for 95·6% (43 of 45) in both groups.

Clinical and laboratory features

Table I shows the clinical and laboratory findings at the time of diagnosis. There were no statistically significant differences in these findings between DS-AMKL and non-DS-AMKL patients. The initial leucocyte count, BM blast cell count, and lactic dehydrogenase activity tended to be higher in patients with non-DS-AMKL than in those with DS-AMKL, but differences were not significant. Nine patients (DS-AMKL, eight; non-DS-AMKL, one) had less than 20% blasts in the BM; the BM of all nine patients was difficult to aspirate, contributing to the low estimates of percentage of blasts. Six patients (DS-AMKL, five; non-DS-AMKL, one) underwent BM biopsy, which confirmed the presence of more than 20% blast cells and the diagnosis of acute leukaemia. Three DS-AMKL patients had more than 20% blasts in the PB; which supported the diagnosis of acute leukaemia.

Morphological features

Forty-two of 45 BM smears were studied, as three smears of patients with non-DS-AMKL were unevaluable. The leukaemic cells of all 42 patients were negative for myeloperoxidase, chloroacetate esterase, and alpha naphthyl butyrate esterase activity. The morphology of blast cells was extremely varied and was categorized it into three groups according to the stage of megakaryocyte maturation: type 1, completely undifferentiated blasts with nucleolus or vacuoles in the cytoplasm (Fig 1A and B); type 2, intermediately differentiated blasts with cytoplasmic blebs, sometimes a large cytoplasm and azurophilic

Table I. Clinical and laboratory findings at the time of diagnosis.

Feature	DS-AMKL (n = 24)	non-DS-AMKL (n = 21)	P
Median age, months (range)	21 (8–38)	15 (2–185)	0·52
Sex			
M/F ratio	12:12	8:13	0·42
Clinical findings			
Fever, n (%)	12 (50)	14 (67)	0·33
Lymphadenopathy, n (%)	2 (8·3)	6 (29)	0·12
Hepatomegaly, n (%)	16 (67)	14 (67)	1
Splenomegaly, n (%)	11 (46)	9 (43)	1
Laboratory findings			
Haemoglobin, g/l (median, range)	83 (52–130)	82 (36–110)	0·38
Platelet count, $\times 10^9$/l (median, range)	26·0 (2–143)	24·0 (3–492)	0·72
Leucocyte count, $\times 10^9$/l (median, range)	6·8 (2·4–107·0)	17·9 (0·1–134·8)	0·07
Circulating blast cells, % (median, range)	15·0 (0–88)	13·0 (0–82)	0·95
Bone marrow blast cells, % (median, range)	25·0 (2–90)	50·0 (10–81)	0·09
Serum LDH, IU/L (median, range)	662 (260–6450)	1639 (338–8660)	0·11

DS-AMKL, Down syndrome-associated acute megakaryoblastic leukaemia; LDH, lactic dehydrogenase.

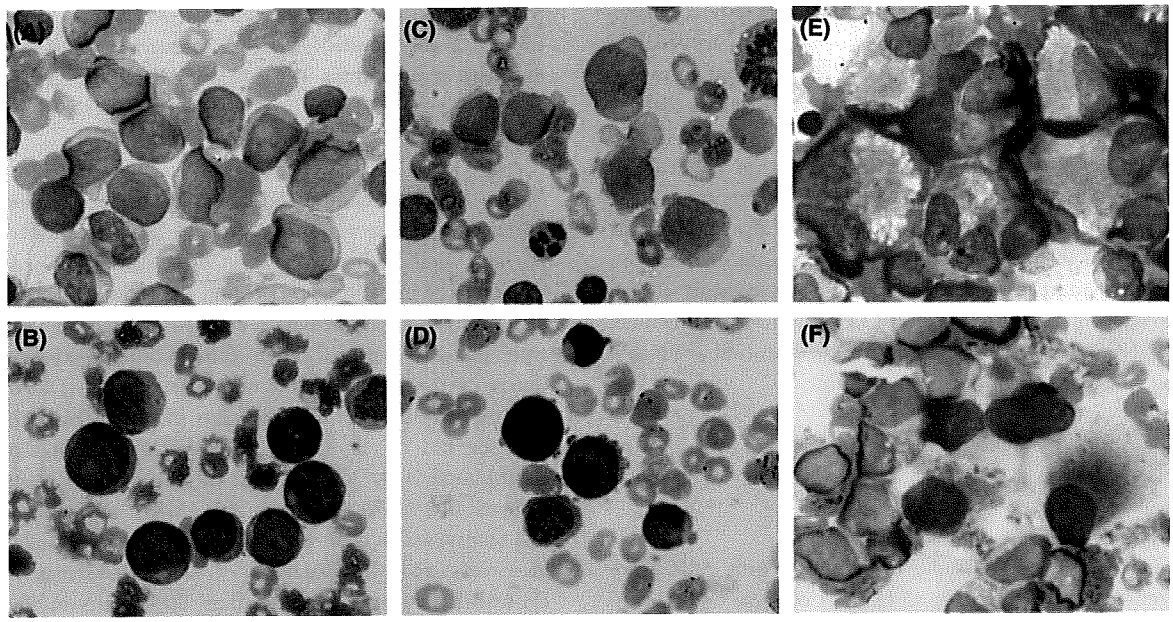

Fig 1. Morphological categories of the blasts. May-Giemsa staining of the bone marrow smears. (A) Type 1a: completely undifferentiated blasts. (B) Type 1b: completely undifferentiated blasts with deep blue cytoplasm. (C) Type 2a: intermediately differentiated blasts with cytoplasmic blebs. (D) Type 2b: intermediately differentiated blasts with cytoplasmic blebs and deep blue cytoplasm. (E) Type 3: blasts with dysmegakaryocytopoiesis. (F) Type 3: blasts with micromegakaryocytes. Original magnification ×1000 for all panels.

granules (Fig 1C and D); and type 3, blasts with dysmegakaryocytopoiesis (Fig 1E) including the presence of micromegakaryocytes (Fig 1F). The blast cells with deep blue cytoplasm (type 1b, 2b) (Fig 1B and D) were distinguishable from type 1a or 2a blasts (Fig 1A and C). The morphology of blast cells in DS-AMKL and non-DS-AMKL were distributed as follows: type 1 (63%, 39%), type 2 (25%, 39%), and type 3 (12%, 22%) (Tables II and III). Type 1b and 2b blasts were detected in eight of 24 patients (33%) with DS-AMKL. The blast cells tended to be less mature in DS-AMKL than in non-DS-AMKL in terms of morphology. Seven patients presented with type 3 morphology: four patients (DS-AMKL, one; non-DS-AMKL, three) had increased numbers of micromegakaryocytes with type 1 blasts, two patients with DS-AMKL had increased numbers of dysmegakaryocytopoiesis, and one non-DS-AMKL patient with t(16;21) showed type 3 blasts with emperipolesis and cytophagocytosis, which are characteristic in patients with t(16;21) (Imashuku *et al*, 2000). Dysplasia in trilineage blood cells was seen in two patients (DS-AMKL, one; non-DS-AMKL, one). Prominent dyserythropoiesis without involvement of the myeloid cell lineage was found in five patients (DS-AMKL, three; non-DS-AMKL, two). Emperipolesis was observed in three patients (DS-AMKL, two; non-DS-AMKL, one).

Immunophenotyping

All but one patient had immunophenotyping studies performed (Tables II and III); the BM sample of one patient with non-DS-AMKL who showed CD41 expression in blast cells by histopathological examination and translocation t(1;22) was insufficient and could not evaluated. The leukaemic cells of 44 patients expressed at least one platelet-associated antigen (CD36, CD41, CD42, or CD61). The blast cells with low expression of platelet-associated antigens in two patients (DS-AMKL, one; non-DS-AMKL, one) were positive for PPO. Among the myeloid antigens, CD13/CD33 was expressed by leukaemic blast cells in 78%/53% and 60%/78% of patients with DS-AMKL and non-DS-AMKL, respectively. Atypical expression of lymphoid-associated antigens CD7 was detected in 88% and 53% of patients with DS-AMKL and non-DS-AMKL, respectively ($P = 0{\cdot}003$). Glycophorin A was detected only on the leukaemic cells of 47% of patients with DS-AMKL ($P = 0{\cdot}009$). Interestingly, 86% of the type 1b and 2b blasts were positive for glycophorin A.

Cytogenetic findings

Cytogenetic studies were performed for 21 patients with DS-AMKL and 21 patients with non-DS-AMKL (Tables II and III). Three patients with DS-AMKL had insufficient BM samples. Cytogenetic abnormalities of leukaemic cells were classified into seven categories: normal karyotype including constitutional trisomy 21 in DS-AMKL; numerical abnormalities only; t(1;22)(p13;q13); 3q21q26 abnormalities; t(16;21)(p11;q22); −5/del(5q) and/or −7/del(7q); and other structural changes. Normal karyotype including constitutional trisomy 21 was found in five patients with DS-AMKL and in two patients with non-DS-AMKL. Numerical chromosomal abnormalities were common in non-DS-AMKL. Patients with non-DS-AMKL had trisomy 8 (six patients), trisomy 19 (five

Table II. Morphological classification, immunophenotype and karyotype of the 24 patients with Down syndrome-associated acute megakaryoblastic leukaemia.

Patient no.	Age (months)	Sex	Morphological classification	Immunophenotype (%)						Karyotype
				CD7	CD13	CD36	CD41	CD42	GlyA	
A: Constitutional trisomy 21 only										
1	32	F	3+Tri	57	19	42	10	14	NA	47,XX,+21c
2	21	F	1a	68	36	42	12	NA	13	47,XX,+21c
3	21	M	2b	72	17	55	56	NA	20	47,XX,+21c
4	21	F	3+Ep	18	45	NA	31	44	NA	47,XX,+21c
5	18	M	2b	53	78	81	43	47	24	47,XX,+21c
B: Numerical abnormalities only										
6	21	M	1a	83	20	NA	41	42	2	51,XY,+8,+19,+21c,+21,+22
7	8	M	1a	56	10	NA	16	23	NA	48,XY,+8,+21c
C: −5/del(5q) and/or −7/del(7q)										
8	20	F	1b	78	30	35	24	34	NA	47,XX,del(6)(q23q25),−7,+ring(7?),+21c
9	23	F	1a	36	29	83	76	75	NA	46,XX,−1,+der(1)t(1;?),−5,+der(5)t(5;7),−7,+21c
10	24	F	1a+dE	90	30	35	24	34	NA	47,XX,del(7)(q32),+21c
11	17	F	3+m	40	75	NA	14	12	12	47,XX,add(5)(p15),−7,+8,+21c
12	20	F	2b	14	79	NA	4	25	99	90,idem×2,−3,−7,−9,del(11)(q?),−18
13	38	M	2b+dE	81	83	NA	15	20	4	47,XY,−7,+8,+21c,+rl
D: Other structural changes										
14	20	F	1a	83	27	NA	59	87	NA	48,X,der(X)t(X;1)(q28;q25),+11,+21c
15	18	M	1a	70	12	34	2	2	38	47,XY,der(3)t(3;3)(p25;p10),i(7q), der(17)t(1;17)(q25;q25),+21c
16	17	F	1a	51	4	NA	11	9	28	48,idem,add(5)(q1?),+11
17	12	M	1a	89	36	NA	74	19	15	47,XY,der(7)t(1;7)(q23;q36),del(20)(q11q13.1),+21c
18	29	M	2b	60	43	69	14	34	62	47,XY,del(11)(p?),+21c
19	25	F	1a+dE	71	23	NA	41	NA	84	47,XX,t(5;12)(p15;q21),add(7)(p11),+21c,add(22)(q13)
20	18	M	1b	52	37	NA	10	26	32	48,idem,+add21
21	22	F	1b+Ep	99	78	88	59	66	93	47,XX,add(7)(p11),add(19)(p13),+21c
22	8	M	1a	72	53	64	72	75	4	NA
23	21	M	2a	87	12	33	20	23	4	NA
24	19	F	1a	87	26	NA	40	NA	11	NA

GlyA, glycophorin A; Tri, trilineage dysplasia; NA, not available; Ep, emperipolesis; dE, dyserythropoieis; m, micromegakaryocytes.

patients), trisomy 21 (seven patients), and monosomy 7 (two patients). Six patients with DS-AMKL had −7/del(7q) and one of them had both monosomy 5 and 7. The translocation t(1:22) was found in two patients with non-DS-AMKL and 3q21q26 abnormalities, which are common in adult AMKL (Lu *et al*, 1993; Tallman *et al*, 2000; Dastugue *et al*, 2002; Duchayne *et al*, 2003), was found in one patient with non-DS-AMKL. The translocation t(16;21) was found in one patient with non-DS-AMKL. These recurrent structural changes were not observed in patients with DS-AMKL. The 11q23 abnormalities and the Philadelphia chromosomes were not detected in either group. Other structural changes, such as t(5;12)(p15;q21) was found in DS-AMKL and t(2;7)(p12;p22), t(2;11;19)(q31;q13;q13) were found in non-DS-AMKL.

GATA1 mutations

GATA1 mutation analysis was performed in 17 of 24 patients with DS-AMKL and 11 of 21 patients with non-DS-AMKL. *GATA1* mutations were observed in all patients with DS-AMKL and one patient with non-DS-AMKL. Ten of 17 patients with *GATA1* mutations in DS-AMKL were previously reported (Hirose *et al*, 2003).

Outcome

Twenty-three of 24 (96%) DS-AMKL patients achieved CR. Three patients relapsed and died, and two other patients with congenital heart anomalies died of congestive heart failure. Of the 24 patients with DS-AMKL, 19 (79%) are currently alive. Of the 21 patients with non-DS-AMKL, 16 (76%) achieved CR. One patient with t(1;22) did not receive induction therapy because of multiple organ failure on the day of admission. Three of four non-responders to induction therapy underwent successful allogeneic SCT and one patient died of pneumonia. Twelve patients received BMT in the first CR, four of whom relapsed and three of whom died. Five patients received chemotherapy only, four of whom have remained in CR. Overall, 16 of 21 patients (76%) are currently alive.

Table III. Morphological classification, immunophenotype and karyotype of the 21 patients with non-Down syndrome-associated acute megakaryoblastic leukaemia.

Patient no.	Age (months)	Sex	Morphological classification	Immunophenotype (%)						Karyotype
				CD7	CD13	CD36	CD41	CD42	GlyA	
A: Normal karyotypes										
1	147	F	3+m	66	95	42	53	19	1	46,XX
2	18	M	2a+Tri	25	54	40	4	2	4	46,XY
B: Numerical abnormalities only										
3	3	F	NA	1	88	NA	77	54	8	48,XX,+21,+22
4	15	F	2a	42	9	NA	68	10	NA	58,XX,+X,+2,+2,+6,+7,+8,+10,+13,+15,+19,+19,+22
5	38	F	1a	38	30	NA	10	4	NA	49,XX,+12,+18,+22
6	12	F	NA	NA	30	NA	95	NA	NA	57,XX,+2,+4,+6,+7,+8,+10,+14,+15,+19,+19,+22
7	45	M	1a	95	3	NA	86	3	1	48,idem,+8
8	2	M	2a	19	32	NA	46	18	5	51,XY,+6,+7,+8,+19,+21
C: t(1;22)(p13;q13)										
9	2	F	2a+dE	NA	NA	NA	NA	NA	NA	46,XX,t(1;22)(p13;q13)
10	12	M	1a	5	13	NA	67	70	NA	51,XY,+der(1)t(1;22)(p13;q13),t(1;22)(p13;q13),+6,+7,+10,+19
D: 3q21q26 abnormalities										
11	12	M	1a	14	3	64	48	47	NA	46,XY,−11,der(11)t(3;11)(q21;p15)
E: t(16;21)(p11;q22)										
12	38	M	3+Ep	3	69	NA	33	33	NA	46,XY,t(16;21)(p11;q22)
F: −7										
13	8	F	1a	30	7	NA	82	80	NA	46,XX,−7,−7,del(11)(p11),+2mar,inc
14	41	M	3+m	66	43	NA	28	32	8	47,XY,−7,+21,+ring(1)
G: Other structural changes										
15	7	M	1a	12	65	NA	57	NA	NA	46,XY,t(2;7)(p12;p22)
16	28	F	1a	NA	NA	NA	30	17	1	51,XX,+X,6p+,6p+,−13,+21,+21,+2mar
17	35	F	3+m	3	18	2	78	1	NA	47,XX,add(16)(p13),+21
18	12	F	2a+dE	30	5	62	67	55	5	46,XX,t(2;11;19)(q31;q13;q13.4),del(3)(q23),13q−
19	6	F	NA	NA	10	NA	52	53	NA	51,XX,4p+,11q+,14q+,+17,+19,+21,+22,+mar
20	33	F	2a	5	2	12	5	1	1	46,XX,del(2)(q11),del(2)(q31),der(5)t(2;5)(q11;q22),der(5)t(5;13)(q35;q14),−13,add(16)(p13),+mar
21	185	F	2a	59	90	NA	16	4	NA	49,XX,+5,+8,i(17)(q10),+21

GlyA, glycophorin A; Tri, trilineage dysplasia; NA, not available; Ep, emperipolesis; dE, dyserythropoiesis; m, micromegakaryocytes.

The estimate of 10-year OS was 79% (95% CI: 54–90) for patients with DS-AMKL and 76% (95% CI: 58–91) for patients with non-DS-AMKL with a median follow-up of 78 months (range, 20–243 months) (P = 0·81, Fig 2). The estimate of 10-year EFS was 79% (95% CI: 58–91) for patients with DS-AMKL and 57% (95% CI: 36–77) for patients with non-DS-AMKL (P = 0·09, Fig 3). The outcome of DS-AMKL and non-DS-AMKL was comparable. In non-DS-AMKL, the estimated OS of the 15 children who received SCT (79%, 95% CI: 51–93) did not differ from five children treated with chemotherapy alone (80%, 95% CI: 30–97) (P = 0·95).

Discussion

In the current series, 23·2% of patients with AML were identified as having AMKL; this frequency was higher than those found in several collaborative group studies of childhood AML (Ravindranath *et al*, 1992; Gamis *et al*, 2003; Creutzig *et al*, 2005; Zeller *et al*, 2005; Rao *et al*, 2006). The relative frequency of AMKL has varied markedly, ranging from 4·1% to 15·3%. There are possible explanations for the high proportion of AMKL in our series. One is the difference in prevalence of DS-AMKL. The proportion of DS-AMKL in this study was 53·3%, which was much higher than those of other institutions. For example, the ratio of DS-AMKL to non-DS-AMKL was 6:35 in the report from St. Jude Children's Research Hospital (Athale *et al*, 2001). In the 1980s and early 1990s, the outcome of patients with DS-AMKL was generally poor (Levitt *et al*, 1990). Most DS-AMKL patients have not been enrolled in clinical studies. In the German (Berlin-Frankfurt-Münster; BFM) co-operative group studies, the percentage of patients with DS has gradually increased since study 78 (1·9%), study 83 (5·6%), study 87 (8·1%), study 93 (9·7%) and study 98 (12·9%) (Creutzig *et al*, 2005). On the other hand, among patients registered in the population-based Nordic study between 1984 and 2001, 72 of 515 (14·0%) children with AML had DS (Zeller *et al*, 2005), which is similar to the percentage in our study, in which 25 of 194 patients (12·9%)

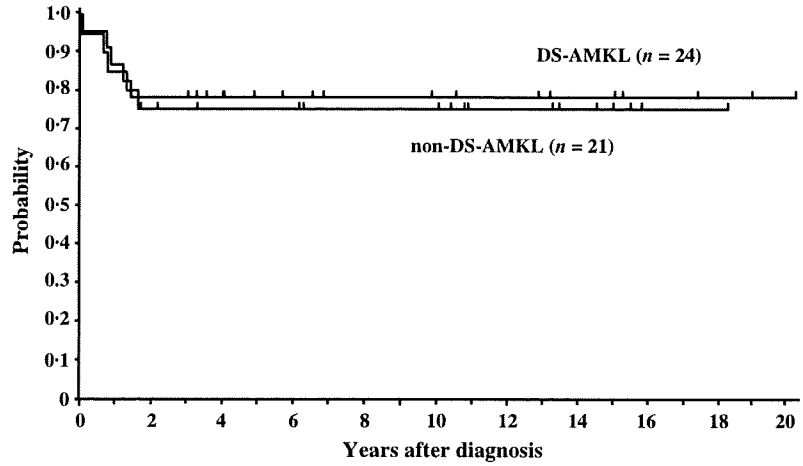

Fig 2. Estimated probability of overall survival for 24 patients with AMKL associated with Down syndrome (DS-AMKL) and 21 patients with non-DS-AMKL. The estimate of 10-year overall survival was 79% [95% confidence interval (CI): 54–90] for patients with DS-AMKL and 76% (95% CI: 58–91) for patients with non-DS-AMKL ($P = 0{\cdot}81$).

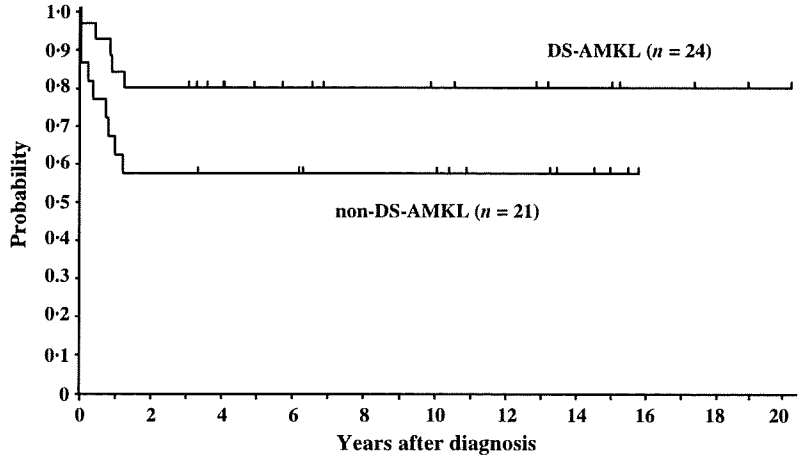

Fig 3. Estimated probability of event-free survival for 24 patients with AMKL associated with Down syndrome (DS-AMKL) and 21 patients with non-DS-AMKL. The estimate of 10-year event-free survival was 79% (95% CI: 58–91) for patients with DS-AMKL and 57% (95% CI: 36–77) for patients with non-DS-AMKL ($P = 0{\cdot}09$).

with AML had DS. Our clinical trial for DS-AMKL used a less intensive regimen that had been specifically designed for DS-AMKL in the mid-1980s (Kojima *et al*, 1990, 1993, 2000). Therefore, patients with DS-AMKL were not excluded from the data file, which may account for the higher proportion of DS-AMKL in our series. In a report from Mexico, 29 of 152 (19·1%) children with AML were diagnosed as AMKL among whom only one patient had DS (Paredes-Aguilera *et al*, 2003).

The incidence of non-DS-AMKL was 12·4% in our non-DS-AML series, which might also be higher than those of other reports. Immunophenotyping with platelet-associated antigens and electron microscopic identification of PPO were introduced over 15 years ago in our hospitals (Kojima *et al*, 1990). The incidence of AMKL might have been underestimated because of its diverse clinical presentation and requirement of specific laboratory methods in early reports. As previously described, 19·1% of children with AML had AMKL in a report from Mexico (Paredes-Aguilera *et al*, 2003). The incidence of AMKL might be different between western countries and non-western countries. We speculate that AMKL represents approximately 10% of all cases of non-DS-AML in children.

In the current study, the morphology of blast cells was categorized into three groups (type 1, type 2, or type 3) according to the stage of megakaryocyte maturation, modelled according to the FAB classification of myeloid leukaemia (Fig 1). The blast cells with deep blue cytoplasm (type 1b, 2b) were only detected in patients with DS-AMKL and were positive for glycophorin A in all but one patient. Erythroid-specific mRNAs encoding γ-globin and erythroid δ-amino levulinate synthase were expressed in blasts from all patients

with DS-AMKL (Ito *et al*, 1995). These findings suggest that type 1b and 2b blasts arise from bipotent megakaryocyte/erythrocyte progenitors. A high incidence of the co-expression of the T cell-associated marker CD7 in patients with DS-AMKL compared with patients with non-DS-AMKL was also observed. In addition to mature T cells, the CD7 antigen is expressed on immature haematopoietic cells (Kita *et al*, 1993; Creutzig *et al*, 1995). In terms of morphology and immunophenotype, the blast cells were more immature in patients with DS-AMKL than in those with non-DS-AMKL.

The cytogenetic profile of AMKL is complex, which reflects the heterogeneity of the disease. In the current study, seven cytogenetic groups were identified. The translocation t(1;22)(p13;q13), which produces the *RBM15-MKL1* fusion gene (Ma *et al*, 2001; Mercher *et al*, 2001, 2002), was detected in two patients (4·3%) with non-DS-AMKL. This frequency was lower than in another study (Dastugue *et al*, 2002; Duchayne *et al*, 2003). Dastugue *et al* (2002) reported that *RBM15-MKL1* transcript was detected in one patient with a normal karyotype, suggesting that the molecular determination method may increase the detection of t(1;22) translocation. The 3q21q26 abnormalities were observed in one patient with non-DS-AMKL. The 3q21q26 abnormalities are rare in childhood AMKL (6·7% in Dastugue *et al*, 2002), while they are seen in 17% to 20% of adult AMKL patients (Lu *et al*, 1993; Tallman *et al*, 2000; Dastugue *et al*, 2002; Duchayne *et al*, 2003). The translocation t(16;21)(p11;q22) generating a *FUS-ERG* transcript (Kong *et al*, 1997) was found in one patient with non-DS-AMKL. This translocation has been found in all subtypes of AML except M3, including several cases with AMKL. The patient with t(16;21) in our series morphologically showed type 3 blasts with emperipolesis and cytophagocytosis. These morphological findings of BM are characteristic of patients with t(16;21), regardless of FAB classification (Imashuku *et al*, 2000). These recurrent structural changes were not observed in patients with DS-AMKL. On the other hand, monosomy 7/del(7q) was more frequent in patients with DS-AMKL (29%) than non-DS-AMKL (9·5%). Monosomy 5/del(5q) was found in only one patient with DS-AMKL who had also monosomy 7. In non-DS-AMKL, trisomies (+8, +19, +21) were more frequent than DS-AMKL, as previously reported (Lu *et al*, 1993; Dastugue *et al*, 2002; Duchayne *et al*, 2003; Reinhardt *et al*, 2005). Acquired trisomy 21 is a common chromosome gain of childhood AMKL, reported in 23% to 43% of patients with non-DS-AMKL (Ribeiro *et al*, 1993; Athale *et al*, 2001). In our study, acquired trisomy 21 was found in 33% of patients with non-DS-AMKL. Coupled with other reports, acquired trisomy 21 seems to have a higher incidence in non-DS-AMKL in children than in other childhood non-DS-AML. The 11q23 abnormalities are often detected in childhood AMKL (Athale *et al*, 2001; Reinhardt *et al*, 2005), although not in the current study. The Philadelphia chromosomes or i(12)(p10) with mediastinal germ cell tumour, which were mainly found in adult AMKL (Dastugue *et al*, 2002; Duchayne *et al*, 2003), were also not found in the current study.

GATA1 mutation analysis was performed in 17 of 24 patients with DS-AMKL and 11 of 21 patients with non-DS-AMKL. *GATA1* mutations were observed in all patients with DS-AMKL as previously reported (Wechsler *et al*, 2002; Hirose *et al*, 2003). In contrast to DS-AMKL, *GATA1* mutations were rarely found in patients with non-DS-AMKL. To date, only four children with *GATA1* mutations in non-DS-AMKL have been reported (Rainis *et al*, 2003; Bourquin *et al*, 2006). Interestingly, all of them had acquired trisomy 21 in their leukaemic cells. Our non-DS-AMKL patient with *GATA1* mutation did not have acquired trisomy 21 in his leukaemic cells.

Before the 1990s, most patients with DS-AML were treated outside of clinical studies and received suboptimal therapies, resulting in poor outcomes (Levitt *et al*, 1990). Following the recognition of the favourable outcome when treated with protocols of the collaborative study group for AML (Ravindranath *et al*, 1992), there has been an increase in recruitment into protocol studies. However, it has become apparent that resistant disease is rare but treatment-related deaths are frequent in most series (Creutzig *et al*, 1996; Lange *et al*, 1998), and several collaborative groups adapted their AML protocols for DS-AML by reducing the dose of chemotherapeutic agents (Creutzig *et al*, 2005; Zeller *et al*, 2005; Rao *et al*, 2006). In recent reports, 5-year survival rate have been in excess of 80%, largely because of reductions in treatment-related deaths with a decrease from 30% to 40% in the early 1990s to around 10% in recent studies (Creutzig *et al*, 2005; Zeller *et al*, 2005; Rao *et al*, 2006). Since the mid-1980s, we have used a less intensive regimen specifically designed for DS-AML (Kojima *et al*, 1990, 1993, 2000). The excellent outcome of DS-AMKL may originate from early use of a regimen specific for DS-AML.

The 10-year OS in our series was 76% (95% CI: 58–91) for patients with non-DS-AMKL, which was superior to those of other reports (Ribeiro *et al*, 1993; Athale *et al*, 2001; Reinhardt *et al*, 2005). The prognosis for children with non-DS-AMKL was poor in previous reports. According to the report from St Jude Children's Research Hospital, the 2-year OS was only 14%, which was significantly higher after allogeneic SCT (30%) than after chemotherapy alone (0%) (Athale *et al*, 2001). The result of a recently published report on AMKL from the European Group for Blood and Marrow Transplantation was excellent (Garderet *et al*, 2005). Three-year OS was 82% in 19 children after allogeneic SCT and 61% in 38 children after autologous SCT. The authors recommended allogeneic SCT when an HLA-matched sibling is available; otherwise, autologous SCT should be used for children with AMKL in first CR. However, this report included 11 children with DS and analysed the outcome of children with DS and without DS together, which confused the interpretation of the results. In the current study, 5-year OS was 79% in patients who received SCT, which did not differ from patients achieving CR and being treated with chemotherapy alone (80%, $P = 0{\cdot}98$). In a recent report from the BFM collaborative group, the 5-year OS was 43% in the SCT group and 54% in the chemotherapy

group ($P = 0{\cdot}37$) (Reinhardt *et al*, 2005). The recent use of intensified chemotherapy may abrogate the indication of allogeneic SCT for children with non-DS-AMKL.

In conclusion, our study shows the diverse heterogeneity of childhood AMKL and the differences in the clinical and biological presentation between DS-AMKL and non-DS-AMKL. Subclassification according to megakaryocyte maturation and cytogenetic abnormalities in childhood AMKL is warranted.

References

Athale, U.H., Razzouk, B.I., Raimondi, S.C., Tong, X., Behm, F.G., Head, D.R., Srivastava, D.K., Rubnitz, J.E., Bowman, L., Pui, C.H. & Ribeiro, R.C. (2001) Biology and outcome of childhood acute megakaryoblastic leukemia: a single institution's experience. *Blood*, **97,** 3727–3732.

Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A., Gralnick, H.R. & Sultan, C. (1985) Criteria for the diagnosis of acute leukemia of megakaryocyte lineage (M7). A report of the French-American-British Cooperative Group. *Annals of Internal Medicine*, **103,** 460–462.

Bernstein, J., Dastugue, N., Haas, O.A., Harbott, J., Heerema, N.A., Huret, J.L., Landman-Parker, J., LeBeau, M.M., Leonard, C., Mann, G., Pages, M.P., Perot, C., Pirc-Danoewinata, H., Roitzheim, B., Rubin, C.M., Slociak, M. & Viguie, F. (2000) Nineteen cases of the t(1;22)(p13;q13) acute megakaryblastic leukaemia of infants/children and a review of 39 cases: report from a t(1;22) study group. *Leukemia*, **14,** 216–218.

Bourquin, J.P., Subramanian, A., Langebrake, C., Reinhardt, D., Bernard, O., Ballerini, P., Baruchel, A., Cave, H., Dastugue, N., Hasle, H., Kaspers, G.L., Lessard, M., Michaux, L., Vyas, P., van Wering, E., Zwaan, C.M., Golub, T.R. & Orkin, S.H. (2006) Identification of distinct molecular phenotypes in acute megakaryoblastic leukemia by gene expression profiling. *Proceedings of the National Academy of Sciences of the United States of America*, **103,** 3339–3344.

Carroll, A., Civin, C., Schneider, N., Dahl, G., Pappo, A., Bowman, P., Emami, A., Gross, S., Alvarado, C., Phillips, C., Krischer, J., Crist, W., Head, D., Gresik, M., Ravindranath, Y. & Weinstein, H. (1991) The t(1;22) (p13;q13) is nonrandom and restricted to infants with acute megakaryoblastic leukemia: a Pediatric Oncology Group Study. *Blood*, **78,** 748–752.

Creutzig, U., Harbott, J., Sperling, C., Ritter, J., Zimmermann, M., Loffler, H., Riehm, H., Schellong, G. & Ludwig, W.D. (1995) Clinical significance of surface antigen expression in children with acute myeloid leukemia: results of study AML-BFM-87. *Blood*, **86,** 3097–3108.

Creutzig, U., Ritter, J., Vormoor, J., Ludwig, W.D., Niemeyer, C., Reinisch, I., Stollmann-Gibbels, B., Zimmermann, M. & Harbott, J. (1996) Myelodysplasia and acute myelogenous leukemia in Down's syndrome. A report of 40 children of the AML-BFM Study Group. *Leukemia*, **10,** 1677–1686.

Creutzig, U., Reinhardt, D., Diekamp, S., Dworzak, M., Stary, J. & Zimmermann, M. (2005) AML patients with Down syndrome have a high cure rate with AML-BFM therapy with reduced dose intensity. *Leukemia*, **19,** 1355–1360.

Dastugue, N., Lafage-Pochitaloff, M., Pages, M.P., Radford, I., Bastard, C., Talmant, P., Mozziconacci, M.J., Leonard, C., Bilhou-Nabera, C., Cabrol, C., Capodano, A.M., Cornillet-Lefebvre, P., Lessard, M., Mugneret, F., Perot, C., Taviaux, S., Fenneteaux, O., Duchayne, E. & Berger, R. (2002) Cytogenetic profile of childhood and adult megakaryoblastic leukemia (M7): a study of the Groupe Francais de Cytogenetique Hematologique (GFCH). *Blood*, **100,** 618–626.

Duchayne, E., Fenneteau, O., Pages, M.P., Sainty, D., Arnoulet, C., Dastugue, N., Garand, R. & Flandrin, G. (2003) Acute megakaryoblastic leukaemia: a national clinical and biological study of 53 adult and childhood cases by the Groupe Francais d'Hematologie Cellulaire (GFHC). *Leukaemia & Lymphoma*, **44,** 49–58.

Gamis, A.S. (2005) Acute myeloid leukemia and Down syndrome evolution of modern therapy–state of the art review. *Pediatric Blood Cancer*, **44,** 13–20.

Gamis, A.S., Woods, W.G., Alonzo, T.A., Buxton, A., Lange, B., Barnard, D.R., Gold, S. & Smith, F.O. (2003) Increased age at diagnosis has a significantly negative effect on outcome in children with Down syndrome and acute myeloid leukemia: a report from the Children's Cancer Group Study 2891. *Journal of Clinical Oncology*, **21,** 3415–3422.

Garderet, L., Labopin, M., Gorin, N.C., Polge, E., Baruchel, A., Meloni, G., Ortega, J., Vossen, J., Bunjes, D., Leverger, G., Blaise, D., Ferrant, A., Brune, M., Dore, E., Gadner, H., Zintl, F., Yaniv, I., Dini, G. & Frassoni, F. (2005) Hematopoietic stem cell transplantation for de novo acute megakaryocytic leukemia in first complete remission: a retrospective study of the European Group for Blood and Marrow Transplantation (EBMT). *Blood*, **105,** 405–409.

Harris, N.L., Jaffe, E.S., Diebold, J., Flandrin, G., Muller-Hermelink, H.K., Vardiman, J., Lister, T.A. & Bloomfield, C.D. (1999) World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. *Journal of Clinical Oncology*, **17,** 3835–3849.

Hirose, Y., Kudo, K., Kiyoi, H., Hayashi, Y., Naoe, T. & Kojima, S. (2003) Comprehensive analysis of gene alterations in acute megakaryoblastic leukemia of Down's syndrome. *Leukemia*, **17,** 2250–2252.

Imashuku, S., Hibi, S., Sako, M., Lin, Y.W., Ikuta, K., Nakata, Y., Mori, T., Iizuka, S., Horibe, K. & Tsunematsu, Y. (2000) Hemophagocytosis by leukemic blasts in 7 acute myeloid leukemia cases with t(16;21)(p11;q22): common morphologic characteristics for this type of leukemia. *Cancer*, **88,** 1970–1975.

Ito, E., Kasai, M., Hayashi, Y., Toki, T., Arai, K., Yokoyama, S., Kato, K., Tachibana, N., Yamamoto, M. & Yokoyama, M. (1995) Expression of erythroid-specific genes in acute megakaryoblastic leukaemia and transient myeloproliferative disorder in Down's syndrome. *British Journal Haematology*, **90,** 607–614.

Kaplan, E. & Meier, P. (1958) Nonparametric estimation from incomplete observations. *Journal of the American Statistical Association*, **53,** 457–481.

Kita, K., Miwa, H., Nakase, K., Kawakami, K., Kobayashi, T., Shirakawa, S., Tanaka, I., Ohta, C., Tsutani, H., Oguma, S., Kyo, T., Dohy, H., Kamada, N., Nasu, K. & Uchino, H. (1993) Clinical importance of CD7 expression in acute myelocytic leukemia. The Japan Cooperative Group of Leukemia/Lymphoma. *Blood*, **81,** 2399–2405.

Kojima, S., Matsuyama, T., Sato, T., Horibe, K., Konishi, S., Tsuchida, M., Hayashi, Y., Kigasawa, H., Akiyama, Y., Okamura, J., Nakahata, T., Bessho, F., Eguchi, M., Nakazawa, S. & Ueda, R. (1990) Down's syndrome and acute leukemia in children: an analysis of phenotype by use of monoclonal antibodies and electron microscopic platelet peroxidase reaction. *Blood*, **76,** 2348–2353.

Kojima, S., Kato, K., Matsuyama, T., Yoshikawa, T. & Horibe, K. (1993) Favorable treatment outcome in children with acute myeloid leukemia and Down syndrome. *Blood*, **81,** 3164.

Kojima, S., Sako, M., Kato, K., Hosoi, G., Sato, T., Ohara, A., Koike, K., Okimoto, Y., Nishimura, S., Akiyama, Y., Yoshikawa, T., Ishii, E., Okamura, J., Yazaki, M., Hayashi, Y., Eguchi, M., Tsukimoto, I. & Ueda, K. (2000) An effective chemotherapeutic regimen for acute myeloid leukemia and myelodysplastic syndrome in children with Down's syndrome. *Leukemia*, **14,** 786–791.

Kong, X.T., Ida, K., Ichikawa, H., Shimizu, K., Ohki, M., Maseki, N., Kaneko, Y., Sako, M., Kobayashi, Y., Tojou, A., Miura, I., Kakuda, H., Funabiki, T., Horibe, K., Hamaguchi, H., Akiyama, Y., Bessho, F., Yanagisawa, M. & Hayashi, Y. (1997) Consistent detection of TLS/FUS-ERG chimeric transcripts in acute myeloid leukemia with t(16;21)(p11;q22) and identification of a novel transcript. *Blood*, **90,** 1192–1199.

Lange, B.J., Kobrinsky, N., Barnard, D.R., Arthur, D.C., Buckley, J.D., Howells, W.B., Gold, S., Sanders, J., Neudorf, S., Smith, F.O. & Woods, W.G. (1998) Distinctive demography, biology, and outcome of acute myeloid leukemia and myelodysplastic syndrome in children with Down syndrome: Children's Cancer Group Studies 2861 and 2891. *Blood*, **91,** 608–615.

Levitt, G.A., Stiller, C.A. & Chessells, J.M. (1990) Prognosis of Down's syndrome with acute leukaemia. *Archives of Disease in Childhood*, **65,** 212–216.

Lion, T., Haas, O.A., Harbott, J., Bannier, E., Ritterbach, J., Jankovic, M., Fink, F.M., Stojimirovic, A., Herrmann, J., Riehm, H.J., Lampert, F., Ritter, J., Koch, H. & Gadner, H. (1992) The translocation t(1;22)(p13;q13) is a nonrandom marker specifically associated with acute megakaryocytic leukemia in young children. *Blood*, **79,** 3325–3330.

Lu, G., Altman, A.J. & Benn, P.A. (1993) Review of the cytogenetic changes in acute megakaryoblastic leukemia: one disease or several? *Cancer Genetics and Cytogenetics*, **67,** 81–89.

Ma, Z., Morris, S.W., Valentine, V., Li, M., Herbrick, J.A., Cui, X., Bouman, D., Li, Y., Mehta, P.K., Nizetic, D., Kaneko, Y., Chan, G.C., Chan, L.C., Squire, J., Scherer, S.W. & Hitzler, J.K. (2001) Fusion of two novel genes, RBM15 and MKL1, in the t(1;22)(p13;q13) of acute megakaryoblastic leukemia. *Nature Genetics*, **28,** 220–221.

Mercher, T., Coniat, M.B., Monni, R., Mauchauffe, M., Nguyen Khac, F., Gressin, L., Mugneret, F., Leblanc, T., Dastugue, N., Berger, R. & Bernard, O.A. (2001) Involvement of a human gene related to the Drosophila spen gene in the recurrent t(1;22) translocation of acute megakaryocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, **98,** 5776–5779.

Mercher, T., Busson-Le Coniat, M., Nguyen Khac, F., Ballerini, P., Mauchauffe, M., Bui, H., Pellegrino, B., Radford, I., Valensi, F., Mugneret, F., Dastugue, N., Bernard, O.A. & Berger, R. (2002) Recurrence of OTT-MAL fusion in t(1;22) of infant AML-M7. *Genes, Chromosomes and Cancer*, **33,** 22–28.

Paredes-Aguilera, R., Romero-Guzman, L., Lopez-Santiago, N. & Trejo, R.A. (2003) Biology, clinical, and hematologic features of acute megakaryoblastic leukemia in children. *American Journal of Hematology*, **73,** 71–80.

Rainis, L., Bercovich, D., Strehl, S., Teigler-Schlegel, A., Stark, B., Trka, J., Amariglio, N., Biondi, A., Muler, I., Rechavi, G., Kempski, H., Haas, O.A. & Izraeli, S. (2003) Mutations in exon 2 of GATA1 are early events in megakaryocytic malignancies associated with trisomy 21. *Blood*, **102,** 981–986.

Rao, A., Hills, R.K., Stiller, C., Gibson, B.E., de Graaf, S.S., Hann, I.M., O'Marcaigh, A., Wheatley, K. & Webb, D.K. (2006) Treatment for myeloid leukaemia of Down syndrome: population-based experience in the UK and results from the Medical Research Council AML 10 and AML 12 trials. *British Journal Haematology*, **132,** 576–583.

Ravindranath, Y., Abella, E., Krischer, J.P., Wiley, J., Inoue, S., Harris, M., Chauvenet, A., Alvarado, C.S., Dubowy, R., Ritchey, A.K., Land, V., Steuber, C.P. & Weinstein, H. (1992) Acute myeloid leukemia (AML) in Down's syndrome is highly responsive to chemotherapy: experience on Pediatric Oncology Group AML Study 8498. *Blood*, **80,** 2210–2214.

Reinhardt, D., Diekamp, S., Langebrake, C., Ritter, J., Stary, J., Dworzak, M., Schrauder, A., Zimmermann, M., Fleischhack, G., Ludwig, W.D., Harbott, J. & Creutzig, U. (2005) Acute megakaryoblastic leukemia in children and adolescents, excluding Down's syndrome: improved outcome with intensified induction treatment. *Leukemia*, **19,** 1495–1496.

Ribeiro, R.C., Oliveira, M.S., Fairclough, D., Hurwitz, C., Mirro, J., Behm, F.G., Head, D., Silva, M.L., Raimondi, S.C., Crist, W.M. & Krance, R. (1993) Acute megakaryoblastic leukemia in children and adolescents: a retrospective analysis of 24 cases. *Leukaemia & Lymphoma*, **10,** 299–306.

San Miguel, J.F., Gonzalez, M., Canizo, M.C., Ojeda, E., Orfao, A., Caballero, M.D., Moro, M.J., Fisac, P. & Lopez Borrasca, A. (1988) Leukemias with megakaryoblastic involvement: clinical, hematologic, and immunologic characteristics. *Blood*, **72,** 402–407.

Shimada, A., Taki, T., Tabuchi, K., Tawa, A., Horibe, K., Tsuchida, M., Hanada, R., Tsukimoto, I. & Hayashi, Y. (2006) 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*, **107,** 1806–1809.

Tallman, M.S., Neuberg, D., Bennett, J.M., Francois, C.J., Paietta, E., Wiernik, P.H., Dewald, G., Cassileth, P.A., Oken, M.M. & Rowe, J.M. (2000) Acute megakaryocytic leukemia: the Eastern Cooperative Oncology Group experience. *Blood*, **96,** 2405–2411.

Taub, J.W., Huang, X., Matherly, L.H., Stout, M.L., Buck, S.A., Massey, G.V., Becton, D.L., Chang, M.N., Weinstein, H.J. & Ravindranath, Y. (1999) Expression of chromosome 21-localized genes in acute myeloid leukemia: differences between Down syndrome and non-Down syndrome blast cells and relationship to in vitro sensitivity to cytosine arabinoside and daunorubicin. *Blood*, **94,** 1393–1400.

Taub, J.W., Huang, X., Ge, Y., Dutcher, J.A., Stout, M.L., Mohammad, R.M., Ravindranath, Y. & Matherly, L.H. (2000) Cystathionine-beta-synthase cDNA transfection alters the sensitivity and metabolism of 1-beta-D-arabinofuranosylcytosine in CCRF-CEM leukemia cells in vitro and in vivo: a model of leukemia in Down syndrome. *Cancer Research*, **60,** 6421–6426.

Wechsler, J., Greene, M., McDevitt, M.A., Anastasi, J., Karp, J.E., Le Beau, M.M. & Crispino, J.D. (2002) Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. *Nature Genetics*, **32,** 148–152.

Zeller, B., Gustafsson, G., Forestier, E., Abrahamsson, J., Clausen, N., Heldrup, J., Hovi, L., Jonmundsson, G., Lie, S.O., Glomstein, A. & Hasle, H. (2005) Acute leukaemia in children with Down syndrome: a population-based Nordic study. *British Journal Haematology*, **128,** 797–804.

Zipursky, A., Peeters, M. & Poon, A. (1987) Megakaryoblastic leukemia and Down's syndrome: a review. *Pediatric Hematology and Oncology*, **4,** 211–230.

Prospective multicenter trial comparing repeated immunosuppressive therapy with stem-cell transplantation from an alternative donor as second-line treatment for children with severe and very severe aplastic anemia

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We conducted a prospective multicenter study to compare the efficacy of repeated immunosuppressive therapy (IST) with stem-cell transplantation (SCT) from an alternative donor in children with acquired aplastic anemia (AA) who failed to respond to an initial course of IST. Patients with severe (n = 86) and very severe disease (n = 119) received initial IST consisting of antithymocyte globulin (ATG) and cyclosporine. Sixty patients failed to respond to IST after 6 months from the initial IST and were eligible for second-line treatment. Among them, 21 patients lacking suitable donors received a second course of IST. Three patients developed an anaphylactoid reaction to ATG and could not complete the second IST. A trilineage response was seen in only 2 of 18 (11%) evaluable patients after 6 months. Thirty-one patients received SCT from an alternative donor. At 5 years from the initiation of second-line therapy, the estimated failure-free survival (FFS), defined as survival with response, was 83.9% (± 16.1%, SD) in the SCT group compared with 9.5% (± 9.0%) in the IST group ($P = .001$). These results suggest that SCT from an alternative donor offers a better chance of FFS than a second IST in patients not responding to an initial IST. (Blood. 2008;111: 1054-1059)

Introduction

Acquired aplastic anemia (AA) is a heterogeneous disorder characterized by pancytopenia of peripheral blood and hypocellular marrow. Currently 2 effective treatments are available for this disorder: hematopoietic stem cell transplantation (SCT) and immunosuppressive therapy (IST). There are several reports comparing bone marrow transplantation (BMT) and IST as first-line treatment for AA.[1-4] These studies indicate that allogeneic BMT from an HLA-matched sibling donor is the treatment of choice for young patients. IST consisting of antithymocyte globulin (ATG) and cyclosporine (CyA) with or without granulocyte-colony stimulating factor (G-CSF) has been successfully used for patients with AA who lack an HLA-matched sibling donor or who are not eligible for SCT. Several reports indicate that 2- to 5-year survival following IST is between 60% and 90%.[5-7] We reported results of a multicenter trial of IST for children younger than 18 years with AA (AA-92 trial).[8] In the AA-92 trial, 119 children with newly diagnosed AA were enrolled, and the response rate at 6 months was 71%, with the probability of survival at 4 years greater than 90%. However, approximately 30% of the patients did not respond to an initial course of IST. Moreover, a significant proportion of patients subsequently relapsed and required second-line therapy.[9] The optimal treatment for such patients has not been established.

A repeated course of IST has been used for patients who fail to respond to, or who have relapsed after an initial course of, IST. Tichelli et al reported the results of a Basel study that consisted of repeated courses of IST, using ATG from the same species (horse) for nonresponders.[10] In their study, repeated IST was well tolerated and the response rate was 63%. An Italian group reported the results of repeated IST using ATG from different species (horse to rabbit), where the response rate was also high.[11] Investigators at the National Institutes of Health (NIH) recently reported the results of retreatment with rabbit ATG and CyA in 22 patients refractory to horse ATG and CyA. Contrary to the reports from Europe, the overall response rate was only 27% and no patients achieved complete response.[12]

SCT from an alternative donor has also been used as salvage therapy for patients not responding to IST because recent progress

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in the management of patients who undergo SCT, and better selection of donors by DNA typing of HLA loci, has improved the outcome for these patients.[13,14] However, no prospective study has been performed to date comparing repeated IST versus SCT from an alternative donor as second-line therapy. Therefore, we conducted a prospective multicenter trial to compare these 2 treatment options for pediatric patients with severe and very severe AA who had failed to respond to initial IST.

Methods

Patients

This multicenter study was designed by the Japan Childhood Aplastic Anemia Study Group and involved 79 hospitals in Japan. The eligibility criteria were as follows: age younger than 18 years, diagnosis less than 180 days before registration, no specific prior treatment for AA, and severe to very severe disease. The definition of disease severity was determined according to currently used criteria.[15] The disease was considered severe if at least 2 of the following were noted: a neutrophil count less than 0.5×10^9/L, a platelet count less than 20×10^9/L, and a reticulocyte count less than 20×10^9/L with hypocellular bone marrow. AA was considered very severe if the criteria for severe disease were fulfilled and the neutrophil count was less than 0.2×10^9/L. Patients were excluded if they had congenital AA. Patients were screened for paroxysmal nocturnal hemoglobinuria (PNH) by flow cytometry using anti-CD55 and anti-CD59 antibodies. Bone marrow cytogenetic studies were performed in all patients. Allogeneic SCT was recommended for patients with severe or very severe disease who had an HLA-matched sibling: these patients were not included in AA-97 study.

Treatment protocol

Patients with very severe disease were treated with IST, which consisted of horse ATG (Lymphoglobulin; IMTIX-SANGSTAT, Lyon, France) 15 mg/kg per day on days 1 through 5; CyA 6 mg/kg per day from day 1 until at least day 180, with subsequent adjustment according to whole blood CyA concentration between 100 and 200 ng/mL; methylprednisolone (MePred) 2 mg/kg per day for 5 days, with subsequent halving of the dose every week until discontinuation on day 28 for prophylaxis of allergic reaction of ATG; and G-CSF (Filgrastim, Kirin, Tokyo, Japan) 400 $\mu g/m^2$ per day from day 1, with responding patients (neutrophil count $> 10^9$/L) receiving the same dose 3 times a week for 60 days (ATG/CyA/MePred/G-CSF). Patients with severe disease were given the same treatment regimen, with the exception that G-CSF was not given unless severe infection was documented (ATG/CyA/MePred).

The hematologic response was evaluated at 6 months after the initiation of therapy. A complete response (CR) was defined for all patients as a neutrophil count more than 1.5×10^9/L, a platelet count more than 100×10^9/L, and a hemoglobin level more than 11.0 g/dL.[8] A partial response (PR) was defined as a neutrophil count more than 0.5×10^9/L, a platelet count more than 20×10^9/L, a hemoglobin level more than 80 g/L (8.0 g/dL) and no requirement of blood transfusions. Patients with very severe or severe disease who failed to respond to initial IST underwent SCT if they had a serologically HLA-matched unrelated donor, HLA-one antigen mismatched family donor, or HLA-matched or HLA-one antigen mismatched unrelated cord blood donor at the time of evaluation. Those lacking a suitable donor received a second course of IST. A second course of IST consisted of the same regimen (horse ATG/CyA/MePred) used in the initial treatment of each patient. To reduce the risk of an anaphylactoid reaction to treatment with horse ATG, patients were initially given a 100-fold diluted dose of ATG as a test dose. An antihistamine was administered to all patients receiving a second course of IST to suppress allergic reactions.

The recommended conditioning regimen for SCT from an alternative donor consisted of cyclophosphamide (CY, 120 mg/kg), rabbit ATG (Thymoglobulin, IMTIX-SANGSTAT, 10 mg/kg), and total body irradiation (TBI, 10 Gy) or CY (3000 mg/m^2), rabbit ATG (10 mg/kg), fludarabine (100 mg/m^2), and local field irradiation (3 Gy).[16,17] Prophylaxis against graft versus host disease (GVHD) consisted of a combination of CyA (3mg/kg per day) or tacrolimus (0.02mg/kg per day) plus short-term methotrexate. CyA dose were adjusted to maintain whole blood concentration of 100 to 200 ng/mL and tacrolimus dose 5 to 10 ng/mL, respectively.

Informed written consent was obtained from all patients or their parents in accordance with the Declaration of Helsinki. The study was approved by the ethics committee of each participating hospital. The list of participating hospitals can be found in Document S1, (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Table 1. Pretreatment characteristics

	SAA	VSAA
Registered	86	119
Evaluable	84	117
Sex (M/F)	48/36	65/52
Median age, y (range)	8 (0-17)	9 (0-15)
Cause of AA		
Idiopathic	73	91
Hepatitis	8	24
Viral infection	1	2
Drug	2	0
Median days from diagnosis to treatment (range)	13 (1-94)	19 (1-179)

SAA indicates severe aplastic anemia; VSAA, very severe aplastic anemia

Statistical analysis

The primary end point of this study was failure-free survival (FFS) after second-line therapy, which was defined as survival with response. Death, no response by 6 months, disease progression requiring clinical intervention, or relapse were considered treatment failures.[18] Overall survival and FFS were analyzed using the Kaplan-Meier method. Differences between the 2 arms of the study were evaluated by the log-rank test. P less than .05 was considered statistically significant.

Results

Patient characteristics

From October 1997 to April 2004, 205 patients with newly diagnosed severe (n = 86) and very severe AA (n = 119) were enrolled in the AA-97 study (Table 1). An interim analysis was performed in April 2005. Four patients were excluded from further analysis for the following reasons: IST without ATG (2 patients) or stem cell transplantation within 4 months of diagnosis (2 patients). Two patients without any granulocytes were not treated with ATG because of severe infections; both of them died of fungal pneumonia within 2 months of diagnosis. Both patients who underwent SCT within 4 months of diagnosis died of graft rejection or cardiac toxicity to the preconditioning regimen. There were 2 further deaths within 6 months of patient registration: hemolysis of unknown cause and aspiration pneumonia. None of the patients was diagnosed with PNH at the time of registration. Severe and very severe AA were associated with hepatitis in 32 patients, with other viral infection in 3 patients, and with medication use in 2 patients. The median days (range) from diagnosis to treatment of severe and very severe AA were 13 (1-94) days and 19 (1-179) days, respectively (Table 1).

Trilineage hematologic response

At 3 months after the initiation of therapy, 49 patients (58%) with severe AA and 46 patients (39%) with very severe AA had