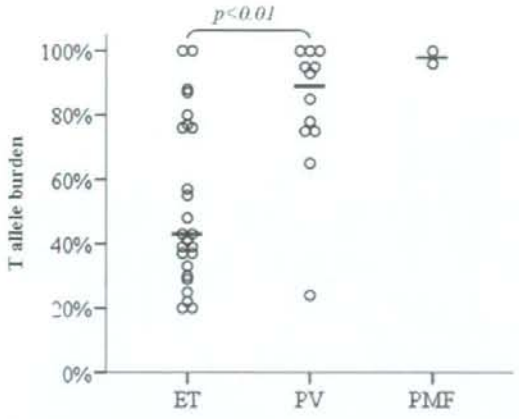

Figure 2. Distribution of the T allele burden according to the disease phenotypes. The T allele burden of each patient with ET group (24 cases), PV group (13 cases) and PMF group (two cases) was plotted with the median value shown by the horizontal line in each group. Mann–Whitney *U* test was done between ET group and PV group.

amount of the mutant allele expression of *JAK2* gene correlates with disease phenotypes such as PV or ET.

A case of PV with a rare JAK2-exon12 mutation

As recent reports have described the cases having various subtypes of *JAK2*-exon12 mutation, we searched such mutations in *JAK2*-V617F-negative patients with erythrocytosis (PV three cases, IE 16 cases) and found just one case of IE who revealed a peculiar *JAK2*-exon12 mutation. In the wild type the nucleotide sequence coding H538K539 is 5′-CACA AA-3′, whereas that of our case showed the mutation of 5′-CAGTTA-3′ at one allele and corresponding amino acid sequence was H538QK539L. The other *JAK2* allele showed the wild type. To our knowledge only one case with H538QK539L mutation was previously reported by Scott et al. [15], but their case showed the nucleotide sequence of 5′-CAATTA-3′ unlike our case.

Discussion

JAK2-V617F mutation is invariably associated with PV and considered to be a responsible gene for the disease etiology [4,6,7]. Accordingly, the revised 2008 WHO classification of MPN has incorporated such a molecular information [1]. In this study, we made a diagnosis of 75 suspected MPN patients based on both diagnostic criteria of the 2001 WHO classification and the revised 2008 WHO classification, and found that both criteria show a quite similar diagnostic power. *JAK2* mutation is a sensitive diagnostic marker for discriminating PV and IE, and the outcome of IE diagnosed by the 2008 WHO criteria is in the favourable status without treatment. These results suggest that the revised 2008 WHO criteria are useful in the medical management of the patients with erythrocytosis.

Today, clinical and biological relevance of *JAK2* mutations were gradually clarified by several studies. Vannucchi et al. [11] reported that *JAK2*-V617F homozygous patients represent high WBC count, high hematocrit value and a similar or low platelet count as compared with *JAK2*-V617F heterozygous patients. Further, it was reported that *JAK2*-V617F homozygous patients have a tendency to undergo thrombosis, myelofibrosis with splenomegaly, to need cytoreductive therapy [11]. Most of such findings correspond to our patient data and even to the report using mouse models [12,20–22].

Considering that the amount of the mutant allele expression might determine the disease phenotypes [6,10,12–14], we have established the method to distinguish between *JAK2*-V617F(high) group and *JAK2*-V617F(middle) group by the PCR and direct sequencing of the genomic DNA from purified neutrophil fraction, and we calculated the ratio of the mutant allele to the total allele in the clinical samples and examined its correlation with clinical/laboratory findings.

The T allele burden correlated well with laboratory findings, and as for disease phenotypes the PV group revealed significantly higher T allele burden than the ET group. These data, together with the previous reports, suggest that the amount of the mutant allele expression or the balance between the mutant allele and the normal allele might define the disease phenotypes, although it is not sufficient to explain the diversity of MPN phenotypes. *JAK2*-V617F (high) mutation also correlated with elevated serum LDH, elevated NAP score and incidence of splenomegaly. It was reported that PV patients showing higher mutant allele load tend to present high NAP expression at the cell membrane [23]. The relation of *JAK2*-V617F mutation with the risk of thrombosis and with need for cytoreductive chemotherapy is in controversy [9–11,24–26]. Vannucchi et al. [11] reported that *JAK2*-V617F mutation is a risk factor of thrombosis in ET patients but not in PV patients. Kittur et al. [27] reported that the presence of *JAK2*-V617F significantly increases the incidence of venous thrombosis in ET patients. *JAK2*-V617F mutation correlated well with the need for chemotherapy by multivariate analysis of PV patients but did not in the case of ET patients [11]. These problems remain to be solved by large-scale studies in the future.

The incidence of *JAK2*-exon12 mutation except V617F mutation is reported to be 2–3% out of total

PV patients [16,17]. Some IE patients also represent this mutation. To date, 10 subtypes of variations and 52 cases in total have been reported regarding *JAK2*-exon12 mutations [19]. Most of them reveal heterozygous mutation and still such patients express erythrocytosis exclusively without leukocytosis/thrombocytosis. In this study, we searched *JAK2*-exon12 mutations in three *JAK2*-V617F-negative PV patients and sixteen IE patients, and found a patient of PV with H538QK539L mutation. This patient is the second case of H538QK539L mutation ever reported, next to the case by Scott et al. [15], but the corresponding nucleotide sequence (5′-CAGTTA-3′) was a novel pattern. Because of its scarcity, *JAK2*-exon12 mutation seems to be often missed within *JAK2*-V617F-negative patients who are diagnosed as or suspected of MPN. As none of IE patients progressed into MPN nor acute leukemia in our series, the 2008 WHO criteria will successfully exclude the IE patients with a good prognosis, unlike the genuine MPN cases.

In summary, we validated the 2008 WHO diagnostic criteria of MPN for 75 suspected MPN patients and confirmed that the new criteria are useful in diagnosis and management of the patients with MPN. Thereafter, we examined the relation between the *JAK2*-V617F mutant allele burden and the clinical/laboratory features, and suggested that the amount of the mutant allele expression or the balance between the mutant allele and the normal allele defines the disease phenotypes. In addition, we found a PV patient who did not represent *JAK2*-V617F mutation but *JAK2*-exon12 mutation due to a novel nucleotide sequence pattern. Taken together, multidirectional analysis of *JAK2* gene is necessary for molecular diagnosis of MPN, and the revised 2008 WHO criteria with *JAK2* gene analysis are useful for precise diagnosis of MPN and the patients with erythrocytosis.

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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 ($>0.5 \times 10^9$/L) and at a median of 49 days (range, 24–138 days), eight patients had an unsupported platelet count of $>50 \times 10^9$/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.

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ORIGINAL ARTICLE

Clinicopathological characteristics of erythroblast-rich RAEB and AML M6a in children

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Abstract The distinction between RAEB, RAEB-T and AML M6a is difficult when erythroblasts in the bone marrow (BM) exceed 50%. We analyzed 19 children (2 RAEB, 13 RAEB-T and 4 AML M6a) enrolled in a prospective pathological central review in Japan and divided them into two groups according to the myeloblasts percentage among non-erythroid cells in BM: group A ($n = 8$), 5–19% myeloblasts; group B ($n = 11$), 20% or more myeloblasts. Their characteristics were very similar except for the number of myeloblasts. The median WBC was in the range of 1.0–5.0 $\times$ 10^9 L^{-1}, the median Hb was around 7.5 g/dL, the median MCV was greater than 90 fL and both group had Auer rods at 60–65%. Severe multilineage dysplasia was observed in most of the patients in two groups. Six with group A and seven with group B treated with AML type chemotherapy achieved complete remission. Five with group A and seven with group B undergoing SCT are alive at a median of 3 years after diagnosis. Erythroblast-rich RAEB and AML M6a in children have similar characteristics and may belong to a single disease entity.

Keywords RAEB · AML M6a · Myelodysplastic syndrome · Children · Erythroblast · Auer rods

1 Introduction

Myelodysplastic syndrome (MDS) is a heterogeneous group of clonal stem cell disorders characterized by peripheral cytopenias and dysplasia of marrow progenitors. Several issues regarding the classification of the disease has been recently updated. In the FAB classification [1],

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acute myeloid leukemia (AML) was diagnosed when the number of myeloblasts exceeded 30%, while the new WHO classification [2] proposed to reduce this percentage to 20% and removed the category of refractory anemia with excess of blasts in transformation (RAEB-T). The majority of patients previously belonging to this category are now classified as having AML with multilineage dysplasia. However, the validity of this approach has not been confirmed in pediatric MDS and AML [3].

Rosati et al. [4] previously argued that the distinction between MDS and AML could be particularly difficult in regard to three specific situations: (1) when the blast count is borderline, (2) when the BM has more than 50% erythroid precursors, and (3) when the cytogenetic abnormality shows a karyotype known to be associated with AML.

MDS occurs predominantly in the elderly, and rarely in childhood. Recently, the number of reports related to pediatric MDS has been increasing [5, 6], and a modified classification of pediatric MDS has been proposed [3]. Japanese children suspected of having MDS have been enrolled on the prospective diagnostic registry since 1999 [6]. There were 51 patients of whom the erythroid precursors exceeded 50% in the entire nucleated cell population and 19 of them were diagnosed with refractory anemia with excess of blasts (RAEB), RAEB-T, or AML M6a. We found that it was very difficult to differentiate MDS from AML M6a only according to the number of myeloblasts. We therefore analyzed in detail patients who were diagnosed with RAEB, RAEB-T or AML M6a.

2 Patients and methods

Clinical and laboratory data of children with MDS were retrospectively analyzed through the MDS Committee of the Japanese Society of Pediatric Hematology. A total of 450 children who were suspected of having MDS were enrolled in a prospective pathological central review from 1999 to 2006. Bone marrow (BM) and peripheral blood (PB) samples were examined prior to treatment at each institution and were sent to the central review system. Then, the diagnosis was made by the two independent investigators of the MDS Committee. Auer rod-positivity was defined as the unequivocal presence of at least one Auer rod in either a PB or BM smear. Cytogenetic analyses of BM samples were performed at the individual institutions. Basically, the diagnosis was made according to the FAB classification

In this registry, 120 patients had primary MDS, 98 patients had myeloproliferative syndrome including juvenile myelomonocytic leukemia (JMML), 35 patients had AML and 16 patients had secondary MDS/AML out of 450 cases [7].

The Japanese Childhood MDS Study Group Trial MDS99 has recommended AML type chemotherapy for children with RAEB with more than 10% of blasts, RAEB-T, and AML M6a since 1999 [8]. We also sent questionnaires enquiring about the clinical and laboratory findings at diagnosis, treatment plans, and clinical outcome to the physicians who treated these children. A response was defined by the IWG response criteria [9].

3 Results

3.1 Characteristics of children with erythroblast-rich RAEB and AML M6a

There were 51 patients in whom the erythroid precursors exceeded 50% in the BM. They consisted of refractory anemia (RA, $n = 8$), JMML ($n = 8$), secondary MDS ($n = 4$), overlap of aplastic anemia (AA) and RA ($n = 2$), AA ($n = 3$), congenital BM failure ($n = 3$), other non-malignant disease ($n = 4$), RAEB ($n = 2$), RAEB-T ($n = 13$), and AML M6a ($n = 4$) categorized by FAB Classification. We selected 19 of these 51 patients, who were diagnosed with RAEB, RAEB-T, and AML M6a with 50% or more erythroblasts in BM, to determine their similarities and differences. Nineteen patients were divided into two groups according to the myeloblasts percentage among non-erythroid cells (NEC): group A consisted of 8 patients with 5–19% myeloblasts of NEC; group B consisted of 11 patients with 20% or more myeloblasts in NEC.

The characteristics of two groups are shown in Table 1. Group A includes two RAEB and six RAEB-T and group B includes seven RAEB-T and four AML M6a according to the FAB classification. The median age of patients among groups A and B was 6 (range 0–15) and 12 (range 3–15), respectively. Seven group A patients were female, while 6 of the 11 patients in group B were male. The median leukocyte count was $4.9 \times 10^9\ L^{-1}$ in group A and $1.7 \times 109\ L^{-1}$ in group B. The median hemoglobin concentration was 8.3 g/dL in group A and 7.3 g/dL in group B. The median MCV was 94 fL in group A and 102 fL in group B. The median platelet count was $33.5 \times 10^9\ L^{-1}$ in group A and $15 \times 10^9\ L^{-1}$ in group B. In the PB, a small number of myeloblasts (0–37%) were identified. The median percentage of erythroid precursors in the BM of patients among group A and B was 60% (range 53–86%) and 67% (range 55–88%), respectively. The median percentage of myeloblasts in the total BM cells of patients among groups A and B was 3.5% (range 1–6%) and 8% (range 3–25%), while the median percentage of myeloblasts in the non-erythroid BM cells of patients among groups A and B was 9% (range 5–15%) and 27% (range

Table 1 Characteristics of the patients

Case no.	sex/age (years)	Diagnosis (FAB)	Peripheral blood (PB)					Bone marrow (BM)				
			WBC ($\times 10^9$ L^{-1})	Blast (%)	Hb (g/dL)	MCV (fL)	Plt ($\times 10^9$ L^{-1})	Blast (%)	Erythroid (%)	NEC blast(%)	Dysplasia lineage	Auer rods
A												
1	M/1	RAEB	9.5	2.0	8.9	83.9	2.3	2	71	7	3	(−)
2	F/9 months	RAEB	7.1	2.0	6.5	79	0.3	1	86	9	2	(−)
3	F/10	RAEB-T	2.7	2.0	7.5	95	37.0	4	58	9	3	(+)
4	F/11	RAEB-T	1.9	0.0	4.7	109	30.0	4	62	10	3	(+)
5	F/4	RAEB-T	2.8	2.0	10.5	93	51.0	6	53	13	3	(+)
6	F/15	RAEB-T	1.6	0.0	9.6	109	56.0	6	58	15	3	(+)
7	F/2	RAEB-T	11.5	8.0	8.7	79	5.1	1	82	5	3	(−)
8	F/8	RAEB-T	6.9	7.0	7.9	100	380.0	3	58	7	3	(+)
B												
9	M/14	RAEB-T	1.2	0.0	5.1	102	15.0	5	75	21	3	(+)
10	F/3	RAEB-T	19.0	3.0	7	83	0.8	4	82	21	3	(−)
11	M/6	RAEB-T	4.1	2.0	7.3	93	7.9	8	60	21	3	(−)
12	M/15	RAEB-T	2.2	3.5	7.6	105	24.0	10	58	25	3	(+)
13	M/14	RAEB-T	1.0	7.4	8.5	102	15.0	8	67	25	3	(+)
14	F/14	RAEB-T	1.2	0.0	4.5	106	44.0	3	88	27	2	(+)
15	F/12	RAEB-T	1.7	1.0	8.2	73	5.4	8	70	28	2	(−)
16	M/4	M6a	4.1	2.5	7.9	102	65.0	10	67	30	3	(+)
17	F/10	M6a	1.1	3.0	6.4	100	33.0	14	56	31	3	(+)
18	F/14	M6a	1.4	0.0	11.9	96	14.1	14	55	31	3	(−)
19	M/12	M6a	1.9	37.0	6.8	104	150.0	25	59	61	3	(+)

Group A 5–19% myeloblasts among non-erythroid cells in bone marrow; *group B* 20% or more myeloblasts among non-erythroid cells in bone marrow

The diagnosis was made according to the FAB classification

NEC non-erythroid bone marrow cells

21–61%). Notably, Auer rods were detected in 5 of the 8 children in group A and 7 of the 11 children in group B.

Karyotypic abnormality was detected in 2 of the 8 patients in group A and 6 of the 11 patients in group B. Severe multilineage dysplasia was observed in most of the patients in both groups. Bone marrow biopsy was performed in eight cases. They were usually hypercellular or normocellular. Except for the percentage of marrow blasts, the marrow aspirate and biopsy specimens from their patients in two groups were similar. It was not helpful for discriminating between RAEB, RAEB-T, and AML M6a (Fig. 1).

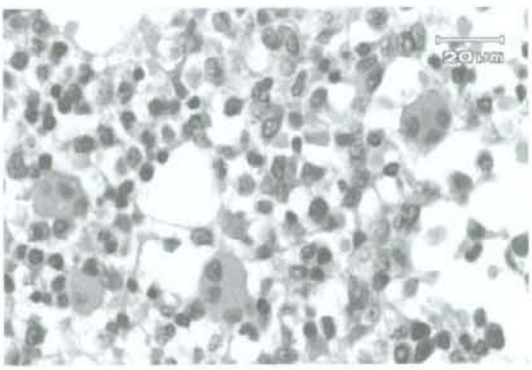

Fig. 1 Bone marrow biopsy specimen in a patient with RAEB/AML M6 syndrome. Erythroid precursors and megakaryocytes are clustered and the numbers of these cells are large. Megakaryocytes are dysplastic with separate nuclei. An increase in myeloblastsis not seen

3.2 Treatment outcome of children erythroblast-rich RAEB and AML M6a

The treatment and outcome of these patients are summarized in Table 2. Seven of 8 patients in group A and 8 of the 11 patients in group B were treated with AML type chemotherapy and all of them had achieved complete remission (CR) except for two patients (cases 1, 9). In cases 3 and 8, the disease progressed to AML 7 and 3 months later. The number of blasts of cases 12 and 17 also had been increasing within 3 months. Death occurred

Table 2 Cytogenetic findings, treatment and outcome

Case no.	Cytogenetic data[a]	Induction therapy		SCT		Overall survival (months)	Cause of death
		Regimen	IWG response	Donor source	IWG response		
A							
1	46,XY,del(12)(p11.2–12.2)	AML type	NA (non-CR)	UBMT	CR	16+	–
2	Normal	AML type	CR	UCBT	CR	42+	–
3	46 XX,t(5;12;18)(q35;q13;q21)	AML type	CR	UCBT	CR	60+	–
4	Normal	None	–	Allo PBSCT	CR	66+	–
5	Normal	AML type	CR	UBMT	CR	21	Complication
6	Normal	AML type	CR	UBMT	CR	84+	–
7	Normal	AML type	CR	UCBT	Failure	8	Complication
8	Normal	AML type	CR	RBMT (mismatch)	Failure	28	Complication
B							
9	47 XY, +8	AML type	NA (non-CR)	UBMT	CR	18+	–
10	46 XY,del(5)(q?)	AML type	CR	UBMT	CR	24+	–
11	46 XY, del (9)(q?)	AML type	CR	UCBT	CR	6+	–
12	47 XY, +8	None	–	RBMT	CR	84+	–
13	Normal	None	–	Allo PBSCT	CR	26	Relapse
14	Normal	AML type	CR	UBMT	CR	60+	–
15	46 XX,der (1;7)(q10;p10)	None	–	RBMT(mismatch)	CR	9+	–
16	48 XY, add(6),+X,+19	AML type	CR	UCBT	Failure	14	Complication
17	Normal	AML type	CR	None	–	20	Relapse
18	Normal	AML type	CR	UBMT	CR	32+	–
19	Normal	AML type	CR	None	–	36	Relapse

Group A 5–19% myeloblasts among non-erythroid cells in bone marrow; *group B* 20% or more myeloblasts among non-erythroid cells in bone marrow

SCT stem cell transplantation, *IWG* International Working Group, *NR* no response, *CR* complete remission *NA* not applicable, *UCBT* unrelated cord blood transplantation, *UBMT* unrelated bone marrow transplantation, *Allo PBSCT* allogeneic peripheral blood stem cell transplantation, *RBMT* related bone marrow transplantation

[a] Demonstrating at least two metaphases with an identical abnormal karyotype

at 21, 8, 28, 26, 14, 20, and 36 months after diagnosis in cases 5, 7, 8, 13, 16, 17, and 19. Two patients (cases 17, 19) who did not receive stem cell transplant (SCT) relapsed after complete remission 1 (CR1) and one patient (case 13) who underwent allogeneic PB stem cell transplantation (PBSCT) from a sibling donor had AML M1 2 years later. Four out of 17 patients died of complications after SCT. As of writing, 12 patients are alive at a median of 3.1 years after diagnosis.

4 Discussion

It is not easy to differentiate MDS from AML M6a when the percentage of erythroid precursors in the BM far exceeds beyond 50%, because as the erythroid component increases in the BM cells, the distinction between AML and MDS will solely rest on fewer non-erythroid cells. We investigated two cases of RAEB, 13 cases of RAEB-T and four cases of AML M6a diagnosed from 1999 to 2006. We found that their characteristics were very similar except for the number of myeloblasts. The median WBC was in the range of 1.0–5.0 $\times$ 10^9 L^{-1}, the median Hb was around 7.5 g/dL, the median MCV was greater than 90 fL and both group had Auer rods at 60–65%. In the PB, 17 patients had at least two-lineage dysplasia and the percentage of myeloblasts among total nucleated cells was below 10% in 17 cases. BM was normocellular or hypercellular and showed severe dysplasia.

It is considered that the presence of Auer rods is a hallmark of AML but is rarely seen in MDS or chronic myelomonocytic leukemia (CMML). In the FAB classification, patients had been assigned to the RAEB-T category if they had myeloblasts with Auer rods, irrespective of the percentage of blasts [1]. However, in the new WHO system, Auer rods are no longer regarded as relevant for the classification of MDS [2]. Strupp suggests that the presence of Auer rods does not necessarily indicate a particularly bad prognosis and that it does not justify placing patients into the high-risk category of MDS [10]. On the other hand, Willis et al. [11] found that patients with RAEB-T with Auer rods had a higher incidence of mortality and concluded that the presence of Auer rods indicated an aggressive biology in MDS with a low blast count. We observed a high frequency of the presence of Auer rods in our cases (63%), a finding that has never been reported in adult series of patients with erythroid-rich RAEB/AML M6a. Accordingly, this may be a specific characteristic of children with such phenotypes.

The karyotype at diagnosis was abnormal in one patient with RAEB and one patient with RAEB-T among group A, and in five patients with RAEB-T and one patient with AML M6a among group B. Previous reports showed that cytogenetic abnormalities, including chromosomes 5 or 7, were the most frequent abnormalities in AML M6a adult patients and complex karyotypic abnormalities indicated a more aggressive phenotype, but such abnormalities were not found in our pediatric series.

There have been few reports examining children with AML M6a, but children with AML M6a apparently had a poorer outcome [12, 13]. In our study, 7 of 19 patients died and two patients treated without SCT relapsed. However, the other 12 children who received SCT were alive without disease. This implies that allogeneic SCT should be indicated for children erythroid-rich RAEB/AML M6a.

The role of AML-like induction chemotherapy for patients with MDS is controversial. The European Working Group on MDS in children indicated that it was of no benefit for the patients with RAEB and RAEB-T before BMT [14], whereas some current evidences would support its use [15]. The MDS Committee of the Japanese Society of Pediatric Hematology also reported that patients with lower blast count (20–30%) could be treated with AML-type chemotherapy [6]. We assume that intensive chemotherapy may improve the prognosis for children with RAEB and RAEB-T/AML M6a, because response to induction chemotherapy was unexpectedly good (87%). However, most patients subsequently underwent SCT; accordingly the role of AML therapy for such patients remains to be determined.

Park showed that the typical AML M6a and RAEB-T with >50% erythroblasts had identical characteristics, including karyotype abnormality and the terms of survival [16, 17]. Barnard also reported that childhood AML M6 more resembled MDS than true de novo AML (M0-5) in presentation and outcomes [13]. AML M6a with >30% of myeloblasts were only three in our analysis, and therefore, we were not able to conclude that AML M6a is the same entity as RAEB-T or RAEB with more than 50% of erythroblasts because of a small number of patients. In principle, the abnormal cone of MDS expands slower than the AML clone. However, we did not find nature in our series of patients. In fact, 4 out of 19 patients developed overt AML within a few months. It is possible that a proportion if immature erythroblasts may be different between erythroblast-rich RAEB and AML M6a. However, we did not find this tendency (data not shown). The clinical, morphological, cytogenetic, and biological borders of these diseases are overlapping.

In conclusion, we found that erythroid-rich RAEB/AML M6a have similar characteristics and may belong to a single disease entity. A further prospective study is needed to support our hypothesis and the unique pathogenesis of this entity should be elucidated in future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2007.10.018.

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Mutation analysis of AML1 gene in pediatric primary myelodysplastic syndrome and juvenile myelomonocytic leukemia

To the Editor,

Chromosomal translocation of *t*(8;21) is frequently found in patients with acute myeloid leukemia-M2. In 1991, Miyoshi et al. first sequenced the AML1 gene on chromosome 21, which is a frequent target of chromosomal translocation associated with leukemia [1]. They found three forms of the gene transcript, which they named AML 1a (250 amino acids), AML 1b (453 amino acids) and AML 1c (480 amino acids) [2]. The three proteins share a Runt domain encoded within exons 3 through 5 of the AML1 gene. AML 1 protein is one of the alpha subunits of the transcription factor polyomavirus enhancer binding protein 2 (PEBP2). The alpha subunit binds DNA via a Runt domain. AML 1b and 1c also have a long C-terminal region encoded by exons 6, 7B and 8, which also encode a transcription activation domain. The Runt domain is located between the 50th and 177th amino acids of the AML1b protein [3].

We examined bone marrow DNA specimens from eight patients – five boys and three girls – ranging in age from 5 days to 13 years, with primary myelodysplastic syndrome (MDS) or juvenile myelomonocytic leukemia (JMML) for mutations in exons 3 through 8 by PCR-SSCP and direct sequencing (predicted PCR products <207 bases), or standard PCR and direct sequencing (predicted PCR products >207

Table 1
Oligonucleotide primers used for amplification of the AML1 gene and the predictable products

Exon	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing temperature (°C)	Predictable PCR products
Exon 3	CAAGCTAGGAAGACCGACCC	TGCAGGGTCCTAACTCAATC	61	440 bases
Exon 4	ACTTCGACCGACAAACCTGA	CCTGATGTCTGCATTTGTCC	63	186 bases
Exon 5	GTGTACCAGCCCCAAGTGGA	GCCACCAACCTCATTCTGTT	63	178 bases
Exon 6	GCAGTGGGCTCCATCTGGTA	CTGATCTCTTCCCTCCCTCC	64	279 bases
Exon 7A	CCACATTCTGCCTTCCTCAT	TTTCTCCCTGGTCACACATG	63	193 bases
Exon 7B	AGAATGTGTTTTCAAGTGGC	GACCTTTCTGATTCTCTTCA	55	207 bases
Exon 8	TGACCTACAGCGAGATCCTG	CCGCAACCTCCTACTCACTT	63	684 bases

Table 2
Cases and mutation characteristics of the patients with pediatric primary MDS or JMML

Age (years)	Gender	Genome mutation of AML 1b	Amino acid mutation of AML 1b protein (single-letter amino acid codes)
13	GIRL	142 G>A	48 D>N
2.9	BOY	441 T>G	147 T>T

DDBJ accession number: D43968.

bases). Six patients suffered from JMML and two suffered from refractory anemia. The DNA specimens were extracted from paraffin-embedded clot materials that had been stocked by the MDS Committee of the Japanese Society of Pediatric Hematology. The PCR primers used in this study and the predicted PCR products for each primer pair are listed in Table 1. Single nucleotide polymorphisms detected are listed in Table 2. The two mutations were not identified in bone marrow DNA specimens from healthy volunteers. A silent mutation was found in one JMML patient, whereas a missense mutation causing amino acid transposition was detected in one patient with refractory anemia. The transposition occurred at only the 3rd amino acid from the Runt domain of the AML1 protein. It is possible that this amino acid transposition influenced the Runt domain because aspartic acid is acidic and asparagine is relatively neutral.

AML-1 gene mutations have been frequently observed in patients with secondary MDS [4–6]. Harada et al. reported that 17% of adult patients with primary MDS showed AML-1 gene mutation, compared with half of adult patients with secondary MDS. Moreover, they mentioned that 8% of adult primary MDS showed mutations in the Runt domain, whereas in 9% the mutations occurred in the C-terminal region [5]. The two mutations detected in our study did not occur in the Runt domain of the AML-1 gene. The rate of mutation of the AML-1 gene in patients with pediatric primary MDS or JMML (2/8 = 25%) was higher than that in adult patients, although most cases of primary MDS, both adults and children, do not have AML-1 gene mutation(s) and the number of cases we studied was lower than those of adult MDS examined by Harada et al. [5]. Our data indicate that AML-1 gene mutation may be more closely related to the pathological backgrounds of pediatric primary MDS or JMML than to that of adult primary MDS. It remains to be clarified whether the relation of the AML1 gene to pediatric primary MDS and JMML is different from that to adult primary MDS or not.

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Retinoic acid syndrome after first dose of ATRA and ileal perforation secondary to promyelocytes infiltration

Acute promyelocytic leukemia (APML) accounts for 10% of acute myeloid leukemia [1]. Retinoic acid syndrome (RAS) is characterized by increasing white blood cell count with fever, weight gain, dyspnea, pleural effusion, and pulmonary infiltrates on chest radiograph and, in some patients, renal failure, hypotension, and pericardial effusion. In absence of definitive criteria of RAS the incidence has ranged from 6% to 27% [2]. Here we report a patient of APML who developed RAS after first dose of ATRA and a recurrence after restarting of ATRA. On day 18 of his illness he developed ileal perforation due to infiltration by leukemic cells.

A 19-year-old male labourer presented with 20 days history of bleeding from the gums, ecchymotic patches and low-grade fever for 2 days prior to the admission. At admission Hb was 4.5 g/L, total leukocyte count (TLC)—2900/mm^3, differential leukocyte count—blast 28%, promyelocytes 68% and lymphocytes 4% and platelet count—15,000/mm^3. Bone marrow aspiration with cytochemistry was consistent with the diagnosis of APML and PCR confirmed the presence of PML RAR with bcr3 transcript. Twelve hours after starting of ATRA, patient developed drowsiness, with pulse rate of 130/min, blood pressure 86/40 mm Hg, respiratory rate (RR) 40/min and arterial blood gases showed PO_2—66 mm Hg and SO_2—80% at room air. On auscultation bilateral coarse crackles were present. X-ray chest revealed bilateral fluffy opacities. As the absolute neutrophil count (ANC) was 200/mm^3, he was started on broad-spectrum antibiotics. In view of rapid development of tachycardia, tachypnea, hypotension and hypoxemia with chest abnormalities, RAS was suspected. Injection dexamethasone 10 mg q12 h was started and ATRA was withheld. After 12 h patient's general condition showed no further deterioration. Daunorubicin was given from day +3 to day +5. On day +6, X-ray chest showed almost complete resolution of the fluffy opacities. ATRA was then restarted at 50% of the dose. On day +10 at a tapering dose of steroid patient developed high-grade fever along with tachypnea (RR—38/min) and bilateral crepts which necessitated the withdrawal of ATRA. Dexamethasone was then increased to full dose of 10 mg q12 h. Patient improved within 12 h of increasing the dose of steroids. On day +18 patient developed severe abdominal pain. Examination revealed board like rigidity of the abdomen; erect X-ray abdomen and ultrasound were suggestive of perforation peritonitis. His ANC was zero. Urgent laparotomy was done which showed an ileal perforation 130 cm proximal to ileocecal junction with clean edges and no stigmata of typhoid or tuberculosis. Multiple biopsies were taken from the perforation site. Four days later patient developed hypotension and succumbed to his illness. At this time his TLC 1000/mm^3, ANC was zero and there were no blasts or promyelocytes on peripheral smear. Ileal biopsy revealed infiltration of promyelocytes into lamina as the cause of perforation.

1. Discussion

This case has three unusual features: (a) development of RAS syndrome after first dose of ATRA in a patient with low TLC on presentation, (b) recurrence of symptoms on rechallenge at day 10 at TLC of 500/mm^3, and (c) infiltration by promyelocytes of ileal wall 15 days after initiating chemotherapy, leading to perforation and death.

Frankel et al. gave the first description of this syndrome in 9 of 35 (25%) newly diagnosed APML patients they treated with ATRA [3]. Signs occurred after 2–21 days of treatment with ATRA. Median time for occurrence of RAS was 7–10 days in published series [4]. Occurrence of RAS after first dose has been reported by Battistella et al. but the leukocyte count in their patient was 94.8×10^3/mm^3 at presentation

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Identification and functional characterization of novel telomerase variant alleles in Japanese patients with bone-marrow failure syndromes

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Abstract

As the incidence of bone-marrow failure syndromes (BMFS) is 2–3× higher in East Asia than in the West, we examined peripheral blood or marrow cells of 100 Japanese patients for possible pathogenic mutations in the two main components of the telomere-synthesizing enzyme telomerase (hTERC RNA and hTERT protein) that have recently been implicated in the disease pathogenesis. We analyzed samples collected from 34 patients with acquired aplastic anemia (AA), 66 patients with myelodysplastic syndromes (MDS) and 120 healthy controls. In addition to two polymorphic germ-line sequence changes (n-771A/G and n-714 C insertion) in the promoter region of *hTERC* and eleven *hTERT* polymorphisms that were identified in both patients and healthy individuals, we found a novel germ-line C323T mutation in the hTERC RNA in an MDS patient only. This heterozygous C323T mutation abolished telomerase enzymatic activity and functioned in a haploinsufficiency manner to modulate telomerase activity in cells. In summary, this study reports a novel telomerase natural variant that abolishes telomerase function, which may lead to telomere shortening and marrow hypocellularity in patients with BMFS. This study also highlights the rarity of genetic alterations in BMFS patients in Japan, which suggests that other factors may play a more prominent role in the disease pathogenesis in East Asia.

Keywords: Telomeres; Telomerase; Aplastic anemia; Myelodysplastic syndromes; Bone-marrow failure syndromes; hTERC; hTER; hTR; hTERT

Introduction

Dyskeratosis congenita (DKC) is an inherited bone-marrow failure syndrome (BMFS) typified by reticulated skin pigmentation, nails dystrophy and mucosal leucoplakia [1]. About 35% of the cases are X-linked recessive, 5% are autosomal dominant and the rest of the cases are with unidentifiable pattern of inheritance [2]. Whereas the gene responsible for X-linked recessive cases is *DKC1* [3], those responsible for autosomal dominant cases are the telomerase *hTERC* and *hTERT* [4–7].

Telomerase is a ribonucleoprotein (RNP) complex with two main components: a protein (hTERT) with RNA-dependent DNA polymerase activity and an integral hTERC RNA, which provides a template to synthesize telomeric DNA repeats [8]. Telomeres are structural elements that seal and protect the ends of linear chromosomes from illegitimate recombination, end-to-end fusion or being recognized as damaged DNA [8]. In human somatic cells, telomeres typically consist of more than 1000 simple repetitive DNA and associated proteins [8]. These repeats are gradually lost with cellular replication and aging, owing to the inability of DNA polymerase to fully replicate the 3′ end of DNA. Telomere attrition eventually leads to critically short

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telomeres, inducing cellular proliferative senescence and/or apoptosis possibly due to genomic instability [8]. It is thought that telomeres are shortened as a result of pathogenic mutations in *DKC1* or telomerase gene components that lead to an impairment in the proliferative capacity of hematopoietic stem cells in patients with BMFS [4,7,9]. Furthermore, an association has been established between the degree of telomere shortening and that of disease severity and the age of onset [7,9].

The existence of possibly cryptic DKC in patients who develop the disease later in life [10] and many cases of aplastic anemia (AA) with significantly shortened telomeres can also be attributed to mutations in telomerase gene components [11–13]. Several groups, including our own, have recently reported that some patients with paroxysmal nocturnal hemoglobinuria (PNH), myelodysplasia (MDS), in addition to those with AA or DKC, carry heterozygous mutations in the telomerase *hTERC* or *hTERT* gene [5,14–18]. In vitro functional analyses of these mutations revealed that the mutations functioned either as dominant negatives or haploinsufficiency to attenuate telomerase enzymatic activity, which could explain the short telomeres in patients [6,17,19–21]. The largest controlled epidemiologic studies reported that the incidence of AA in the West is 2 per million per year and is about 2- to 3-fold higher in Asia [22]. The subjects of previous studies have mostly been those with Caucasian, Black or Hispanic ancestry. Screening for telomerase mutations among Asian populations has rarely been done [18,23]. Therefore, we carried out an investigation to determine whether mutations in *hTERC* and *hTERT* genes are associated with the disease in our cohort of Japanese BMFS patients.

Materials

Patients and healthy controls

We examined mononuclear cells (MNC) of peripheral blood or bone marrow from 100 BMFS patients with acquired AA ($n=34$) or with MDS (RA) ($n=66$) diagnosed between 1993 and 2005 at the Nippon Medical School and its affiliated hospital. These patients were diagnosed with AA based on the blood count criteria of the International Study of AA and agranulocytosis with severity determined by the criteria of Camitta et al. [24]. We excluded AA patients who had achieved complete remission or good response to immunosuppressive therapy. Good response was defined as a resolution of all blood transfusion requirements and a more than 2 g/dL increase in hemoglobin as compared with pretreatment levels. Most AA patients (91.1%) received ATG and cyclosporine A combination therapy and showed either only partial or no response to treatment (Table 1). For a diagnosis of MDS, patients were subclassified according to the French–American–British (FAB) nomenclature [25]. MDS (RA) classification might include several heterogeneous BMF or other hematologic diseases. However, we excluded patients who had developed MDS (RAEB) or MDS leukemia for more than 3 years from original diagnosis. Most of selected patients with MDS (RA) were treated with blood transfusion or anabolic steroid. As normal controls, we analyzed blood samples from 120 healthy individuals. Our volunteers provided informed consent prior to genetic testing as approved by our institutional review board.

Table 1
Patients' clinical background

		AA ($n=34$)	MDS (RA) ($n=66$)
Sex	Male	14	34
	Female	20	32
Age (range)		13–77	19–90
Family history	+	0	2
	–	34	64
Chromosome abnormality	Trisomy 8	0	4
	7q-	1	1
	del (20)	0	3
	Complex	0	2
Treatment			
Immunosuppressive therapy			
Partial response		17	5
No response		14	9
Others			
Blood transfusion only		0	27
Metenolone		2	22
Stem cell transplantation		1	3

Mutational analysis

Mononuclear cells (MNC) from bone marrow or peripheral blood were isolated by density gradient centrifugation using lymphocyte separation medium (Organon, Durham, NC). The genomic DNA of MNC was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) amplification of telomerase gene components (*hTERC* and *hTERT*) was carried out essentially as described previously [16,17]. The Advantage GC2 PCR amplification kit (BD Biosciences Clontech, CA, USA) and the TaKaRa Ex Taq DNA polymerase (Takara, Shiga, Japan) were used to amplify the genes from genomic DNA. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced bidirectionally using the Big Dye Termination 3.1 kit and the ABI Prism 310 system (Perkin-Elmer Cetus, CA, USA). Specific sequences of primers used for sequencing are available upon request. To validate the sequencing results, PCR products were inserted into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, CA, USA). Recombinant plasmids isolated from 8 to 12 white colonies were sequenced.

In vivo reconstitution of telomerase enzymatic activity

Wild-type or mutant pcDNA3-hTERC DNAs (2 μg) were transfected into VA13+hTERT cells (at approximately 70% confluency) in 6-well polystyrene dishes using SuperFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In certain cases, two different versions of the *hTERC* gene, each on a separate vector and at 1 μg, were co-expressed simultaneously in the VA13+hTERT cells. To monitor transfection efficiency, a plasmid (peGFP-N1) (Strategene, CA, USA) expressing green fluorescent protein was transfected in a parallel transfection reaction. The eGFP