厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

Congenital dyserythropoietic anemia (CDA)の効果的診断法の確立に関する　研究

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研究要旨：Congenital dyserythropoietic anemia(CDA)は先天的に赤血球系細胞に形成異常があり、慢性の不応性貧血、無効造血および続発性ヘモクロマトーシスを伴う稀な疾患群である。本邦での実態は未だ十分に把握されていないため、小児血液学会の中央診断と疾患登録事業による予後等の追跡調査ならびに患者検体の遺伝子検索を行い、日本におけるの全体像を把握する。これにより効果的診断法を開発し、CDAに特異的で有効な治療法開発の基盤となる研究を行う。

A．研究目的

本研究は、小児血液学会の中央診断と疾患登録事業による予後等の追跡調査ならびに患者検体の遺伝子検索を行い、日本における Congenital dyserythropoietic anemia(CDA)の全体像を把握し、効果的診断法を開発することを目的とする。

B．研究方法

他の先天性骨髄不全研究班と合同で、一次調査を行い、CDA およびその疑い例を抽出する。抽出された症例について、２次調査を行い、臨床像、治療の実態を把握する。また名古屋大学小児科で遺伝子解析を行う。

（倫理面への配慮）

小児血液学会疾患登録事業は、疫学研究倫理指針に準拠した臨床研究として、すでに学会倫理審査委員会で承認されている。患者検体を用いた研究の実施にあたっては、小児血液学会の倫理委員会の承諾を得るほか、検体の採取にあたっては、患者および家族に事前に十分な説明を行い、文書による同意を得る。ヒト遺伝子研究に該当する場合は、ヒトゲノム遺伝解析に関する倫理指針を従う。

C．研究結果

報告者の協力に基づいて作成された調査票に基づいて１次調査が行われ、CDAおよび疑い例が抽出された。症例ありと回答された施設に対し、２次調査、遺伝子診断を行う。

D．　考察

今回の調査により本邦におけるCDAの実態が把握できると考えられる。

E．　結論

１次調査により本邦におけるCDA症例が抽出された。今後２次調査、名古屋大学で遺の伝子解析の結果を解析し、日本におけるCDAの全体像を把握する。

F．　研究発表

１．論文発表　　なし
２．学会発表　　なし

G．知的財産権の出願・登録状況

１．特許取得　　なし
２．実用新案登録　　なし

Ⅲ　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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Villalobos IB, Takahashi Y, Akatsuka Y, Muramatsu H, Nishio N, Hama A, Yagasaki H, Saji H, Kato M, Ogawa S, Kojima S.	Relapse of leukemia with loss of mismatched HLA due to uniparental disomy following haploidentical hematopoietic stem cell transplantation.	Blood	Epub ahead of print		2010

Ⅳ　研究成果の刊行物・別刷

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Correlation of Clinical Features With the Mutational Status of GM-CSF Signaling Pathway-Related Genes in Juvenile Myelomonocytic Leukemia

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ABSTRACT: Mutations in *RAS*, neurofibromatosis type 1 (*NF1*), and *PTPN11*, constituents of the granulocyte-macrophage colony-stimulating factor signaling pathway, have been recognized in patients with juvenile myelomonocytic leukemia (JMML). We assessed 71 children with JMML for *NRAS*, *KRAS*, and *PTPN11* mutations and evaluated their clinical significance. Of the 71 patients, three had been clinically diagnosed with neurofibromatosis type 1, and *PTPN11* and *NRAS/KRAS* mutations were found in 32 (45%) and 13 (18%) patients, respectively. No simultaneous aberrations were found. Compared with patients with *RAS* mutation or without any aberrations, patients with *PTPN11* mutation were significantly older at diagnosis and had higher fetal Hb levels, both of which have been recognized as poor prognostic factors. As was expected, overall survival was lower for patients with the *PTPN11* mutation than for those without (25 *versus* 64%; $p = 0.0029$). In an analysis of 48 patients who received hematopoietic stem cell transplantation, *PTPN11* mutations were also associated with poor prognosis for survival. Mutation in *PTPN11* was the only unfavorable factor for relapse after hematopoietic stem cell transplantation ($p = 0.001$). All patients who died after relapse had *PTPN11* mutation. These results suggest that JMML with *PTPN11* mutation might be a distinct subgroup with specific clinical characteristics and poor outcome. (***Pediatr Res* 65: 334–340, 2009**)

Juvenile myelomonocytic leukemia (JMML) is a rare clonal myelodysplastic/myeloproliferative disorder that affects young children. It is characterized by specific hypersensitivity of JMML cells to granulocyte-macrophage colony-stimulating factor (GM-CSF) *in vitro*, but is thought to be a genetically and phenotypically heterogeneous disease (1–4). JMML seems to have its genesis in dysregulation of GM-CSF signal transduction, and gene mutations interfering with downstream components of the GM-CSF signaling pathway can be identified in approximately 70% of children with this disorder (2,5–11). Constitutional mutations of *NF1* occur in approximately 10% of patients with JMML (2,5,12). *NF1* is known to be the causative gene of neurofibromatosis type 1 (NF1), an autosomal dominant cancer predisposition syndrome. *NF1* codes for neurofibromin, a GTPase activating protein for Ras, and acts as a tumor suppressor (13). Similarly, oncogenic *RAS* mutations at codons 12, 13, and 61 have been identified in approximately 20–25% of patients with JMML (2,6–8). These mutations lead to elevated levels of Ras-GTP, the active form of Ras, resulting in constitutive activation of the signal transduction pathway (14).

Somatic mutations in *PTPN11*, which encodes the protein tyrosine phosphatase SHP-2, a molecule that also relays the signal from the GM-CSF receptor to Ras, have been reported in approximately 35% of patients with JMML (9–11). Germline mutations in *PTPN11* were first observed in Noonan syndrome (NS) (15), and somatic mutations have also been identified in hematological malignancies (9–11,16,17). SHP-2 is a positive regulator in this signal transduction pathway and *PTPN11* mutations cause gain of function in SHP-2, resulting in inappropriate activation of the GM-CSF pathway (10). *PTPN11* mutations have been found without coexisting *NRAS*, *KRAS*, or *NF1* mutations (9,11,16). These alterations are thought to be responsible for GM-CSF hypersensitivity and the clinical features associated with this condition. Given this information, mutational analysis of *PTPN11* and *RAS*, or

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Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; **HbF,** fetal hemoglobin; **HSCT,** hematopoietic stem cell transplantation; **JMML,** juvenile myelomonocytic leukemia; **NF1,** neurofibromatosis type 1; **NS,** Noonan syndrome; **OS,** Overall survival

either identification of an *NF1* mutation or a clinical diagnosis of NF1, are currently key diagnostic procedures for JMML. However, we have a poor understanding of the relationship between mutational status and the clinical features of JMML.

Most patients with JMML usually experience an aggressive clinical course and die from progressive disease unless treated with hematopoietic stem cell transplantation (HSCT) (1,4,18,19). Recent studies have shown that children with JMML have better outcomes when they undergo HSCT early in the course of the disease (20,21). In contrast, there is a certain proportion of patients who have a stable clinical course for a considerable period of time, and disease that sometimes spontaneously resolves without any treatment (1,22,23). Therefore, information on prognostic factors that can be used to identify patients requiring early HSCT is important in developing a treatment plan.

Although several clinical characteristics have been reported as prognostic factors for JMML, including age at diagnosis, sex, fetal Hb (HbF) level, platelet count, and cytogenetic abnormality (1,19–21,23–26), the relationship between prognosis and particular genetic aberrations is unclear and needs to be clarified. Thus, in the current study, we assessed 71 children with JMML for *NRAS*, *KRAS*, and *PTPN11* mutations and analyzed the association between mutational status and previously recognized prognostic factors for JMML, then evaluated the clinical significance of these mutations to clarify whether genotype-phenotype correlations exist.

MATERIALS AND METHODS

Patients. A total of 71 children with JMML diagnosed between 1987 and 2006 in 30 institutions throughout Japan were studied retrospectively. The diagnosis of JMML was based on the internationally accepted criteria previously published (27). We excluded patients with NS, a JMML-like myeloproliferative disease characterized by spontaneous regression of the disease. The clinical and hematological characteristics of the 71 patients are summarized in Table 1. The median age at diagnosis was 24 mo (range, 1–69 mo).

Table 1. *Patients characteristics*

No. of patients	71
Median age at diagnosis, mo (range)	24 (1–69)
Male/female	43/28
Peripheral blood	
Median Hb at diagnosis, g/dL (range)	9.3 (4.9–13.0)
Percentage of HbF at diagnosis (range)	19.0 (1.0–78.0)
Median WBC count at diagnosis, $\times 10^9$/L (range)	31.8 (7.6–563.0)
Median monocyte count at diagnosis, $\times 10^9$/L (range)	4.2 (1.0–84.5)
Median platelets count at diagnosis, $\times 10^9$/L (range)	42.0 (1.4–320.0)
Hepatomegaly (yes/no)	67/4
Splenomegaly (yes/no)	68/3
Cytogenetic study, no.of patients	
Normal	55
Monosomy 7	9
Other abnormalities	2
+8	1
−Y	1
+X,+13	1
Inv(4)(p14p16)	1
t(3;18)(q25;q21)	1
Del(6)(q?),−20	
No. of patients with clinical evidence of NF1	3
No. of patients received HSCT	48

WBC, white blood cell.

Karyotypic abnormalities were detected in 16 patients, including nine patients with monosomy 7. Three children had clinical evidence of NF1. Treatment was planned in the institute responsible for each child and 48 of 71 patients had been treated with HSCT. The source of grafts was bone marrow from a related donor for 15 patients, bone marrow from an unrelated donor for 20, unrelated cord blood for 11, and related peripheral blood for two. Total body irradiation, TBI, was used in half of the patients and the remainder underwent a non-TBI regimen in which the drug dosage varied widely. Approval for this study was obtained from the Ethics Committee of Nagoya University Graduate School of Medicine.

Screening for mutations of the PTPN11, NRAS, and KRAS genes. Written informed consent was obtained from the parents of each patient, and bone marrow or peripheral blood samples were obtained at initial diagnosis. Mononuclear cells were isolated using Ficoll-Hypaque density gradient centrifugation and they were cryopreserved until use. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Chatsworth, CA). To screen for *PTPN11* mutations, we amplified genomic DNA corresponding to exons 2, 3, 4, 7, 8, 12, and 13 of *PTPN11* using the PCR with cycling parameters and primers as previously reported (28,29). The PCR products were purified and directly sequenced on an ABI Prism 3100 DNA Analyzer (Applied Biosystems, Foster City, CA) using a BigDye terminator cycle sequencing kit (Applied Biosystems).

NRAS and *KRAS* mutations of codons 12, 13, and 61 were identified as previously described (11,30) and were confirmed by sequencing.

Analysis of correlations between clinical characteristics and mutational status. To assess the correlations between clinical characteristics and mutational status, patients were subdivided into four groups: those with a *PTPN11* mutation, a *RAS* mutation, a clinical diagnosis of NF1, or none of these. However, the number of patients with NF1 was too small to allow subgroup analysis, so these patients were excluded from this analysis. Then, for the three remaining groups, we performed a comparison of clinical and laboratory findings.

Statistical analysis. The date of analysis was January 30, 2008. Survival probabilities were estimated by the Kaplan-Meier method and comparisons between probabilities for different patient groups were performed using the two-sided log-rank test. All results are expressed as 5-y probabilities with a 95% confidence interval (CI). Overall survival (OS) for all patients was defined as the time from diagnosis to death or last follow-up. OS in patients who received HSCT was defined as the time from transplantation to death or last follow-up. Relapse incidence was defined as the probability of experiencing a relapse and death without relapse was considered a competing event. The parameters for univariate analyses of OS and relapse incidence included age at diagnosis, sex, platelet count, percentage of HbF, karyotype, and mutational status. For multivariate analyses, the Cox proportional hazard regression model was used. To evaluate correlations between clinical characteristics and mutational status, differences in continuous variables were analyzed using the Mann-Whitney U test and differences in frequencies were tested using the χ^2 test. When appropriate (because of small sample size), Fisher's exact test was used. p values less than 0.05 were considered statistically significant. These statistical analyses were performed with StatView-J 5.0 software (Abacus Concepts Inc., Berkeley, CA).

RESULTS

Mutation analysis. The results of the *PTPN11* and *NRAS/KRAS* mutational screening for the 71 Japanese children with JMML are listed in Table 2. We found *PTPN11* mutations in 32 of 71 (45%) patients. All mutations were missense changes, 30 of which were in exon 3 and two of which were in exon 13. Thirteen of 71 (18%) patients had *RAS* mutations, 11 of which were in *NRAS* and two of which were in *KRAS*. Ten of 13 patients with *RAS* mutations had been reported in a previous study (22). Three (4%) patients were clinically diagnosed with NF1. No patient with NF1 was found to have mutations in *PTPN11* or *RAS*, and 23 (32%) patients had neither a *PTPN11* mutation nor a *RAS* mutation, nor a clinical diagnosis of NF1.

Comparison of clinical characteristics of patients according to mutational status. We compared the laboratory and clinical parameters for patients with a *PTPN11* mutation, with a *RAS* mutation, and without any aberration (Table 3). In the

group corresponding to individuals with a *PTPN11* mutation, quite distinct characteristic features were evident, whereas there was no difference in the clinical characteristics displayed by individuals in the groups with a *RAS* mutation and no aberration. Patients with *PTPN11* mutations were significantly older at diagnosis (median: 35 mo) than those with *RAS* mutations (median: 10 mo; $p < 0.0001$) or those without any aberrations (median: 10 mo; $p = 0.0037$), and the presence of the *PTPN11* mutation in infants was rare (only two of 32 patients). In addition, the HbF level was significantly higher in the *PTPN11* mutation group than in the *RAS* mutation group or the group with no aberration (25.6 versus 8.6%; $p = 0.0026$ or versus 9.8%; $p = 0.0014$). The patients with monosomy 7 are known to have normal or only slightly elevated HbF levels (1). When the patients with monosomy 7 were excluded from the analysis, strong correlations of higher HbF level with *PTPN11* mutation compared with *RAS* mutation or no aberration were still observed ($p = 0.0004$ or $p = 0.0014$, respectively). Even after the groups other than the *PTPN11* group were combined (including the patients with NF1), the factors of older age at diagnosis and higher HbF level were still significantly different between the *PTPN11* mutation group and the other group. Karyotypic aberrations other than monosomy 7 occurred only in patients with the *PTPN11* mutation. Patients with a *PTPN11* mutation were more likely to receive HSCT than those with a *RAS* mutation or without any aberrations. As shown in Table 3, no correlation was observed between mutational subgroup and sex ratio, white blood cell count, or platelet count.

Because of the small number of patients in the NF1 group, we excluded these three patients from subgroup analysis. However, consistent with previous findings (1), children with NF1 had been given a diagnosis at an older age except for one patient with a family history (JMML was diagnosed in two girls with NF1 at 7 and 47 mo and in one boy with NF1 at 69 mo) but no other clinical parameters differed from those of the other mutational subgroups.

Prognostic impact of the GM-CSF signaling pathway-related genes. For all 71 children, the OS probability at 5 y was 43% (95% CI: 35–51), and the median follow-up time for all living patients was 59 mo (range, 13–240 mo). Given the quite distinct clinical characteristics of the *PTPN11* mutation group, which associated with recognized poor prognostic factors, we compared the clinical outcomes for patients with or without a *PTPN11* mutation. The survival of patients with a *PTPN11* mutation was significantly inferior to survival of patients without (25 *versus* 64%; $p = 0.0029$) as shown in Figure 1. Of the patients without *PTPN11* mutation, survival

Table 2. *PTPN11, NRAS, and KRAS mutations in 71 children with JMML*

Gene	No. of patients	Nucleotide substitution	Amino acid substitution
PTPN11	2	179G>T	Gly60Val
	4	181G>T	Asp61Tyr
	3	182A>T	Asp61Val
	1	214G>A	Ala72Thr
	5	215C>T	Ala72Val
	11	226G>A	Glu76Lys
	1	226G>C	Glu76Gln
	3	227A>G	Glu76Gly
	2	1508G>C	Gly503Ala
NRAS	3	34G>A	Gly12Ser
	2	34G>T	Gly12Cys
	1	35G>A	Gly12Asp
	3	38G>A	Gly13Asp
	1	181C>A	Gln61Lys
	1	182A>T	Gln61Leu
KRAS	1	35G>A	Gly12Asp
	1	35G>T	Gly12Val

Table 3. *Correlation between mutational status and clinical characteristics in JMML*

	Mutational group				
	PTPN11, $n = 32$ (45%)	*NRAS/KRAS*, $n = 13$ (18%)	p*	No aberrations, $n = 23$ (32%)	p*
Median age at diagnosis, mo	35	10	<0.0001	10	0.0037
Older than 24 mo, no.	24	1	<0.0001	8	0.0067
24 mo or younger, no.	8	12		15	
Gender, male/female					
Male, no.	22	8	NS	12	NS
Female, no.	10	5		11	
Median HbF level, %	25.6	8.6	0.0026	9.8	0.0014
More than 10%, no.	29	5	0.0008	11	0.0023
10% or less, no.	3	8		12	
Median WBC count, $\times 10^9$/L	27.7	29.4	NS	36.0	NS
Median platelets count, $\times 10^9$/L	38.5	55.0	NS	45.0	NS
Less than 40 $\times 10^9$/L, no.	17	5	NS	10	NS
40 $\times 10^9$/L or more, no.	15	8		13	
Cytogenetics					
Abnormal karyotype, no.	11	2	NS	2	NS
Monosomy 7, no.	4	2	NS	2	NS
Other abnormalities, no.	7	0		0	
Normal karyotype, no.	21	11		21	
No. of patients received HSCT	28	6	0.0107	11	0.0023

* These were compared with those of *PTPN11* group.
WBC, white blood cell; NS, not significant.

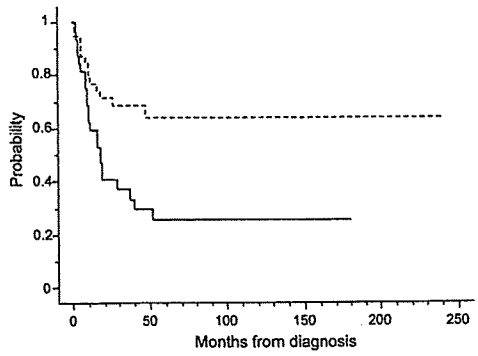

Figure 1. Kaplan-Meier estimate of overall survival in all patients according to *PTPN11* mutation status. *PTPN11*-mutation group (n = 32): *solid lines* *PTPN11* wild-type group (n = 39): *broken line*. The survival of *PTPN11*-mutation group was significantly inferior to survival of *PTPN11* wild-type group: 25% (95% CI: 17–33) *vs* 64% (95% CI: 56–72); p = 0.0029.

Table 4. *Probability of 5-y overall survival (OS) in 71 patients with JMML*

Variable	No. of patients	Probability (%)	95% CI	p
Mutational status				
PTPN11 mutation	32	25	17–33	0.0029
PTPN11 wild type	39	64	56–72	
RAS mutation	13	61	45–78	
No aberration	23	65	55–75	
Age at diagnosis				
Older than 24 mo	35	33	25–42	0.0030
24 mo or younger	36	58	48–67	
Cytogenetics				
Abnormal karyotype	16	22	11–34	0.0125
Normal karyotype	55	53	46–61	
Platelets count				
Less than 40×10^9/L	34	43	34–52	NS
40×10^9/L or more	37	49	40–58	
HbF level				
More than 10%	47	41	33–49	NS
10% or less	24	57	45–69	
Gender				
Male	43	43	35–51	NS
Female	28	49	38–60	

NS, not significant.

values for the *RAS* mutation group and the no aberration group were 61 and 65%, respectively. The three patients with NF1 all received HSCT. One patient died because of transplantation-related toxicity and the others survived without the disease. The prognostic significance of the initial clinical and laboratory parameters, together with mutational status, is shown in Table 4. In the univariate analysis, age greater than 24 mo (p = 0.0030) and presence of cytogenetic abnormality (p = 0.0125) were associated with poor prognosis, as was the presence of *PTPN11* mutation. Of particular interest cytogenetically is the fact that patients with monosomy 7 had a comparable outcome to that of children with a normal karyotype. However, all seven patients with an abnormal karyotype other than monosomy 7 died, and all had a *PTPN11* mutation. Multivariate analysis showed that none of the variables influenced survival (Table 6).

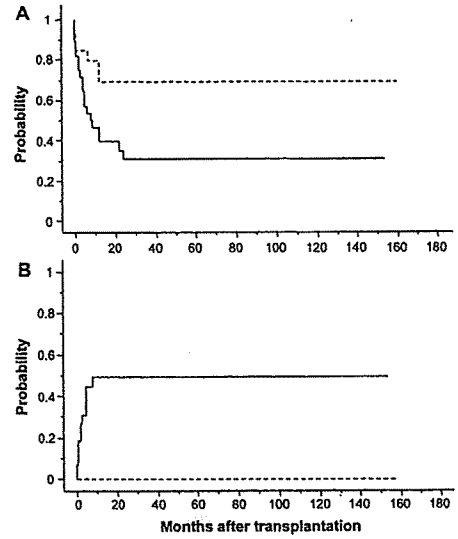

Figure 2. Kaplan-Meier estimate of overall survival and probability of relapse after HSCT in 48 patients according to *PTPN11* mutation status. *PTPN11*-mutation group (n = 28): *solid lines*. *PTPN11* wild-type group (n = 20): *broken lines*. (*A*) Overall survival. *PTPN11*-mutation group had significantly lower survival than *PTPN11* wild-type group: 30% (95% CI: 21–39) *vs* 69% (95% CI: 59–80); p = 0.018. (*B*) Relapse incidence. Whereas no relapse was observed in *PTPN11* wild-type group, the relapse incidence of *PTPN11*-mutation group was 49% (95% CI: 39–60); p = 0.001.

We then analyzed the prognostic value of *PTPN11* mutations in the 48 of 71 patients who received HSCT. *PTPN11* mutations were found in 28 of 48 (58%) patients, and of the 48 patients, 25 patients died after HSCT. As shown in Figure 2*A*, patients with a *PTPN11* mutation had significantly lower survival than patients without also in this cohort. (30 *versus* 69%; p = 0.018). We found that the presence of a *PTPN11* mutation was the most significantly associated factor with OS after HSCT, and followed by age greater than 24 mo and presence of cytogenetic abnormality (Fig. 3*A* and Table 5). No variables significantly associated with inferior survival after HSCT in a multivariate model (Table 6). In addition, we compared the probability of relapse after HSCT between patients with and without *PTPN11* mutations and found that the patients with a *PTPN11* mutation had significantly higher risk for relapse (p = 0.001) (Fig. 2*B*). No other variables including older age and cytogenetic abnormality arose statistically significant difference with the probability of relapse after HSCT (Fig. 3B and Table 5). Twelve patients died of relapse after transplantation and 13 died of transplantation-related toxicity. Notably, all 12 patients who died after relapse had a *PTPN11* mutation.

All four patients with a *PTPN11* mutation who did not receive HSCT died (at 3, 4, 19, and 29 mo after diagnosis), whereas 12 of 19 patients without a *PTPN11* mutation who did not receive HSCT remain alive, with a median follow-up of 80 mo (range, 21–240 mo) from diagnosis.

DISCUSSION

Since the discovery of *PTPN11* mutations in JMML (9), biomedical and molecular research on this disease has pro-

gressed rapidly, and data on molecular aberrations are now of great importance in the diagnosis of JMML. In the current study, we confirmed that *PTPN11* mutations are the most frequent molecular aberrations (45%) in Japanese children with JMML. If a *PTPN11* mutation is present, it is important to rule out the possibility of NS, especially in infants, because the JMML-like disorder in these patients may spontaneously disappear without therapy, so it is considered distinct from common JMML (31). All mutations detected in our cohort were located in exons 3 and 13 of the *PTPN11* gene, which accords with previous findings that mutations associated with JMML exist only in these two exons (9,16). In contrast, mutations in NS are located in a much broader range of locations, in exons 2, 3, 4, 7, 8, and 13 (29). Kratz *et al.* (32) clearly demonstrated that a different spectrum of *PTPN11* mutations between JMML and JMML-like disorder with NS. According to Kratz *et al.*, all mutations in the present study were those associated with JMML, not a JMML-like disorder. These findings suggest that our study population included only patients with common JMML and that the observed mutations are somatic changes.

The prevalence of *PTPN11* mutations in our cohort was slightly higher than that reported previously (9–11), and the prevalence of *RAS* mutations was comparable with that found in previous studies (2,6–8). In other studies, the proportion of patients with clinically diagnosed NF1 has been found to be 9 and 14% (1,33), but in our cohort, the proportion was smaller, only 3 of 71 (4%) patients. A similar NF1 prevalence (4 of 83 patients; 5%) was observed in an ongoing prospective study conducted by the MDS Committee of the Japanese Society of Pediatric Hematology, so NF1 might be less prevalent in the Japanese population. Another possibility is that NF1 was under diagnosed because of the paucity of signs and symp-

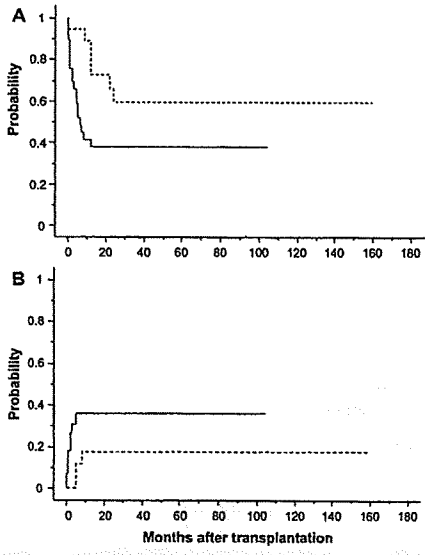

Figure 3. Kaplan-Meier estimate of overall survival and probability of relapse after HSCT in 48 patients according to age at diagnosis. Age >24 mo (n = 29): *solid lines.* Age ≦24 mo (n = 19): *broken lines.* (*A*) Overall survival. Age ≦24 mo: 59% (95% CI: 47–71) *vs* Age >24 mo: 38% (95% CI: 29–47); p = 0.033. (*B*) Relapse incidence. Age ≦24 mo: 19% (95% CI: 9–29) *vs* Age >24 mo: 35% (95% CI: 26–45); p = NS.

Table 6. *Multivariate analysis of survival for all 71 patients and 48 patients who received HSCT*

	Relative risk	95% CI	p
All patients (n = 71)			
PTPN11 mutation	1.854	0.852–5.139	NS
Older than 24 mo	2.011	0.925–4.790	NS
Abnormal karyotype	1.793	0.800–5.401	NS
Patients received HSCT (n = 48)			
PTPN11 mutation	2.226	0.852–5.814	NS
Older than 24 mo	1.707	0.742–3.925	NS
Abnormal karyotype	1.863	0.764–4.542	NS

NS, not significant.

Table 5. *Univariate analysis of 5-y overall survival (OS) and relapse incidence (RI) after HSCT in 48 patients with JMML*

		OS			RI		
Variable	No. of patients	Probability (%)	95% CI	p	Probability (%)	95% CI	p
Mutational status							
PTPN11 mutation	28	30	21–39	0.0181	49	39–60	0.0012
PTPN11 wild type	20	69	59–80		0	0–0	
RAS mutation	6	63	41–83		0	0–0	
No aberration	11	72	59–86		0	0–0	
Age at diagnosis							
Older than 24 mo	29	38	29–47	0.0331	35	26–45	NS
24 mo or younger	19	59	47–71		19	9–29	
Cytogenetics							
Abnormal karyotype	14	17	4–30	0.0474	48	32–64	NS
Normal karyotype	34	56	47–64		23	15–30	
Platelets count							
Less than 40×10^9/L	27	50	41–60	NS	22	13–30	NS
40×10^9/L or more	21	40	29–51		41	29–53	
HbF level							
More than 10%	37	45	37–54	NS	33	24–42	NS
10% or less	11	56	40–72		26	10–42	
Gender							
Male	29	40	30–49	NS	33	23–42	NS
Female	19	56	44–68		24	13–34	

NS, not significant.

toms in young children. Niemeyer *et al.* (1) found that patients with NF1 were more likely to have higher platelet counts and normal karyotypes. In our patients with NF1, no clinical parameters except for age seemed to differ from the other groups, although the number of patients was too small to draw any conclusions. The outcome of patients with NF1 remains unclear; therefore, further accumulations of prognostic data in this condition are needed.

Our analysis showed a striking correlation between mutational status and clinical and laboratory findings of known prognostic factors. Compared with the *RAS* mutation group and the no aberration group, age and HbF level at diagnosis were significantly higher in the *PTPN11* mutation group. Given that in previous reports older age at diagnosis and elevated HbF level have been repeatedly described as risk factors for survival (1,19–21,23–26), these results suggest that JMML with *PTPN11* mutation is a distinct subgroup and that the outcome for patients with this condition might be poorer. In this study, both *PTPN11* mutation and age were the strongest predictors of the probability of survival in univariate analyses. The poor survival of the *PTPN11* mutation group was also observed when only the patients who had been treated with HSCT were included in the analysis. Because multivariate analysis did not discriminate between age and *PTPN11* mutation, it remains unclear whether mutation in *PTPN11* is an independent predictor for poor survival. However, this could possibly be ascribed to the strong relationship between the *PTPN11* mutation group and older age. Poor outcome in patients with a *PTPN11* mutation may be due to the presence of several unfavorable factors, suggesting that previously recognized prognostic factors might reflect the genetic status.

Presently, HSCT is the only curative treatment for JMML; however, disease recurrence remains the major cause of treatment failure. Notably, mutation in *PTPN11* was the only risk factor for relapse after HSCT in our study. Previously published studies have found that older age, elevated HbF level, and abnormal karyotype are patient-specific risk factors for relapse after HSCT (20,21). The finding that our patients with a *PTPN11* mutation had an association with all these factors and our results on risk factors for relapse also support the idea that the genetic status may be an explanation of previous prognostic factors.

In our study, all 12 patients who relapsed after HSCT had a *PTPN11* mutation, suggesting that patients with *PTPN11* mutation may experience an aggressive clinical course. In addition, patients with *PTPN11* mutation were more likely to receive HSCT, also suggesting that there was a bias attributable to the aggressive clinical course in these patients. Indeed, all patients in the *PTPN11* mutation group who did not receive HSCT died, whereas five of seven patients in the *RAS* mutation group and seven of 12 patients in the no aberration group were alive without HSCT. Moreover, all patients with an abnormal karyotype other than monosomy 7 had a *PTPN11* mutation, and all died, suggesting that clones with a *PTPN11* mutation might be more likely to acquire additional chromosomal alterations.

To the best of our knowledge, this is the first report to investigate the prognostic relevance of the GM-CSF signaling pathway-related genes in patients with JMML and demonstrate the correlation between mutational status and recognized prognostic factors. The finding that mutations in *PTPN11* or *RAS* and a clinical diagnosis of NF1 were mutually exclusive is consistent with the idea that these molecules act in the same pathway. Nonetheless, the clinical features were quite different in these groups. This difference might be caused by distinct gain-of-function effects of each gene on the GM-CSF pathway and unknown additional genetic alterations may cooperate with these mutations. Furthermore, considering the present and previous findings together, the previously recognized prognostic factors might reflect the genetic status of this pathway. Further biologic studies are necessary to clarify what kind of genetic alterations cooperate with altered GM-CSF pathway-related genes during the development of JMML.

In conclusion, JMML with mutation in *PTPN11* seems to be a distinct subgroup with specific clinical characteristics and poor outcome. Consideration should be given to early HSCT therapy in this group of patients and better strategies to lower the risk of relapse in these patients are warranted.

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Treatment of Children With Refractory Anemia: The Japanese Childhood MDS Study Group Trial (MDS99)

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Background. Although hematopoietic stem cell transplantation (HSCT) is offered as a curative therapy for pediatric myelodysplastic syndrome (MDS), it may cause severe complications and mortality. Several reports have shown the efficacy of immunosuppressive therapy (IST) in adult patients with refractory anemia (RA), but its safety and efficacy remains to be fully elucidated in childhood RA. ***Procedure.*** Eleven children diagnosed with RA and enrolled on a prospective multicenter trial conducted by the Japanese Childhood MDS Study Group were eligible for analysis. If patients showed transfusion dependent or suffered from infection due to neutropenia, they received IST consisting of antithymocyte globulin (ATG), cyclosporine (CyA), and methylprednisolone (mPSL). ***Results.*** Eight children received IST, 2 received only supportive therapy, and one underwent HSCT without IST. Five (63%) of eight children who received IST showed hematological response. Of note, one patient showed the disappearance of monosomy 7 after IST. Responders were significantly younger than non-responders (29 months vs. 140 months; $P = 0.03$). No severe adverse events related to IST were reported in this study. Of 6 children with chromosomal abnormalities who received IST, four showed hematological response. The probability of failure-free and overall survival at 5 years was $63 \pm 17\%$ and $90 \pm 9\%$ respectively. ***Conclusion.*** IST is likely to be a safe and effective modality for childhood RA. Pediatr Blood Cancer 2009;53:1011–1015. © 2009 Wiley-Liss, Inc.

Key words: myelodysplastic syndrome; refractory anemia; children; immunosuppressive therapy

INTRODUCTION

Myelodysplastic syndrome (MDS) is a hematopoietic stem cell disorder and rarely occurs in childhood [1,2]. Refractory anemia (RA) is a subgroup of MDS with less than 5% of blasts in the bone marrow (BM) and little is known about childhood RA because of its rarity. European Working Group of MDS in Childhood (EWOG-MDS) retrospectively analyzed the clinical characteristics of children with RA [3]. They found that neutropenia and thrombocytopenia were more prominent than anemia [3,4] and karyotype had a strong impact on prognosis in children with RA [3]. Children with monosomy 7 were significantly more likely to progress to advanced disease and they recommended hematopoietic stem cell transplantation (HSCT) for this unfavorable group as early as possible, whereas, appropriate treatment for children with chromosomal abnormalities other than monosomy 7 and those with normal karyotypes remained to be determined.

Disturbance of the immune system may play a role in pathogenesis in some adults and children with RA [5–7]. Several reports have shown positive effects of immunosuppressive therapy (IST) in adult patients with RA [8–12]. The hematological response rate of IST was reported as 30–80% but IST could not restore the cytogenetic abnormalities or dysplastic features. Recently, EWOG-MDS reported the results of IST consisting of antithymocyte globulin (ATG) and cyclosporine A (CyA) in children with hypoplastic refractory cytopenia (RC) and normal karyotype or trisomy 8 who were thought as being at low risk of progression to advanced MDS [13]. However, the role of IST in children with RA has not been fully elucidated because the above study selected children with favorable predictive factors for a positive response to IST.

This study reports the outcome of 11 children with RA enrolled on a prospective multicenter trial (MDS99) conducted by the Japanese Childhood MDS Study Group, which applied IST with ATG and CyA to unselected patients who needed intervention.

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PATIENTS AND METHODS

Patients

Eleven children younger than 16 years of age were enrolled onto MDS99 from September 1999 to March 2004. They were diagnosed as having RA according to the French-American-British (FAB) classification [14] and diagnosis was confirmed by the central review of morphology by two independent investigators [15]. Cytogenetic analysis of the bone marrow cells was performed in each institution. There were no patients who had undergone previous chemotherapy or radiotherapy, nor patients with a history of congenital bone marrow failure syndrome or aplastic anemia in the analysis. The study was approved by the Steering Committee of the Japanese Childhood MDS Study Group and the institutional review boards of the participating institutions or the equivalent organization. Informed consent was obtained from the guardians of the patients.

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The authors report no potential conflicts of interest.

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Treatment Protocol

Each patient with RA required repetitive bone marrow aspiration at 6–8 weeks intervals in order to confirm the diagnosis. If the disease was stable and blood transfusion was not required, patients were observed closely without any therapy. If patients showed transfusion dependent or suffered from infection due to neutropenia, IST was administered as follows: horse ATG (15 mg/kg/day) for 5 days as a slow intravenous infusion over 12 hr, CyA (6 mg/kg/day given orally as an initial dose, and the dose was adjusted to achieve a whole blood trough level of 100–200 ng/ml) was started on day 1 and continued until day 180, and methylprednisolone (mPSL; 2 mg/kg/day) was administered intravenously on days 1–7, then mPSL was administered orally and slowly tapered from day 8 to end on day 29. In this study, the use of G-CSF was not restricted. HSCT was recommended when a patient showed no response to IST and required further intervention because of cytopenia or progression to more advanced disease.

Evaluation and Statistical Analysis

Response to IST was evaluated at 6 months. Complete response (CR) was defined as a neutrophil count $>1.5 \times 10^9$/L, platelet count $>100 \times 10^9$/L, and hemoglobin (Hb) level of >11.0 g/dl. Partial response (PR) was defined as a neutrophil count $>0.5 \times 10^9$/L, platelet count $>20 \times 10^9$/L, and Hb level of >8.0 g/dl. When neither the CR nor the PR criteria were met, a patient was considered as no response (NR) to IST.

Mann–Whitney test and Fisher's exact test were applied to evaluate the differences between patients that responded to IST and those who did not. Failure-free survival (FFS) was calculated from the date of initiating IST to the date of treatment failure as follows; death, no response to IST at 6 months, HSCT, a second course of IST, acquisition of chromosomal abnormality, progression to advanced disease, or relapse. Overall survival (OS) was calculated from the date of diagnosis to the date of death or last follow-up. Both FFS and OS were estimated by the Kaplan–Meier method.

RESULTS

Patient Characteristics

Eleven children, 6 males and 5 females, were analyzed in this study (Table I). The median age at diagnosis was 67 months (range, 9 months to 15 years). Eight of 11 children had neutrophil counts of less than 1.5×10^9/L. All except 1 patient had Hb levels below 10 g/dl. Eight patients had platelet counts below 50×10^9/L. In total, one patient had anemia only, five had bi-cytopenia (anemia and neutropenia 2, anemia and thrombocytopenia 2, and neutropenia and thrombocytopenia 1), and five had pancytopenia at diagnosis. Since bone marrow biopsy specimen was available in only 6 of 11 cases, we determined cellularity by central pathological review from bone marrow smear rather than biopsy specimens and used a more suitable term, cell content, instead of cellularity in this report. Overall, there were only three patients in whom BM cell content was low. All patients showed dysplasia in multilineage series, which was compatible with the definition of refractory cytopenias with multilineage dysplasia (RCMD) in the World Health Organization (WHO) classification [16]. Data on the cytogenetic analyses at diagnosis were available for all patients. Karyotype was normal in three patients, and of the remaining eight patients, two had monosomy 7, two had trisomy 8, and four had other abnormalities; del (7)(q11), i(8)(q10), 20q-, and +der(1;19)(q10;q10). Of four patients in whom presence of paroxysmal nocturnal hemoglobinuria (PNH) cells was assessed by flow cytometry, none showed an expansion of PNH clone. Of five patients in whom data on HLA-DR was available, only one patient showed DR2 antigen, which is a broad antigen of DR15 and DR16.

TABLE I. Patients Characteristics

	Median (range)
Age	5y7m (9m to 15y5m)
Gender	M/F = 6:5
WBC ($\times 10^9$/L)	3.8 (1.1–12.5)
Neutrophil ($\times 10^9$/L)	0.94 (0.16–8.1)
PB blast (%)	0 (0)
Hb (g/dl)	6.2 (3.6–11.7)
Reticulocyte (%)	2 (1–44)
Reticulocyte ($\times 10^9$/L)	41.7 (12.3–572.0)
MCV (fl)	104 (84–123)
Plt ($\times 10^9$/L)	23.0 (3.0–117.0)
BM blast (%)	1.0 (0–4.8)
BM cell content	Low 3, normal 5, high 3
Chromosome	Normal/abnormal = 3:8
Cytopenia[a]	Anemia only 1, bi-cytopenia 5, pancytopenia 5

[a]Cut-off; neutrophils <1,500/μl, Hb < 10.0 g/dl, Plt < 50,000/μl.

Observation Without Intervention

Figure 1 shows the outcome of the 11 patients analyzed. Of 3 patients who initially received only supportive therapy, one with normal karyotype was still stable without therapy, one with trisomy 8 showed spontaneous improvement of anemia but the chromosomal abnormality remained. One with 20q- (UPN 046) showed stable disease for 2 years, but cytopenia deteriorated and IST was initiated at 968 days after diagnosis.

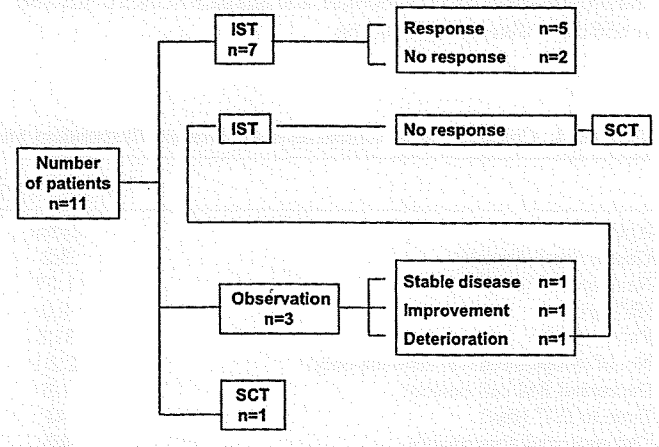

Fig. 1. Outcome of 11 patients with refractory anemia. SCT, stem cell transplantation; IST, immunosuppressive therapy.

Immunosuppressive Therapy

Seven patients received IST as the first-line treatment and one (UPN 046) received IST because of recurrence of cytopenia after 2-year observation. IST was given at a median of 42 (range 0–968) days after the diagnosis of RA. Five of eight patients showed response to IST at 6 months after the initiation of treatment (response rate was 63%; CR 2, PR3). Of five responders, three were able to successfully discontinue IST and remained disease-free, and the remaining two patients have been continuing therapy. Of note, the disappearance of a monosomy 7 clone after IST was observed in UPN 035 [17] and the patient is still in remission after 63 months. Of three non-responders, one was lost to follow up, one responded to a second course of IST, and one (UPN 046) underwent HSCT 3 months after initiating IST.

To address predictive factors for response to IST, the characteristics were compared between children who responded to IST and those who did not (Table II). The age at diagnosis was significantly younger in responders than in non-responders (median 29 months vs. 140 months; $P=0.03$), whereas there was no statistically significant associations between response to IST and sex, neutrophil count, Hb level, platelet count, interval from diagnosis to IST, chromosomal abnormality, BM cell content, or number of cytopenia. Serious adverse events related to IST were not observed, including the progression to advanced disease. The most frequent adverse event in this study was pyrexia.

Hematopoietic Stem Cell Transplantation

Two children underwent HSCT in this series. One patient with 20q- (UPN 046) received bone marrow transplantation (BMT) from her human leukocyte antigen (HLA) 1-locus-mismatched father at 1,088 days after diagnosis because of non-response to IST. This patient suffered from adenoviral colitis, salmonella colitis, herpes zoster, and grade III acute GVHD of the skin, however, she is still alive without disease 23 months after BMT. One other patient with monosomy 7 (UPN 053) received BMT from a matched unrelated donor on 537 days after diagnosis without IST by physician's decision. His post-transplant course was uneventful, but disease relapsed 151 days after transplantation. A BM specimen at relapse showed severe fibrosis and progression to overt leukemia, and this patient died of disease at 656 days after transplantation.

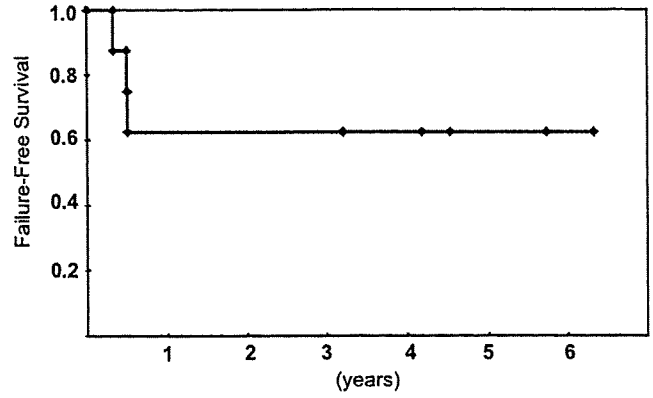

Fig. 2. Kaplan–Meier estimate of failure-free survival of patients who received immunosuppressive therapy. Failure-free survival was calculated from the date of initiating IST to the date of treatment failure as follows; death, no response to IST at 6 months, HSCT, a second course of IST, acquisition of additional chromosomal abnormality, progression to advanced disease, or relapse. The 5-year failure-free survival was $63 \pm 17\%$ (n = 8). Median follow-up was 1,346 days.

Chromosomal Abnormality

There were eight children with chromosomal abnormality in this study. Of those, six received IST and four showed responses to IST, including one with cytogenetic response (UPN 35).

Survival

Of eight children who received IST, three non-responders were considered as treatment failure. No patient died with IST after a median follow-up of 1,346 days; the 5-year FFS was $63 \pm 17\%$ (Fig. 2). Of total, 10 patients are alive after a median follow-up of 1,685 days; the 5-year OS was $90 \pm 9\%$ (Fig. 3).

DISCUSSION

Although HSCT is the curative modality for children with MDS, it may cause severe complications, mortality, and late sequelae. Several reports have shown encouraging results from the use of IST in adults with RA, and the hematological response rate to IST was 30–80% [8–12]. Yoshimi et al. [13] reported on 31 children with hypoplastic RC and normal karyotype or trisomy 8 treated with IST, which resulted in a response rate at 6 months of 71%, 3-year OS of

TABLE II. Comparison of Characteristics Between Responders and Non-Responders to IST

	Responder (n = 5)	Non-responder (n = 3)	*P*-value
Age[a]	2y5m	11y8m	0.03
Gender (male/female)	3:2	1:2	n.s.
Neutrophils[a] ($\times 10^9$/L)	1.27	0.63	n.s.
Hb[a] (g/dl)	8.0	6.2	n.s.
Plt[a] ($\times 10^9$/L)	31.0	20.0	n.s.
No. of cytopenia (tri-/bi-/anemia only)	2:2:1	2:1:0	n.s.
Decreased BM cell content	1/5	2/3	n.s.[b]
Time to IST[a] (day)	42	42	n.s.[b]
Chromosomal abnormality	4/5	2/3	n.s.[b]

[a]Median; [b]Evaluated by Mann–Whitney test and Chi-square test.

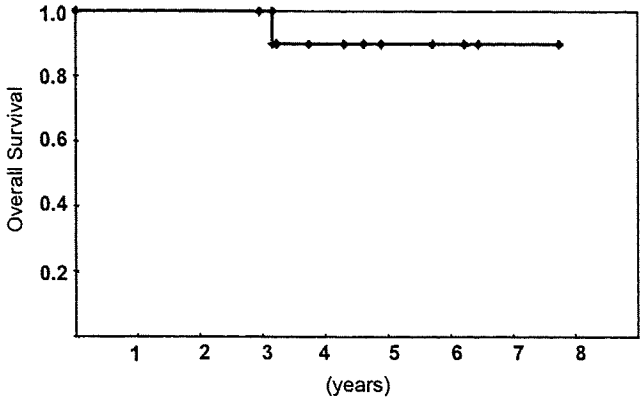

Fig. 3. Kaplan–Meier estimate of overall survival of all evaluable patients. Overall survival was calculated from the date of diagnosis to the date of death or last follow-up. The 5-year overall survival was 90 ± 9% (n = 11). Median follow-up was 1,685 days.

88%, and 3-year FFS of 57%. In contrast to the larger series by Yoshimi et al. children with RA and karyotypic abnormalities or non-hypoplastic marrow were also enrolled in this study. Overall, 5 of 8 patients (63%) responded to IST, and similar responses were observed in two-thirds of patients with chromosomal abnormalities. Patients whose BM cell content was not low also responded to IST (responder 4, non-responder 1); however, the significance of cellularity in pediatric RA still needs further study. No severe adverse events, disease progression, or death due to any cause after IST was reported. Only one death in this study was due to disease progression after HSCT, which was not related with IST. As a whole, the 5-year OS and FFS were 90% and 63%, which were comparable with the previous study in adult MDS and superior to our previous retrospective analysis of children with RA (4-year OS was 79%) [2]. Therefore, although the number of subjects was limited, we infer from these results that the IST is effective and safe for children with MDS.

The rationale for IST used as treatment of RA is based on previous studies, which suggested that alterations in the immune system might contribute to the pathogenesis in some subgroups of RA [5–7]. Dysregulated T cells are thought to destroy normal hematopoietic cells as bystanders as well as MDS clones [6]. IST can reduce MDS clone-specific T cells and improve normal hematopoiesis, but cytogenetic abnormalities and dysplastic features often persist [9,11,12]. However, in this study one patient showed the disappearance of karyotypic abnormalities. In addition, three of the responders were able to successfully discontinue IST. These results might be explained by the findings that the residual healthy stem cells can compensate for the loss of stem cells after the immune-mediated destruction is interrupted by IST in the setting of aplastic anemia [18,19]. Recovery of healthy hematopoiesis might outstrip MDS clones in these patients. In the patient with monosomy 7 who experienced cytogenetic response another mechanism could be speculated. The investigators from the EWOG-MDS reported that almost half of children with RA had monosomy 7 and they were likely to experience disease progression [3]. In contrast, anecdotal case reports described a decline or disappearance of a monosomy 7 clone [20]. Sloand et al. [21] reported paradoxical responses of monosomy 7 cells to G-CSF. Namely, high concentrations of G-CSF induced significant proliferation of monosomy 7 cells, but survival and proliferation of monosomy 7 cells were inferior to those of diploid cells at lower G-CSF levels. Thus, there is a possibility that the recovery of normal hematopoiesis after the administration of IST might affect the intrinsic level of G-CSF and survival of monosomy 7 cells. However, the interpretation of the present results still needs caution because most patients with RA and monosomy 7, including another case in this study, showed poor prognosis.

Previous studies on IST in adult RA found some factors that could predict good responders to IST, such as younger age, shorter duration of transfusion dependence, HLA-DR15, and presence of an expanded clone of PNH cells [8,10–12]. In this study, age was the only factor that showed a statistically significant difference between responders and non-responders to IST. The European study published by Yoshimi et al. [13] also contained older patients, but the proportion and treatment responses of older patients were not shown. Therefore, the effects of patient age on pathophysiology of pediatric RA and treatment response remain to be elucidated. Of the limited cases who were examined, no patient showed an expansion of PNH clone and only one patient had HLA-DR2 antigen, who responded to IST well. We did not systemically examine the immunological status such as TCR Vbeta repertoire [7] in this study. Clinical trials, including systematic studies on immunological status, are required to investigate prognostic factors more precisely in childhood RA because the sample size in this study was small.

Thus, a significant drawback of our study was small size of registered patients. We assumed that considerable number of patients with RA did not enter this study and might have received HSCT without IST. In fact, retrospective analysis of pediatric MDS in Japan showed that 52 patients with RA were diagnosed by the central morphological review between 1999 and 2006 [22]. Consecutive enrollment on both diagnostic and therapeutic trials would be essential for a future trial. It might allow the determination of biologic parameters that correlated with clinical characteristics.

In conclusion, the present results suggest the efficacy and safety of IST for children with RA. Disease-free status might be expected with IST in a subset of patients. Chromosomal aberration was not an absolute contraindication for IST, whereas using this approach for patients with monosomy 7 has not been substantiated. A larger prospective study including biological surrogate markers for therapeutic interventions would be important to elucidate the clinical characteristics of this rare disease as well as the prognostic factors and mechanism of IST.

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Downregulation of *GATA-2* and overexpression of adipogenic gene-PPARγ in mesenchymal stem cells from patients with aplastic anemia

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Aplastic anemia (AA) is characterized by a reduced number of hematopoietic stem cells and fatty replacement in the bone marrow. Transcriptional factor *GATA-2* plays several important roles in both hematopoiesis and adipogenesis. Decreased levels of *GATA-2* compromise the proliferation and survival of hematopoietic stem cells. *GATA-2* suppresses adipocyte differentiation through direct inhibition of adipogenic factors, including peroxisome proliferator-activated receptor−γ *(PPARγ)*. Previous studies have shown that expression of *GATA-2* is decreased in marrow CD34-positive cells in AA. To elucidate the mechanisms of fatty marrow replacement, we evaluated the mRNA expression for *GATA-2* and *PPARγ* in mesenchymal stem cells (MSCs) from patients with AA by quantitative real-time polymerase chain reaction. *GATA-2* expression by MSCs from AA patients was significantly lower than in normal subjects. Conversely, expression of *PPARγ* was significantly higher in AA patients. Western blot analysis demonstrated that protein levels of *GATA-2* were lower in AA patients than those in normal subjects. Moreover, incubation with interferon-γ induced downregulation of *GATA-2* levels in MSCs from normal subjects. These findings indicate that fatty marrow replacement in AA patients can be explained by downregulation of *GATA-2* and overexpression of *PPARγ* in MSCs. Decreased expression of *GATA-2* might be responsible for the pathogenesis and development of the clinical features of the disease. © 2009 ISEH - Society for Hematology and Stem Cells. **Published by Elsevier Inc.**

Aplastic anemia (AA) is characterized by a reduced number of hematopoietic stem cells (HSCs) and fatty replacement in bone marrow (BM), resulting in pancytopenia. To date, most efforts by investigators have concentrated on the elucidation of immune-mediated mechanisms of hematopoietic cell destruction [1–3]. Although replacement of hematopoietically active marrow with fat cells is another characteristic feature of AA, the fat cells themselves have received little attention, and the mechanisms of fatty marrow replacement remain unclear.

The transcription factor *GATA-2* is expressed in HSCs and early progenitors and plays a critical role in hematopoiesis [4]. Decreased levels of *GATA-2* compromise proliferation and survival of HSCs [5,6]. Considering the critical role of *GATA-2* in hematopoiesis, Fujimaki et al. quantified the levels of mRNA of *GATA-2*, which were markedly lower in AA patients when compared with normal subjects [7]. GeneChip analysis also confirmed that *GATA-2* gene expression was downregulated in CD34-positive marrow cells from AA patients [8]. These findings suggest that aberrant expression of *GATA-2* is responsible for the pathogenesis of AA.

Adipocytes are differentiated from preadipocytes, which are thought to emerge from multipotent mesenchymal stem cells (MSCs) [9]. *GATA-2* is specifically expressed in not only hematopoietic tissues, but also preadipocytes, and is known to be an important adipogenic regulator. Preadipocytes are the main components of the BM microenvironment and *GATA-2* suppresses adipocyte differentiation, keeping the cells at the preadipocyte stage. Suppression of adipocyte differentiation by *GATA-2* is mediated through

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direct inhibition of adipogenic transcriptional factors, including peroxisome proliferator-activated receptor−γ (*PPARγ*) [10]. *PPARγ* has been shown to be expressed in both preadipocytes and MSCs [11]. These observations prompted us to investigate *GATA-2* and *PPARγ* mRNA expression in MSCs derived from AA patients. We show here that *GATA-2* expression by MSCs from AA patients was reduced significantly, while expression of *PPARγ* was significantly elevated in AA patients when compared with normal subjects. Our findings may help to clarify the mechanisms of fatty marrow replacement in AA patients.

Materials and methods

Patients

Thirty-four AA patients and 15 normal subjects were included in the study. Clinical characteristics of these patients are summarized in Table 1. Diagnosis of AA was established by peripheral blood counts and BM findings, and disease severity was classified using international criteria [12]. Patients were screened for Fanconi anemia and paroxysmal nocturnal hemoglobinuria (PNH) by chromosome breakage analysis and flow cytometry using anti-CD55 and anti-CD59 antibodies. Cytogenetic studies were performed to exclude myelodysplastic syndrome. Aplasia was secondary to acute hepatitis in 4 patients and the cause was unknown in 30 patients. Median age at diagnosis was 10 years, ranging from 2 to 21 years. Of the 19 immunosuppressive therapy (IST)–treated patients, 11 responded and were transfusion-independent at the time of study, while the remaining patients were nonresponders.

Generation of MSCs

BM was collected either at the time of diagnosis or after IST with antithymocytoglobulin and cyclosporine. Response criteria were as described previously [13]. Normal BM samples were obtained from 15 allogenic BM transplantation donors ranging in age from 4 to 45 years. The Institutional Review Board of the Nagoya University Graduate School of Medicine approved the study and written informed consent was obtained from patients or their guardians.

Mononuclear cells were isolated from BM samples by Ficoll-Hypaque density gradient centrifugation. A total of 1×10^7 BM mononuclear cells were cultured in MesenCult Basal Medium (Stem Cell Technologies, Vancouver, Canada) supplemented with human mesenchymal stem cell supplements (Stem Cell Technologies) in a T25 tissue culture flask (Falcon, Franklin Lakes, CA, USA), followed by incubation at 37°C and 5% CO_2 in an incubator for 48 hours. Whole medium and nonadherent cells were then removed and replaced with fresh medium until confluence. At the end of the culture period, residual nonadherent cells and medium were removed by washing with phosphate-buffered saline (PBS) before trypsinization. Adherent cells were trypsinized, washed, and expanded to the second generation. Third-generation MSCs were used in all experiments.

Table 1. Clinical characteristics of patients with aplastic anemia

Clinical characteristics	
No. of patients	34
Median age (y) at diagnosis (range)	10 (2 – 21)
Gender: male/female	16/18
Etiology	
Idiopathic	30
Hepatitis	4
Disease severity at diagnosis	
Very severe	7
Severe	19
Nonsevere	8
Treatment at the time of study	
None	15
After IST	
Responder	11
Nonresponder	8
Duration (mos) from diagnosis to the time of study, median (range)	28 (0 – 308)

IST = immunosuppressive therapy.

Characterization of MSC

MSC immunophenotype was determined using a FACSCaliber flow cytometer (Becton Dickinson Biosciences, San Diego, CA, USA). Anti-human CD13-fluorescein isothiocyanate (FITC), anti-human CD14-FITC, anti-human CD44-PE, and anti-human CD45-FITC were purchased from Immunotech (Marseille, France); anti-human CD29-PE, anti-human CD73-PE, anti-human CD90-PE, anti-human CD106-PE, anti-human CD166-PE, and anti-human human leukocyte antigen–D-related FITC were purchased from Becton Dickinson Biosciences. Anti-human CD34-FITC and anti-human CD105-PE were purchased from DAKO (Glostrup, Denmark) and Beckman Coulter (Marseille, France), respectively. After trypsinization, MSCs (1×10^5) were stained with 5 μg each monoclonal antibody for 30 minutes at 4°C. Cells were washed with 1% fetal calf serum containing PBS and resuspended in 300 μL PBS before analysis.

MSCs demonstrated adipocytogenic and osteocytogenic differentiation as follows. For differentiation into adipocytes, MSCs were seeded at 2×10^5/T25 flask in MesenCult Basal Medium supplemented with human adipocytogenic supplements (Stem Cell Technologies). Culture medium was refreshed every 3 days. After 3 weeks, morphological changes were examined under an inverted microscope. Typical adipocytes had lipid droplets and were stained with Oil Red O. For differentiation into osteoblasts, osteogenic supplement (Stem Cell Technologies) was used instead of adipogenic supplement. Cell layers were stained with silver nitrate to detect calcium deposition.

Quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNA was extracted from third-passage MSCs using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and concentrations of extracted RNA were measured by spectrophotometry. Synthesis of complementary DNA (cDNA) was performed using a Thermoscript RT-PCR system (Invitrogen, San Diego, CA, USA) according to manufacturer's instructions.

QRT-PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Branchburg, NJ, USA). Ready-made primers and TaqMan probes for *glyceraldehyde phosphate dehydrogenase* (*GAPDH*) (HS99999905_m1), *PPARγ* (Hs00234592_m1), and *GATA-2* [7] were purchased from Applied Biosystems.

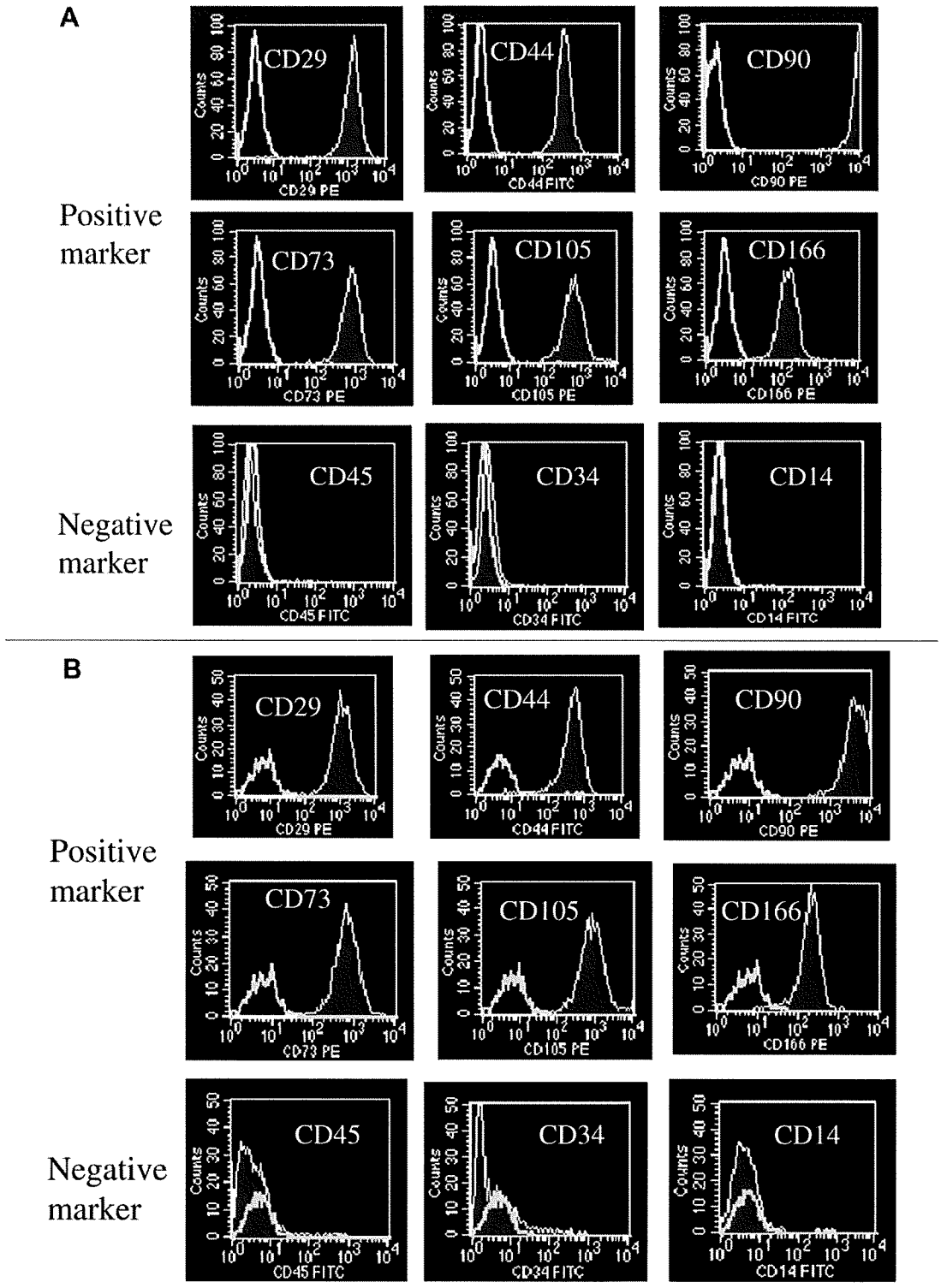

Figure 1. Representative phenotype of mesenchymal stem cells (MSCs) from a normal subject (**A**) and a patient with aplastic anemia (**B**). MSCs express adhesion markers CD29, CD44, CD90, CD73, CD105, and CD166, but not hematological markers CD34, CD45, and CD14. There were no significant differences in the expression of MSC markers between aplastic anemia patients and normal subjects.

PCR was performed in a total volume of 15 μL, containing each fluorogenic probe at 100 nM, each primer at 0.2 μM, 1 × TaqMan Universal PCR Mastermix (Applied Biosystems) and 10 ng sample cDNA or various amounts (0.01, 0.1, 1, 10, or 100 ng) of standard cDNA. Standard RNA was extracted from the K562 cell line for *GATA-2* and MOLM-13 cell line for *PPARγ*. Each test and standard cDNA was amplified in triplicate. PCR conditions were as follows: 2 minutes at 50°C; 10 minutes at