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). Germ-line 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

Received July 7, 2008; accepted October 6, 2008.

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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
<i>PTPN11</i>	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
	3	34G>A	Gly12Ser
	2	34G>T	Gly12Cys
<i>NRAS</i>	1	35G>A	Gly12Asp
	3	38G>A	Gly13Asp
	1	181C>A	Gln61Lys
	1	182A>T	Gln61Leu
	1	35G>A	Gly12Asp
<i>KRAS</i>	1	35G>A	Gly12Asp
	1	35G>T	Gly12Val

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

	Mutational group				
	<i>PTPN11</i> , <i>n</i> = 32 (45%)	<i>NRAS/KRAS</i> , <i>n</i> = 13 (18%)	<i>p</i> *	No aberrations, <i>n</i> = 23 (32%)	<i>p</i> *
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				
<i>PTPN11</i> mutation	32	25	17–33	0.0029
<i>PTPN11</i> wild type	39	64	56–72	
<i>RAS</i> 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 $40 \times 10^9/L$	34	43	34–52	NS
$40 \times 10^9/L$ 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 2A, 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. 3A 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. 2B). 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); $p = \text{NS}$.

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

	Relative risk	95% CI	<i>p</i>
All patients ($n = 71$)			
<i>PTPN11</i> 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 ($n = 48$)			
<i>PTPN11</i> 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

Variable	No. of patients	OS			RI		
		Probability (%)	95% CI	<i>p</i>	Probability (%)	95% CI	<i>p</i>
Mutational status							
<i>PTPN11</i> mutation	28	30	21–39	0.0181	49	39–60	0.0012
<i>PTPN11</i> wild type	20	69	59–80		0	0–0	
<i>RAS</i> 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.

Acknowledgments. The authors thank all of the patients, families, and referring physicians who contributed samples to the investigators.

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