improvement was 6.3 weeks) in all Japanese patients with anemia in low- or intermediate-1-risk MDS with del 5q, and transfusion independence in patients who were transfusion dependent at baseline, demonstrating that lenalidomide can effectively improve anemia in Japanese MDS patients. Moreover, the effect of lenalidomide was persistent in all the patients at the data cutoff date (median duration of the study, 48.1 weeks; range, 28.1–56.1 weeks). The median duration of improvement in anemia was not estimable.

The effect of anemia improvement observed in the present study compare favorably with the results from the US pivotal clinical study MDS-003 [9].

Of note is not only the achievement of transfusion independence in patients with RBC transfusion-dependent anemia, but also the increase of blood hemoglobin concentration to at least 10 g/dL in this study. In most western countries, the threshold for receiving an RBC transfusion for anemia associated with MDS is a blood hemoglobin concentration less than 10 g/dL [15]. In Japan, in contrast, RBC transfusion is generally avoided whenever possible, initiated only when the blood hemoglobin concentration is below 7 g/dL. Such a clinical practice is also apparent in the present study, as patients with blood hemoglobin concentration as low as 4.7 g/dL at baseline had not been heavily transfused. Improvement of anemia in Japanese MDS patients not receiving RBC transfusion, thus, is as clinically important as successful achievement of independence from RBC transfusion in western countries.

Histopathologic findings confirmed improvement of the dysplasia of megakaryocytes, paralleling the improvement in anemia from lenalidomide. Moreover, cytogenetic evaluation demonstrated improvement of chromosome abnormality with del(5q) in 10 patients on day 85, which suggests that the cytogenetic response and the Hb increase run parallel, although a relation between maintenance of the anemia improvement and the cytogenetic response was not observed.

In the cytogenetic evaluation, a new complex karyotype, del(20q),del(5q), was detected in one patient. The incidence of del(5q) accompanied with del(20q) was reported in 8% [16]. Considering more than a 2-year history of MDS in this patient, it would be likely that a progression to this complex karyotype was the natural course of MDS. The del(5q) accompanied with del(20q) is presumed to be less sensitive to lenalidomide than the isolated del(5q). It may be considered that the del(5q),del(20q) became obvious in this study, since the original del(5q) abnormality disappeared due to the lenalidomide treatment. In these 2 years, lenalidomdie-inducing risks of clonal evolution and AML progression have been discussed, but several recent studies reported that the risk of AML progression for patients who received lenalidomide was similar to those in the historical data [17, 18].

In a previous US study MDS-003, approximately half of the patients reported lenalidomide-related neutropenia and thrombocytopenia of grade 3 or higher (by the Common Toxicity Criteria of the National Cancer Institute, Version 2.0), which were the most common reasons for the dose adjustment of lenalidomide [9]. In the present study, the adverse events, most notably neutropenia, did not lead to treatment discontinuation, and were manageable with dose reduction, dose interruption or symptomatic treatment.

Lenalidomide was absorbed with maximum plasma concentrations occurring at approximately 3 h following oral administration and then rapidly eliminated from plasma ($t_{1/2}$ was less than 4 h and $CL/F$ was about 190 mL/min). Multiple dose (10 mg $\times$ 4 days) did not cause accumulation of lenalidomide ($C_{max}$/AUC ratios were 1.16 and 1.04 for day1 and day4, respectively). Other PK parameters were similar between day 1 and day 4. It was also reported that lenalidomide was rapidly adsorbed and eliminated in healthy volunteers. Moreover, multiple dosing did not result in drug accumulation [19].

Oral lenalidomide confers less burden on the patients than transfusion therapy and can improve the quality of life. The results of the present study indicate that lenalidomide can be a useful drug for the treatment of MDS with del(5q) in Japanese patients as well. In the absence of approved ESA for MDS, such as erythropoietin, lenalidomide is potentially a new therapeutic option of great clinical significance for the treatment of anemia in Japanese patients with del(5q) MDS.

## References

1. Heaney ML, Golde DW. Myelodysplasia. N Engl J Med. 1999; 340:1649–60.
2. Greenberg PL. Myelodysplastic syndrome. In: Hoffman R, Benz Jr EJ, Shatil SJ, Furie B, Silberstein LE, McGlave P, editors. Hematology: basic principles and practice. 3rd ed. New York: Churchill Livingstone; 2000. p. 1106–29.
3. Rose EH, Abels RI, Nelson RA, McCullough DM, Lessin L. The use of r-HuEpo in the treatment of anaemia related to myelodysplasia (MDS). Br J Haematol. 1995;89:831–7.
4. Hellström-Lindberg E, Negrin R, Stein R, Krantz S, Lindberg G, Vardiman J, et al. Erythroid response to treatment with G-CSF plus erythropoietin for the anaemia of patients with myelodysplastic syndromes: proposal for a predictive model. Br J Haematol. 1997;99:344–51.
5. Malcovati L, Porta MG, Pascutto C, Invernizzi R, Boni M, Travaglino E, et al. Prognostic factors and life expectancy in myelodysplastic syndromes classified according to WHO criteria: a basis for clinical decision making. J Clin Oncol. 2005;23: 7594–603.
6. Takatoku M, Uchiyama T, Okamoto S, Kanakura Y, Sawada K, Tomonaga M, et al. Retrospective nationwide survey of Japanese patients with transfusion-dependent MDS and aplastic anemia highlights the negative impact of iron overload on morbidity/mortality. Eur J Haematol. 2007;78:487–94.

7. Galustian C, Labarthe MC, Bartlett JB, Dalgleish AG. Thalidomide-derived immunomodulatory drugs as therapeutic agents. Expert Opin Biol Ther. 2004;4:1963–70.
8. Greenberg P, Cox C, Le Beau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood. 1997;89:2079–88.
9. List A, Dewald D, Bennett J, Giafounidis A, Raza A, Feldman E, et al. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. N. Engl. J. Med. 2006;355:1456–65.
10. National Comprehensive Cancer Network® (NCCN) (2009). Clinical practice guideline in oncology™. Myelodysplastic syndromes. Version 1. Available at http://www.nccn.org/professionals/physician_gls/PDF/mds.pdf.
11. Tasaka T, Tohyama K, Kishimoto M, Ohyashiki K, Mitani K, Hotta T, et al. Myelodysplastic syndrome with chromosome 5 abnormalities: a nationwide survey in Japan. Leukemia. 2008;22:1874–81.
12. Chen B, Zhao W-L, Jin J, Xue Y-Q, Cheng X, et al. Clinical and cytogenetic features of 508 Chinese patients with myelodysplastic syndrome and comparison with those in western countries. Leukemia. 2005;19:767–75.
13. Cheson BD, Bennett JM, Kantarjian H, Pinto A, Schiffer CA, Nimer SD, et al. Report of an international working group to standardize response criteria for myelodysplastic syndromes. Blood. 2000;96:3671–4.
14. An international system for human cytogenetic nomenclature. Shaffer LG, Tommerup N, editors. Basel: S Karger; 2005.
15. Alessandrino EP, Amadori S, Barosi G, Cazzola M, Grossi A, Liberato LN, et al. Italian Society of Hematology: evidence- and consensus-based practice guidelines for the therapy of primary myelodysplastic syndromes, a statement from the Italian Society of Hematology. Haematologica. 2002;87:1286–306.
16. Hasse D. Cytogenetic features in myelodysplastic syndromes. Ann Hematol. 2008;87:515–26.
17. List AF, Giagounidis A, Brandenburg N, Wride K, Glasmacher A, Germing U. Risk factors for AML transformation and mortality in transfusion-dependent deletion 5q MDS. Haematologica. 2008;93(s1):287. Abstract.
18. Giagounidis A, Hasse S, Platzbecker U, Germing U, Lohrbacher V, Aul C. Risk factors fpr disease progression in del(5q) myelodysplastic syndromes patients treated with lenalidomide. Blood. 2007;110:1460. Abstract.
19. Celgene Corporation REVLIMID® (lenalidomide) packaging insert. Available at http://www.celgene.com/products/revlimid-thamolid-home.aspx.

ORIGINAL ARTICLE

# The impact of cytogenetic abnormalities on the prognosis of primary myelofibrosis: a prospective survey of 202 cases in Japan

Tomonori Hidaka[1], Kotaro Shide[1], Haruko Shimoda[1], Takurou Kameda[1], Keiko Toyama[1], Keiko Katayose[1], Youko Kubuki[1], Kenji Nagata[1], Katsuto Takenaka[2], Koichi Akashi[2], Takashi Okamura[3], Yoshiyuki Niho[2,4], Hideaki Mizoguchi[5], Mitsuhiro Omine[6], Keiya Ozawa[7], Mine Harada[2], Kazuya Shimoda[1]

[1]Department of Gastroenterology and Hematology, Miyazaki University, Miyazaki, Japan; [2]Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan; [3]Division of Hematology, Department of Medicine, and Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, Kurume, Fukuoka, Japan; [4]Chihaya Hospital, Fukuoka, Japan; [5]Saitama Prefecture Red Cross Blood Center, Hidaka, Saitama, Japan; [6]Department of Hematology, Fujigaoka Hospital, Showa University, Yokohama, Kanagawa, Japan; [7]Division of Hematology, Department of Medicine, Jichi Medical School, Tochigi, Japan

## Abstract

Cytogenetic abnormalities were often observed in primary myelofibrosis patients. The presence of specific cytogenetic abnormalities, such as sole abnormalities of chromosome 13q), 20q), or )7/7q), is reported to have the influence on the prognosis of primary myelofibrosis. We analyzed the data from the prospective survey of Japanese primary myelofibrosis patients which was conducted from 1999 to clarify the impact of cytogenetic abnormalities on the prognosis of primary myelofibrosis. A total of 202 primary myelofibrosis patients had the cytogenetic and the prognostic data. Eighty (40%) out of 202 cases had cytogenetic abnormalities, and an association was evident for platelet counts. Although the presence of an abnormal karyotype did not affect the prognosis, primary myelofibrosis patients with cytogenetic abnormalities other than 13q) and 20q) showed an inferior prognosis compared to patients with a normal karyotype or sole 13q) or 20q) abnormalities. Patients with an unfavorable cytogenetic profile (abnormal cytogenetics other than 13q) or 20q)) also had a greater tendency to transform to leukemia than patients with a favorable cytogenetic profile (normal cytogenetics, sole abnormalities of either chromosome 13q), or 20q)). Abnormal cytogenetics other than 13q) or 20q) in primary myelofibrosis patients has the poor prognostic effect for both survival and the risk of leukemic transformation.

Key words primary myelofibrosis; cytogenetic abnormalities; 13q); 20q); )7/7q)

Correspondence Kazuya Shimoda, Department of Gastroenterology and Hematology, Miyazaki University, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan. Tel: +81 985 85 9121; Fax: +81 985 85 5194; e-mail: kshimoda@med.miyazaki-u.ac.jp

Accepted for publication 12 June 2009

doi:10.1111/j.1600-0609.2009.01298.x

Primary myelofibrosis is a clonal stem cell disorder that is characterized by bone marrow histologic changes including collagen fibrosis, osteosclerosis, and angiogenesis (1), and is a type of myeloproliferative neoplasma along with polycythemia vera and essential thrombocythemia (2). Activated JAK2 mutation (V617F) or the activated myeloproliferative leukemia virus oncogene (MPL) (receptor for thrombopoietin) mutation was observed in about half or about 5% of primary myelofibrosis patients, respectively (3–9). This suggests that there is significant heterogeneity in the molecular basis of primary myelofibrosis, as the etiology remains unknown in the remaining 50% of patients without JAK2 or MPL mutations.

Cytogenetic abnormalities were often observed in primary myelofibrosis patients. Some studies suggested that the presence of cytogenetic abnormalities influenced the prognosis of primary myelofibrosis (10, 11), whereas other studies did not find such association (12, 13). Not only the presence or absence of cytogenetic abnormalities, some reports suggested that the specific cytogenetic



© 2009 John Wiley & Sons A/S

abnormalities, such as sole abnormalities of chromosome 13q), 20q) (14, 15), or )7/7q) (16), might have the influence on the prognosis of primary myelofibrosis. To clarify the impact of cytogenetic abnormalities on the prognosis in primary myelofibrosis patients, we analyzed the data from the prospective survey of Japanese primary myelofibrosis patients which was conducted from 1999. Cytogenetic result as well as clinical and laboratory evaluation were obtained from 202 primary myelofibrosis patients between 1999 and 2008. We found that primary myelofibrosis patients with cytogenetic abnormalities other than 13q) and 20q) had an inferior prognosis compared to patients with a normal karyotype or sole 13q) or 20q) abnormalities.

## Materials and methods

The Research Committee for Idiopathic Hematopoietic Disorders in Japan conducted the prospective survey in collaboration with hematologists at 587 medical institutes throughout Japan, all of which were approved as designated institutes by the Japanese Society of Hematology. We sent a questionnaire to hematologists in December of each year, and collected information on patients with newly diagnosed primary myelofibrosis in the reference year. The diagnostic criteria for primary myelofibrosis were an increased number of atypical megakaryocytes, the presence of marrow fibrosis and osteosclerosis as determined by bone marrow biopsy, and the exclusion of secondary myelofibrosis, including other hematologic malignancies, cancer metastasis, and inflammatory or metabolic disorders (17). Myelodysplastic syndrome with fibrosis and secondary myelofibrosis preceding polycythemia vera or essential thrombocythemia were not included in this study. Cytogenetic studies were done at the time of diagnosis, and both direct technique and unstimulated 24-h culture methods were used to harvest 20 metaphases using bone marrow or peripheral blood cells. All data were analyzed using STATVIEW 5.0 (Abacus Concepts, Inc., Berkley, CA, USA) or SPSS software (SPSS Inc., Chicago, IL, USA). All P values were two-tailed and values $P < 0.05$ were considered statistically significant. Comparisons between categorical variables were performed by chi-squared statistics, and comparisons between categorical and continuous variables were performed using the Mann–Whitney U-test.

The mutational analysis for V617F JAK2 was approved by the Miyazaki University Institutional Review Board, and performed using DNA derived from peripheral blood granulocytes. Genomic DNA was purified, and exon 12 of JAK2 was amplified using primers 5t-TATAGTCATGCTGAAAGTAGG-3t and 5t-TAACTGAATAGTCCTACAGTG-3t. The PCR products were sequenced directly using an ABI DNA analyzer.

The influence of karyotypic abnormalities on survival was examined. In addition, based on previous studies (14–16), cytogenetic findings of sole 13q) or 20q) abnormalities, abnormalities other than 13q) and 20q), or )7/7q) were evaluated for an association with prognosis. Survival was estimated using Kaplan–Meier plots that analyzed the interval from the diagnosis date to death or last contact, and then examined for significance by the log-rank test. The comparison of leukemic transformation rates among different cytogenetic groups was estimated using a similar procedure. A Cox proportional hazards regression analysis was used to assess the following variables for the prognostic relevance of primary myelofibrosis patients; hemoglobin levels of <10 g/dL, a leukocyte count of either >30 or $<4 \cdot 10^9$/L, circulating blasts of 31% or the presence of constitutional symptoms in addition to the presence of cytogenetic abnormalities or specific cytogenetic abnormalities.

## Results

The Research Committee for Idiopathic Hematopoietic Disorders began conducting a prospective survey of primary myelofibrosis in 1999, and a total of 202 cases had both cytogenetic and prognosis data during the 10-yr period until 2008. The median age of the patients was 65 yr. Males were 1.8 times more likely to be affected than females. During this period, 148 patients (73%) have received treatment for primary myelofibrosis including hydeoxyurea in 43 patients, melphalan in 11 patients, anabolic steroid in 51 patients, prednisolone in 34 patients, thalidomide in six patients and splenic irradiation in eight patients. Eighty-one patients received blood transfusions.

Eighty (40%) out of the 202 cases had cytogenetic abnormalities, including 31 patients with a complex abnormality or more than one abnormal chromosomes. Among 49 patients having a single abnormality, numerous chromosomes were affected, including del(20) (11 cases), del(13) (11 cases), +8 (3 cases) and del(12) (3 cases). Translocation, including chromosomes 12, 15, 8, 9, and others were seen in 9 cases. The V617F JAK2 mutation was analyzed only in 24 cases, and 12 out of them were positive for this mutation.

Cytogenetic abnormalities were examined for a significant association with other clinical and laboratory variables as shown in Table 1. After a univariate analysis, an association was only evident for platelet counts ($P < 0.05$). The presence of abnormal cytogenetics at the time of diagnosis had no significant impact on age, Hb level, WBC counts, the requirement of treatment, the ratio of leukemic transformation, or the prognosis.

Although the presence of abnormal cytogenetics did not influence survival of the patients, specific cytogenetic abnormalities might affect the prognosis. Sole

ª 2009 John Wiley & Sons A/S

Table 1 Comparison of clinical and laboratory features of patients with and without cytogenetic abnormalities

| Characteristics | All patients (n = 202) | Normal cytogenetics (n = 122) | Abnormal cytogenetics (n = 80) | P value |
|---|---|---|---|---|
| Age in years | 63.8 ± 12.1 | 64.0 ± 11.8 | 63.6 ± 12.6 | 0.7686 |
| Median | 65 | 64 | 65 | |
| Range | 13–95 | 36–95 | 13–84 | |
| Sex, M/F | 129/73 | 81/41 | 48/32 | 0.3549 |
| Hemoglobin level (g/L) | 8.9 ± 2.6 | 9.1 ± 2.7 | 8.6 ± 2.4 | 0.1285 |
| White blood cell level (/ l L) | 16400.0 ± 32148.7 | 19675.4 ± 38944.2 | 11405.0 ± 16273.6 | 0.0688 |
| Platelet count ( $\cdot$ $10^4$) | 29.0 ± 26.6 | 32.7 ± 26.2 | 23.4 ± 26.3 | 0.0015 |
| Treatment (yes/no) | 148/54 | 89/33 | 59/21 | 0.9001 |
| Transformation to leukemia (yes/no) | 15/187 | 6/116 | 9/971 | 0.0932 |
| Outcome (alive/death) | 117/85 | 72/50 | 45/35 | 0.6969 |
| Follow-up years | 2.3 ± 2.2 | 2.6 ± 2.3 | 2.0 ± 1.9 | 0.0712 |

Bold value indicates significant P values.

abnormalities of either chromosome 13q) or 20q) is reportedly associated with a favorable prognosis (14, 15). As shown in Fig. 1A, 22 cases with sole 13q) or 20q) abnormalities showed superior prognosis than the 58 cases with cytogenetic abnormalities other than 13q) and 20q) (P = 0.02), but not than patients with a normal karyotype (P = 0.17). The prognosis of primary myelofibrosis in patients having cytogenetic abnormalities other than 13q) and 20q) was inferior to all patients except for those having cytogenetic abnormalities other than 13q) or 20q) (P = 0.02) (Fig. 1B). Chromosome 7 deletions were previously reported to be associated with an unfavorable prognosis in myelofibrosis (16). In our study, 11 out of the 202 cases had del(7) or )7. The prognosis of primary myelofibrosis in patients with )7/7q) was same as that of patients having normal cytogenesis (P = 0.76).

We also examined the effect of extensively used adverse risk factor (hemoglobin level of < 10 g/dL, a leukocyte count of either > 30 or < 4 · $10^9$/L, circulating blasts of 31%, or the presence of constitutional symptoms) on the prognosis of primary myelofibrosis patients. Consistent with previous observation, univariate analysis confirmed the adverse prognostic relevance to overall survival of a leukocyte count of either > 30 or < 4 · $10^9$/L (P = 0.033) and circulating blasts of 31% (P = 0.04) in addition to the presence of cytogenetic abnormalities other than 13q) or 20q) (P = 0.02). The presence of constitutional symptoms, hemoglobin level of < 10 g/dL, or the requirement of therapy did not affect the prognosis of primary myelofibrosis patients in this cohort. On multivariate analysis, both leukocyte count (P = 0.02) and the presence of cytogenetic abnormalities other than 13q) or 20q) (P = 0.02) retained their significance. Circulating blasts of 31% lost its significance.

Fifteen patients converted to leukemia during the follow-up period. Six cases had normal cytogenetics, and 9 cases had the cytogenetic abnormalities other than 13q) or 20q). None of the patients having sole abnormalities of either chromosome 13q) or 20q) converted to leukemia. As mentioned above, the presence of abnormal cytogenetics did not influence the tendency of leukemic transformation (Table 1). Patients with abnormal cytogenetics other than 13q) or 20q) had a greater tendency to transform to leukemia than patients with normal cytogenetics or sole abnormalities of either chromosome 13q) or 20q) (P = 0.002).

## Discussion

In primary myelofibrosis patients, numerous chromosomes are affected; 40% of primary myelofibrosis patients had cytogenetic abnormalities in our study. The most frequently reported cytogenetic abnormalities include 13q), 20q), +8, and abnormalities of chromosomes 1, 7, and 9 (10, 12, 15, 18, 19). In our study, the total incidence of these major abnormalities is up to 71% in patients with a single chromosome abnormality, although the incidence of each individual lesion is less than 25%. The fact that there is no consistent cytogenetic abnormality in primary myelofibrosis patients might be because the range of abnormalities reflects the genomic instability present in primary myelofibrosis.

Cytogenetic abnormalities were only associated with platelet counts, and were not significantly associated with age, Hb level, WBC counts, leukemic transformation, or prognosis (Table 1). There is some discrepancy among previous reports regarding the prognostic relevance of cytogenetic findings in primary myelofibrosis (10, 11, 13, 15, 18). Some studies suggested that the presence of cytogenetic abnormalities influenced the prognosis of primary myelofibrosis (10, 11), whereas other studies did not find such association (12, 13). The idea that specific cytogenetic abnormalities have a differential effect on prognosis might account for this discrepancy. In reports that have linked specific chromosomal abnormalities to



ª 2009 John Wiley & Sons A/S

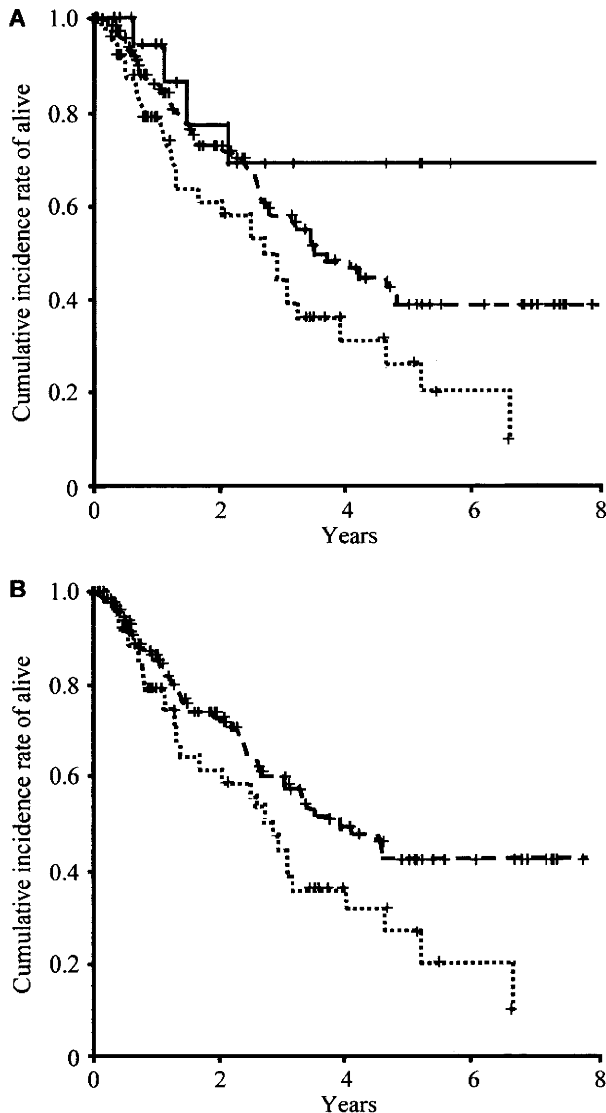



Figure 1 Survival rate curves for primary myelofibrosis patients according to cytogenetic groups. (A) ·····→ Normal cytogenetics (n = 122). Either 13q) or 20q) lesion (n = 22). ——→ cytogenetic abnormalities other than 13q) and 20q) (n = 58). ——→ The prognosis of primary myelofibrosis in patients having cytogenetic abnormalities other than 13q) and 20q) was inferior to patients having cytogenetic abnormalities with sole 13q) or 20q) (P = 0.02). (B) ·····→ Normal cytogenetics, sole abnormalities of either chromosome 13q) or 20q) (n = 144). ——→ cytogenetic abnormalities other than 13q) and 20q) (n = 58). The prognosis of primary myelofibrosis in patients having cytogenetic abnormalities other than 13q) and 20q) was inferior to all patients except for those having cytogenetic abnormalities other than 13q) or 20q) (P = 0.02)

prognosis, sole abnormalities of either chromosome 13q) or 20q) was correlated with an indolent prognosis, and cytogenetic abnormalities other than 13q) and 20q) were associated with an inferior prognosis (14, 15). As in previous reports, the prognosis of patients with cytogenetic abnormalities other than 13q) and 20q) is poorer than those with sole 13q) or 20q) abnormalities (Fig. 1A). Tefferi et al. also reported that the sole abnormalities of either 13q) or 20q) was linked to a reduced risk of transformation in addition to the indolent nature (14). In our study, none of the 22 patients with sole abnormality of either chromosome 13q) or 20q), 9 of the 58 patients with cytogenetic abnormalities other than 13q) and 20q), and 6 of the 116 patients with normal cytogenetics converted to acute leukemia. Patients with an unfavorable cytogenetic profile (abnormal cytogenetics other than 13q) or 20q)) had a greater tendency to transform to leukemia than patients with a favorable cytogenetic profile (normal cytogenetics, sole abnormalities of either chromosome 13q), or 20q)) (P = 0.002).

The genes located on 13q or 20q have not been identified. Although 13q) is also observed in multiple myeloma patients (20, 21), the gene located on deleted 13q may be different in these two diseases. In most myeloma patients with 13q), RB1, the powerful tumor suppressor gene, is deleted (21), whereas the lesion on 13q in bcr/abl-negative chronic myeloid disorders does not involve RB1 (22). Differences in the involved gene may account for the prognostic value of 13q) in myeloma and primary myelofibrosis; 13q) is associated with an inferior prognosis for myeloma (20), but not for primary myelofibrosis (14, 15). In myelodysplastic syndrome, 20q) abnormalities have been associated with prolonged survival (23). The common deleted lesion on 20q) in myeloid malignancies is being investigated (24), and the h-l (3) mbt gene, which encodes a member of the polycomb group family of proteins and is the human homologue of a tumor suppressor gene in Drosophia (25), is a candidate for the responsible gene (24).

Strasser-Weippl et al. previously reported that Chromosome 7 deletions are associated with an unfavorable prognosis in myelofibrosis (16). Chromosome 7 deletions also tend to be associated with other chromosomal aberrations. Six of 7 patients in their report (16), and 9 of 11 patients harboring del(7) or )7 in our study had other chromosomal aberrations. Contrary to the previous report, )7/7q) is not associated with an unfavorable prognosis in our study. One possible reason for this discrepancy in the prognostic value of )7/7q) is that our survey is limited to primary myelofibrosis patients, while the report showing the unfavorable prognostic value of )7/7q examined secondary myelofibrosis (postpolycytemia vera or essential thrombocytosis) patients in addition to primary myelofibrosis patients (16). This possibility is unlikely, because it is very difficult to distinguish between a primary myelofibrosis and a secondary myelofibrosis in clinical practice. The other possibility might depend on the sensitivity of the assay used to

ª 2009 John Wiley & Sons A/S

detect the chromosome abnormality. We performed a G-banding stain in metaphase cells, whereas they adapted interphase fluorescence in situ hybridization (FISH) analysis to identify the chromosomal abnormality. To detect a specific chromosomal abnormality, FISH is much more informative than a G-banding stain because FISH can be used for interphase cytogenetic analysis. In fact, we detected abnormal metaphase in only 40% of patients using a G-banding stain, while they found cytogenetic abnormalities in 56% of patients using FISH analysis (16). In order to clarify these differences, a larger prospective survey is needed to determine the prognostic value of cytogenetic information and the role of involved genes in pathogenesis.

JAK2 mutation is observed in about half of primary myelofibrosis patients (3–7). We examined JAK2 mutation status in only 24 primary myelofibrosis patients, and 12 were positive for this mutation. This number is too small to evaluate the role of JAK2 mutation on survival or leukemic transformation in primary myelofibrosis. Hussein et al. examined the presence of JAK2 mutation in addition to the cytogenetic studies in 109 primary myelofibrosis patients, and found JAK2 mutations in 63(58%) patients (26). In their study, the presence of JAK2 mutation was inconsequential to survival. JAK2 mutation also may not be the disease-initiating lesion in transformation, because the emergence of JAK2V617F⁾ blast phase from previously positive myeloproliferative neoplasms was observed by Tam et al. (27), Campbell et al. (28) and Theocharides et al. (29), who reported disappearance of JAK2V617F positivity in 7 of 12 (58%), 3 of 4 (75%), and 7 of 17 (41%) patients, respectively. The presence or absence of JAK2 mutation or its allele burden does not appear to carry prognostic information (30).

## Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Health, Labour and Welfare of Japan. We would like to thank Mayu Ota, Kazumi Sato, and Masami Dairaku for their excellent assistance in preparing this manuscript.

## References

1. Tefferi A. Myelofibrosis with myeloid metaplasia. N Engl J Med 2000;342:1255–65.
2. Tefferi A, Vardiman JW. Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. Leukemia 2008;22:14–22.
3. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 2005;365:1054–61.
4. James C, Ugo V, Le Couedic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature 2005;434:1144–8.
5. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M, Skoda RC. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 2005;352:1779–90.
6. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 2005;7:387–97.
7. Jones AV, Kreil S, Zoi K, et al. Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. Blood 2005;106:2162–8.
8. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood 2006;108:3472–6.
9. Pikman Y, Lee BH, Mercher T, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. PLoS Med 2006;3:e270.
10. Reilly JT, Snowden JA, Spearing RL, Fitzgerald PM, Jones N, Watmore A, Potter A. Cytogenetic abnormalities and their prognostic significance in idiopathic myelofibrosis: a study of 106 cases. Br J Haematol 1997;98:96–102.
11. Dupriez B, Morel P, Demory JL, Lai JL, Simon M, Plantier I, Bauters F. Prognostic factors in agnogenic myeloid metaplasia: a report on 195 cases with a new scoring system. Blood 1996;88:1013–8.
12. Cervantes F, Barosi G, Demory JL, Reilly J, Guarnone R, Dupriez B, Pereira A, Montserrat E. Myelofibrosis with myeloid metaplasia in young individuals: disease characteristics, prognostic factors and identification of risk groups. Br J Haematol 1998;102:684–90.
13. Okamura T, Kinukawa N, Niho Y, Mizoguchi H. Primary chronic myelofibrosis: clinical and prognostic evaluation in 336 Japanese patients. Int J Hematol 2001;73:194–8.
14. Tefferi A, Dingli D, Li CY, Dewald GW. Prognostic diversity among cytogenetic abnormalities in myelofibrosis with myeloid metaplasia. Cancer 2005;104:1656–60.
15. Tefferi A, Mesa RA, Schroeder G, Hanson CA, Li CY, Dewald GW. Cytogenetic findings and their clinical relevance in myelofibrosis with myeloid metaplasia. Br J Haematol 2001;113:763–71.
16. Strasser-Weippl K, Steurer M, Kees M, Augustin F, Tzankov A, Dirnhofer S, Fiegl M, Gisslinger H, Zojer N, Ludwig H. Chromosome 7 deletions are associated with unfavorable prognosis in myelofibrosis with myeloid metaplasia. Blood 2005;105:4146.
17. Shimoda K, Shide K, Kamezaki K, et al. The effect of anabolic steroids on anemia in myelofibrosis with myeloid metaplasia: retrospective analysis of 39 patients in Japan. Int J Hematol 2007;85:338–43.
18. Demory JL, Dupriez B, Fenaux P, Lai JL, Beuscart R, Jouet JP, Deminatti M, Bauters F. Cytogenetic studies and their prognostic significance in agnogenic


© 2009 John Wiley & Sons A/S

myeloid metaplasia: a report on 47 cases. Blood 1988;72:855–9.

19. Bench AJ, Nacheva EP, Champion KM, Green AR. Molecular genetics and cytogenetics of myeloproliferative disorders. Bailliere's Clin Haematol 1998;11: 819–48.
20. Tricot G, Barlogie B, Jagannath S, Bracy D, Mattox S, Vesole DH, Naucke S, Sawyer JR. Poor prognosis in multiple myeloma is associated only with partial or complete deletions of chromosome 13 or abnormalities involving 11q and not with other karyotype abnormalities. Blood 1995;86:4250–6.
21. Fonseca R, Oken MM, Harrington D, Bailey RJ, Van Wier SA, Henderson KJ, Kay NE, Van Ness B, Greipp PR, Dewald GW. Deletions of chromosome 13 in multiple myeloma identified by interphase FISH usually denote large deletions of the q arm or monosomy. Leukemia 2001;15:981–6.
22. Pastore C, Nomdedeu J, Volpe G, et al. Genetic analysis of chromosome 13 deletions in BCR/ABL negative chronic myeloproliferative disorders. Genes Chromosomes Cancer 1995;14:106–11.
23. Greenberg P, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 1997;89:2079–88.
24. MacGrogan D, Alvarez S, DeBlasio T, Jhanwar SC, Nimer SD. Identification of candidate genes on chromosome band 20q12 by physical mapping of translocation breakpoints found in myeloid leukemia cell lines. Oncogene 2001;20:4150–60.
25. Koga H, Matsui S, Hirota T, Takebayashi S, Okumura K, Saya H. A human homolog of Drosophila lethal(3)malignant brain tumor (l(3)mbt) protein associates with condensed mitotic chromosomes. Oncogene 1999;18:3799–809.
26. Hussein K, Huang J, Lasho T, Pardanani A, Mesa RA, Williamson CM, Ketterling RP, Hanson CA, Van Dyke DL, Tefferi A. Karyotype complements the International Prognostic Scoring System for primary myelofibrosis. Eur J Haematol 2009;82:255–9.
27. Tam CS, Nussenzveig RM, Popat U, et al. The natural history and treatment outcome of blast phase BCR-ABL- myeloproliferative neoplasms. Blood 2008;112:1628–37.
28. Campbell PJ, Baxter EJ, Beer PA, et al. Mutation of JAK2 in the myeloproliferative disorders: timing, clonality studies, cytogenetic associations, and role in leukemic transformation. Blood 2006;108:3548–55.
29. Theocharides A, Boissinot M, Girodon F, Garand R, Teo S-S, Lippert E, Talmant P, Tichelli A, Hermouet S, Skoda RC Leukemic blasts in transformed JAK2-V617F-positive myeloproliferative disorders are frequently negative for the JAK2-V617F mutation. Blood 2007;110:375–9.
30. Vannucchi AM, Antonioli E, Guglielmelli P, Pardanani A, Tefferi A. Clinical correlates of JAK2V617F presence or allele burden in myeloproliferative neoplasms: a critical reappraisal. Leukemia 2008;22:1299–307.

© 2009 John Wiley & Sons A/S

**Pediatr Blood Cancer 2009;53:1011–1015**

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

**Daisuke Hasegawa, MD,[1]* Atsushi Manabe, MD,[1] Hiroshi Yagasaki, MD,[2] Yoshitoshi Ohtsuka, MD,[3] Masami Inoue, MD,[4] Akira Kikuchi, MD,[5] Akira Ohara, MD,[6] Masahiro Tsuchida, MD,[7] Seiji Kojima, MD,[2] and Tatsutoshi Nakahata, MD[8] on behalf of Japanese Childhood MDS Study Group**

***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.

© 2009 Wiley-Liss, Inc.
**DOI 10.1002/pbc.22121**
**Published online 4 June 2009 in Wiley InterScience (www.interscience.wiley.com)**

## 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.

[1]Department of Pediatrics, St. Luke's International Hospital, Tokyo, Japan; [2]Department of Pediatrics, Nagoya University School of Medicine, Nagoya, Japan; [3]Department of Pediatrics, Hyogo College of Medicine, Nishinomiya, Japan; [4]Division of Hematology/Oncology, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Japan; [5]Division of Hematology-Oncology, Saitama Children's Medical Center, Iwatsuki, Japan; [6]First Department of Pediatrics, Toho University, Tokyo, Japan; [7]Department of Pediatrics, Ibaraki Children's Hospital, Mito, Japan; [8]Department of Pediatrics, Kyoto University, Kyoto, Japan

The authors report no potential conflicts of interest.

*Correspondence to: Daisuke Hasegawa, Department of Pediatrics, St. Luke's International Hospital, 9-1, Akashi-cho, Chuo-ku, Tokyo 104-8560, Japan. E-mail: hase-dai@umin.net

Received 4 February 2009; Accepted 27 April 2009

## 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 $1.5 \times 10^9$/L. All except 1 patient had Hb levels below 10 g/dl. Eight patients had platelet counts below $50 \times 10^9$/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.

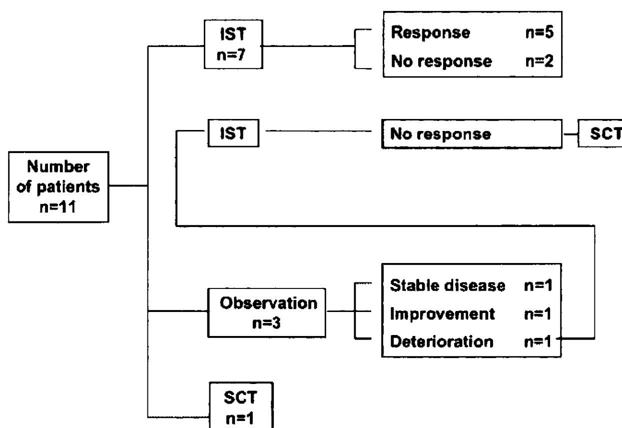



**Fig. 1.** Outcome of 11 patients with refractory anemia. SCT, stem cell transplantation; IST, immunosuppressive therapy.

## 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.

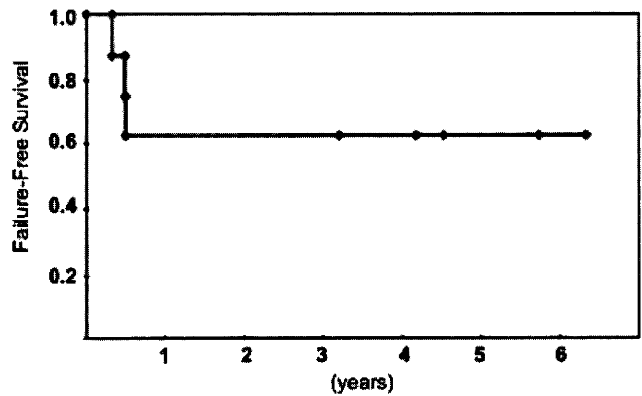



**Fig. 2.** Kaplan–Meier estimate of failure-free survival of patients who received immunosuppressive therapy. Failure-free survival was calculated from the date of initiating IST to the date of treatment failure as follows; death, no response to IST at 6 months, HSCT, a second course of IST, acquisition 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.

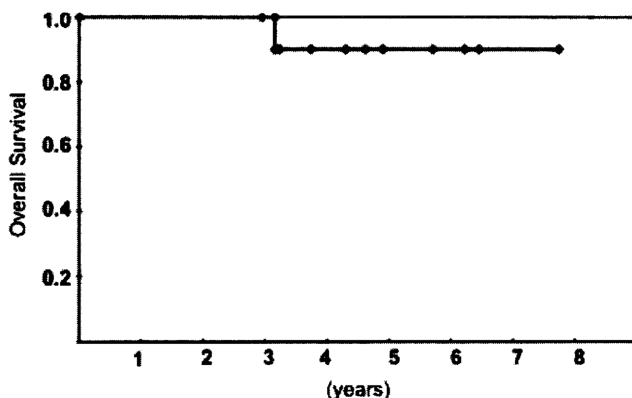



**Fig. 3.** Kaplan–Meier estimate of overall survival of all evaluable patients. Overall survival was calculated from the date of diagnosis to the date of death or last follow-up. The 5-year overall survival was $90 \pm 9\%$ (n = 11). Median follow-up was 1,685 days.

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.

## ACKNOWLEDGMENT

The authors thank Dr. Y. Nagatoshi, Dr. M. Maeda, Dr. T. Kanazawa, Dr. N. Shiba, Dr. M. Nagasawa, Dr. T. Kusuda, Dr. K. Kokawa, Dr. T. Tsuruta, Dr. S. Oana, Dr. Y. Noguchi, Dr. S. Ikushima, and Dr. A. Watanabe for providing patients' information. We also thank Dr. C. Ogawa and Dr. A. Yoshimi for valuable advice.

## REFERENCES

1. Luna-Fineman S, Shannon KM, Atwater SK, et al. Myelodysplastic and myeloproliferative disorders of childhood: A study of 167 patients. Blood 1999;93:459–466.
2. Sasaki H, Manabe A, Kojima S, et al. MDS Committee of the Japanese Society of Pediatric Hematology, Japan. Myelodysplastic syndrome in childhood: A retrospective study of 189 patients in Japan. Leukemia 2001;15:1713–1720.

3. Kardos G, Baumann I, Passmore SJ, et al. Refractory anemia in childhood: A retrospective analysis of 67 patients with particular reference to monosomy 7. Blood 2003;102:1997–2003.
4. Hasle H, Niemeyer CM, Chessells JM, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. Leukemia 2003;17:277–282.
5. Selleri C, Maciejewski JP, Catalano L, et al. Effects of cyclosporine on hematopoietic and immune functions in patients with hypoplastic myelodysplasia: In vitro and in vivo studies. Cancer 2002;95:1911–1922.
6. Sloand EM, Mainwaring L, Fuhrer M, et al. Preferential suppression of trisomy 8 compared with normal hematopoietic cell growth by autologous lymphocytes in patients with trisomy 8 myelodysplastic syndrome. Blood 2005;106:841–851.
7. de Vries AC, Langerak AW, Verhaaf B, et al. T-cell receptor Vbeta CDR3 oligoclonality frequently occurs in childhood refractory cytopenia (MDS-RC) and severe aplastic anemia. Leukemia 2008;22:1170–1174.
8. Molldrem JJ, Caples M, Mavroudis D, et al. Antithymocyte globulin for patients with myelodysplastic syndrome. Br J Haematol 1997;99:699–705.
9. Jonásova A, Neuwirtová R, Cermák J, et al. Cyclosporin A therapy in hypoplastic MDS patients and certain refractory anaemias without hypoplastic bone marrow. Br J Haematol 1998;100:304–309.
10. Saunthararajah Y, Nakamura R, Nam JM, et al. HLA-DR15 (DR2) is overrepresented in myelodysplastic syndrome and aplastic anemia and predicts a response to immunosuppression in myelodysplastic syndrome. Blood 2002;100:1570–1574.
11. Shimamoto T, Tohyama K, Okamoto T, et al. Cyclosporin A therapy for patients with myelodysplastic syndrome: Multicenter pilot studies in Japan. Leuk Res 2003;27:783–788.
12. Stadler M, Germing U, Kliche KO, et al. A prospective, randomised, phase II study of horse antithymocyte globulin vs rabbit antithymocyte globulin as immune-modulating therapy in patients with low-risk myelodysplastic syndromes. Leukemia 2004;18:460–465.
13. Yoshimi A, Baumann I, Führer M, et al. Immunosuppressive therapy with anti-thymocyte globulin and cyclosporine A in selected children with hypoplastic refractory cytopenia. Haematologica 2007;92:397–400.
14. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 1982;51:189–199.
15. Manabe A, Nakahata T. Experiences on MDS and JMML from Japan. In: Lopes LF, Hasle H, editors. Myelodysplastic and myeloproliferative disorders in children. Sao Paulo, Brazil: Lemar Livraria; 2003. pp. 317–324.
16. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 2002;100:2292–2302.
17. Nagasawa M, Tomizawa D, Tsuji Y, et al. Pancytopenia presenting with monosomy 7 which disappeared after immunosuppressive therapy. Leuk Res 2004;28:315–319.
18. Maciejewski JP, Kim S, Sloand E, et al. Sustained long-term hematologic recovery despite a marked quantitative defect in the stem cell compartment of patients with aplastic anemia after immunosuppressive therapy. Am J Hematol 2000;65:123–131.
19. Führer M, Rampf U, Baumann I, et al. Immunosuppressive therapy for aplastic anemia in children: A more severe disease predicts better survival. Blood 2005;106:2102–2104.
20. Mantadakis E, Shannon KM, Singer DA, et al. Transient monosomy 7: A case series in children and review of the literature. Cancer 1999;85:2655–2661.
21. Sloand EM, Yong AS, Ramkissoon S, et al. Granulocyte colony-stimulating factor preferentially stimulates proliferation of monosomy 7 cells bearing the isoform IV receptor. Proc Natl Acad Sci USA 2006;103:14483–14488.
22. Hirabayashi S, Manabe A, Watanabe S, et al. Myelodysplastic syndrome (MDS) and myeloproliferative disease (MPD) in children: A prospective registration of 222 cases. Blood (ASH Annu Meet Abstr) 2008;112:2678.

# Acquired pure red cell aplasia associated with malignant lymphomas: A nationwide cohort study in Japan for the PRCA Collaborative Study Group

Makoto Hirokawa,[1]* Ken-ichi Sawada,[1] Naohito Fujishima,[1] Fumio Kawano,[2] Akiro Kimura,[3] Takashi Watanabe,[4] Ayako Arai,[5] Toshimitsu Matsui,[6] Shinji Nakao,[7] Akio Urabe,[8] Mitsuhiro Omine,[9] and Keiya Ozawa[10]

Pure red cell aplasia (PRCA) has been reported in association with lymphoma as one of the autoimmune diseases seen during the course of lymphoid malignancies. However, the relation of PRCA with the underlying lymphomas remains unclear. The aim of this study was to clarify the histologic subtypes of lymphomas, the chronological sequence of anemia and lymphoma, and the response to treatment. We conducted a nationwide survey in Japan. From a cohort of 185 PRCA patients, 8 patients with lymphoma were evaluated. Histologic subtypes varied and the lymphoma was of the B-cell type in four cases and of the T-cell type in four. Four patients simultaneously developed PRCA and lymphoma. Three patients developed PRCA following lymphoma, two of whom developed anemia during remission of lymphoma. PRCA preceded lymphoma in one patient. Effective chemotherapy was associated with remission of anemia in concurrent lymphoma and PRCA. Overall, anemia responded to chemotherapy and/or immunosuppressive therapy in seven patients. In four responding patients, PRCA remained in durable remission without maintenance immunosuppressive therapy, which is different from a recurrent feature of idiopathic PRCA. We suggest that the mechanism of lymphoma-associated PRCA is heterogeneous and that durable maintenance-free remission of anemia can be obtained in some patients. Am. J. Hematol. 84:144–148, 2009. © 2008 Wiley-Liss, Inc.

## Introduction

Malignant lymphomas are often accompanied by autoimmune diseases including Sjögren's syndrome, systemic lupus erythematosus and autoimmune hemolytic anemia [1,2]. Lymphomas can also occur resulting from immunosuppressive therapy for autoimmune diseases. Rheumatoid arthritis patients may develop Epstein-Barr virus-associated lymphoma during immunosuppressive therapy such as methotrexate [3].

Pure red cell aplasia (PRCA) has been reported in association with malignant lymphomas as one of the autoimmune diseases often seen during the course of lymphoid malignancies [4]. However, due to the limited number of reported cases of PRCA associated with malignant lymphomas, the relationship between PRCA and the underlying lymphomas remains uncertain, and standard treatment has yet to be established.

The efficacy of immunosuppressive therapy for secondary PRCA could differ among the underlying diseases. We conducted a nationwide survey in Japan between 2004 and 2006 in order to investigate the long-term outcome following immunosuppressive therapy in acquired chronic PRCA in adults. From a cohort of 185 PRCA patients consisting of 73 idiopathic and 112 secondary PRCA cases, we evaluated 8 patients with lymphomas for this report. We also reviewed reported cases of lymphoma-associated PRCA in order to clarify the relation of PRCA to lymphoma.

## Results

### Histologic subtypes of lymphomas and chronological sequence

Patient age at the onset of PRCA ranged from 47 to 82 years (median age, 68 years) with an equal male to female ratio (Table I). The patient designated as UPN56 has been previously reported elsewhere [5]. Histologic subtypes varied and the lymphoma was of the B-cell type in four cases and of the T-cell type in four. Six of eight patients had advanced disease. One patient developed PRCA four months before the onset of diffuse large B-cell lymphoma (UPN187). In four patients, PRCA and lymphoma simultaneously occurred (UPN166, 18, 57, and 34) (Table I). Three other patients developed PRCA after the onset of lymphoma (UPN56, 128 and 33), in two of whom lymphoma was in complete remission following chemotherapy (Table

[1]Division of Hematology and Oncology, Department of Medicine, Akita University School of Medicine, Akita, Akita, Japan; [2]Department of Medicine, National Hospital Organization Kumamoto Medical Center, Kumamoto, Kumamoto, Japan; [3]Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Hiroshima, Japan; [4]Medical Oncology Division, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan; [5]Department of Hematology, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan; [6]Division of Hematology and Oncology, Kobe University Graduate School of Medicine, Kobe, Japan; [7]Department of Cellular Transplantation Biology, Kanazawa University Graduate School of Medicine, Kanazawa, Ishikawa, Japan; [8]Division of Hematology, NTT Kanto Medical Center, Shinagawa, Tokyo, Japan; [9]Internal Medicine, Showa University Fujigaoka Hospital, Yokohama, Kanagawa, Japan; [10]Division of Hematology, Department of Medicine, Jichi Medical School, Kawachi, Tochigi, Japan

Contract grant sponsors: Idiopathic Disorders of Hematopoietic Organs Research Committee of the Ministry of Health, Labor and Welfare of Japan; "Global Center of Excellence Program (COE)" of the Ministry of Education, Science, Technology, Sports, and Culture of Japan.

*Correspondence to: Makoto Hirokawa, Division of Hematology and Oncology, Department of Medicine, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan. E-mail: mhirokawa@hos.akita-u.ac.jp

Received for publication 10 December 2008; Accepted 12 December 2008

Am. J. Hematol. 84:144–148, 2009.

Published online 22 December 2008 in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/ajh.21354

© 2008 Wiley-Liss, Inc.

TABLE I. Patient Characteristics

| UPN | Age/Sex | Histologic subtypes | Clinical stage | IPI | Days from lymphoma to PRCA | Disease status of lymphoma at the onset of PRCA | Hematological data at the diagnosis of PRCA | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Hb (g/dl) | Ret (%) | Erythroid cells in BM (%NCC) |
| 187 | 76/F | DLBCL | II | L | 2114 | – | 5.7 | 1.0 | 0.4 |
| 166 | 75/M | DLBCL | III | L-I | 213 | On disease | 6.4 | 0.2 | 0.2 |
| 57 | 82/F | MZL | IV | L-I | 0 | On disease | 3.0 | 13.5 | 4.5 |
| 18 | 62/M | ATLL | IV | H | 0 | On disease | 4.5 | 0.6 | 0.2 |
| 34 | 58/F | AILT | IV | H-I | 35 | On disease | 5.4 | 1.0 | 0 |
| 128 | 64/M | T-LBL | II | L-I | 205 | CR | 4.8 | 1.2 | Hypoplastic |
| 56 | 47/M | FL | IV | H-I | 720 | On disease | 6.8 | 0.1 | 0.4 |
| 33 | 71/F | AILT | III | H | 801 | CR | 8.1 | 1.8 | 4.8 |

UPN, unique patient number; DLBCL, diffuse large B-cell lymphoma; ATLL, adult T-cell leukemia/lymphoma; MZL, marginal zone lymphoma; AILT, angioimmunoblastic T-cell lymphoma; FL, follicular lymphoma; T-LBL, precursor T lymphoblastic lymphoma; IPI, international prognostic index; L, low; L-I, low-intermediate; H-I, high-intermediate; H, high; Hb, hemoglobin; Ret, reticulocyte; BM, bone marrow; NCC, nucleated cell count.

TABLE II. Induction and Maintenance Therapy for Lymphoma-Associated PRCA

| Type of induction therapy for PRCA | UPN | Lymphoma, disease status | Induction for PRCA (dose)[a] | Response of lymphoma | Response of PRCA | Salvage therapy for PRCA (dose) and response | Relapse of anemia | Remission duration for anemia | Maintenance (maintenance-free period) |
|---|---|---|---|---|---|---|---|---|---|
| Chemo | 166 | DLBCL, on disease | R-CHOP | PR | NR | CsA (150mg), CR | No | 33 M | No (27 M) |
| | 34 | AILT, on disease | CHOP | CR | CR | | No | 95 M | No (76 M) |
| | 56 | FL, on disease | CHOP | CR | CR | | No | 126 M | No (127 M) |
| IST | 187 | DLBCL, before onset | PSL (40 mg) | N/A | CR | N/A | No | 15 M | Yes, PSL 5 mg |
| | 57 | MZL | PSL (10 mg) | NR | NR | None | N/A | N/A | N/A |
| | 18 | ATLL | CsA (50 mg) | N/A | CR | N/A | No | 5 M | Yes, CsA 50 mg |
| | 128 | T-LBL, CR | PSL (60 mg) | N/A | PR | N/A | Yes | 3 M | No (salvaged by CsA, 97 M for CR2) |
| | 33 | AILT, CR | PSL-CsA[b] (60, 200 mg) | N/A | PR/PR | N/A | No | 17 M | Yes, PSL 10 mg |

[a] The daily doses per body are shown in parenthesis.

[b] Administered in combination later on.

Chemo, chemotherapy; IST, immunosuppressive therapy; R-CHOP, CHOP chemotherapy plus rituximab; PSL, prednisolone; CsA, cyclosporine A; CR, complete response; PR, partial response; NR, no response; N/A, not applicable. M, months.

I). The median hemoglobin concentration at the diagnosis of PRCA was 5.6 g/dl with a range of 3.0 to 8.1 g/dl.

## Response of PRCA to chemotherapy and immunosuppressive therapy

Three patients with concurrent PRCA and active lymphoma received chemotherapy as the induction treatment (UPN166, 34, and 56; Table II). In a patient with DLBCL (UPN166), chemotherapy by itself achieved partial response of lymphoma but not response of PRCA. The subsequent administration of cyclosporine achieved CR of PRCA (Table II). In angioimmunoblastic T-cell lymphoma (AILT) and follicular lymphoma patients, chemotherapy alone induced remission for both lymphoma and anemia (UPN34 and 56). All of these three patients remained in CR without maintenance therapy (27, 76, 127 months) (Table II).

Five patients received immunosuppressive therapy as the initial induction treatment of PRCA (UPN187, 57, 18, 128, and 33; Table II). In a patient who developed PRCA before the onset of diffuse large B-cell lymphoma (UPN187), prednisolone was given and resulted in CR. Subsequent lymphoma showed complete response to chemotherapy (CVP). Low dose corticosteroid was given in a patient with splenic marginal zone lymphoma with neither response of lymphoma nor anemia (UPN57). In a patient with adult T-cell leukemia/lymphoma (ATLL) (UPN18), cyclosporine achieved remission of PRCA. The patient then developed an acute crisis and died of disease progression despite of chemotherapy (CHOP). In two patients developing PRCA following successful treatment of lymphoma, corticosteroid was given with partial response (UPN128 and 33). These two patients received chemotherapy with a L10M protocol [6] and a CHOP regimen, respectively. Relapse of anemia was seen in one patient (UPN128) and was salvaged by cyclosporine. This patient was free of PRCA without maintenance immunosuppressive therapy for 97 months.

Taken together, PRCA responded to chemotherapy and/or immunosuppressive therapy in seven of eight patients, and four patients were free of anemia without maintenance immunosuppressive therapy (UPN166, 34, 56 and 128) (Table II). The median time for transfusion-independence from the start of therapy was 95 days with a range of 27 to 450 days.

## Outcome of lymphoma-associated PRCA

Four patients were alive, and two of these four remained in remission for both lymphoma and PRCA (Table III). One patient died of infection while lymphoma and PRCA was maintained in remission (UPN33).

## Literature search

We found twenty-two reported cases of lymphoma-associated with PRCA and these can be classified into two groups based on the chronological sequence in the onset of lymphoma and anemia. Twelve patients presented with lymphoma and PRCA simultaneously (Table IV) [7–18], 10 patients developed PRCA following lymphoma (Table V) [5,19–27]. Histologic subtypes of lymphoma varied. Three important points can be noted from an extensive review of the literatures. First, effective induction chemotherapy for lymphoma was associated with remission of anemia in patients with concurrent lymphoma and PRCA. Second, a significant fraction of patients were positive for Coombs test. Third, four cases were infected with human parvovirus B19 following chemotherapy including monoclonal antibody (Table V).

TABLE III. Outcome of Lymphoma-Associated PRCA

| UPN | Age/sex | Histologic subtypes | Disease status at last observation | | Outcome[a] | Cause of death |
|---|---|---|---|---|---|---|
| | | | Lymphoma | PRCA | | |
| 187 | 76/F | DLBCL | Non-CR | CR | Alive (117 M) | |
| 166 | 75/M | DLBCL | Non-CR | CR | Alive (144 M) | |
| 57 | 82/F | MZL | Non-CR | Non-CR | Dead (42 M) | Undetermined |
| 18 | 62/M | ATLL | Non-CR | CR | Dead (8 M) | Disease progression |
| 34 | 58/F | AILT | CR | CR | Alive (181 M) | |
| 128 | 64/M | T-LBL | CR | CR | Alive (1128 M) | |
| 56 | 48/M | FL | Non-CR | CR | Dead (159 M) | Disease progression |
| 33 | 71/F | AILT | CR | CR | Dead (45 M) | Infection |

[a] Months from the diagnosis of lymphoma.

TABLE IV. Reported Cases of PRCA with Concurrent Lymphoma

| Reference | Age/sex | Histologic subtypes of lymphomas | Therapy for lymphomas | Response of lymphoma | Response of anemia to chemotherapy | Others |
|---|---|---|---|---|---|---|
| [7] | 16/M | Hodgkin | MOPP | Yes | Yes | |
| [8] | 19/M | Hodgkin | COPP,VEPA | Yes | Yes | |
| [9] | 66/F | Diffuse, mixed | COPP | Yes | Yes | |
| [10] | 61/M | Well-differentiated lymphocytic | COP, Cy, PSL, MACOP-B | Yes | Yes | Coombs (1) |
| [11] | 46/M | AILT | Chemotherapy | Yes | Yes | |
| [12] | 53/M | DLBCL | CHOP | Yes | Yes | Coombs (1) |
| [13] | 54/F | Follicular | R-CHOP | Yes | Yes | Coombs (1) |
| [14] | 71/F | AILT | THP-COP | Yes | Yes | Coombs (1) |
| [15] | 54/F | DLBCL | R-CHOP | Yes | Yes | Coombs (1) |
| [16] | 57/F | Marginal zone | Rituximab | Yes | Yes | |
| [17] | 82/F | DLBCL | Rituximab | Yes | Yes | |
| [18] | 75/F | DLBCL | R-CHOP | Yes | Yes | Coombs (1) |

Cy, cyclophosphamide; PSL, prednisolone.

TABLE V. Reported Cases of PRCA with Preceding Lymphoma

| Reference | Age/sex | Histologic subtypes of lymphomas | Therapy for lymphomas | Response of lymphoma | Effective therapy for anemia | Others |
|---|---|---|---|---|---|---|
| [19] | 25/F | Hodgkin | MOPP/ABV | Yes | CsA, EPO | |
| [20] | 37/F | Hodgkin | Irradiation, MOPP/ABV | Not described | Immunoab, steroid | |
| [21] | 58/M | Follicular | COP | Yes | Spontaneous resolution | |
| [22] | 48/M | Follicular | PROVECIP, BEAC, autologous stem cell transplant | Yes | EPO | |
| [5] | 47/M | Follicular | CHOP, others | Yes | Chemotherapy | |
| [23] | 45/M | Follicular | R-CHOP | Yes | IVIG | Parvo B19 |
| [24] | 56/F | Diffuse, mixed | CHOP | Yes | Pred | Coombs (1) |
| [25] | 26/F | DLBCL | R-CHOP | Yes | IVIG | Parvo B19 |
| [26] | 56/F | Mycosis fungoides | Alemtuzumab | Not described | IVIG | Parvo B19 |
| [27] | 40/F | Follicular | CHOP, R-FND | Yes | IVIG | Parvo B19 |

PROVECIP, procarbazine, vinblastine and cyclophosphamide, prednisone; R-FND, rituximab, fludarabine, mitoxantrone and dexamethasone; immunoab, immunoabsorbance; CsA, cyclosporine A; EPO, erythropoietin; IVIG, intravenous immunoglobulin.

## Discussion

In our patient cohort, immunosuppressive therapy and/or chemotherapy were effective for improving anemia in the majority of patients with lymphoma-associated PRCA. The median time to response after the start of therapy for PRCA was 85 days, which is similar to that of idiopathic PRCA [28]. Wöhrer et al. have reported that rituximab-combined CHOP therapy given for lymphoma treatment is effective for therapy of concurrent rheumatic diseases in non-Hodgkin's lymphoma [29]. Since chemotherapy for malignant lymphoma is immunosuppressive to some extent, the efficacy of chemotherapeutic agents in PRCA patients is not surprising.

However, it is intriguing that four of seven patients maintained remission of anemia without immunosuppressive therapy, which differs from the other form of PRCA [28,30–32]. We have recently reported that discontinuing maintenance immunosuppressive therapy was strongly correlated with relapse of anemia in acquired primary idiopathic PRCA [28,33], and that cyclosporine was quite effective in thymoma-associated PRCA but most patients were receiving maintenance therapy [31]. These results suggest that durable maintenance-free remission of anemia may be obtained in lymphoma-associated PRCA and that PRCA may occur as paraneoplastic syndrome of lymphoid malignancy.

From the point of view regarding the pathogenesis of lymphoma-associated PRCA, it is interesting that positive Coombs test is often associated with PRCA, which suggests the role of autoreactive antibody in lymphoma-associated PRCA. Hauswirth et al. have analyzed more than 100 reported cases in the literatures of non-CLL non-Hodgkin's lymphoma associated with autoimmune hemolytic anemia (AIHA) or Evans' syndrome, and they have reported that warm antibody mediated AIHA was more frequent in B-cell lymphomas, while cold antibody mediated AIHA predominantly occurred in T-cell lymphomas [34]. Some reports demonstrated that serum immunoglobulin showed an inhibitory activity against erythropoiesis in vitro [7,9,21]. Theoretically, the pathogenesis of PRCA in lymphoma patients can

be explained by three potential mechanisms. First, PRCA may occur as a paraneoplastic syndrome of lymphoid malignancy, as described above. Malignant B cell clone might produce autoreactive antibody against erythroid progenitors, and neoplastic T cells might directly or indirectly inhibit erythroid differentiation of hematopoietic stem cells. Cytotoxic therapy may eliminate these pathogenic clones. Second, cytotoxic therapy may cause profound immunosuppression sufficient to inhibit host immunity against parvovirus B19 infection, although our patient cohort did not include this type of patients. In immunocompromised hosts such as recipients of organ transplantation or patients infected with human immunodeficiency virus (HIV) [35–37], acute or chronic anemia can be developed following B19 infection due to the lack of the production of specific antibodies. Third, some patients may develop PRCA via autoimmune mechanisms irrelevant of lymphoma itself following successful chemotherapy, which is supported by the efficacy of immunosuppressive therapy for anemia (UPN128, 33).

One crucial question is how much risk of developing PRCA is present in lymphoma patients. In order to address this issue, it is necessary to establish the cohort of the patients with lymphomas and estimate the risk of developing PRCA. Unfortunately, however, the registry data of lymphoma patients are not available at present in Japan, and thus we need to establish the cancer registration system suitable for this type of analysis.

In conclusion, we have elucidated that the maintenance-free remission is achievable in some PRCA patients associated with lymphomas. Chemotherapy should be introduced for patients with coexisting lymphoma and PRCA. Additional immunosuppressive therapy may be necessary for PRCA that has failed to respond to chemotherapy. However, physicians should be also cautious about unexplained anemia during chemotherapy, combined with monoclonal antibody in particular, because this anemia might be due to persistent parvovirus B19 infection which is treatable with intravenous immunoglobulin [35].

## Methods

Collection of the data and patient characteristics. The first questionnaires were sent to 109 institutions in Japan to estimate the number of patients aged 15 and above who had been newly diagnosed as having acquired PRCA between 1990 and 2006. The diagnosis of PRCA was based on the absence of erythroid cells in the bone marrow and the absence of circulating reticulocytes. Morphological diagnosis of bone marrow was done by hematologists at each institution. Human parvovirus B19 infection-associated PRCA was excluded, because the initial aim of the present study was to evaluate the efficacy of immunosuppressive therapy for acquired chronic PRCA in adults. Eligible patients were limited to those who had been diagnosed during the designated period in order to minimize the effect of transfusion-associated hepatitis C virus infection. Overall, 273 patients were enrolled from 45 institutions. Secondary questionnaires were then sent to these institutions to collect data regarding underlying diseases, laboratory findings including peripheral blood cell counts and leukocyte differentials, bone marrow examination, immunologic and cytogenetic parameters, efficacy of immunosuppressive therapy and outcome. A total of 185 patients were enrolled in response to the second questionnaires. Of 185 collected patients, 73 patients were classified as having idiopathic PRCA and 112 patients as having secondary PRCA.

The classification of PRCA was based on the criteria proposed by the Hematopoietic Organs Research Committee of the Ministry of Health, Labor and Welfare of Japan in 2005 [28]. This classification was fundamentally based on the criteria proposed by Dessypris and Lipton [4]. Eight patients demonstrated both malignant lymphoma and PRCA. Personal information was protected by giving each data set a unique patient number at each participating institution. This study was approved by the institutional review board, and performed according to the Declaration of Helsinki and the Ethical Guidelines for Epidemiological Research of the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan.

Definition of the response and data analysis. Complete response (CR), partial response (PR), and no response (NR) were defined as the achievement of normal hemoglobin levels without transfusion, the presence of anemia but without transfusion dependence, and the continued presence of transfusion-dependence, respectively [28]. The date of remission was defined as that of the final transfusion after the initiation of remission induction therapy. The minimum period required for evaluation of response to agents was defined as two weeks; therefore, agents added within a two-week period were included into a simultaneous combination with the preceding agents. The agents for remission induction and salvage therapy were defined as those used initially and those used either sequentially or in later combination, respectively. The agent for maintenance therapy was defined as that used or tapered off after successful remission induction. Relapse was defined as the reappearance of transfusion requirement.

Literature search. National Library of Medicine's search service (PubMed) was used to find reported cases of PRCA accompanied by lymphoma published in the literature. The reports that described the outcome of both lymphoma and PRCA were included in this study. Small lymphocytic lymphoma was excluded because this type of lymphoma is often indistinguishable from chronic lymphocytic leukemia, which is well known to be associated with autoimmune hemolytic anemia, immune thrombocytopenia and PRCA [38].

## Acknowledgments

The authors are grateful to all physicians of the institutions listed in the Appendix for their contribution to the present study.

## References

1. Smedby KE, Hjalgrim H, Askling J, et al. Autoimmune and chronic inflammatory disorders and risk of non-Hodgkin lymphoma by subtype. J NCI 2006;98:51–60.
2. Smedby KE, Vajdic CM, Falster M, et al. Autoimmune disorders and risk of non-Hodgkin lymphoma subtypes: A pooled analysis within the InterLymph Consortium. Blood 2008;111:4029–4038.
3. Salloum E, Cooper DL, Howe G, et al. Spontaneous regression of lymphoproliferative disorders in patients treated with methotrexate for rheumatoid arthritis and other rheumatic diseases. J Clin Oncol 1996;14:1943–1949.
4. Dessypris EN. Pure Red Cell Aplasia. Baltimore, London: The Johns Hopkins University Press; 1988. pp 41–113.
5. Kuramoto K, Oda K, Katsutani S, et al. Acquired pure red cell aplasia associated with relapsed non-Hodgkin's lymphoma: A case report-improvement of PRCA after acute hepatitis. Rinsho Ketsueki 1998;39:290–296.
6. Boldt DH, Kopecky KJ, Head D, et al. Expression of myeloid antigens by blast cells in acute lymphoblastic leukemia of adults. The Southwest Oncology Group experience. Leukemia 1994;8:2118–2126.
7. Morgan E, Pang KM, Goldwasser E. Hodgkin disease and red cell aplasia. Am J Hematol 1978;5:71–75.
8. Natori Y, Yamagishi A, Maeda S. Hodgkin's disease associated with pure red cell aplasia-report of a case. Rinsho Ketsueki 1986;27:219–224.
9. Hirosawa S, Kamiyama R, Dan K, Kuriya S, Nomura T. A case malignant lymphoma associated with pure red cell aplasia. Rinsho Ketsueki 1982;23:1463–1467.
10. Vukelja SJ, Krishnan J, Link CM, et al. Resolution of pure red cell aplasia and lymphoma: Response to intravenous gammaglobulin and combination chemotherapy. Am J Hematol 1989;32:129–133.
11. Tsujimura H, Sakai C, Takagi T. Pure red cell aplasia complicated by angioimmunoblastic T-cell lymphoma: Humoral factor plays a main role in the inhibition of erythropoiesis from CD34(1) progenitor cells. Am J Hematol 1999;62:259–260.
12. Katayama H, Takeuchi M, Yoshino T, et al. Epstein-Barr virus associated diffuse large B-cell lymphoma complicated by autoimmune hemolytic anemia and pure red cell aplasia. Leuk Lymphoma 2001;42:539–542.
13. Nakashima Y, Abe Y, Ohtsuka R, et al. Follicular lymphoma complicated with autoimmune hemolytic anemia and pure red cell aplasia. Rinsho Ketsueki 2004;45:1208–1210.
14. Mizobe T, Tsukada J, Higashi T, et al. Angioimmunoblastic T-cell lymphoma accompanied by pure red cell aplasia. Rinsho Ketsueki 2005;46:211–216.
15. Kuroda H, Matsunaga T, Iyama S, et al. De novo CD5-positive diffuse large B-cell lymphoma associated with autoimmune hemolytic anemia presenting as erythroid hypoplasia. Rinsho Ketsueki 2006;47:633–638.
16. Narra K, Borghaei H, Al-Saleem T, et al. Pure red cell aplasia in B-cell lymphoproliferative disorder treated with rituximab: Report of two cases and review of the literature. Leuk Res 2006;30:109–114.
17. Suzuki T, Kumagai T, Kitano A, et al. Pure red cell aplasia successfully treated with rituximab in a patient with relapsed diffuse large B-cell lymphoma. Rinsho Ketsueki 2007;48:67–70.
18. Sumi M, Ichikawa N, Shimizu I, et al. Primary diffuse large B-cell lymphoma of the bone marrow complicated with autoimmune hemolytic anemia and erythroid hypoplasia. Rinsho Ketsueki 2007;48:571–575.

19. Reid TJ III, Mullaney M, Burrell LM, et al. Pure red cell aplasia after chemotherapy for Hodgkin's lymphoma: In vitro evidence for T cell mediated suppression of erythropoiesis and response to sequential cyclosporin and erythropoietin. Am J Hematol 1994;46:48–53.
20. Mohri H, Harano H, Okubo T. Concomitant association of myasthenia gravis and pure red cell aplasia after chemotherapy for Hodgkin's disease. Am J Hematol 1997;54:343.
21. Alter R, Joshi SS, Verdirame JD, Weisenburger DD. Pure red cell aplasia associated with B cell lymphoma: Demonstration of bone marrow colony inhibition by serum immunoglobulin. Leuk Res 1990;14:279–286.
22. Martelli M, Ponchio L, Beguin Y, et al. Pure red cell aplasia following peripheral stem cell transplantation: Complete response to a short course of high-dose recombinant human erythropoietin. Haematologica 1994;79:456–459.
23. Sharma VR, Fleming DR, Slone SP. Pure red cell aplasia due to parvovirus B19 in a patient treated with rituximab. Blood 2000;96:1184–1186.
24. Toyota S, Nakamura N, Dan K. Coexistence of pure red cell aplasia and autoimmune hemolytic anemia occurring during remission of malignant lymphoma. Rinsho Ketsueki 2002;43:493–495.
25. Song KW, Mollee P, Patterson B, et al. Pure red cell aplasia due to parvovirus following treatment with CHOP and rituximab for B-cell lymphoma. Br J Haematol 2002;119:125–127.
26. Herbert KE, Prince HM, Westerman DA. Pure red-cell aplasia due to parvovirus B19 infection in a patient treated with alemtuzumab. Blood 2003;101:1654.
27. Isobe Y, Sugimoto K, Shiraki Y, et al. Successful high-titer immunoglobulin therapy for persistent parvovirus B19 infection in a lymphoma patient treated with rituximab-combined chemotherapy. Am J Hematol 2004;77:370–373.
28. Sawada K, Hirokawa M, Fujishima N, et al. Long-term relapse-free survival and overall survival of patients with acquired primary idiopathic PRCA receiving cyclosporine A. A nationwide cohort study in Japan for the PRCA Collaborative Study Group. Haematologica 2007;92:1021–1028.
29. Wöhrer S, Troch M, Zwerina J, et al. Influence of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone on serologic parameters and clinical course in lymphoma patients with autoimmune diseases. Ann Oncol 2007;18:647–651.
30. Clark AD, Dessypris EN, Krantz SB. Studies on pure red cell aplasia. XI. Results of immunosuppressive treatment of 37 patients. Blood 1984;63:277–286.
31. Hirokawa M, Sawada K, Fujishima N, et al. Long-term response and outcome following immunosuppressive therapy in thymoma-associated pure red cell aplasia: A nationwide cohort study in Japan for the PRCA Collaborative Study Group. Haematologica 2008;93:27–33.
32. Fujishima N, Sawada K, Hirokawa M, et al. Long-term responses and outcomes following immunosuppressive therapy in large granular lymphocyte leukemia-associated pure red cell aplasia: A Nationwide Cohort Study in Japan for the PRCA Collaborative Study Group. Haematologica 2008;93:1555–1559.
33. Sawada K, Fujishima N, Hirokawa M. Acquired pure red cell aplasia: Updated review of treatment. Br J Haematol 2008;142:505–514.
34. Hauswirth AW, Skrabs C, Schützinger C, et al. Autoimmune hemolytic anemias, Evans' syndromes, and pure red cell aplasia in non-Hodgkin lymphomas. Leuk Lymphoma 2007;48:1139–1149.
35. Frickhofen N, Abkowitz JL, Safford M, et al. Persistent B19 parvovirus infection in patients infected with human immunodeficiency virus type 1 (HIV-1): A treatable cause of anemia in AIDS. Ann Intern Med 1990;113:926–933.
36. Ahsan N, Holman MJ, Gocke CD, et al. Pure red cell aplasia due to parvovirus B19 infection in solid organ transplantation. Clin Transpl 1997;11:265–270.
37. Wong TY, Chan PK, Leung CB, et al. Parvovirus B19 infection causing red cell aplasia in renal transplantation on tacrolimus. Am J Kidney Dis 1999;34:1132–1136.
38. Diehl LF, Ketchum LH. Autoimmune disease and chronic lymphocytic leukemia: Autoimmune hemolytic anemia, pure red cell aplasia, and autoimmune thrombocytopenia. Semin Oncol 1998;25:80–97.

## Appendix

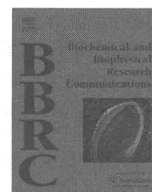
The following institutions participated in the Collaborative Study Group: Aichi Medical School, Akita University, Asahikawa Medical School, Chiba University, Dokkyo Medical School, Ehime University, Fujita Health University, Fukui University, Fukui National Hospital, Fukuoka University, Fukushima Medical University, Gifu University, Gunma University, Hamamatsu Medical School, Hirosaki University, Hiroshima University, Hokkaido University, Hyogo Medical University, Iwate Medical School, Jichi Medical School, Jikei University, Juntendo University, Kagawa Childrens' Hospital, Kagawa University, Kagoshima University, Kanazawa University, Kanazawa Medical School, Kansai Medical University, Kawasaki Medical School, Keio University, Kinki University, Kitazato University, Kobe University, Kochi University, Kumamoto University, Kurume University, Kyoto Prefectural University, Kyoto University, Kumamoto Medical Center, Kyushu University, Mie University, Nagasaki University, Nagoya City University, Nagoya Medical Center, Nagoya University, Nara Medical University, National Cancer Center, National Institute of Infectious Diseases, Niigata University, Nishi Sapporo National Hospital, Nippon Medical School, Nippon University, NTT Kanto Medical Center, Oita University, Okayama Medical Center, Okayama University, Osaka City University, Osaka Medical School, Osaka National Hospital, Osaka University, Ryukyu University, Saga University, Saitama Medical School, Sapporo Medical School, Sendai Medical Center, Shimane University, Shinsyu University, Showa University, St. Marianna University, Teikyo University, Toho University, Tohoku University, Tokai University, Tokushima University, Tottori University, Tokyo Medical Center, Tokyo Medical School, Tokyo Medical and Dental University, Tokyo University, Tokyo Women's Medical School, Tsukuba University, University of Occupational and Environmental Health, Wakayama Medical University, Waseda University, Yamagata University, Yamaguchi University, Yamanashi University, and Yokohama City University.

Biochemical and Biophysical Research Communications 383 (2009) 411–416

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc



# p27 deregulation by *Skp2* overexpression induced by the JAK2V617 mutation

A. Furuhata [a,1], A. Kimura [b,1], K. Shide [c], K. Shimoda [c], M. Murakami [a], H. Ito [a], S. Gao [a], K. Yoshida [a], Y. Tagawa [a], K. Hagiwara [d], A. Takagi [a], T. Kojima [a], M. Suzuki [e], A. Abe [f], T. Naoe [f], T. Murate [a,*]

[a] *Nagoya University Graduate School of Medicine, Nagoya University School of Health Sciences, Nagoya, Japan*
[b] *Nagoya City University School of Medicine, Department of Laboratory Medicine, Nagoya, Japan*
[c] *Division of Gastroenterology and Hematology, Department of Internal Medicine, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan*
[d] *National Hospital Organization, Nagoya Medical Center, Nagoya, Japan*
[e] *Division of Molecular Carcinogenesis, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[f] *Department of Hematology/Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan*

ARTICLE INFO

*Article history:*
Received 4 April 2009
Available online 11 April 2009

*Keywords:*
Myeloproliferative disease
*JAK2V617F*
Signal transducer and transcriptions (STATs)
$p27^{Kip1}$
Skp2
Promoter analysis
EMSA
ChIP assay

ABSTRACT

*Janus kinase 2 (JAK2) V617F* mutation has been regarded as the major cause of myeloproliferative disorders (MPD). However, the mechanisms of abnormal cell growth by *JAK2V617F* have not been elucidated. In this study, cell cycle regulatory protein expression was analyzed using *JAK2V617F*-Ba/F3 and mock-Ba/F3. *JAK2V617F*-Ba/F3, but not mock-Ba/F3, showed IL-3 independent cell growth and constitutive STATs activation. Deregulation of $p27^{Kip1}$, the cell cycle regulator at the G1 to S transition, was observed in *JAK2V617F*-Ba/F3 but not in mock-control. $p27^{Kip1}$ deregulation was not due to $p27^{Kip1}$ mRNA level but due to high *Skp2* expression, a subunit of ubiquitin E3 ligase, through the STAT binding in the *Skp2* promoter. Like *JAK2V617F* overexpression, constitutively active *STAT5* or *STAT3* induced aberrant $p27^{Kip1}$ expression of Ba/F3 cells. Similar findings were observed in *BCR/ABL*-transfected Ba/F3. Our results elucidate the regulatory mechanism by which *JAK2V617F* modulates *Skp2* gene expression through the STAT transcription factors.

© 2009 Elsevier Inc. All rights reserved.

## Introduction

The Janus kinase (JAK)/signal transducers and activators of transcription (STATs) pathway plays an important role in hematopoiesis. Recently, a mutation that substitutes valine with phenylalanine at position 617 (V617F) of the JAK2 protein has been identified in patients with myeloproliferative disorders (MPD) [1]. A subset of patients, most commonly with polycythemia vera (PV), have homozygous *JAK2V617F* mutations, which are the result of mitotic recombination and duplication of the mutant allele [2]. Valine 617 locates in the pseudokinase domain (JH2) of JAK2. It is thought that the pseudokinase domain serves an auto-inhibitory role similar to the juxtamembrane domain of receptor tyrosine kinases such as FLT3 [3]. Biochemical evidence also supports the notion that *JAK2V617F* is a constitutively active tyrosine kinase [4]. When *JAK2V617F* was expressed in Ba/F3 cells with the erythropoietin (EPO) receptor, Ba/F3 cells resulted in EPO independent growth and in EPO hypersensitivity [5].

It has been suggested that elevated JAK2 tyrosine kinase activity contributes to transformation, probably in part through STAT transcription factors. However, the detailed molecular targets of JAK2 activation, which characterizes the MPD phenotype, are still obscure. In the current study, we compared profiles of cell cycle regulatory protein between *JAK2V617F*-Ba/F3 and mock-Ba/F3 cells. Based on these results, we focused on a cell cycle regulatory protein of $p27^{Kip1}$ [6]. Our current results suggested that aberrant expression of $p27^{Kip1}$ might be a cause of IL-3 independent cell growth of *JAK2V617F*. We further found that reduced $p27^{Kip1}$ expression was due to its increased degradation by SKP2, an F-box protein of the E3 ligase, $SCF^{SKP2}$.

We also observed that similar deregulation occurs in *BCR/ABL*-BaF3 cells, which has been recently reported by others [7,8]. Constitutively active STAT3 and STAT5 mimic the expression of $p27^{Kip1}$ observed in *JAK2V617F*-Ba/F3. The involvement of these proteins in MPD as well as CML pathogenesis was discussed.

## Materials and methods

*Cell lines and reagents.* Mock, *JAK2* wild type (Wt) and *JAK2V617F* transfectants of Ba/F3 (*JAK2V617F-Ba/F3*) were reported previously [9]. Ba/F3, mock-Ba/F3 and Wt-Ba/F3 were maintained in RPMI 1640 medium supplemented with 10% FCS and 3% conditioned

* Corresponding author. Address: Nagoya University School of Health Sciences, Medical Technology, Daiko-minami 1-1-20, Higashi-ku, Nagoya, Aichi 461-8673, Japan. Fax: +81 527191186.
*E-mail address:* murate@met.nagoya-u.ac.jp (T. Murate).
[1] These two authors are contributed equally to this work.

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.04.015

medium from WEHI 3B cells (WEHI-CM) as a source of IL-3. *JAK2V617F*-Ba/F3 cells were maintained in the absence of IL-3. JAK Inhibitor I (Calbiochem) was used at a final concentration of 0.5 mM. Murine *STAT5 1*6* mutant (constitutively active) and wild type *STAT5* expression pMX retrovirus vectors [10] were provided by Dr. T. Kitamura (University of Tokyo, Tokyo, Japan). pEFHA STAT3 [11] was provided by Dr. T. Hirano (Osaka University, Osaka, Japan). Constitutive active STAT3, DMsam STAT3C-IRES-EGFP [12], was provided by Dr. A. Iwama (Chiba University, Chiba, Japan). Wild-type and constitutively active *STAT3* cDNA were cut out from the original vector and inserted into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA), and used for DNA transfection. To establish stable transfectants of *STAT3* and *STAT5*, Fugene 6 (Roche Diagnostics, Basel, Swiss) was used for DNA transfection and transformants were selected by G418. Retroviral *BCR/ABL* expression vector [13] and its control vector were a generous gift from Prof. C. Eaves (Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, Canada). *BCR/ABL*-Ba/F3 and MIG-mock-Ba/F3 were established by transfection followed sorting of GFP positive clones by flow cytometry.

*Western blotting.* Western blotting was performed as described previously [14]. The antibodies used were anti-JAK2 (M-126, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-STAT3 (S5933, SIGMA, St. Louis, MO, USA), anti-STAT5A (S6058, SIGMA), anti-p-STAT5A/B (S5058, SIGMA), anti-$p27^{Kip1}$ (C-19, Santa Cruz), anti-p15 (4822, Cell Signaling, Beverly, MA, USA), anti-CDK4 (2906, Cell Signaling), anti-CDK6 (3136, Cell Signaling), anti-$p21^{WAF1}$ (C-19, Santa Cruz), anti-Cyclin D1 (2926, Cell Signaling), anti-Cyclin D3 (2936, Cell Signaling), anti-β-actin (Cytoskelton Inc., Denver, CO, USA), anti-p-JAK2 (Cell Signaling), anti-p-STAT3 (Cell Signaling) and anti-SKP2 (Santa Cruz, H-435).

*Semi-quantitative RT-PCR.* Semi-quantitative reverse transcription (RT)-PCR was performed according to a method described previously [14]. Primer sets are described in the Supplementary materials. The mRNA levels of *$p27^{Kip1}$* and *Skp2* are expressed as the ratio of *$p27^{Kip1}$/β-actin* and *Skp2/β-actin*, respectively.

*Rapid amplification of 5′-cDNA ends (5′-RACE).* The transcription initiation sites of mouse *$p27^{Kip1}$* and *Skp2* of Ba/F3 cells were determined with the RNA ligase-mediated rapid amplification method of 5′-cDNA ends (5′-RACE) using a Gene Racer kit (Invitrogen). Primer set was described in the Supplementary materials.

*Cloning of promoter regions.* The luciferase vector containing *Skp2* 5′-promoter (pGL2–2275) was a generous gift of Prof. K. Nakayama (Kyushu University Graduate School of Medicine, Fukuoka, Japan). This fragment was inserted into the SmaI and BglII sites of the pGL3 basic vector. Truncation and mutation of the *Skp2* promoter were prepared by a PCR-based method. The upper primers for truncated luciferase vectors of −1247, −916, −307, −167, −116 and −101 bp 5′-promoter/luc were 5′-GGGGCTAGCAAGAAGTAACTG ATGCAAAGA-3′, 5′-GGGGCTAGCATTCTAGGACAGGCTGTGGATT-3′, 5′-GGGGCTAGCAGTCCCGGGCGACCGTG-3′, 5′-CCTCCTCCTCCTTCA ATCC-3′, 5′-AGGGTTGGTCCGAAATCAG-3′ and 5′-GGGGCTAGC TCA GAGTGGAAGAACCCAG-3′. The lower primer was the pGL primer 2 of the pGL3 basic vector. A single underline denotes an added NheI enzyme site. The fragment was ligated to the NheI and NcoI (−1247, −916, −307 and −101 bp 5′-promoter/luc) or SmaI and NcoI (−167 and −116 bp 5′-promoter/luc) sites of the pGL3 basic vector. To introduce mutated STAT binding sites (Fig.. 3B), the following primer sets were prepared, and PCR was performed using the −916 bp 5′-promoter/luc as a template.

Primers (A) and (B) (for distal STAT binding site), 5′-CAGG TTTTCCTGGGCCCGGCGCTCAC-3′ and 5′-GTGAGCGCCGGGCCCAG GAAAACCT-3′.

Primers (C) and (D) (for proximal STAT binding site), 5′-CCTCCA GATACCCACGGCTCCCTGCG-3′ and 5′-CGCAGGGAGCCGTGGGTATC TGGAGG-3′.

A double underline denotes the mutated STATS site. To introduce the mutated distal and proximal STAT sites, a two-step PCR was performed. To obtain a distal STAT site mutation, PCR amplification was performed using the two primer sets, (RV primer 3 of the pGL3 basic vector and A, and B and pGL primer 2) with 916 bp 5′-promoter/luc as the template. Each PCR product was purified, and the mixture of these two PCR products was used for the second PCR template with the primer set of RV primer 3 and pGL primer 2. Proximal STAT mutation was obtained using a similar method. For the first PCR, two primer sets, RV primer 3 and C, and D and pGL primer 2, were used. Then, for the second primer set, RV primer 3 and pGL primer 2, was used with the first PCR products as the template.

*Promoter analysis.* Ba/F3 cells ($1 \times 10^6$) were transfected with 5 μg of reporter plasmid containing various lengths of the 5′-promoter and 2 μg of β-galactosidase expression vector (Promega) using Lipofectin reagent (Invitrogen). After 24 or 48 h, cell lysates were prepared. Promoter activity was normalized with the β-galactosidase activity and was expressed as Luc/β-gal.

*Electrophoresis mobility shift assay of Skp2.* EMSA was performed as described previously [15]. For the supershift experiment, anti-STAT5 or anti-STAT3 antibody was added to the nuclear extract for 15 min at room temperature before mixing with biotin-labeled probes as described below. Forward, 5′-GGGAGTTGTGGGTATCTGG A-3′; reverse: 5′-TCCAGATACCCACAACTCCC-3′ (STAT binding motifs were underlined). In some experiments, mutated oligo was used. *Skp2* mutated *STAT* forward, 5′-GGGAGCCGTGGGTATCTGGA-3′; reverse, 5′-TCCAGATACCCACGGCTCCC-3′ (the mutated STAT binding motifs were double underlined). A biotin label was attached to the 3′ end of each forward probe (Sigma Genosys, Hokkaido, Japan).

*Chromatin immunoprecipitation (ChIP) assay of Skp2.* ChIP assay was performed as described previously [15]. *JAK2V617F*-Ba/F3 cells were used for the crosslinking with formaldehyde. For the immunoprecipitation, normal mouse IgG, anti-STAT3-antibody (final concentration: 3 μg/μl) or anti-STAT5-antibody (final concentration: 3 μg/μl) was added and incubated at 4 °C overnight. Immunocomplexes were extracted, and crosslinking was reversed by heating elutes at 65 °C overnight. Eluates were then digested with proteinase K at 50 °C for 5 h and extracted with phenol/chloroform/isoamyl alcohol. DNA was purified by ethanol precipitation. The promoter region was amplified by PCR using primers 5′-CTCCTCCTCCTCCTCCTCCT-3′ (forward) and 5′-TGCCTGCTGGGA-ATTGTAGT-3′ (reverse).

*Statistical analysis.* The statistical significance was analyzed by one-way factorial analysis of variance and multiple comparison test or Student's *t*-test using Statview ver 5 (SAS Institute Inc., Cary, NC, USA).

## Results

### *Characterization of JAK2V617F-Ba/F3*

While mock- and Wt-Ba/F3 did not grow at all and gradually died in the absence of IL-3, *JAK2V617F*-Ba/F3 acquired the ability to proliferate in an IL-3-independent manner (Supplementary Fig. 1a). When *JAK2V617F*-Ba/F3 was cultured with JAK Inhibitor I, IL-3-independent proliferation and cell viability of *JAK2V617F*-Ba/F3 were inhibited remarkably. *JAK2V617F* as well as *BCR/ABL-Ba/F3* activated JAK2, STAT3 and STAT5 (Supplementary Fig. 2).

### *Cell cycle regulator, $p27^{Kip1}$, was upregulated in mock- and Wt- but not in JAK2V617F-Ba/F3*

We analyzed the expressions of various cell cycle regulatory proteins. After 48 h of IL-3 deprivation, the cell cycle inhibitor $p27^{Kip1}$