Toxicities

The toxicities observed during this study are provided in Table 2. Hematological toxicities were the most common, but grade 3–4 toxicities, including neutropenia (37.5%), thrombocytopenia (5.0%), and anemia (2.5%) were relatively modest. There were only two cases of febrile neutropenia (5.0%). Grade 1 nausea, fatigue, alopecia, neuropathy, and angialgia occurred with a greater frequency than the non-hematologic toxicities. Grade 3–4 non-hematologic toxicities were not seen except in cases of pulmonary toxicity. Two patients (5.0%) developed interstitial pneumonitis (grade 3; one patient, grade 4; one patient), and were responsive to steroid therapy.

Efficacy of treatment

The median number of cycles administered per patient was 4, and the number of cycles ranged from 1 to 8. Twenty-two patients exhibited a partial response. The overall response rate was 55% (22/40) [95% confidence interval (CI): 38.2–71.8%]. Stable disease was achieved in 14 patients (35%), and 4 patients (10%) had progressive disease. All 40 patients were included in the survival analysis. The overall median survival time was 11.9 months (95% CI: 10.3–14 months). The 1-year survival rate was 47.5% (19/40). The median time to disease progression was 6.4 months. Thirty patients (75%) received chemotherapy, and 4 patients (10%) received thoracic irradiation as second-line treatment.

Discussion

Although a standard regimen of first-line chemotherapy for advanced NSCLC is being established, it is important to develop a more active and well-tolerated regimen. Several published randomized studies reported that non-platinum-based chemotherapy in advanced NSCLC was as effective and less toxic than platinum-based regimens [13, 15, 18, 29]. Georgoulias et al. [13] compared the combination of a cisplatin and docetaxel regimen with the GEM and docetaxel regimen. Objective response rates were similar in the two groups, with 32.4% in the former and 30.2% in the latter. The two groups did not differ in the overall survival or 1- or 2-year survival rates. They concluded that both drug combinations had comparable activity and the non-platinum-based regimen had the more favorable profile.

Generally, non-cisplatin-containing treatment does not require supplemental hydration as does standard cisplatin-based chemotherapy. This may be advantageous for elderly patients, patients with poor PS, and patients with renal or cardiac impairment. Recchia et al. [22] conducted a trial of PTX plus GEM in advanced NSCLC patients with a low PS. The chemotherapy regimen consisted of 200 mg/m^2 PTX on day 1 plus 1,000 mg/m^2 GEM on days 1 and 8, repeated every 3 weeks, for a maximum of eight cycles. They achieved a reasonable response rate of 41.3%. Median overall survival time was 13.6 months; the authors concluded that a satisfactory clinical benefit could be obtained with GEM plus PTX regimen in NSCLC patients with a poor PS.

Thus, non-platinum-based chemotherapy may be used as alternative to platinum-based regimens. We conducted a phase II trial was designed to examine the efficacy and tolerance of the non-platinum-based combination of weekly PTX and GEM for patients with untreated advanced NSCLC. Results including an overall response rate of 55%, a median survival time of 11.9 months, and a 1-year survival probability rate of 47.5% suggested that this regimen might have anti-tumor activity equal to that of platinum-based regimens.

Weekly chemotherapy for lung cancer has recently been carried out at several facilities, and favorable results were reported [9, 16, 26, 30]. Compared to standard chemotherapy with administration of drugs at intervals of 3–4 weeks, weekly chemotherapy appears acceptable for the reduction of a single dose level of anti-cancer drugs with fewer side effects. In addition, weekly dose level is more easily adjusted according to the general clinical condition of individual patients or if hematologic toxicity develops. Belani et al. [6] conducted a randomized phase II trial of a 3-week schedule of GEM plus PTX (ArmA) versus a weekly schedule of GEM plus PTX (ArmB) in the treatment of NSCLC. It was concluded that a weekly schedule resulted in improved survival and lower hematologic toxicity than the 3-week schedule.

The clinical outcomes of weekly PTX and GEM therapy found in the literature [3, 6, 7, 11, 12, 14, 19, 28] and in our results are summarized in Table 3. The response rate ranges were from 23.1 to 55%; overall median survival time was 4.9–11.9 months; and 1-year survival rates were 26–53%. Most adverse reactions were hematologic (such as leukope-

Table 2 Maximum toxicity over 40 patients

	CTCAE v 3.0 grade (no. of patients)		Grade 3 or 4 (%)
	Grade 3	Grade 4	
Leukopenia	11	1	12 (30)
Neutropenia	11	4	15 (37.5)
Febrile neutropenia	2	0	2 (5.0)
Anemia	1	0	1 (2.5)
Thrombocytopenia	2	0	2 (5.0)
Pneumonitis	1	1	2 (5.0)

CTCAE v 3.0: Common Terminology Criteria for Adverse Events version 3.0

Table 3 PG regimens used as first-line treatment of advanced NSCLC

First author (ref.)	No. of patients		Regimen and schedule	Response rate (%)	Survival median	One-year (%)
Belani et al. [6]	50	Arm A	P 200 mg/m^2 day 1 q3w G 1 g/m^2 days 1, 8 q3w	28.2	7.5	34
	50	Arm B	P 100 mg/m^2 days 1, 8 q 3w G 1 g/m^2 days 1, 8 q3w	26.8	9.6	42
Bhatia et al. [7]	39		P 110 mg/m^2 days 1, 8, 15 q 4w G 1 g/m^2 days 1, 8, 15 q4w	38.2	4.9	26
De Pas et al. [12]	54		P 100 mg/m^2 days 1, 8, 15, 22 q 4w G 1 g/m^2 days 1, 8, 15, 22 q4w	46	9.6	53
Akerley et al. [3]	39		P 85 mg/m^2 days 1, 8, 15, 22, 29, 36 q 8w G 1 g/m^2 days 1, 8, 15, 22, 29, 36 q8w	23.1	7.5	32
Gillenwater et al. [14]	39		P 100 mg/m^2 days 1, 8, 15, 21 q 4w G 1 g/m^2 days 1, 8, 15, 21 q4w	35	4.9	35
Kosmidis et al. [19]	225		P 200 mg/m^2 day 1 q 3w G 1 g/m^2 days 1, 8, q3w	31	9.3	42
Treat et al. [28]	312		P 200 mg/m^2 day 1 q 3w G 1 g/m^2 days 1, 8, q3w	43.6	8.4	33
Our study	40		P 100 mg/m^2 days 1, 8, q 3w G 1 g/m^2 days 1, 8 q3w	55	11.9	47.5

NSCLC non-small-cell lung cancer, *P* paclitaxel, *G* demcitabine

nia and neutropenia of grade 3 or greater occurrence) in 28–53%. Variable toxicities may be due to population-related pharmacogenomics [11]. Overall, the non-hematologic toxicity was mild, and there were few adverse reactions of grade 3 or greater. A few patients had pneumonitis which was not responsive to steroid therapy. The treatment in our current study was reasonably tolerated, especially in the area of non-hematologic toxicity. Nausea, vomiting, and fatigue, which are often seen in cisplatin-containing regimens, were relatively mild; no patients developed renal toxicity.

In conclusion, weekly chemotherapy with PTX and GEM is a well-tolerated and effective regimen for previously untreated patients with advanced NSCLC. Further studies are expected for the application of this regimen to the elderly, and patients with a poor PS or suspected vulnerability to platinum compound toxicity.

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Conflict of interest statement None.

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ORIGINAL ARTICLE

Expression of myeloperoxidase enhances the chemosensitivity of leukemia cells through the generation of reactive oxygen species and the nitration of protein

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Myeloperoxidase (MPO), a pivotal lineage marker for acute myeloid leukemia (AML), has been also shown to have a prognostic value: a high percentage of MPO-positive blasts correlates to favorable prognosis. To understand the relationship between the expression of MPO in leukemia cells and the response to chemotherapeutic agents, we established MPO-expressing K562 leukemia cell lines and then treated them with cytosine arabinocide (AraC). Cells expressing wild-type MPO, but not mutant MPO that could not mature, died earlier of apoptosis than control K562 cells. Reactive oxygen species (ROS) were generated more in leukemia cells expressing MPO, and the generation was abrogated by MPO inhibitors or antioxidants. Tyrosine nitration of cellular protein also increased more in MPO-expressing K562 cells than control cells after treatment with AraC. In clinical samples, CD34-positive AML cells from high-MPO cases showed a tendency to be sensitive to AraC in the colony-formation assay, and the generation of ROS and the nitration of protein were observed only when the percentage of MPO-expressing cells was high. These data suggest that MPO enhances the chemosensitivity of AML through the generation of ROS and the nitration of proteins.

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Keywords: myeloperoxidase; reactive oxygen species; chemosensitivity; nitrotyrosine; acute myeloid leukemia

Introduction

It is widely accepted that the expression of myeloperoxidase (MPO), a microbiocidal protein, is a golden marker for the diagnosis of acute myeloid leukemia (AML) utilized by the French–American–British and WHO classifications[1,2] to determine the hematopoietic lineage of immature blasts as myeloid. Apart from its role in the diagnosis of AML, MPO has also been shown to have a prognostic value by several groups including ours.[3–5] These reports demonstrated that the percentage of MPO (or Sudan black B)-positive blasts assessed by cytochemical methods was related to the prognosis of AML patients; those with a higher percentage of MPO-positive blasts had better survival rates. Our previous report[5] showed significant differences in complete remission rate, disease-free survival and overall survival using multivariate analysis. However, so far there is no clear explanation as to how the expression of MPO relates to the prognosis of AML.

Chemotherapeutic agents create various reactions in leukemia cells when administered. One of the effects triggered by chemotherapy is the production of reactive oxygen species (ROS).[6,7] ROS are known to modulate the regulators of a wide variety of cellular biological processes including calcium signaling, protein phosphorylation, gene expression, cell growth and differentiation, and chemotaxis.[8,9] They also induce cellular damage associated with lipid peroxidation and alteration of proteins and nucleic acids.[10] Mainly on the basis of *in vitro* studies, it is believed that ROS produced by chemotherapeutic agents play a role in the induction of apoptosis in target cells, which could directly relate to the efficacy of chemotherapy.[7] MPO catalyzes the production of hypochlorous acid using hydrogen peroxide (H_2O_2) as a substrate.[11] Since hypochlorous acids are highly toxic for cells, it is presumed that higher amounts of hypochlorous acids produced by MPO would result in higher toxicity for cells. For example, in the HL60 leukemia cell line, the amount of MPO in cells was directly related to cytotoxicity elicited by chemotherapeutic agents.[12] MPO in HL60 cells was also demonstrated to be involved in the induction of apoptosis by H_2O_2.[13]

The clinical and experimental importance of MPO in the cytotoxicity of chemotherapeutic agents prompted us to directly evaluate the influence of MPO on the efficacy of cytosine arabinocide (AraC), an important antileukemia drug for AML, on leukemia cells. We generated MPO-expressing K562 leukemia cell lines that were originally negative for MPO expression to test for changes in sensitivity to AraC. In this report, we show that the activity of MPO directly enhanced the cytotoxicity of AraC by producing increased amounts of ROS and nitrated tyrosine residues in cellular proteins. In accordance with the observation on leukemia cell line, in samples from AML patients, AraC inhibited colony formation of AML cells more efficiently when MPO expression was high. The production of ROS and nitrated tyrosine was also partly related to the percentage of MPO-positive blasts in clinical samples. These observations suggest important roles for MPO in the cytotoxicity of chemotherapeutic agents during the treatment of AML.

Materials and methods

Vectors, cDNA constructs and mutagenesis

Full-length cDNA for human *MPO* (kindly provided by Dr Nagata, Institute of Medical Science, University of Tokyo)[14] was cloned into pCI-neo, a mammalian expression vector (Promega,

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Madison, WI, USA). The R569W mutation of the *MPO* gene (arginine at the 569th amino-acid position was changed into tryptophan) was generated by PCR-based methods that replaced the C nucleotide at the 1868 bp position by T. Mutagenesis was confirmed by using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM3100 Genetic analyzer (Applied Biosystems). All PCR experiments were performed using the GeneAmp PCR System9700 and GeneAmp High Fidelity Enzyme Mix (Applied Biosystems).

Cell culture and electroporation

The human leukemia cell line, K562, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; CCL-243) was maintained in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (SAFC biosciences, Lenexa, KA, USA) and antibiotics at 37 °C under 5% CO_2. In some experiments, cytosine arabinoside (Sigma, St Louis, MO, USA), H_2O_2 (Wako Pure Chemical Industries, Osaka, Japan), *N*-acetylcysteine (Sigma) or 4-aminobenzoic hydrazide (Sigma) were added alone or in various combinations into the culture medium. Peroxynitrite tetramethylammonium (Alexis Biochemicals, San Diego, CA, USA) was used as a source of reactive nitrogen species (RNS). Cell growth was assessed using the Premix WST-1 Cell Proliferation Assay System (Takara Biochem., Tokyo, Japan). pCI-neo carrying normal or mutated cDNA for MPO was transfected into log-phase K562 cells by electroporation. In brief, cells suspended at the concentration of 1×10^7 cells per ml in Nucleofector solution (Amaxa biosystems, Gaithersburg, MD, USA) were mixed with 1 μg of plasmid DNA and then electroporation was performed with Nucleofector (program T-16; Amaxa biosystems). Stable lines that were transfected with various plasmids were selected as a single clone in the presence of 800 μg ml^{-1} of G418 (Sigma).

Flow cytometry analysis

For the detection of Annexin V, cells were stained with an Annexin V Fluos staining kit (Roche, Mannheim, Germany). To measure the mitochondrial membrane potential, cells were incubated with the J-aggregate-forming cationic dye, JC-1 (Molecular Probes, Karlsruhe, Germany), at a concentration of 10 μg ml^{-1} for 10 min at 37 °C. ROS in cells were measured by flow cytometry using 2-[6-(4′-amino)phenoxy-3*H*-xanthen-3-on9-yl]benzoic acid (APF; Daiichi pure chemicals, Tokyo, Japan) fluorescence and 2-[6-(4′-hydroxy) phenoxy-3*H*-xanthen-3-on9-yl] benzoic acid (HPF; Daiichi pure chemicals) fluorescence. APF reacts with hydroxyl radicals, peroxinytrite and hypochlorous acid. HPF reacts with hydroxyl radicals and peroxinytrite, but not with hypochlorous acid. For the detection of nitric oxide, diaminofluorescein-2 diacetate (Daiichi pure chemicals) was used. All flow cytometric measurements were performed with a FACScan flowcytometer (Becton Dickinson, San Jose, CA, USA). Data were analyzed using CellQuest software (Becton Dickinson).

Morphological analysis

Cells spread on slide glasses were stained with standard May–Grunwald Giemusa staining and the diaminobenzidine (DAB) method for the detection of MPO activity. For analysis of MPO activity with electron microscopy (JEM-1210 electron microscope; JEOL, Tokyo, Japan), cells fixed with 1.25% glutaraldehyde were incubated with DAB.

Western blot analysis

After disruption in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P40, 1 mM EDTA, 10 μg ml^{-1} of aprotinin, 10 μg ml^{-1} of leupeptin and 1 mM phenylmethylsulfonyl fluoride), samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). Target proteins were visualized using a rabbit polyclonal antibody against MPO (DakoCytomation, Carpinteria, CA, USA), a rabbit polyclonal antibody to nitrotyrosine (Chemicon, Temecula, CA, USA), mouse monoclonal antibody to β-actin (Abcam, Cambridge, UK) or to heat-shock protein 90α/β (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with peroxidase-labeled secondary antibodies (Amersham Bioscience, Buckinghamshire, UK) and an enhanced chemiluminescence system (ECL Advance Western Blotting Detection Kit; GE Healthcare Bio-Sciences, Buckinghamshire, UK).

Patients' samples for ROS and nitrotyrosine detection

Peripheral blood or bone marrow samples were obtained from 14 AML patients prior to treatment with informed consent. CD34-positive (+) cells were selected using an immunomagnetic column (Miltenyi Biotech, Auburn, CA, USA). The purity of CD34 + cells was assessed by flow cytometry, demonstrating that more than 95% of cells was CD34-positive after selection. In six cases, CD34-positive AML cells (1×10^5 per well in a 24-well culture plate) with or without 20 nM AraC were cultured in semisolid media (MethoCalt GF H4434; StemCell Technologies, Vancouver, BC, Canada). The number of colonies containing 30 or more cells was counted 7–14 days after plating. In other eight cases, cells were cultured in Iscove's modified Dulbecco's medium with 10% fetal bovine serum incubated with 10 μM AraC or saline as a control for 6 h with or without H_2O_2, and then analyzed for the detection of ROS. Using four out of eight samples treated with 10 μM AraC up to 6 h, the nitration of tyrosine residues was assessed by western blot analysis with anti-nitrotyrosine antibody as mentioned above. Quantification of bands of western blot was performed using FluoChem IS-8800 and AlphaEase FC Software (Alpha Innotech Corp., san Leandro, CA, USA). The intensity of bands was shown as an average value (AVG). The pixel value and area of each band were counted; then AVG was calculated as follows: AVG = [Σ(each pixel value−background)]/area, which was suggested by the system manual. Expression of MPO in CD34 + cells was examined by flow cytometry.

Statistical analysis

Results are presented as the mean ± s.d. of three independent experiments. Differences between experimental groups were compared using one-way analysis of variance followed by the Scheffe's multiple comparison procedure. Statistical significance was considered at a *P*-value of 0.05.

Results

Establishment of cell lines expressing wild-type or mutant MPO

K562 cell lines expressing wild-type and mutant (R569W) MPO were established as single clones. R569W mutation of the MPO

protein,[15] originally found in an MPO-deficient person, resulted in a defective maturation process. MPO protein with the R569W mutation attains apopro-myeloperoxidase status but cannot mature further; it remains in the non-functional stage. Western blot analysis demonstrated the presence of immature MPO protein (apopro-myeloperoxidase, 89 kDa) in both wild-type and mutant MPO-expressing cell lines (MPO-21 and R569W-2, respectively; Figure 1). On the other hand, as expected, the α-subunit of mature MPO protein at 64 kDa and the β-subunit at 14 kDa were detected only in MPO-21 cells since these subunits are generated at the late maturation process of MPO. No apparent difference in the morphological features of MPO-21 and R569W-2 were detected by May-Grunwald Giemusa staining (Figure 2a). Cytochemical analysis using light microscopy detected MPO activity in MPO-21 but not R569W-2 cells (Figure 2a). Other two MPO-expressing lines (MPO-6 and MPO-18) also had the same-size MPO protein as MPO-21 and showed the MPO activity (Supplementary data, Figure 1). Electron microscopy revealed that enzymatically active MPO protein was localized to the cytoplasm (Figure 2b).

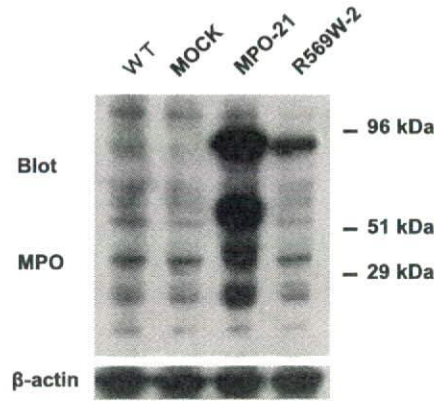

Figure 1 Western blot analysis of myeloperoxidase (MPO) protein in K562 cells. Immature MPO protein (apo-pro MPO, 89 kDa) was detected in K562 cells expressing wild-type MPO (MPO-21) and mutant MPO (R569W-2). Mature MPO protein (64 and 14 kDa) was seen only in MPO-21 cells. MOCK, K562 cells transfected with control plasmid; WT, wild-type K562 cells.

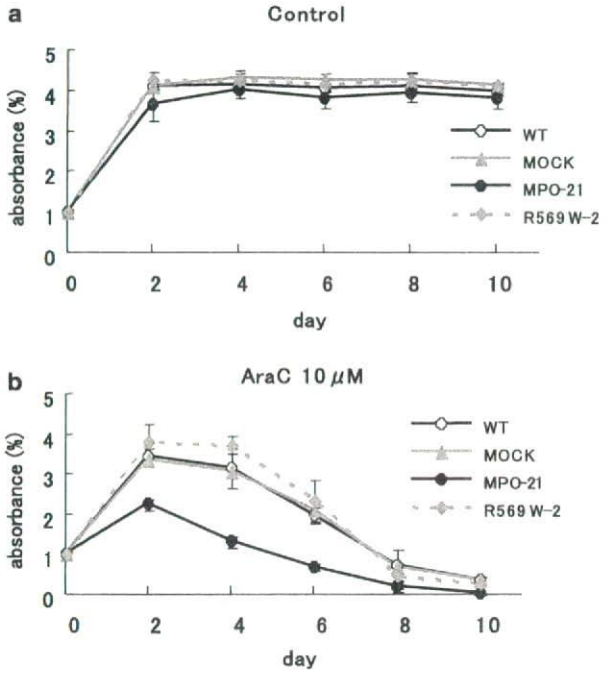

Figure 3 WST-1 analysis of K562 cells. Wild-type K562 (WT), MOCK (transfected with control vector), MPO-21 (wild-type myeloperoxidase (MPO)-expressing K562) and R569W-2 (mutant MPO-expressing K562) cells showed a similar growth pattern in the steady state (**a**). In the presence of cytosine arabinocide (AraC), MPO-21 cells demonstrated an earlier decline than other three cell lines (**b**). The mean value of three independent experiments at each point is shown with the standard deviation.

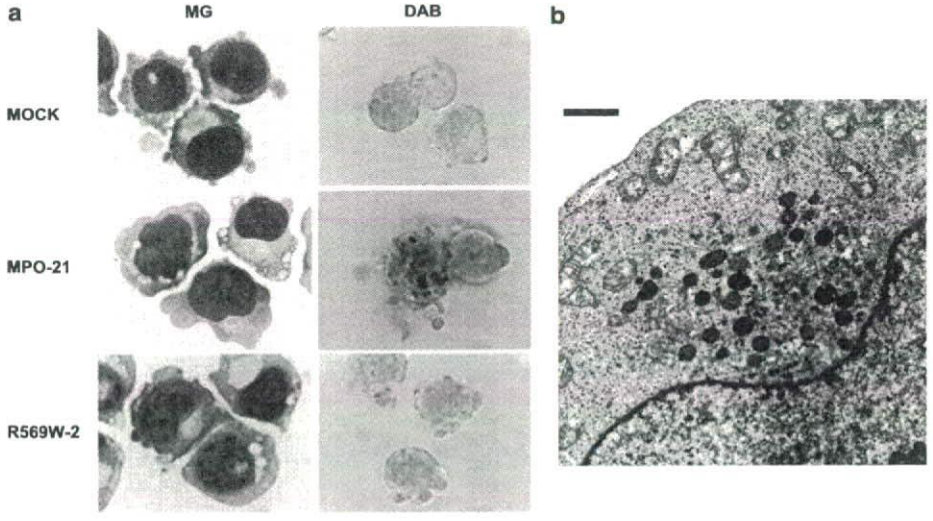

Figure 2 Morphological and cytochemical features of myeloperoxidase (MPO)-expressing K562 cells. May-Grunwald Giemusa (MGG) staining and diaminobenzidine (DAB) staining of K562 cells transfected with control vector (MOCK), wild-type MPO (MPO-21) and mutant MPO (R569W-2) (**a**). DAB was observed only in MPO-21 cells. (**b**) DAB-positive granules (dark granules) were seen in the cytoplasm of MPO-21 cells using electron microscope (scale bar represents 1 μm).

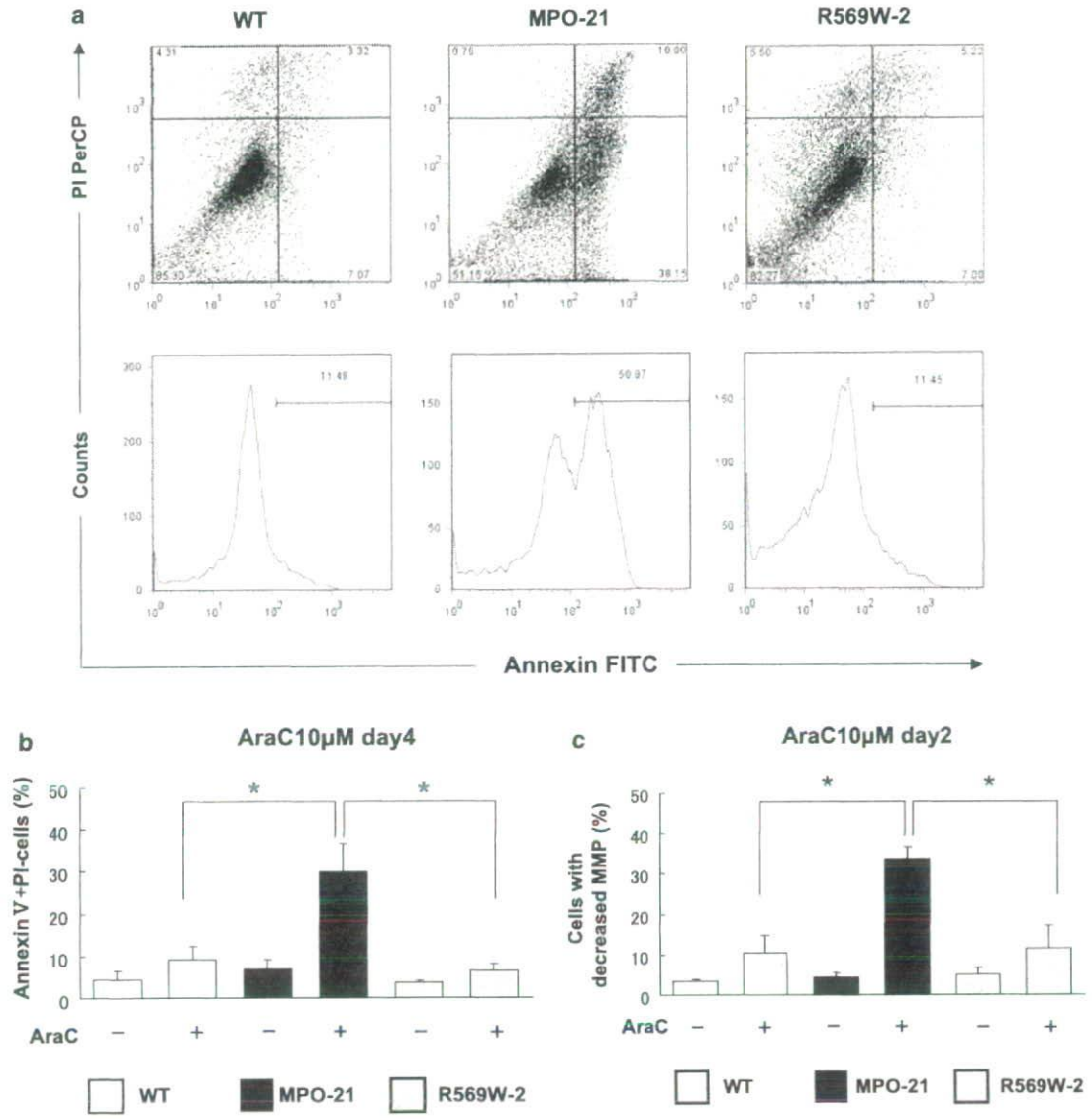

Figure 4 Apoptosis is accelerated in MPO-21 cells. Histogram of Annexin V expression on day 4 of cytosine arabinocide (AraC) treatment (**a**). A larger proportion of MPO-21 cells expressed Annexin V in a propidium iodide (PI)-negative fraction than wild-type K562 (WT). Data of three independent experiments are shown (**b**). There was a statistically significant difference (*$P<0.05$). Change of mitochondria membrane potential (MMP) was probed by JC-1 on day 2 of AraC treatment and the results of three independent experiments are shown (**c**). The decrease of MMP was significant in MPO-21 cells (*$P<0.05$).

MPO activity enhanced the cytotoxic effect of AraC by inducing apoptosis

Proliferation of wild-type K562 (WT), control K562 transfected with an empty vector (MOCK), MPO-21 and R569W-2 cells was similar as assessed by WST-1 assay, keeping maximum absorbance that showed a log phase in growth after day 2 of culture (Figure 3a). However, when cells were treated with AraC at 10 μM, MPO-21 showed an earlier decline than others (Figure 3b). MPO-6 also showed similar pattern as MPO-21 (Supplementary data, Figure 2). Since it is known that AraC induces apoptosis in leukemia cells,[16] we next analyzed whether the introduction of MPO in K562 cells accelerated this process or not. As shown in Figure 4a, on day 4 of AraC treatment, a larger proportion of MPO-21 cells (38%) were found to have Annexin V (and propidium iodide-negative) on their surface than wild-type K562 cells (7%). On the other hand, no change was observed between wild-type K562 and R569W-2. Data from three independent experiments showed statistical differences in the expression of Annexin V between MPO-21 and wild-type K562 or R569W-2 ($P<0.05$, Figure 4b). An earlier marker for apoptosis, the change of mitochondrial membrane potential detected using JC-1, was also significantly increased in MPO-21 cells than other two lines on day 2 ($P<0.05$, Figure 4c).

MPO enhanced the generation of ROS

Since MPO catalyzes the formation of hypochlorous acid, an ROS, we examined whether the generation of ROS was enhanced by the expression of MPO using fluorescent markers

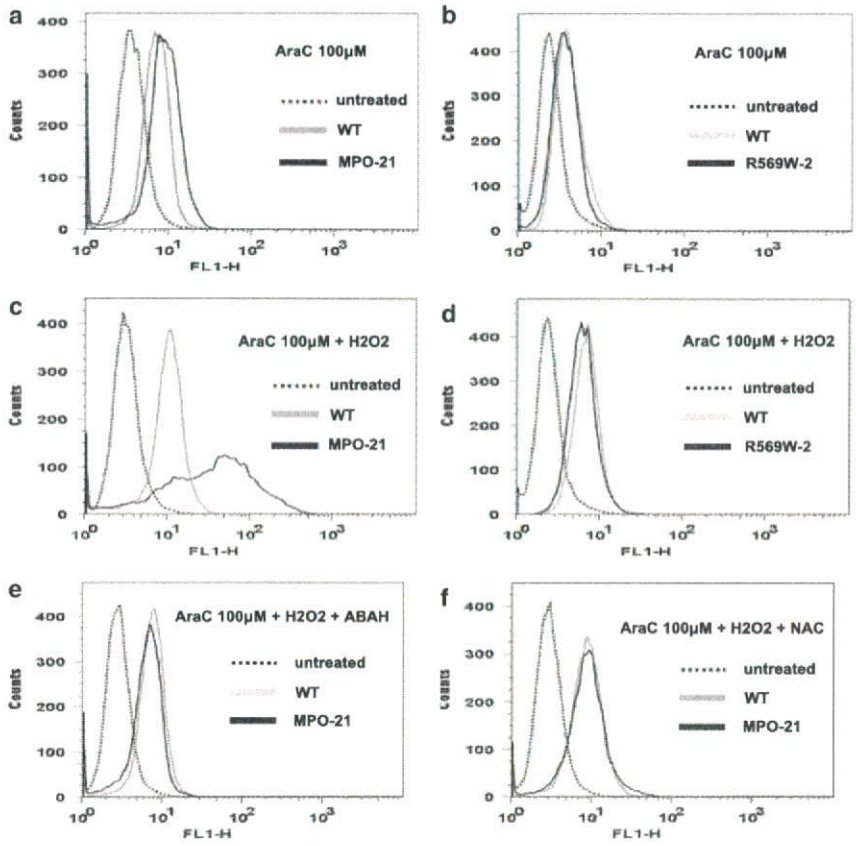

Figure 5 Reactive oxygen species (ROS) generation in K562 cells expressing myeloperoxidase (MPO). After treatment with cytosine arabinocide (AraC), ROS production probed with APF was increased in wild-type K562 (WT) cells, and was still stronger in MPO-21 cells (**a**). There was no difference in ROS production between WT and R569W-2 treated with AraC (**b**). In the presence of H_2O_2, ROS generation was enhanced more strongly in MPO-21 cells than WT or R569W-2 cells (**c** and **d**). These changes were completely abrogated by 4-aminobenzoic hydrazide (ABAH), an inhibitor of MPO (**e**), and *N*-acetylcysteine (NAC), an antioxidant (**f**). FL1-H represented the fluorescent intensity of APF.

for ROS. After treatment with AraC, the amount of ROS detected by APF but not by HPF was increased in MPO-21 cells when compared to wild-type K562 cells or R569W-2, suggesting the production of hypochlorous acid among ROS (Figures 5a and b). To clarify differences in ROS production, H_2O_2 was added into the culture medium to enhance MPO-dependent ROS production. ROS production was increased with H_2O_2 alone (Supplementary data, Figures 3a and b); however, combining H_2O_2 (40 μM) with AraC significantly enhanced the generation of ROS in MPO-21 cells but not in wild-type K562 cells or R569W-2 (Figures 5c and d). ROS were also generated in other MPO-expressing cell lines, MPO-6 and MPO-18 (Supplementary data, Figures 3c–f). The increase of ROS was completely abolished by 4-aminobenzoic hydrazide (100 μM), an inhibitor of MPO (Figure 5e) or by *N*-acetylcysteine (1 mM), a thiol antioxidant (Figure 5f). These results suggested that the activity of MPO was directly related to the production of ROS when cells were treated with AraC. We did not observe any change in the fluorescent intensity of diaminofluorescein-2 diacetate, a probe for nitric oxide, even after treatment with H_2O_2 and AraC (data not shown).

To further analyze the effect of H_2O_2 on cell growth, we treated wild-type K562 and MPO-21 cells with H_2O_2 for a short period in the presence or absence of AraC. As shown in Figure 6a, after treatment with 40 μM H_2O_2 for 45 min, the value of WST-1 assay decreased in both wild-type K562 and MPO-21 cells transiently and recovered on day 2. However, in the presence of AraC, the same treatment with H_2O_2 suppressed cell growth more significantly in MPO-21 than in K562 cells (Figure 6b). In addition, only wild-type K562 cells recovered from the suppression. In this system, the combination of AraC and H_2O_2 was not enough to suppress the growth of leukemia cells; MPO was also necessary.

Generation of nitrotyrosine was enhanced by MPO

Since MPO was shown to catalyze the generation of not only ROS but also nitrotyrosine in the presence of nitrogen dioxide, we next examined whether the introduction of MPO in K562 cells also changed the amount of nitrotyrosine. Western blot analysis using an anti-nitrotyrosine antibody detected strong nitration of proteins in the positive control lysate of wild-type K562 cells incubated with RNS (Figure 7). Without AraC or RNS, wild-type K562, R569W-2 and MPO-21 cells showed similar patterns and intensities in the expression of nitrotyrosine, which were all much weaker than those of the positive control. After treatment with AraC, the intensity of bands only increased in MPO-21 cells.

Colony formation of AML cells in semisolid media

In six AML cases, colony formation of CD34 + AML cells was tested in the presence or absence of AraC (Table 1). CD34 + cells were selected from the bone marrow or peripheral blood to avoid the influence of MPO present in mature myeloid cells. The number of colonies generated was increased among three cases with low MPO (3, 6 and 10%) than three with high MPO positivity (90, 96 and 100%). AraC (20 nM in culture) suppressed colony formation in three cases with high MPO compared to low-MPO cases: the number of colonies decreased to 0–10% of control in the presence of AraC, whereas 33–89% of control in low-MPO cases.

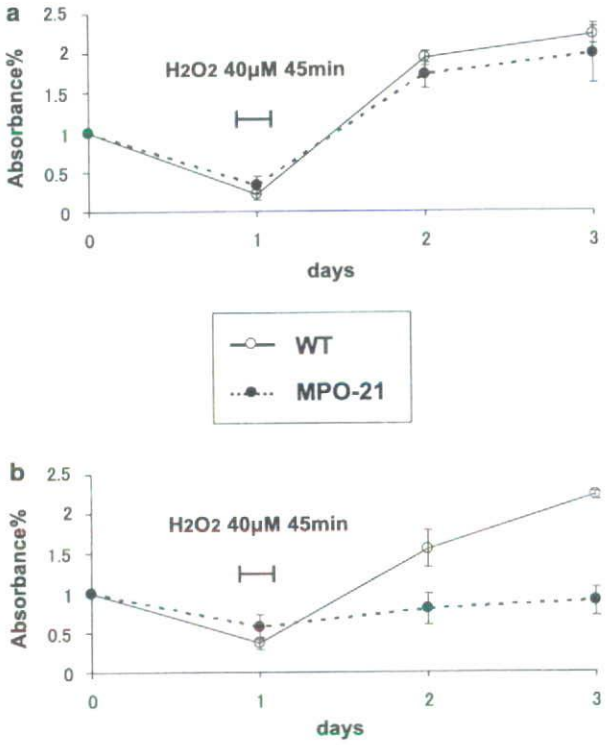

Figure 6 Combination of cytosine arabinocide (AraC), hydrogen peroxide (H_2O_2) and myeloperoxidase (MPO) showed the strong suppression of proliferation as measured by WST-1 assay. WT and MPO-21 cells were treated with H_2O_2 for 45 min with or without AraC. Transient treatment with H_2O_2 alone resulted in no difference between WT and MPO-21 (**a**). Addition of AraC suppressed proliferation of MPO-21 cells only (**b**). The mean value of three independent experiments at each point is shown with the standard deviation.

Production of ROS and nitrotyrosine in AML cells treated with AraC

We next examined whether the expression of MPO in CD34 + AML cells related to the production of ROS when treated with AraC. As shown in Figures 8a and b, in one out of eight samples tested, ROS production was increased by AraC in the presence of H_2O_2. The MPO positivity in this case was 98% by flow cytometry. In other seven cases, regardless of the percentage of MPO-positive cells (0, 0.4, 94, 1, 5.5, 96 and 25% among CD34 + cells), no ROS were detected. Figures 8c and d are the representative histograms of negative samples in which ROS were not detected in leukemia cells even after treatment with AraC, H_2O_2 or the combination of both.

Nitration of tyrosine residues was tested with western blot analysis among four cases of AML: two with high MPO (case 8,

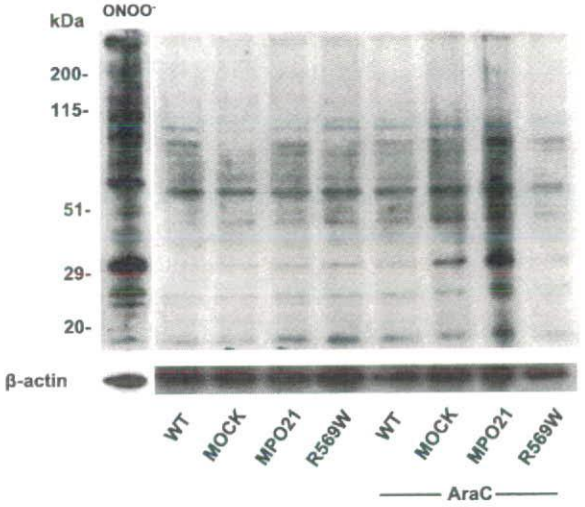

Figure 7 Nitration of tyrosine residues after cytosine arabinocide (AraC) treatment in MPO-21 cells. The generation of nitrotyrosine was examined in cells treated with reactive nitrogen species (RNS) or AraC. Positive control samples treated with RNS (peroxynitrite tetramethylammonium ($ONOO^-$)) and AraC-treated MPO-21 cells showed a clear increase in nitrotyrosine when compared to untreated controls. WT, wild-type control.

Table 1 Inhibition of colony formation of CD34-positive AML cells by AraC

Case number	*Diagnosis*	*% MPO+cells*	*Number of cells per well*	*Number of colonies per well*		*Percentage of colony number in AraC 20*
				Control	*AraC (20 nM)*	
1	M4	6	1×10^5	19	17	89
2	MDS/AML	3	1×10^5	63	55	87
			2×10^5	111	89	80
3	M5b	10	1×10^5	141	47	33
			2×10^5	TMTC	66	NA
4	M1	100	1×10^5	0	0	NA
			2×10^5	4	0	0
5	M2	96	1×10^5	61	6	10
6	M2	90	1×10^5	8	0	0

Abbreviations: AML, acute myeloid leukemia; AraC, cytosine arabinocide; MDS, myelodysplastic syndromes; MPO, myeloperoxidase; NA, not available; TMTC, too many to count.

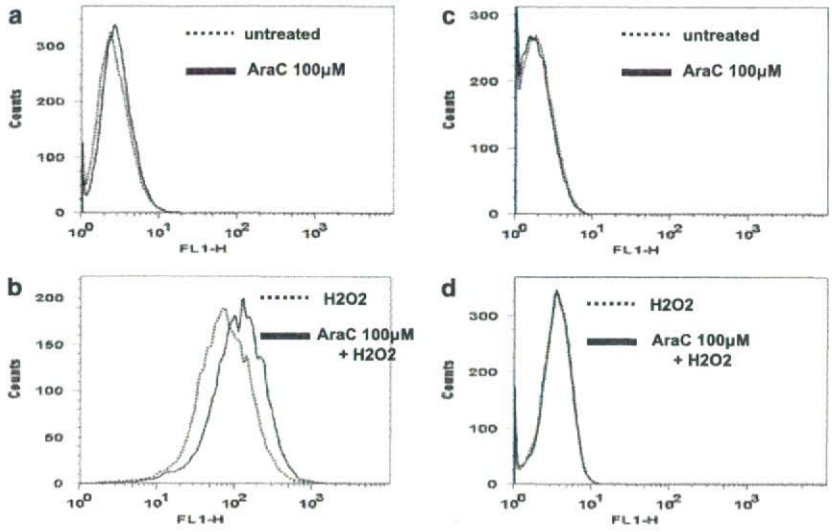

Figure 8 Generation of reactive oxygen species (ROS) in acute myeloid leukemia samples. CD34+ cells were selected and were treated with 100 μM cytosine arabinocide (AraC) and 40 μM hydrogen peroxide (H_2O_2) for 3 h. ROS generation was examined with flow cytometry. Histograms of a positive case (**a** and **b**, high myeloperoxidase (MPO) expression) and a negative case (**c** and **d**, low MPO expression) are shown.

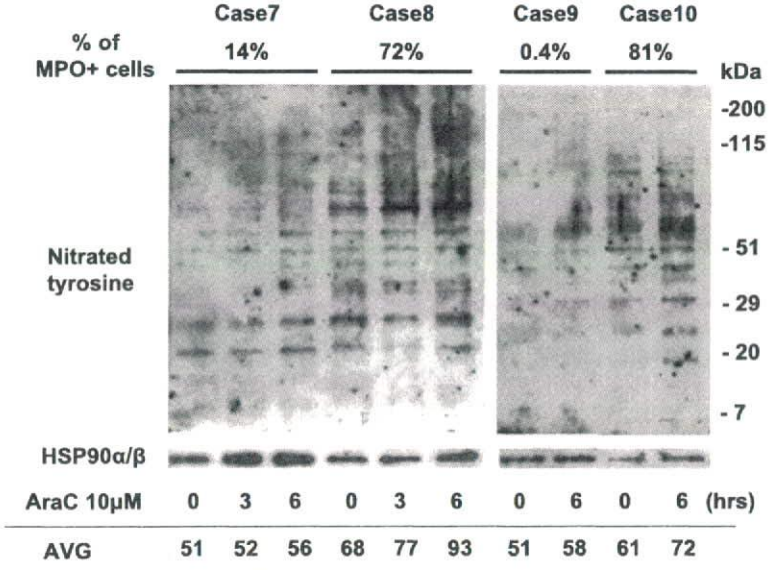

Figure 9 Nitration of tyrosine residues in acute myeloid leukemia (AML) samples. CD34+ cells from four AML cases were treated with 10 μM cytosine arabinocide (AraC) for up to 6 h. Two cases had high myeloperoxidase (MPO) expression (cases 8 and 10), and two had low MPO (cases 7 and 9). Nitrotyrosine was detected by western blot analysis. The intensity of bands in each lane was shown as average value (AVG) in this figure. The percentage of MPO-positive cells was assessed using flow cytometry.

72% of MPO positivity, and case 10, 81%) and two with low MPO (case 7, 0.4%, and case 9, 14%). The intensity of bands in each lane was measured as described in Materials and Methods, then shown as an average intensity (AVG) in Figure 9 (raw data of this procedure is in Supplementary data, Figure 4 and Table 1), which increased along with the incubation time with AraC. The increment of AVG after 6 h of treatment was larger among cases with high MPO (136 and 118% in cases 8 and 10, respectively) than among those with low MPO (110 and 113% in cases 7 and 9, respectively).

Discussion

In this study, we demonstrated that MPO-expressing K562 leukemia cells showed an increased sensitivity to AraC when compared to wild-type or non-functional MPO-expressing K562 cells. After treatment with AraC, these cells generated a higher amount of ROS and nitrated tyrosine residues, resulting in an earlier induction of apoptosis. These reactions were abrogated by inhibitors of MPO or ROS. The results above strongly suggested the relationship between the expression of MPO and

the production of ROS or tyrosine nitration in leukemia cells when treated with AraC. Since ROS and protein nitration were already shown to be toxic for target cells, it is likely that the active MPO protein itself worked with AraC to increase its cytotoxicity. Accordingly, using fresh AML cells, the inhibition of colony formation by AraC tended stronger in cases with high MPO than in those with low MPO expression. It is interesting that the number of colony in high-MPO cases was less than that in low-MPO cases inspite of the fact that the forced expression of MPO in K562 did not influence their proliferation. It seemed that MPO itself does not change growth of cells, but the characteristics of AML cells that express MPO might relate to one of the many factors that control their growth, at least, in some cases. The generation of ROS and the nitration of tyrosine residues, though not so apparent as in colony-formation experiments, were observed only when CD34+ blasts expressed MPO at high levels. It is conceivable that similar reactions were triggered by AraC in high-MPO AML cells as in MPO-expressing K562 cells. MPO did not enhance the fluorescence of diaminofluorescein-2 diacetate, which reacts with NO, in MPO-expressing K562 cells after AraC treatment; however, the nitration of tyrosine residues in these cells was observed by western blot analysis. It seemed that the ROS generated by MPO were involved in the nitration of tyrosine residues as reported previously.[17–19]

Clinical observation has repeatedly shown a significant impact of the percentage of MPO-positive blasts on the prognosis of AML patients.[3–5] From data in this study, we postulate that MPO itself could enhance the cytotoxicity of chemotherapeutic agents through the generation of ROS or the nitration of cellular proteins, and that it could contribute, at least in part, to favorable responses to chemotherapy. It is very interesting that AML cases with favorable karyotypes such as t(15;17), t(8;21) and inv(16) usually have a high percentage of MPO-positive blasts.[20,21] Recently, a polymorphism in the promoter region of the *MPO* gene was shown to relate to survival of breast cancer patients after chemotherapy:[22] patients having lower transcriptional activity of *MPO* (G to A conversion at the −463 nucleotide of the *MPO* gene) showed significantly worse prognosis. The authors of this report concluded, in concordance with our current observation, that the oxidative stress would modify prognosis after chemotherapy for breast cancer.

Leukemia stem cells that consist of a small fraction of the overall leukemia cell population have been reported to maintain leukemia.[23] It is highly possible that the chemosensitivity of leukemia stem cells is an important and vital factor for obtaining a good response to chemotherapy leading to a favorable prognosis. We previously reported that expression of the *MPO* gene in CD133-positive leukemia cells related to the prognosis of AML.[24] As the CD133-positive fraction of AML cells contained leukemia stem cells,[25] the results of the present study could be interpreted as events occurring in the growth fraction of AML cells.

Myeloperoxidase cannot be the sole marker of a good response to chemotherapy. For example, defenses against oxidative stress would also affect the response to ROS generated by anticancer drugs. In this regard, the results in Figures 8 and 9, the ROS and nitrotyrosine generation in clinical samples needed to be re-evaluated. It therefore is necessary to fully understand the biology of the immature (stem cell) fraction of leukemia, including the expression of MPO and defense mechanism against ROS and its relationship with other factors such as the karyotype of leukemia cells and other genetic abnormalities.

Acknowledgements

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Proposals for a Grading System for Diagnostic Accuracy of Myelodysplastic Syndromes

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Abstract

Despite recent advances in cytogenetics and molecular research, universal biomarkers for the diagnosis of the myelodysplastic syndromes (MDS) are still lacking. It is not easy to diagnose MDS by morphology alone, particularly in patients with < 5% blasts in the bone marrow (BM) and normal karyotype. Therefore, the possibility of misdiagnosis and discordance among observers can occur. In order to resolve these problems, we propose a grading system for diagnostic accuracy of MDS. The diagnostic accuracy of MDS is graded into "definite," "probable," or "possible" in addition to "idiopathic cytopenia(s) of uncertain significance (ICUS)." The criteria of grading for diagnostic accuracy are a combination of (1) the frequency of blasts in BM, (2) grade of dysplasia (high, intermediate, or low), and (3) division of cytogenetics (abnormal, normal, or unknown). For quantitative morphologic evaluation of dysplasias, we classified morphologic dysplastic changes into highly specific category A (pseudo–Pelger-Huet anomaly, degranulation of neutrophils, micromegakaryocytes, and ringed sideroblasts) and less specific category B (dysplasias other than those in category A). We believe that diagnostic problems would be reduced by using our grading system and repeating BM examination at suitable intervals for patients who are allocated into the "possible" or "ICUS" categories, and this will make the vague margin of MDS category clearer.

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Key words: Cytogenetics, Dyserythropoiesis, Idiopathic cytopenia of uncertain significance, Pelger-Huet

Introduction

Myelodysplastic syndromes (MDS) are acquired clonal stem-cell disorders characterized by ineffective hematopoiesis with myelodysplasia[1] and are associated with a high risk of progression to acute leukemias.[2] Despite recent advances in cytogenetics and molecular research, universal biomarkers for the diagnosis of MDS are still lacking. It is not easy to diagnose MDS, particularly in patients with < 5% blasts in the bone marrow (BM) and normal karyotype. In such patients, the diagnosis mainly depends on morphologic examinations. Minimal morphologic requirements to diagnose MDS are well established but might not be accurate or leave too much room for subjectivity. Herein, we propose a grading system for the diagnostic accuracy in an attempt to reduce misdiagnosis and improve concordance among observers.

Background for Proposals

Exclusion of nonclonal disorders[3-6] with some myelodysplasia is crucial to the diagnosis of MDS. However, in patients with < 5% blasts in the BM and normal karyotype, it is not easy to distinguish MDS from such nonclonal disorders by morphology alone. In addition, judgments of dysplasia are subjective to a certain extent. Therefore, misdiagnosis and discordance among observers are likely to occur. In patients with hypoplastic BM, it is important to distinguish hypoplastic MDS from aplastic anemia (AA). Dyserythropoiesis (Dys E) is often found in patients with AA and cannot be used alone to distinguish MDS from AA.[7]

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We previously reported that the presence of hyposegmented mature neutrophils (Pelger), degranulation of neutrophils (agranular or hypogranular neutrophils; Hypo-Gr), and micromegakaryocytes (mMgk) in BM or peripheral blood (PB) were found in 76%, 30%, and 74% of MDS cases, respectively, whereas there was no AA case with these dysplasias.[8] We confirmed the specificity of these dysplasias in a different case series showing that Pelger ≥ 10% or mMgk ≥ 10% in BM was not found in the AA group (presented at the Ninth International Symposium on MDS). We also showed that, among patients with refractory anemia (RA) according to the French-American-British (FAB) classification[9] (FAB-RA), excluding MDS associated with isolated deletion of chromosome 5q (5q-syndrome), the presence of Pelger ≥ 10% or mMgk ≥ 10% in BM (15% and 14% of RA cases, respectively) were significantly related to the shorter overall survival (OS) and leukemia-free survival (LFS). The median OS and LFS of cases with Pelger ≥ 10% were 29 months and 36 months, respectively, and were significantly shorter than those without Pelger ≥ 10% (158 months and not reached, respectively; $P < .001$ in both). Micromegakaryocytes ≥ 10% showed similar effect on OS and LFS (23 months vs. 153 months for OS [$P < .001$] and 51 months vs. not reached for LFS [$P < .001$]).[10] The concordance rates of Pelger and mMgk were reasonably high among observers.[11] These dysplasias are much easier to detect, not only for expert morphologists but also for clinical hematologists in general. We considered that misdiagnosis and discordance would be avoided by enumerating these MDS-specific dysplasias.

Idiopathic cytopenia of uncertain significance (ICUS) was first proposed by Mufti et al at the Eighth International Symposium on MDS in Nagasaki, Japan, in 2005. If patients with normal karyotype and < 5% BM blasts do not show morphologic dysplasia (ie, < 10% of any cell lineage) and all other diseases have been ruled out as a cause of cytopenia, the patients are diagnosed with ICUS. The cytopenia(s) should persist for ≥ 6 months without any other cause identified. The criteria for ICUS was proposed in a recent publication by Valent et al.[12] Idiopathic cytopenia of uncertain significance might be a useful category for patients with unexplained cytopenia who do not fulfill the criteria of MDS (either of the FAB classification or the World Health Organization [WHO] classification[13]). Extensive study for this category in terms of MDS pathophysiology, particularly a molecular aspect, will clarify the clinical and pathophysiologic features of the ICUS category.

We previously compared the morphologic features between FAB-RA, excluding 5q-syndrome AA at the Ninth International Symposium on MDS, held in Florence, Italy, in 2007. One hundred patients with FAB-RA, excluding 5q-syndrome, were diagnosed by a joint review of a Japanese and German collaboratory study.[10,14] Forty patients with AA who registered to the Japanese AA and MDS Registration System of the National Research Group on Idiopathic Bone Marrow Failure Syndromes, Japan were diagnosed by the Central Review Working Group. In all patients with FAB-RA, the frequency of dysplasia was ≥ 10% in ≥ 1 lineage. Some (17%) patients with AA showed Dys E ≥ 10% in BM; Hypo-Gr ≥ 10%, Pelger ≥ 10%, or mMgk ≥ 10% were found only in the FAB-RA group. In addition, dysplasia ≥ 10% in ≥ 2 lineages was found only in the FAB-RA group. The number of megakaryocytes was markedly decreased in all patients with AA. The presence (≥ 5%) of blasts in BM was never found in the patients with AA.

Table 1 Prerequisite Criteria

Criteria
A. Constant cytopenia (≥ 6 months) in ≥ 1 of the following lineages:
Hemoglobin < 11 g/dL
Absolute neutrophil count < 1.5×10^9/L
Platelet count < 100×10^9/L
B. Less than 20% blasts in PB or BM and absence of cytogenetic findings related with acute myeloid leukemia with recurrent cytogenetic abnormalities*
C. Less than 1×10^9/L monocytes in PB
D. Exclusion of all other hematopoietic or nonhematopoietic disorders as primary reason for cytopenia
E. Exclusion of aplastic anemia. In case of hypoplastic BM, exclusion of aplastic anemia needs to be considered using morphologic findings and cytogenetic data.

A-E must be fulfilled.
*t(8;21)(q22;q22); (AML1/ETO), t(15;17)(q22;q12); (PML/RARα), and inv(16)(p13;q22) or (t16;16)(p13;q22); (CBFβ/MYH11).

Recently, minimal diagnostic criteria for MDS have been proposed by Valent et al.[12] They did not show a list of dysplastic cells in their criteria. We think a clear and definite list of dysplastic cells is necessary for diagnostic criteria. We propose a grading system for diagnostic accuracy of MDS by combining the results of our morphologic study presented at the Ninth International Symposium on MDS with the criteria proposed by Valent et al.

A Grading System for Diagnostic Accuracy of Myelodysplastic Syndrome

Exclusion of disorders with constant cytopenia(s) and some morphologic dysplasia(s) other than MDS is a prerequisite for diagnosing MDS. We propose that dysplasia(s) be divided into category A (high specificity) and category B (low specificity) for assessment of the frequency of dysplasia(s). A quantitative morphologic evaluation of category A or A + B is essential to start diagnosis of MDS. We then suggest a grading of dysplasia based on the enumeration and a division of cytogenetic findings. The criteria for grading of diagnostic accuracy are a combination of the frequency of blasts in BM, grade of dysplasia, and divisions of cytogenetics. The grades of diagnostic accuracy are divided into "definite," "probable," or "possible" in addition to "ICUS." Patients who are diagnosed as "definite," "probable," or "possible" should be classified according to the WHO classification for MDS.

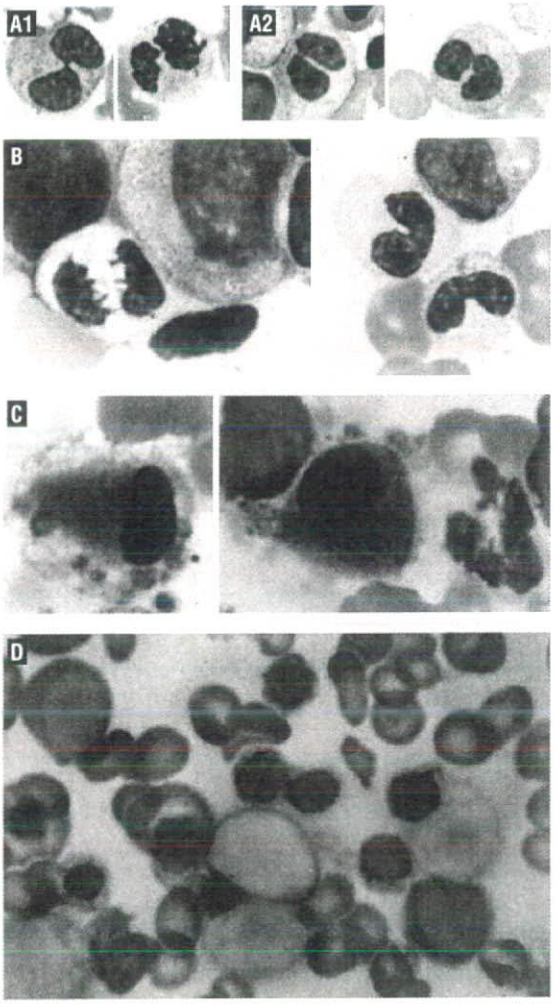
Step I: Exclusion Diagnosis of Disorders Other Than Myelodysplastic Syndrome

We modified the excellent prerequisite criteria proposed by Valent et al.[12] Table 1 shows our prerequisite criteria, consisting of the definition of constant cytopenias (≥ 6 months) and exclusion of disorders with constant cytopenias or some myelodysplasia. Acute myeloid leukemia (AML) should be excluded by frequency of blasts and cytogenetic findings. Bone marrow differential counts should be performed on 500 cells. Counting the number of monocytes in PB is necessary for the exclusion of chronic myelomonocytic

Table 2 Classification of Dysplasia

Category A
Granulocytic series
Hyposegmented mature neutrophils (Pelger)
Degranulation (agranular or hypogranular neutrophils; Hypo-Gr)
Megakaryocytic series
Micromegakaryocytes
Erythroid series
Ringed sideroblasts
Category B
Granulocytic series
Small size
Hypersegmentation
Pseudo–Chediak-Higashi granule
Megakaryocytic series
Nonlobulated nuclei
Multiple, widely separated nuclei
Erythroid series
Nucleus
Budding
Internuclear bridging
Karyorrhexis
Multinuclearity
Megaloblastoid change
Cytoplasm
Vacuolization

Figure 1 Dysplasia of Category A



(A) Hyposegmented mature neutrophils (Pelger). Two lobes are connected with a fine (1) or thin (2) filament. **(B)** Degranulation of neutrophils (agranular or hypogranular neutrophils; Hypo-Gr). **(C)** Micromegakaryocytes. **(D)** Ringed sideroblasts. **(A-D)** Provided by the National Research Group on Idiopathic Bone Marrow Failure Syndromes, Japan.

leukemia. To exclude nonclonal disorders (Table 1D), laboratory studies (eg, serum iron, ferritin, cobalamin, and folic acid levels; Coombs test; anti-nuclear antibody; thyroid function tests; etc) and abdominal ultrasonography are necessary.

For evaluations of BM cellularity, specimens of BM trephine biopsy must be examined. A BM biopsy of good quality and adequate length (≥ 1.5 cm) is necessary. Often, repeat BM examination is required to confirm the diagnosis when there is doubt about initial BM examination or if an inadequate sample was taken.[15] Because BM cellularity is highly age-dependent, hypocellularity is defined as < 30% in patients aged < 60 years or < 20% in patients aged ≥ 60 years.[16,17] In hypocellular BM, microscopic examinations for the assessment of dysplasias should be performed with ≥ 2 BM films, if necessary.

In patients with hypocellular BM, it is absolutely necessary to exclude AA to diagnose hypoplastic MDS (Table 1E). Dysplasia of BM cells, the percentage of blasts in BM, and abnormal localization of immature precursors (ALIP) are useful markers for this differentiation. As mentioned earlier, significant dysplasia in ≥ 2 lineages and > 10% of Hypo-G, Pelger, or mMgk strongly suggest MDS rather than AA. Abnormal localization of immature precursors is usually not found in AA but is found in MDS, and blasts in BM are usually < 5% in AA cases. In this regard, it is very difficult to diagnose hypoplastic RA cases that show dysplasia only in erythroid lineage. Morphologic evaluation alone might not be enough for the diagnosis, and other data such as cytogenetics will provide further useful information when they show MDS-specific karyotype (see Step VI). However, in some cases with hypoplastic BM, in particular those having dysplasia in a single lineage or at low percentage, careful course observation is necessary to make a diagnosis. On the other hand, the presence of paroxysmal nocturnal hemoglobinuria–type cells[18] or dysplasia in erythroid lineage alone does not support the diagnosis of MDS alone.

Step II: Classification of Dysplasia

Table 2 shows a classification of dysplasias into category A (highly specific) and category B (less specific), which is the thrust of our proposal. Dysplasias in Table 2 are modified from those described in the WHO classification, except for the periodic acid-Schiff (PAS) reaction for erythroid cells because the PAS reaction is no longer used routinely

in hematologic laboratories. As described earlier, Pelger, Hypo-Gr, and mMgk are highly specific to MDS when present at levels ≥ 10%. In addition, we think that the diagnostic value of ringed sideroblasts (RS) is similarly specific when present at a level of ≥ 15%. Dysplasias other than Pelger, Hypo-Gr, mMgk, and RS are less specific for MDS but, if present at ≥ 10%, are sufficient to suggest a diagnosis of MDS. Therefore, we think that the classification of dysplasias for the diagnosis of MDS is necessary and helpful for clinical hematologists in general. Quantitative assessment of category A or category B dysplasias is a basis for grading the accuracy of diagnosis of MDS. Four types of category A dysplasias are shown in Figure 1 (A-D) and Table 2. Category B dysplasias are shown in Table 2.

Pelger are hyposegmented (dumbbell-shaped) mature neutrophils. Two lobes are connected with a fine or thin filament (Figure 1A), and their chromatin structure is abnormally coarse. Hypo-Gr are neutrophils with a total or > 80% loss of neutrophilic granules in the cytoplasm (Figure 1B). Micromegakaryocytes are mono- or bi-nulceated megakaryocytes with a size less than that of normal promyelocytes and abundant platelet granule formation (Figure 1C). Ringed sideroblasts are erythroid cells with perinuclear siderotic granules occupying > one third of the nuclear margin or > 5 distinct siderotic granules in the perinuclear region (Figure 1D).

Step III: Assessment of Category-A Dysplasias

For the assessment of Pelger and Hypo-Gr, ≥ 100 mature neutrophils should be examined on BM films. The frequencies of Pelger and Hypo-Gr should be evaluated individually, not the sum of Pelger or Hypo-Gr. Because BM films frequently fail to stain optimally for neutrophil granules, observation of PB films is very helpful in confirming degranulation. In particular, when Hypo-Gr is the sole dysplasia in the absence of other dysplastic features, the assessment of Hypo-Gr should not be evaluated as a positive finding unless confirmed as mentioned earlier. Concerning the frequency of mMgk, ≥ 25 megakaryocytes should be examined on multiple BM films. When the megakaryocyte number is markedly reduced, detection of ≥ 3 mMgks is sufficient to regard this category-A dysplasia as ≥ 10%. In almost all patients with AA, megakaryocytes are absent or very few in number. For RS, ≥ 100 erythroblasts of all stages should be examined. Independent assessment of category-A dysplasias is necessary for grading of diagnostic accuracy of MDS.

Step IV: Assessment of Dysplasia A + B in Each Lineage

Concerning the frequencies of dysplasia A + B in each lineage, we suggest the microscopic methods as follows: ≥ 100 mature neutrophils, ≥ 25 megakaryocytes, and ≥ 100 erythroblasts in BM should be examined. The frequency of dysplasia in each lineage is evaluated by total dysplastic cells (%) showing category A or B. The frequency of Dys E is evaluated by the sum of frequency of RS on iron-stained films and that of category B on May-Giemsa–stained films. For example, when the frequency of RS and that of category B in erythroid lineage are 5% and 10%, respectively, the frequency of Dys E is calculated as 15%. The microscopic examinations for the assessment of dysplasia should be performed with multiple BM films if necessary. If the megakaryocyte number is markedly reduced, detection of ≥ 3 dysplastic megakaryocytes is sufficient to regard dysplasia A + B as ≥ 10%.

Table 3 **Grade of Dysplasia**

Dysplasia Grade
High (Defined as 1 or 2)
1. Pelger ≥ 10% or Hypo-Gr ≥ 10% plus mMgk ≥ 10%
2. RS ≥ 15%
Intermediate
Dysplasia (category A or B) ≥ 10% in 2-3 lineages
Low
Dysplasia (category A or B) ≥ 10% in 1 lineage
Minimal
Dysplasia (category A or B) 1%-9% in 1-3 lineages

Step V: Grade of Dysplasia

As shown in Table 3, the grade of dysplasia is divided into high, intermediate, low, or minimal. High is defined as follows: (1) when Pelger ≥ 10% or Hypo-Gr ≥ 10% plus mMgk ≥ 10% in granulocytic and megakaryocytic lineages or (2) when RS ≥ 15% in erythroid series. In order to classify a case as high by the existence of RS ≥ 15% alone, other sideroblastic anemias such as alcoholic anemia must be excluded. Intermediate is defined as dysplasia A + B ≥ 10% in 2-3 lineages. Low is defined as dysplasia A + B ≥ 10% in a single lineage. Minimal is defined as 1%-9% of dysplasia A + B in 1-3 lineages.

Step VI: Division of Cytogenetic Findings

The divisions of cytogenetic findings are abnormal, normal, or unknown. Abnormal is defined as typical clonal abnormal karyotypes recurrently found in MDS (del[5q], –7/7q–, +8, del[20q], complex, and others) with high frequency as reported by Haase et al.[19] This definition is similar to that of typical chromosome abnormalities proposed by Valent et al.[12] t(8;21)(q22;q22), t(15;17)(q22;q12), inv(16)(p13;q22), and t(16;16)(p13;q22) are not included in the abnormal division even when the blast percentage is < 20%. Patients with these cytogenetic abnormalities are diagnosed with AML with recurrent cytogenetic abnormalities according to the WHO classification. Normal is defined as normal karyotype by analyzing > 10 metaphases. When cytogenetic findings are not available because of poor samples or an absence of metaphases, cases are labeled unknown.

Step VII: Grade of Diagnostic Accuracy

Table 4 shows the criteria for grading the diagnostic accuracy. These criteria are a combination of the frequency of blasts in BM, grade of dysplasia, and division of cytogenetics. The grade of diagnostic accuracy is divided into definite, probable, possible, and ICUS. The reliability of the diagnosis as MDS is high in the following order: definite, probable, and possible. In patients diagnosed as possible or ICUS, the diagnostic accuracy is low; thus, re-examination at suitable intervals is required to confirm the diagnosis. In such cases, the diagnosis might become more accurate when re-examination provides a result of definite or probable or remains possible or ICUS for a long period. The observation of the clinical course of patients with possible or ICUS will provide important information on the pathophysiologic similarity or dissimilarity between these diagnostic groups based on diagnostic grading.

Table 4 Grade of Diagnostic Accuracy for Myelodysplastic Syndromes

Grade	Blasts in BM (%)	Grade of Dysplasia	Division of Cytogenetics
	5-19	High, intermediate, low	Any
MDS Definite	0-4	High, intermediate, low	Abnormal
	0-4	High	Any
MDS Probable	0-4	Intermediate	Normal or unknown
MDS Possible	0-4	Low	Normal or unknown
ICUS	0-4	Minimal or none	Normal or unknown

Step VIII: Subtyping According to the World Health Organization Classification

Patients who are diagnosed as definite, probable, or possible should be classified according to the WHO classification. Patients classified in the possible category in our system are diagnosed as RA- or MDS-unclassified (refractory neutropenia or refractory thrombocytopenia) according to the WHO classification. However, the diagnosis of these patients should be tentative. The diagnosis according to the WHO classification of these patients must be decided by re-examination of BM at suitable intervals.

Discussion

Diagnosis of MDS must be as accurate and consistent as that of acute leukemia. However, the judgment of morphologic dysplasias has the inherent problem of the subjective nature of the morphology, and the objectivity of the evaluation has long been problematic. For the elimination or reduction of these problems, we propose a grading system for diagnostic accuracy of MDS. Category-A dysplasias are much easier for clinical hematologists to detect on routine BM diagnosis. Category-B dysplasias are sufficiently reliable when observed along with category A. Therefore, quantitative, morphologic evaluation by using this system will facilitate the routine diagnosis of MDS.

Exclusion of nonclonal disorders with minimal or no morphologic dysplasia is extremely important for the differential diagnosis of MDS as described in Step I. We believe our system is also useful in this respect. If there is no certain evidence for this exclusion diagnosis of non-MDS disorders despite the careful performance of other laboratory examinations, the possibility of misdiagnosis would likely be markedly reduced by using this grading system for diagnosis. Repeat BM examination at suitable intervals for patients graded as possible or ICUS will make clearer the still-vague margin of MDS as a clinical entity. It is also important to identify differences if present in responses to new drugs such as lenalidomide and hypomethylating agents.

Of course, our diagnostic schema still requires validation and demonstration of reliability, hopefully in 2 populations or a split-sample cohort. Long-term observation of MDS cases diagnosed with our proposal is also necessary for the evaluation of this proposal. Recent techniques in the detection of genetic abnormalities such as fluorescence in situ hybridization and single nucleotide polymorphism arrays[20] expand cytogenetic data of MDS. Although universal biomarkers for the diagnosis of MDS are still lacking, new data on genetic abnormality of MDS will be quite useful for accurate diagnosis and understanding the biology of MDS. In conclusion, until the discovery of universal biomarkers for entire MDS or subtypes of MDS, this diagnostic grading system could be useful for clinical routine work.

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Brief Report

Karyotype at diagnosis is the major prognostic factor predicting relapse-free survival for patients with Philadelphia chromosome-positive acute lymphoblastic leukemia treated with imatinib-combined chemotherapy

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ABSTRACT

To identify factors associated with relapse-free survival (RFS), 80 patients with newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia, enrolled in a phase II study of imatinib-combined chemotherapy, were analyzed. The median follow-up of surviving patients was 26.7 months (maximum, 52.5 months). Twenty-eight out of 77 patients who had achieved CR relapsed. The probability of RFS was 50.5% at 2 years. Multivariate analysis revealed that the presence of secondary chromosome aberrations in addition to t(9;22) at diagnosis constitute an independent predictive value for RFS (p=0.027), and increase the risk of treatment failure by 2.8-fold.

Key words: acute lymphoblastic leukemia, Philadelphia chromosome, BCR-ABL, imatinib, karyotype.

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Introduction

The treatment for Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph$^+$ ALL) has changed dramatically since imatinib, a selective inhibitor of the ABL tyrosine kinase, was introduced.[1,2] Combined with chemotherapy, or even as a single agent, it can produce complete remission (CR) rates of 90% or higher in newly diagnosed patients.[3-9] We previously reported the results of a phase II study by the Japan Adult Leukemia Study Group (JALSG) to test the efficacy and feasibility of imatinib-combined chemotherapy for newly diagnosed Ph$^+$ ALL.[6] The rate of CR reached 96%, and that of BCR-ABL negativity in bone marrow 71%. However, despite a relatively short follow-up period, relapse occurred in a subset of the patients who had achieved CR.

On the other hand, remarkable progress is being made with the development of novel tyrosine kinase inhibitors with more potent *in vitro* and *in vivo* activities than imatinib.[10,11] Given this, we investigated factors associated with relapse-free survival (RFS).

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Design and Methods

Patients and treatments

Eligibility criteria included newly diagnosed Ph+ ALL, age between 15 and 64 years, an Eastern Co-operative Oncology Group performance status between 0 and 3, and adequate liver, kidney and heart function. Written informed consent was obtained from all patients prior to registration.

For remission induction therapy, imatinib was administered from day 8 to day 63 in combination with daunorubicin, cyclophosphamide, vincristine (VCR) and prednisolone (PSL). Consolidation therapy consisted of an odd course (C1) comprising high-dose methotrexate, high-dose cytarabine and methylprednisolone, and an even course (C2) with single-agent imatinib for 28 days. C1 and C2 were alternated for 4 cycles each. After completion of the consolidation therapy, patients received maintenance therapy consisting of VCR, PSL and imatinib for up to 2 years from the date CR had been achieved.[6] The daily dose of imatinib used in this study was 600 mg. Allogeneic hematopoietic stem cell transplantation (HSCT) was recommended if a matched sibling donor was available, and was allowed from an alternative donor.

The protocol was reviewed and approved by the institutional review board of each of the participating centers and was conducted in accordance with the Declaration of Helsinki.

Cytogenetic and molecular analysis

At diagnosis, bone marrow samples were examined for cytogenetic abnormalities with standard banding techniques. Karyotypes were classified according to the International System for Human Cytogenetic Nomenclature.[12] The number of BCR-ABL copies in bone marrow was determined at a central laboratory with the real-time quantitative RT-PCR test according to the previously described method.[13]

Statistical analysis

Kaplan-Meier survival analysis was performed to estimate the probabilities of RFS, event-free survival (EFS), and overall survival (OS), with differences between the groups compared by the log-rank test. Cumulative incidences of relapse were calculated with non-relapse mortality considered as a competing risk, and differences between the groups were compared with the Gray's test. For risk factor analysis, a Cox proportional hazards model was constructed. In multivariate analysis, variables with p values of <0.10 determined by univariate analysis were included in the final model. A hazard ratio (HR) was calculated in conjunction with a 95% confidence interval (CI).

Results and Discussion

A total of 80 patients were recruited between September 2002 and January 2005. The median age was 48 years (range 15-63), with 49 males and 31 females. CR was achieved by 77 (96.2%) patients. During a median follow-up of 26.7 months (maximum 52.5 months), 28 patients relapsed. Of the 17 relapses observed during the consolidation therapy, 13 occurred during the imatinib course. The probabilities of EFS and OS were 48.5±5.7% and 58.1±5.7% at 2 years (Figure 1). For patients who had achieved CR, the probability of RFS was 50.5±5.9% at 2 years. Allogeneic HSCT was performed for 60 patients, including 24 from a sibling donor, 1 from a related donor other than a sibling, 25 from an unrelated donor, and 10 from unrelated cord blood. Disease status at the time of transplantation was first CR for 44 patients, second CR for 4 and non-CR for 12. The 2-year RFS for those who had undergone allogeneic HSCT during first CR was 62.6±7.5% and 62.1±12.3% for those who had not undergone allogeneic HSCT. When allogeneic HSCT was considered as a time-dependent covariate, it was shown to have no significant effect on RFS (HR, 1.03; 95% CI, 0.51-2.09; p=0.934). Major and minor BCR-ABLs were detected in 23 and 56 patients respectively. The transcript type of the remaining patient could not be determined because fluorescent *in situ* hybridization analysis was used instead of the PCR test. Neither transcript types nor copy numbers at diagnosis were associated with RFS (p=0.763 and 0.912). Pre-treatment cytogenetic results were not available for 4 patients because analysis was not performed (n=2) or was not successful (n=2). Of the remaining 76 patients, 22 showed only t(9;22) or variant translocations, 51 showed additional chromosome aberrations, and 3 showed normal karyotype. Additional aberrations exceeding a frequency of 10% comprised +der(22)t(9;22) in 17 patients, abnormalities involving the short arm of chromosome 9 [abn(9p)] in 17, monosomy 7 in 10, and trisomy 8 in 10. Figure 2 compares RFS for patients with and without additional chromosome aberrations. The presence of additional aberrations was significantly associated with shorter RFS (p=0.003). The relapse rate was also higher in patients with additional aberrations (41% *vs.* 20% at 2 years, p=0.0414). Analyses of the 4 recurrent abnormalities mentioned above demonstrated a statistically significant negative impact on RFS for +der(22)t(9;22) and abn(9p) (p<0.001 and p=0.005). Even after allogeneic HSCT, patients with additional aberrations appeared to have a trend for shorter RFS than those without (p=0.080), but this might reflect a larger proportion of transplantation beyond first CR in the former (31% *vs.* 17%). In patients allografted during first CR, there was no difference in cumulative incidences of relapse dated from the day of transplantation between the 2 groups

(16.5% *vs.* 12.5% at 2 years, p=0.546). Variables that showed a significant effect on RFS in the univariate Cox model included additional chromosome aberrations (p=0.005), peripheral blood blasts % (p=0.024) and sex (p=0.03). Results of multivariate analysis are shown in Table 1. The presence of additional chromosome aberrations was identified as the only independent prognostic factor for RFS (p=0.027). These updated data strongly support recent reports showing the feasibility and remarkable efficacy of imatinib-combined chemotherapy for newly diagnosed Ph+ ALL.[3-9,14,15] The main objective of this report was to identify factors affecting RFS, an issue of rapidly increasing importance given the development of novel tyrosine kinase inhibitors which are expected to further expand the treatment options for this disease. Our data indicated that additional chromosome aberrations, particularly +der(22)t(9;22) and abn(9p), were associated with shorter RFS. It is well known that additional chromosome aberrations are seen frequently in Ph+ ALL. Before the imatinib era, some groups reported the prognostic relevance of additional aberrations.[16-18] By contrast, from a large series of 204 patients, Moorman *et al.*[19] recently showed no significant effect of specific additional aberrations, including +der(22)t(9;22) and del(9p), on survival. In this study, analyzing patients treated with imatinib-combined chemotherapy, the 2-year RFS rate exceeded 80% for those without additional aberrations, whereas outcomes for those with additional aberrations were relatively unfavorable.

Acquisition of resistance to imatinib is an emerging problem in the treatment of chronic myeloid leukemia. One of the most common mechanisms of resistance is the mutation involving the ABL kinase domain. Although it has not been confirmed whether such mutations compromise the clinical outcome of Ph+ ALL patients treated with imatinib-combined chemotherapy, our observation that most of the early relapses occurred during the consolidation courses consisting of imatinib alone implies possible imatinib resistance. If that is the case, switching from imatinib to other novel tyrosine kinase inhibitors based on the pre-treatment cytogenetic results soon after achieving CR or even earlier could be an alternative treatment approach for further improving outcome in Ph+ ALL. Lack of mutation analysis is a major limitation of this study. Recently, Pfeifer *et al.*[20] studied the ABL kinase domain mutation status in newly diagnosed Ph+ ALL patients who were treated with imatinib-combined chemotherapy, and showed that even before exposure to imatinib, mutations were detected in 38% of patients. Importantly, the frequency of the mutant allele was low in such patients. However, at the time of relapse, the same mutation was present as the dominant clone in 90% of the relapsing cases.[20] Altogether, further insights will be provided by investigating the association between karyotype and mutation status at diagnosis.

Despite such limitations, the analysis of 80 patients entered into a single trial identified karyotype at diagnosis as a significant prognostic factor for RFS in newly diagnosed Ph+ ALL patients treated with imatinib-combined chemotherapy. Although our results need to be confirmed regarding kinase domain mutation status, these findings may play a critical role in the future treatment of Ph+ ALL.

Table 1. Multivariate analysis of factors associated with relapse-free survival.

P-value	*HR (95% CI)**	*Factors*	
Additional [chromosome aberrations	0.027	2.84 (1.12-7.19) 1.00	Present Absent
Peripheral blood blasts%	0.051	1.12 (1.00-1.22)	Per 10% increase
Sex	0.148 1.00	1.73 (0.82-3.64) Female	Male

*HR, hazard ratio; 95% CI, 95% confidence interval. *Values higher than unity indicate higher risk for failure.*

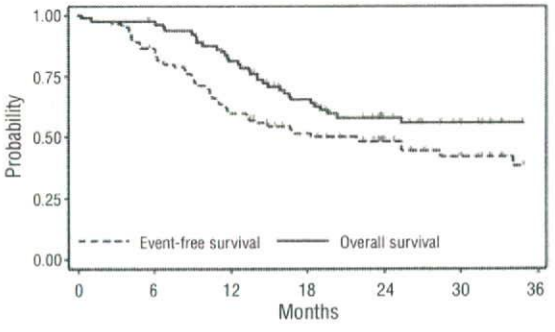

Figure 1. Kaplan-Meier curves for event-free and overall survival. The probabilities of event-free and overall survival at 2 years were 48.5% and 58.1% respectively (n=80).

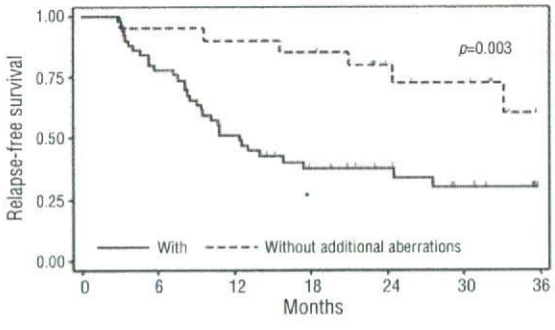

Figure 2. Relapse-free survival for patients with and without additional cytogenetic aberrations. Patients with additional cytogenetic aberrations (n=50) had significantly shorter relapse-free survival than those without (n=20).