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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.*

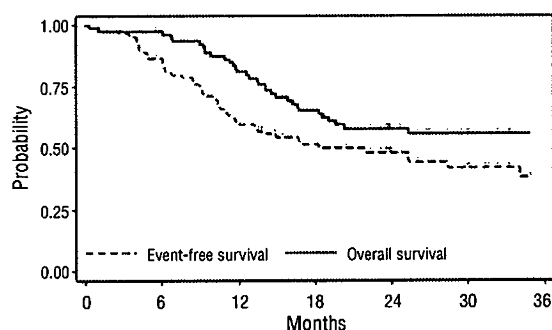

Figure 1. Kaplan-Meier curves for event-free and overall survival. The probabilities of event-free and overall survival at 2 years were 48.5% and 58.1% respectively (n=80).

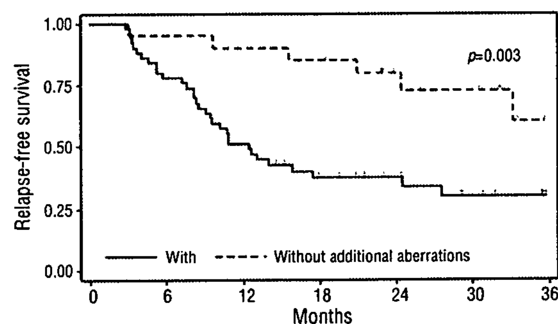

Figure 2. Relapse-free survival for patients with and without additional cytogenetic aberrations. Patients with additional cytogenetic aberrations (n=50) had significantly shorter relapse-free survival than those without (n=20).

Authorship and Disclosures

MY designed and co-ordinated the study, analyzed the data, and wrote the paper; JT, NU, FY, SM, and IJ designed the study, and provided patient sample and clinical data; IS, HA, KN, YU, MT, and AM provided patient sample and clinical data; HN co-ordinated the study, and revised the paper. YM provided patient sample and clinical data, and engaged in data management. SO designed the study, provided patient sample and clinical data, and engaged in data management; KM designed the study, and analyzed the data; TN chaired the study group, co-ordinated the study, and revised the paper; RO served as the principal investigator, chaired the study group, and revised the paper. All authors reviewed the paper, interpreted the results, and approved the final version. The authors reported no potential conflicts of interest.

References

1. Ottmann OG, Wassmann B. Treatment of Philadelphia chromosome-positive acute lymphoblastic leukemia. Hematology Am Soc Hematol Educ Program 2005:118-22.
2. Yanada M, Naoe T. Imatinib combined chemotherapy for Philadelphia chromosome-positive acute lymphoblastic leukemia: major challenges in current practice. Leuk Lymphoma 2006;47:1747-53.
3. Thomas DA, Faderl S, Cortes J, O'Brien S, Giles FJ, Kornblau SM, et al. Treatment of Philadelphia chromosome-positive acute lymphocytic leukemia with hyper-CVAD and imatinib mesylate. Blood 2004;103:4396-407.
4. Towatari M, Yanada M, Usui N, Takeuchi J, Sugiura I, Takeuchi M, et al. Combination of intensive chemotherapy and imatinib can rapidly induce high-quality complete remission for a majority of patients with newly diagnosed BCR-ABL-positive acute lymphoblastic leukemia. Blood 2004;104:3507-12.
5. Lee KH, Lee JH, Choi SJ, Lee JH, Seol M, Lee YS, et al. Clinical effect of imatinib added to intensive combination chemotherapy for newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia. Leukemia 2005;19:1509-16.
6. Yanada M, Takeuchi J, Sugiura I, Akiyama H, Usui N, Yagasaki F, et al. High complete remission rate and promising outcome by combination of imatinib and chemotherapy for newly diagnosed BCR-ABL-positive acute lymphoblastic leukemia: a phase II study by the Japan Adult Leukemia Study Group. J Clin Oncol 2006;24:460-6.
7. Wassmann B, Pfeifer H, Goekbuget N, Beelen DW, Beck J, Stelljes M, et al. Alternating versus concurrent schedules of imatinib and chemotherapy as front-line therapy for Philadelphia-positive acute lymphoblastic leukemia (Ph+ALL). Blood 2006; 108:1469-77.
8. Vignetti M, Fazi P, Cimino G, Martinelli G, Di Raimondo F, Ferrara F, et al. Imatinib plus steroids induces complete remissions and prolonged survival in elderly Philadelphia chromosome-positive patients with acute lymphoblastic leukemia without additional chemotherapy: results of the Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) LAL0201-B protocol. Blood 2007; 109:3676-8.
9. Ottmann OG, Wassmann B, Pfeifer H, Giagounidis A, Stelljes M, Duhrsen U, et al. Imatinib compared with chemotherapy as front-line treatment of elderly patients with Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL). Cancer 2007;109:2068-76.
10. Talpaz M, Shah NP, Kantarjian H, Donato N, Nicoll J, Paquette R, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N Engl J Med 2006;354: 2531-41.
11. Kantarjian H, Giles F, Wunderle L, Bhalla K, O'Brien S, Wassmann B, et al. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. N Engl J Med 2006;354: 2542-51.
12. Mitelman F et al. ISCN 1995: An International System for Human Cytogenetic Nomenclature. Basel, Switzerland: S Karger; 1995.
13. Osumi K, Fukui T, Kiyoi H, Kasai M, Kodera Y, Kudo K, et al. Rapid screening of leukemia fusion transcripts in acute leukemia by real-time PCR. Leuk Lymphoma 2002; 43:2291-9.
14. Lee S, Kim YJ, Min CK, Kim HJ, Eom KS, Kim DW, et al. The effect of first-line imatinib interim therapy on the outcome of allogeneic stem cell transplantation in adults with newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia. Blood 2005;105:3449-57.
15. de Labarthe A, Rousselot P, Huguet-Rigal F, Delabesse E, Witz F, Maury S, et al. Imatinib combined with induction or consolidation chemotherapy in patients with de novo Philadelphia chromosome-positive acute lymphoblastic leukemia: results of the GRAAPH-2003 study. Blood 2007; 109:1408-13.
16. Rieder H, Ludwig WD, Gassmann W, Maurer J, Janssen JW, Gokbuget N, et al. Prognostic significance of additional chromosome abnormalities in adult patients with Philadelphia chromosome positive acute lymphoblastic leukaemia. Br J Haematol 1996;95: 678-91.
17. Thomas X, Thiebaut A, Olteanu N, Danaila C, Charrin C, Archimbaud E, et al. Philadelphia chromosome positive adult acute lymphoblastic leukemia: characteristics, prognostic factors and treatment outcome. Hematol Cell Ther 1998;40:119-28.
18. Wetzler M, Dodge RK, Mrozek K, Stewart CC, Carroll AJ, Tantravahi R, et al. Additional cytogenetic abnormalities in adults with Philadelphia chromosome-positive acute lymphoblastic leukaemia: a study of the Cancer and Leukaemia Group B. Br J Haematol 2004;124: 275-88.
19. Moorman AV, Harrison CJ, Buck GA, Richards SM, Secker-Walker LM, Martineau M, et al. Karyotype is an independent prognostic factor in adult acute lymphoblastic leukemia (ALL): analysis of cytogenetic data from patients treated on the Medical Research Council (MRC) UKALLXII/ Eastern Cooperative Oncology Group (ECOG) 2993 trial. Blood 2007; 109:3189-97.
20. Pfeifer H, Wassmann B, Pavlova A, Wunderle L, Oldenburg J, Binckebanck A, et al. Kinase domain mutations of BCR-ABL frequently precede imatinib-based therapy and give rise to relapse in patients with de novo Philadelphia-positive acute lymphoblastic leukemia (Ph+ ALL). Blood 2007;110:727-34.

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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).

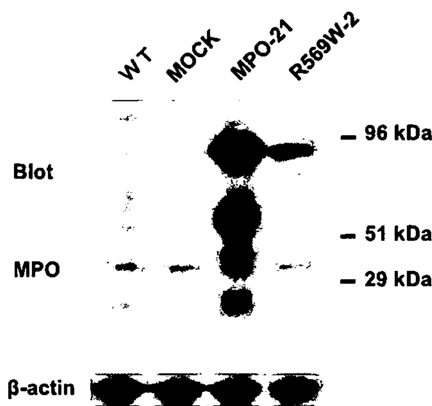

Figure 1 Western blot analysis of myeloperoxidase (MPO) protein in K562 cells. Immature MPO protein (apo-pro MPO, 89 kDa) was detected in K562 cells expressing wild-type MPO (MPO-21) and mutant MPO (R569W-2). Mature MPO protein (64 and 14 kDa) was seen only in MPO-21 cells. MOCK, K562 cells transfected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

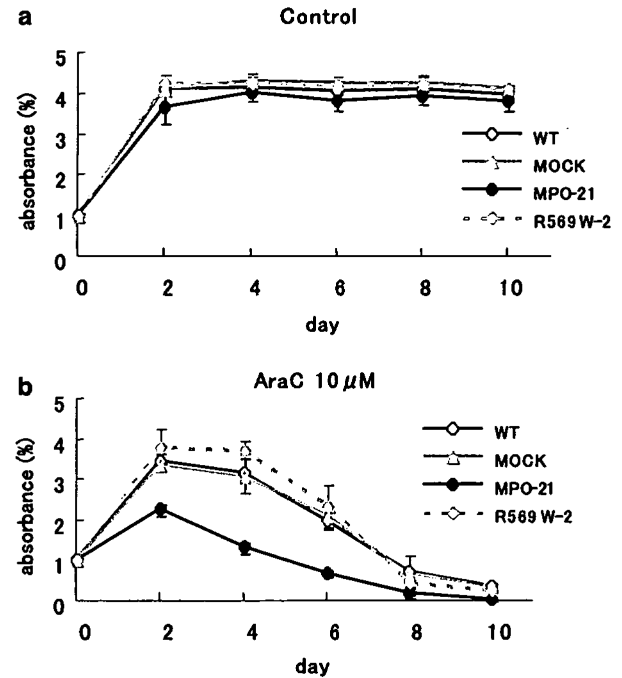

Figure 3 WST-1 analysis of K562 cells. Wild-type K562 (WT), MOCK (transfected with control vector), MPO-21 (wild-type myeloperoxidase (MPO)-expressing K562) and R569W-2 (mutant MPO-expressing K562) cells showed a similar growth pattern in the steady state (**a**). In the presence of cytosine arabinocide (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.

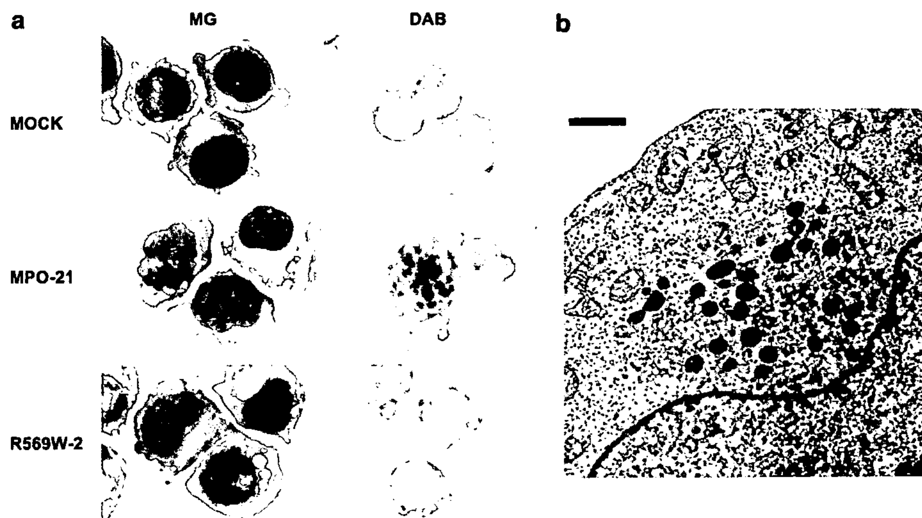

Figure 2 Morphological and cytochemical features of myeloperoxidase (MPO)-expressing K562 cells. May-Grunwald Giemusa (MGG) staining and diaminobenzidine (DAB) staining of K562 cells transfected with control vector (MOCK), wild-type MPO (MPO-21) and mutant MPO (R569W-2) (**a**). DAB was observed only 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).

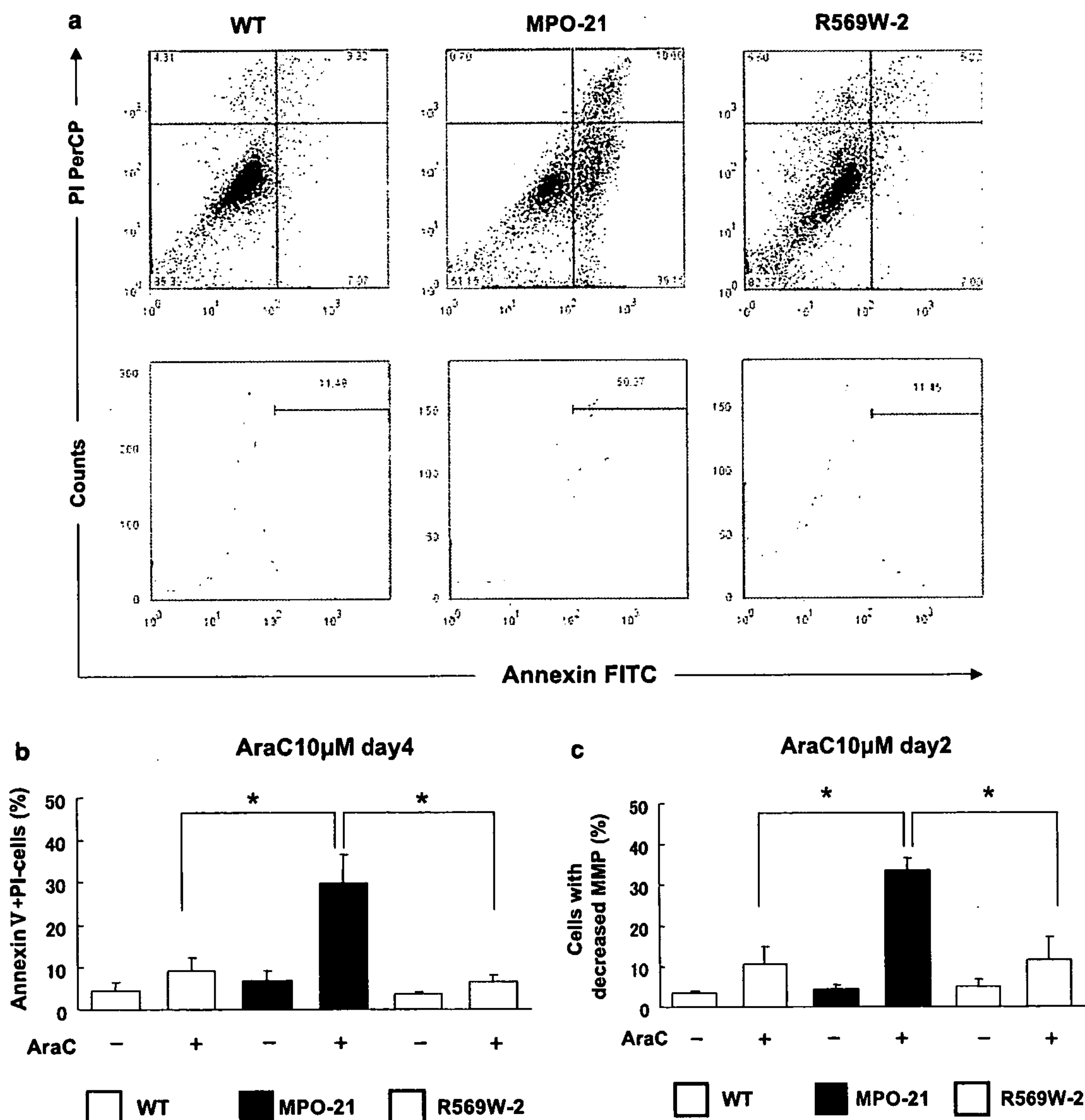

Figure 4 Apoptosis is accelerated in MPO-21 cells. Histogram of Annexin V expression on day 4 of cytosine arabinocide (AraC) treatment (**a**). A larger proportion of MPO-21 cells expressed Annexin V in a propidium iodide (PI)-negative fraction than wild-type K562 (WT). Data of three independent experiments 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

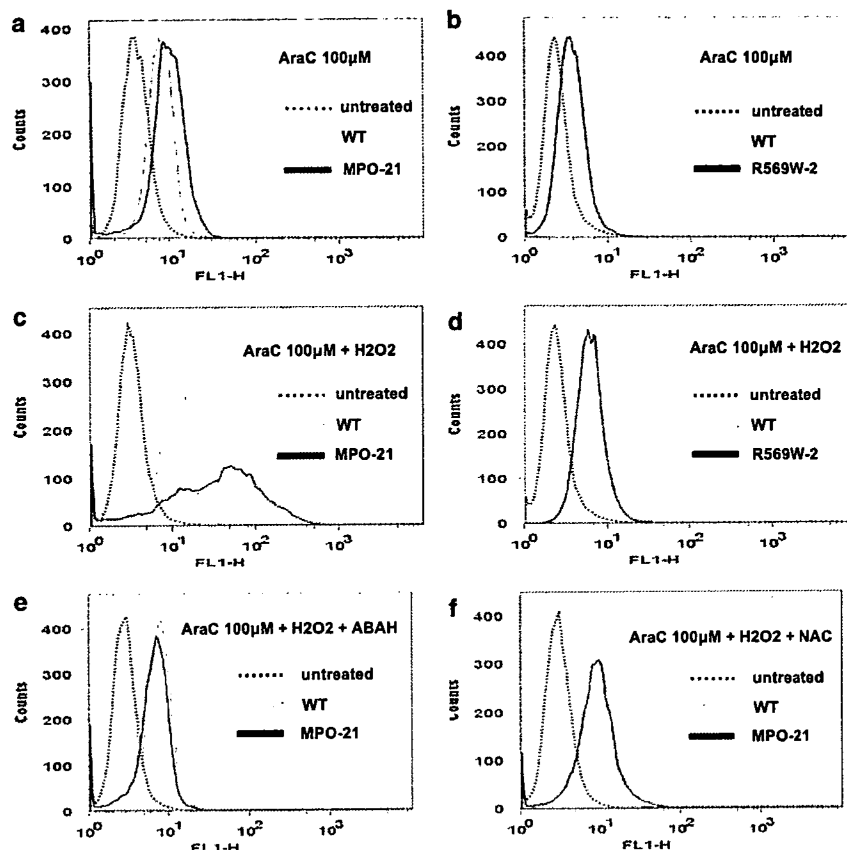

Figure 5 Reactive oxygen species (ROS) generation in K562 cells expressing myeloperoxidase (MPO). After treatment with cytosine arabinocide (AraC), ROS production probed with APF was increased in wild-type K562 (WT) cells, and was still stronger in MPO-21 cells (**a**). There was no difference in ROS 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.

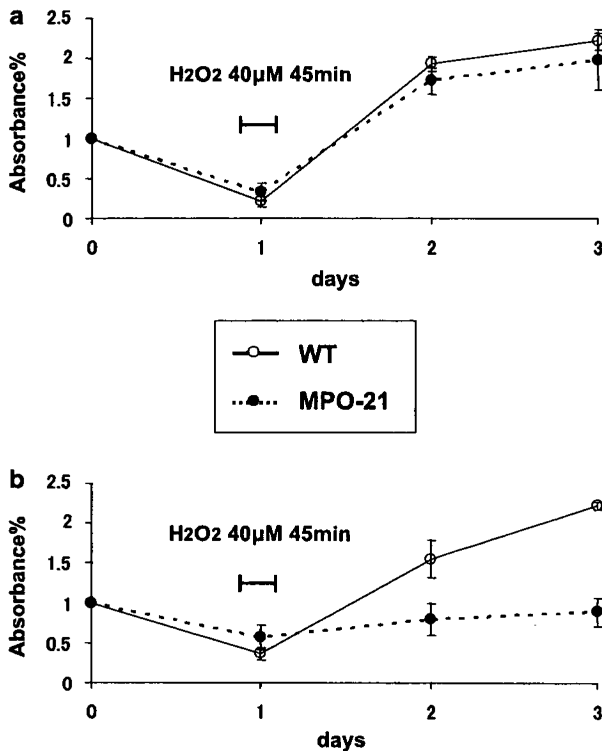

Figure 6 Combination of cytosine arabinocide (AraC), hydrogen peroxide (H_2O_2) and myeloperoxidase (MPO) showed the strong suppression of proliferation as measured by WST-1 assay. WT and MPO-21 cells were treated with H_2O_2 for 45 min with or without AraC. Transient treatment with H_2O_2 alone resulted 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,

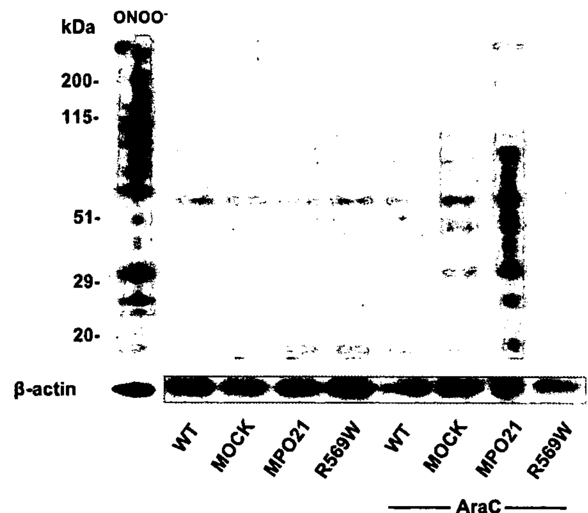

Figure 7 Nitration of tyrosine residues after cytosine arabinocide (AraC) treatment in MPO-21 cells. The generation of nitrotyrosine was examined in cells treated with reactive nitrogen species (RNS) or AraC. Positive control samples treated with RNS (peroxynitrite tetramethylammonium ($ONOO^-$)) and 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.

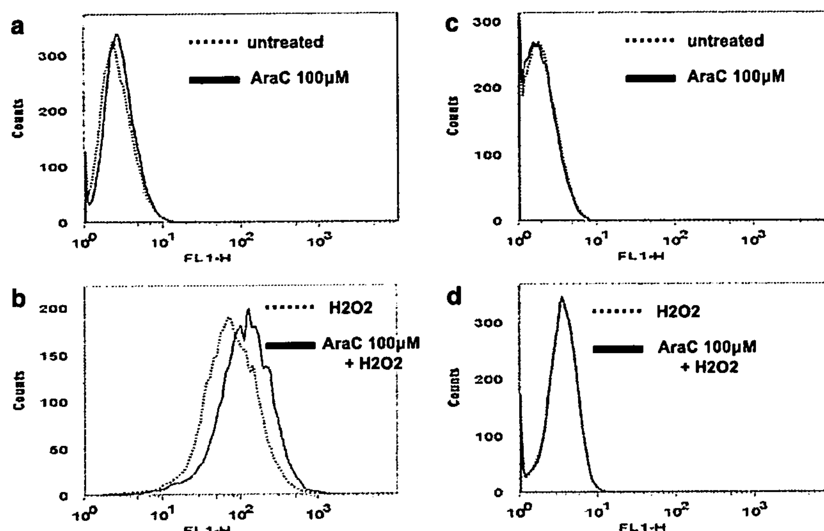

Figure 8 Generation of reactive oxygen species (ROS) in acute myeloid leukemia samples. CD34+ cells were selected and were treated with 100 μM cytosine arabinocide (AraC) and 40 μM hydrogen peroxide (H_2O_2) for 3 h. ROS generation was examined with flow cytometry. Histograms of a positive case (**a**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

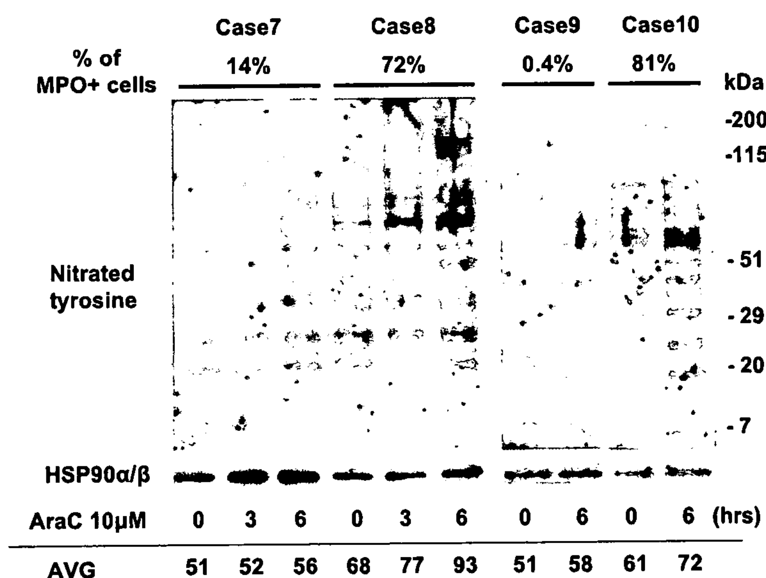

Figure 9 Nitration of tyrosine residues in acute myeloid leukemia (AML) samples. CD34+ cells from four AML cases were treated with 10 μM cytosine arabinocide (AraC) for up to 6 h. Two cases had high myeloperoxidase (MPO) expression (cases 8 and 10), and two had low MPO (cases 7 and 9). Nitrotyrosine 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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References

1 Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR *et al.* Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French–American–British Cooperative Group. *Ann Intern Med* 1985; **103**: 620–625.
2 World Health Organization. Classification of Tumors. In: Jaffe ES, Harris NL, Stein H and Vardiman JW (eds). *Pathology and Genetics of Tumors of Haematopoietic and Lymphoid Tissues.* IARC Press: Lyon, 2001, pp. 79–80.
3 Hoyle CF, Gray RG, Wheatley K, Swirsky D, de Bastos M, Sherrington P *et al.* Prognostic importance of Sudan Black positivity: a study of bone marrow slides from 1386 patients with *de novo* acute myeloid leukaemia. *Br J Haematol* 1991; **79**: 398–407.
4 Matsuo T, Cox C, Bennett JM. Prognostic significance of myeloperoxidase positivity of blast cells in acute myeloblastic leukemia without maturation (FAB: M1): An ECOG study. *Hematol Pathol* 1989; **3**: 153–158.
5 Matsuo T, Kuriyama K, Miyazaki Y, Yoshida S, Tomonaga M, Emi N *et al.* The percentage of myeloperoxidase-positive blast cells is a strong independent prognostic factor in acute myeloid leukemia, even in the patients with normal karyotype. *Leukemia* 2003; **17**: 1538–1543.
6 Modica-Napolitano JS, Singh KK. Mitochondria as targets for detection and treatment of cancer. *Expert Rev Mol Med* 2002; **4**: 1–19.
7 Weijl NI, Cleton FJ, Osanto S. Free radicals and antioxidants in chemotherapy-induced toxicity. *Cancer Treat Rev* 1997; **23**: 209–240.
8 Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* 2000; **279**: 1005–1028.
9 Fruehauf JP, Meyskens Jr FL. Reactive oxygen species: a breath of life or death? *Clin Cancer Res* 2007; **13**: 789–794.
10 Mignotte B, Vayssiere JL. Mitochondria and apoptosis. *Eur J Biochem* 1998; **252**: 1–15.
11 Winterbourn CC, Vissers MC, Kettle AJ. Myeloperoxidase. *Curr Opin Hematol* 2000; **7**: 53–58.
12 Myzak MC, Carr AC. Myeloperoxidase-dependent caspase-3 activation and apoptosis in HL-60 cells: protection by the antioxidants ascorbate and (dihydro) lipoic acid. *Redox Rep* **7**: 47–53.
13 Wagner BA, Buettner GR, Oberley LW, Darby CJ, Burns CP. Myeloperoxidase is involved in H2O2-induced apoptosis of HL-60 human leukemia cells. *J Biol Chem* 2000; **275**: 22461–22469.
14 Morishita K, Kubota N, Asano S, Kaziro Y, Nagata S. Molecular cloning and characterization of cDNA for human myeloperoxidase. *J Biol Chem* 1987; **262**: 3844–3851.
15 Nauseef WM, Brigham S, Cogley M. Hereditary myeloperoxidase deficiency due to a missense mutation of arginine 569 to tryptophan. *J Biol Chem* 1994; **269**: 1212–1216.
16 Bullock O, Ray S, Krajewski S, Ibrado AM, Huang Y, Bhalla K. Intracellular metabolism of Ara-C and resulting DNA fragmentation and apoptosis of human AML HL-60 cells possessing disparate levels of Bcl-2 protein. *Leukemia* 1996; **10**: 1731–1740.
17 Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B *et al.* Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 1998; **391**: 393–397.
18 Eiserich JP, Baldus S, Brennan ML, Ma W, Zhang C, Tousson A *et al.* Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science* 2002; **296**: 2391–2394.
19 Brennan ML, Wu W, Fu X, Shen Z, Song W, Frost H *et al.* A tale of two controversies defining both the role of peroxidases in nitrotyrosine formation *in vivo* using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J Biol Chem* 2002; **227**: 17415–17427.

20 Kuriyama K, Tomonaga M, Kobayashi T, Takeuchi J, Ohshima T, Furusawa S *et al.* Morphological diagnoses of the Japan adult leukemia study group acute myeloid leukemia protocols: central review. *Int J Hematol* 2001; **73**: 93–99.
21 Miyazaki Y, Kuriyama K, Miyawaki S, Ohtake S, Sakamaki H, Matsuo T *et al.* Cytogenetic heterogeneity of acute myeloid leukaemia (AML) with trilineage dysplasia: Japan Adult Leukaemia Study Group-AML 92 study. *Br J Haematol* 2003; **120**: 56–62.
22 Ambrosone CB, Ahn J, Singh KK, Rezaishiraz H, Furberg H, Sweeney C *et al.* Polymorphisms in genes related to oxidative stress (*MPO, MnSOD, CAT*) and survival after treatment for breast cancer. *Cancer Res* 2005; **65**: 1105–1111.
23 Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; **3**: 730–737.
24 Taguchi J, Miyazaki Y, Tsutsumi C, Sawayama Y, Ando K, Tsushima H *et al.* Expression of the myeloperoxidase gene in AC133 positive leukemia cells relates to the prognosis of acute myeloid leukemia. *Leuk Res* 2006; **30**: 1105–1112.
25 Vercauteren SM, Sutherland HJ. CD133 (AC133) expression on AML cells and progenitors. *Cytotherapy* 2001; **3**: 449–459.

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

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ORIGINAL ARTICLE

Diagnosis of acute myeloid leukemia according to the WHO classification in the Japan Adult Leukemia Study Group AML-97 protocol

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Abstract We reviewed and categorized 638 of 809 patients who were registered in the Japan Adult Leukemia Study Group acute myeloid leukemia (AML)-97 protocol using morphological means. Patients with the M3 subtype were excluded from the study group. According to the WHO classification, 171 patients (26.8%) had AML with recurrent genetic abnormalities, 133 (20.8%) had AML with multilineage dysplasia (MLD), 331 (51.9%) had AML not otherwise categorized, and 3 (0.5%) had acute leukemia of ambiguous lineage. The platelet count was higher and the rate of myeloperoxidase (MPO)-positive blasts was lower in AML with MLD than in the other WHO categories. The outcome was significantly better in patients with high ($\geq$50%) than with low (<50%) ratios of MPO-positive blasts ($P < 0.01$). The 5-year survival rates for patients with favorable, intermediate, and adverse karyotypes were 63.4, 39.1, and 0.0%, respectively, and 35.5% for those with 11q23 abnormalities ($P < 0.0001$). Overall survival (OS) did not significantly differ between nine patients with *t*(9;11) and 23 with other 11q23 abnormalities ($P = 0.22$). Our results confirmed that the cytogenetic profile, MLD phenotype, and MPO-positivity of blasts are associated with survival in patients with AML, and showed that each category had the characteristics of the WHO classification such as incidence, clinical features, and OS.

Keywords AML · WHO classification · Myeloperoxidase · Multilineage dyplasia · 11q23 abnormalities

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1 Introduction

The French-American-British (FAB) classification of acute myeloid leukemia (AML), based on morphological and cytochemical findings, was established in 1976 and has since become the standard classification [1, 2]. However, specific chromosomal and genetic abnormalities that have been extracted from analyses of prognostic factors for AML are recognized as important in selecting treatment strategies and are reflected in the AML classification as

factors that are required to establish the disease entity [3]. The 1999 World Health Organization (WHO) classification includes morphological, immunological, cytogenetic, genetic, and clinical features [4–6]. The WHO and FAB classifications differ in several aspects. The blast threshold required for a diagnosis of AML was reduced from 30 to 20%, and new AML categories have been added for cytogenetic abnormalities, the presence of multilineage dysplasia (MLD), as well as a history of chemotherapy and subtypes for acute basophilic leukemia, acute panmyelosis with myelofibrosis, and myeloid sarcoma. The WHO classification comprises more subtypes and is more comprehensive than the FAB classification.

Cytogenetic features are important prognostic factors in AML [3, 7–12]. However, 11q23 abnormalities have not yet been established as a cytogenetic risk classification. Over 30 partner genes with 11q23 abnormalities have been described, and some reports indicate that patients with *t*(9;11) have a relatively more favorable prognosis than those with other partner chromosomes/partner genes [13–16].

In the present study, we reviewed stained smears of blood and bone marrow from patients who were registered in the Japan Adult Leukemia Study Group (JALSG) AML-97 trial, and classified them into FAB subtypes and WHO categories. We also evaluated their survival on the basis of the WHO classification, the myeloperoxidase (MPO)-positivity of blasts, and cytogenetic findings including 11q23 abnormalities.

2 Patients and methods

2.1 Patients

Between December 1997 and July 2001, 809 patients aged from 15 to 66 years with untreated AML (excluding M3) were registered from 103 institutions in the AML-97 trial of the JALSG. The patients were diagnosed with AML according to the FAB criteria at each institution. Patients with a history of MDS, hematological abnormalities before the diagnosis of AML, or a history of chemotherapy were not eligible for the AML-97 trial.

2.2 Treatment strategies

Details of the JALSG AML-97 treatment protocol are described elsewhere [17]. In brief, all patients underwent induction therapy consisting of idarubicin (3 days) and Ara-C (7 days). Patients who achieved complete remission were randomized into one of two arms of consolidation chemotherapy alone or in combination with maintenance chemotherapy. Patients who were placed into intermediate/poor risk groups according to the JALSG scoring system [17] and who had an HLA-identical sibling (≤50 years old) were simultaneously assigned to receive allogeneic hematopoietic stem cell transplantation during their first remission.

2.3 Morphologic and cytochemical analyses

Peripheral blood and bone marrow smears from registered patients were sent to Nagasaki University for staining with May-Giemsa, MPO, and esterase, and the diagnosis was then reevaluated by the Central Review Committee for Morphological Diagnosis. Patients were subsequently categorized according to the FAB and WHO classifications. Dyserythropoietic features were defined as >50% dysplastic features in at least 25 erythroblasts and dysgranulopoietic features including ≥3 neutrophils with hyposegmented nuclei (pseudo-Pelger–Heut anomaly), and hypogranular or agranular neutrophils (>50% of ≥10 neutrophils). Dysmegakaryopoietic features were defined as ≥3 megakaryocytes that were micronuclear, multiseparate nuclear, or large mononuclear [18].

We assessed the ratios (%) of MPO-positive blasts on MPO-stained bone marrow smears using the diaminobenzidine method [19].

2.4 Cytogenetic analysis

Cytogenetic analysis was performed at either laboratories in participating hospitals or authorized commercial laboratories. The karyotypes of leukemic cells were collected through the JALSG AML-97 case report forms and reviewed by the Central Review Committee for Karyotyping. The patients were classified into favorable, intermediate, or adverse risk groups based on karyotypes according to results of the Medical Research Council (MRC) AML 10 trial [3]. The favorable risk group included patients with *t*(8;21) and inv(16), whether alone or in combination with other abnormalities. The intermediate risk group included those with a normal karyotype and other abnormalities that were not classified as either favorable or adverse. The adverse risk group included patients with a complex karyotype with four or more numerical or structural aberrations, −5, deletion (5q), and −7, whether alone or in combination with intermediate risk or other adverse risk abnormalities.

2.5 Statistical analysis

The overall survival (OS) for all patients was defined as the interval from the date of diagnosis to that of death. We applied the Kaplan–Meier method to estimate OS and

Table 1 Patient characteristics

Age (year)	45 (15–66)
Male/female	390/248
WBC count ($\times 10^9$/l)	13.7 (0.4–709)
Hemoglobin (g/dl)	8.3 (3.8–17.2)
Platelet count ($\times 10^9$/l)	52 (0–890)
Bone marrow blasts (%)	56 (6–99)

Values are presented as the median (range)

WBC white blood cell

5-year survival. We compared survival rates between groups using the log-rank test (Stat View J 5.0). Differences were examined by the Chi-square test using Excel software. All *P*-values are two-sided, and values <0.05 were considered significant.

3 Results

3.1 Patient characteristics

Of the 809 registered patients, 638 were consistent with the WHO classification. Data were incomplete for 10 of the 638 patients. Table 1 lists the characteristics of the patients. The median age of all 638 patients (390 males and 248 females) was 45 years (range 15–66 years). The median values of WBC, hemoglobin (Hb), platelets, and the ratio of blasts in the bone marrow were 13.7 $\times$ 10^9/l, 8.3 g/dl, 52.0 $\times$ 10^9/l, and 56.0%, respectively.

3.2 FAB classification

Table 2 shows the FAB classification of the 638 patients. Most were classified as M2 (n = 261; 40.9%), followed by M4 (n = 148; 23.2%), and M1 (n = 109; 17.1%) with M0, M4Eo, M5a, M5b, M6, M7, and acute leukemia of ambiguous lineage comprising the remainder in that order.

3.3 WHO classification and clinical characteristics

Table 3 shows the patients categorized according to the WHO classification. The first category of AML with recurrent genetic abnormalities accounted for 171 patients (26.8%), 133 (20.8%) were in the second category of AML with MLD, 331 (51.9%) were in the fourth category of AML not otherwise categorized, and 3 (0.5%) were categorized as having acute leukemia of ambiguous lineage. Most patients in the second category were identical to those with a de novo MLD phenotype. We found that 144 patients diagnosed with the MLD phenotype comprised 133 (92.4%) in the second category, 10 (7.0%) with 11q23 abnormalities, and 1 (0.7%) with acute leukemia of ambiguous lineage. Figure 1 shows the OS of each category. The 5-year survival rates of the first, second, and fourth categories were 58.2, 22.5, and 40.9% (P < 0.0001), respectively.

Table 2 Number of patients according to the FAB classification

Subtype	Description	No. of patients	%
M0	Minimally differentiated acute myeloid leukemia (AML)	30	4.7
M1	AML without maturation	109	17.1
M2	AML with maturation	261	40.9
M4	Acute myelomonocytic leukemia (AMMoL)	148	23.2
M4Eo	AMMoL with eosinophils	23	3.6
M5a	Acute monoblastic leukemia	19	3.0
M5b	Acute monocytic leukemia	24	3.8
M6	Acute erythroleukemia	16	2.5
M7	Acute megakaryoblastic leukemia	5	0.8
Acute leukemia of ambiguous lineage		3	0.5
Total		638	100

Table 3 Number of patients according to the WHO classification

Category and subtype	No. of patients	%
I. AML with recurrent genetic abnormalities	171	26.8
t(8;21)(q22;q22);(AML1/ETO)	113	17.7
inv(16)(p13;q22) or *t*(16;16)(p13;q22);(CBFβ/MYH11)	26	4.1
t(15;17)(q22;q12)(PML/RARα)	–	–
11q23(MLL)abnormalities	32	5.0
II. AML with multilineage dysplasia	133	20.8
Following MDS	–	–
Without antecedent MDS	133	20.8
III. AML and MDS, therapy-related	–	–
Alkylating agent-related	–	–
Topoisomerase type II inhibitor-related	–	–
Other types	–	–
IV. AML not otherwise categorized	331	51.9
AML, minimally differentiated	25	3.9
AML without maturation	99	15.5
AML with maturation	108	16.9
Acute myelomonocytic leukemia (AMMoL)	63	9.9
AMMoL with eosinophilia	5	0.8
Acute monoblastic leukemia	8	1.3
Acute monocytic leukemia	16	2.5
Acute erythroid leukemia	6	0.9
Acute megakaryoblastic leukemia	1	0.2
Acute leukemia of ambiguous lineage	3	0.5
Total	638	100

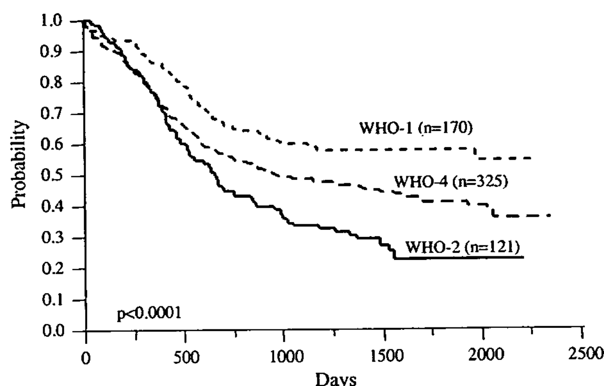

Fig. 1 Overall survival of patients categorized according to the WHO classification

Table 4 compares the clinical features among the WHO categories. The mean values of platelets, WBC, Hb, and the ratio (%) of blasts in bone marrow and of MPO-positive blasts significantly differed, whereas age did not significantly differ. Patients in the second category had a higher platelet count (111.0 × 10^9/l), whereas those with 11q23 abnormalities had a lower count (38.3 × 10^9/l) compared with those of other subtypes.

The WBC count of patients with *t*(8;21) was 1.4 × 10^9/l and lower than in other subtypes. The MPO-positive rate of blasts among patients with *t*(8;21) was higher (93.3%) and that of patients in the second category was lower (34.0%), than in other subtypes. All patients were grouped as high- or low-MPO according to ≥50% or <50% of MPO-positive blasts, respectively. A total of 339 patients (53.1%) were classified as high-MPO, 268 (42.0%) as low-MPO, and the MPO status of blasts could not be assessed in 31 (4.9%). Figure 2 shows the OS of patients with high- or low-MPO. The 5-year survival rate for patients with high or low-MPO was 50.7 and 29.6%, respectively ($P < 0.0001$).

3.4 Cytogenetics

All 638 patients were classified into favorable ($n = 139$; 21.8%), intermediate ($n = 413$; 64.7%), and adverse ($n = 54$; 8.5%) cytogenetic risk groups (Table 5). Figure 3 shows the OS according to this stratification. The 5-year survival rates were 63.4, 39.3, and 0.0% in the favorable, intermediate (except for those with 11q23 abnormalities), and adverse risk groups, respectively, and 35.5% in the group with 11q23 abnormalities ($P < 0.0001$).

The numbers of patients with or without MLD and high- or low-MPO in each cytogenetic risk group are listed in Table 6. None of those with the MLD phenotype were classified into the favorable risk group, while 129 (89.6%) and 15 (10.4%) of 144 patients with MLD were classified into intermediate or adverse risk groups, respectively. Only 15 patients (4.4%) in the high-MPO group were classified as having an adverse risk, while 11 (4.1%) in the low-MPO group were included in the favorable risk group.

The 32 patients with 11q23 abnormalities comprised 11 (34.4%) with *t*(11;19), 9 (28.1%) with *t*(9;11), 5 (15.6%) with del(11)(q23), 4 (12.5%) with *t*(6;11), and 3 (9.4%) with *t*(11;17). Figure 4 shows the OS of the intermediate risk group. The 5-year survival rate was 44.0% in patients with a normal karyotype, 35.5% in those with 11q23 abnormalities, and 30.6% in other patients including those with *t*(7;11), *t*(6;9), and Ph(+) abnormalities, respectively ($P = 0.033$).

Table 7 shows the relationship between *t*(9;11) ($n = 9$) and other 11q23 abnormalities ($n = 23$). More patients with low-MPO, without MLD, or with the FAB M5 subtype were found in the group with *t*(9;11) than with other 11q23 abnormalities. The survival rates between the two groups did not significantly differ ($P = 0.22$, data not shown).

4 Discussion

We attempted to classify selected patients who were reviewed morphologically and had available chromosomal data according to the WHO system. However, our series had some limitations in terms of analysis and patient selection. Although we obtained chromosomal data, genetic data were not available. Patients who were diagnosed with AML M3 or who had *t*(15;17), a history of MDS, or preceding hematological abnormalities, or who had previously undergone chemotherapy, were not eligible for the present study. However, multicenter trials might have some advantages in diagnosing AML according to the WHO classification, because morphological diagnoses and karyotypes are reviewed by the corresponding institutional committees.

The incidence of each category of the WHO classification was similar to those in several reports when patients with *t*(15;17) and therapy-related AML were excluded [20–22]. We and several others have shown that approximately 30% of patients have recurrent genetic abnormalities. Multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) assays have recently been applied to analyze cytogenetic abnormalities [21, 23, 24]. This method might cause the frequency of the first WHO category to increase. Thus, the multiplex RT-PCR assay might have to be incorporated into the WHO system. The JALSG has started a cohort study in which all AML patients in participating hospitals are registered and analyzed according to the WHO classification. That study should clarify the real ratios of the AML subtypes in the WHO classification.

Table 4 Comparison of clinical findings of patients diagnosed according to the WHO classification

Category		Platelets ($\times 10^9$/l ± SE)	WBC ($\times 10^9$/l ± SE)	Hb (g/dl ± SE)	Age (year ± SE)	Blasts in bone marrow (%±SE)	MPO positivity of blasts (%±SE)
I	*t*(8;21)	76.7 ± 56.43 (113)[a]	1.4 ± 0.6 (113)	7.8 ± 0.2 (113)	41.6 ± 1.3 (113)	49.9 ± 2.0 (113)	93.3 ± 3.3 (108)
	inv(16)	57.8 ± 52.03 (26)	6.6 ± 1.2 (26)	9.2 ± 0.5 (26)	44.5 ± 2.6 (26)	50.5 ± 4.1 (26)	66.9 ± 6.7 (26)
	11q23	38.3 ± 30.8 (32)	4.3 ± 1.1 (32)	8.9 ± 0.4 (32)	41.6 ± 2.4 (32)	56.3 ± 3.7 (32)	43.6 ± 6.1 (32)
II		111.0 ± 121.5 (133)	3.0 ± 0.5 (133)	8.3 ± 0.2 (133)	44.2 ± 1.2 (133)	48.0 ± 1.8 (133)	34.0 ± 3.1 (126)
IV		72.8 ± 91.7 (330)	5.1 ± 0.3 (331)	8.8 ± 0.1 (330)	43.8 ± 0.7 (331)	65.7 ± 1.2 (328)	53.7 ± 1.9 (312)
		$P < 0.0001$	$P < 0.0001$	$P = 0.0004$	$P = 0.4077$	$P < 0.0001$	$P < 0.0001$

SE standard error, *WBC* white blood cell, *MPO* myeloperoxidase, *Hb* hemoglobin

[a] Number of patients

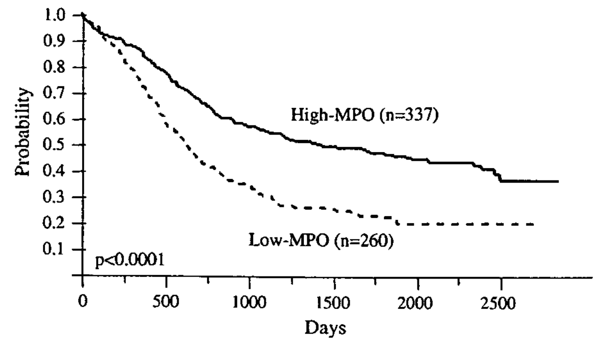

Fig. 2 Overall survival of patients with high or low MPO-positive blasts

Table 5 Distribution of patients classified by cytogenetic risk

Cytogenetic risk group	No. of patients	%
Favorable	139	21.8
t(8;21)	113	17.7
inv(16)	26	4.1
Intermediate	413	64.7
Normal karyotype	267	41.8
11q23	32	5.0
Ph(+)	7	1.1
t(7;11)(p15;p15)	4	0.6
t(6;9)	4	0.6
Other	131	20.5
Adverse	54	8.5
Complex	41	6.4
−7	2	0.3
abn3	5	0.8
del5q	2	0.3
−5	1	0.2
Other	3	0.5
Total	638	100.0

Few reports have included clinical data with the WHO classification. We found that the platelet count was higher among patients in the second category than in other categories. This supports our previous finding that the platelet count is higher in patients with AML accompanied by the MLD phenotype [25]. Among patients with MLD, none were in the favorable risk group, whereas the intermediate or adverse risk ratios among these patients were 89.6 and 10.4%, respectively. These differences might influence the finding that OS was better among patients without than with MLD ($P = 0.0002$, data not shown). Previous studies have also associated the MLD phenotype with a poorer outcome, although MLD is not significantly prognostic on multivariate analysis [18, 26], and a German group showed that dysplastic features correlate with adverse karyotypes

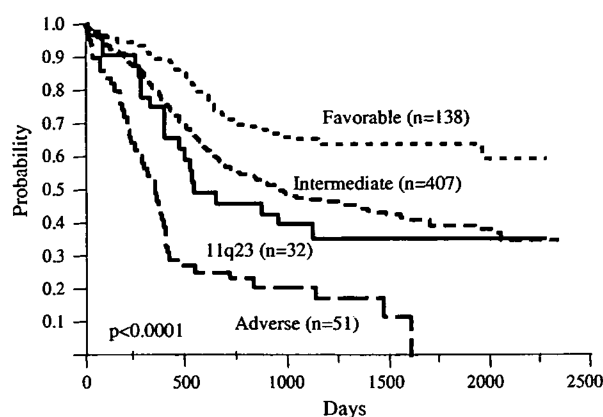

Fig. 3 Overall survival of patients stratified according to cytogenetic risk groups. Significant differences were observed between patients with a favorable, intermediate (except 11q23), and adverse karyotype ($P < 0.0001$)

Table 6 Relationship between cytogenetic risk groups and MLD phenotype or MPO-positive rates of blasts

	Favorable $n = 139$	Intermediate $n = 445$	Adverse $n = 54$	Total
MLD				
+	0	129 (89.5%)	15 (10.4%)	144
−	138 (28.2%)	292 (59.6%)	38 (7.8%)	490
Unknown	1	2	1	4
MPO				
High	123 (36.3%)	201 (59.3%)	15 (4.4%)	339
Low	11 (4.1%)	221 (82.5%)	36 (13.4%)	268
Unknown	5	23	3	31

High- and low-MPO indicates a percentage of myeloperoxidase positive blasts ≥50 or <50%, respectively

MLD multilineage dysplasia

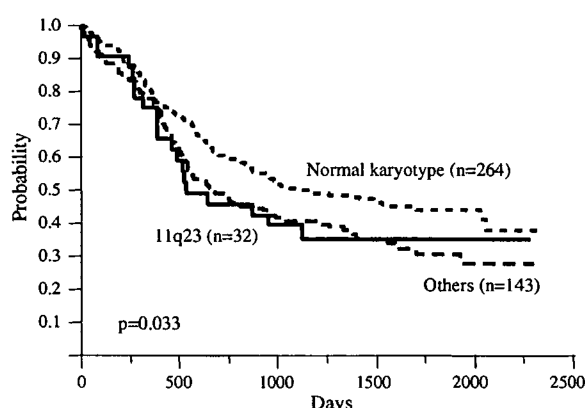

Fig. 4 Overall survival of patients with subtypes of intermediate cytogenetic risk. Significant differences were observed between patients with a normal karyotype and those with 11q23 abnormalities ($P = 0.033$)

[26]. Furthermore, patients in the second category had a lower MPO-positive rate of blasts, whereas those with *t*(8;21) had a higher rate. Patients with high- and low-MPO were more frequently observed in the favorable and adverse risk groups, respectively. Multivariate analysis has shown that MPO is a significant factor affecting OS [19]. We did not assess prognostic factors by multivariate analysis here because the main theme of this study was to categorize patients according to the WHO classification, and we have already examined these in a previous series [18, 19].

Several studies have demonstrated the impact of specific cytogenetic abnormalities on survival in AML [3, 7–12, 20–22]. The cytogenetic risk groups stratified the AML patients in the present study according to the MRC system, as in these reports [3]. Therefore, we confirmed the clinical usefulness of cytogenetics as the first category of the WHO classification. We found that 32 patients had 11q23 abnormalities. The MRC system revealed that de novo and secondary AML patients with 11q23 abnormalities had an intermediate outcome with an OS rate of 45% at 5 years ($n = 60$; median age, 17 years) in a younger cohort [3] and an OS rate of 0% at 5 years ($n = 11$; median age 64 years) in an elderly cohort [7]. In contrast, SWOG/ECOG trials including adult de novo AML patients (age, 16–55 years) assigned those with 11q abnormalities to the unfavorable cytogenetic subgroup [8]. Our data showed that patients with 11q23 abnormalities have an intermediate rather than adverse outcome. The prognostic effect of 11q23 abnormalities might depend on the partner gene. Several studies have shown that 11q23 abnormalities with *t*(6;11) and *t*(10;11) are associated with a poor prognosis, whereas *t*(9;11) is associated with a superior OS and such patients might respond well to intensive treatment, especially when the chemotherapy regimen includes high-dose cytarabine [15, 27–30]. The CALGB study has shown that the median OS of 13.2 months among 23 patients with *t*(9;11) was significantly longer than the 7.7 months among 24 patients with other 11q23 rearrangements ($P = 0.009$) [30]. In a recent CALGB series of 54 patients with 11q23 abnormalities, 27 patients with *t*(9;11) had an intermediate outcome and a median OS of 13.2 months, whereas those with *t*(6;11) or *t*(11;19) had a poor outcome of 7.2 or 8.4 months [15]. Conversely, Schoch et al. showed that 14 patients with *t*(9;11) had a median OS of 10.0 months compared with the 12.8 months of 26 patients with other MLL rearrangements, and that the two cytogenetic groups did not significantly differ [13]. Our data showed that nine patients with *t*(9;11) were more frequently involved in M5. The MPO and MLD features significantly differed between patients with *t*(9;11) and those with other 11q23 abnormalities. However, the CALGB study found no significant differences in myelodysplastic features between the two

Table 7 Comparison of $t(9;11)$ and other 11q23 abnormalities

	No. of patients	Auer		MPO*		MLD*		FAB						Median age (year)	Median survival (day)
		+	–	High	Low	+	–	M1	M2	M4	M4Eo	M5a**	M5b		
$t(9;11)$	9	0	9	1	8	0	9	0	0	3	0	6	0	39	1031.00
Other 11q23	23	5	18	13	10	10	13	1	3	13	1	2	3	48	520.00
Total	32	5	27	14	18	10	22	1	3	16	1	8	3	44.5	531.5

High- and low-MPO indicates a percentage of myeloperoxidase-positive blasts ≥50 or <50%, respectively

MLD multilineage dysplasia

* $P < 0.05$, ** $P < 0.01$

cytogenetic groups [30]. In terms of OS, our results showed no significant differences between patients with $t(9;11)$ and those with other 11q23 abnormalities ($P = 0.22$). Some problems are associated with the analyses of 11q23 abnormalities. We had few patients with these abnormalities, particularly individual translocations, and genetic analysis was not performed. Thus, the prognostic risk of 11q23 abnormalities cannot be concluded from the present study. Nonetheless, these abnormalities were never associated with a favorable risk. To classify 11q23 abnormalities into each prognostic risk group, further investigations and genetic analyses of a large number of patients with 11q23 abnormalities are required.

The fourth WHO category, which is not otherwise categorized, accounted for 52% of patients in the present study. Most of them were classified into the intermediate risk group, and no prognostic subdivisions were valuable. Using cytogenetic features as a prognostic factor in groups with a normal karyotype has limitations, and such patients accounted for 64.6% of the intermediate risk group (data not shown). Additional factors are required to stratify these patients. We and several others suggested that differences could be based on molecular genetic analysis [22, 31–35]. For example, FLT3 mutations are important biomarkers of a normal karyotype and might be valuable for stratifying the intermediate risk group. Further follow-up studies might also shed light on the roles of FLT3 ITD mutations in the development of AML and aid their use as novel molecular targeting agents against AML [22, 32]. Bienz et al. identified CEBPA mutations, FLT3-ITD, and differing levels of BAALC expression as having independent prognostic significance in patients with a normal karyotype [33]. If these genetic markers can be confirmed as being of clinical significance, genetic analyses will probably be incorporated into the WHO classification.

In summary, our results confirmed those of previous studies showing the prognostic significance of cytogenetics, MLD, and MPO-positivity of blasts in AML. Furthermore, we categorized patients with de novo AML according to the WHO classification and showed the clinical characteristics and OS of each category.

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References

1. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol. 1976;33:451–8.
2. Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia: a report of the French-American-British Cooperative Group. Ann Intern Med. 1985;103:620–5.
3. Grimwade D, Walker H, Oliver F, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. Blood. 1998;92:2322–33.
4. Harris NL, Jaffe ES, Diebold J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol. 1999;17:3835–49.
5. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002;100:2292–302.
6. Brunning RD, Matutes E, Harris NL, et al. Acute myeloid leukaemias. In: Jaffe ES, Harris NL, Stein H, Vardiman JW (eds) Pathology and genetics of tumors of haematopoietic and lymphoid tissues. Lyon: IARC Press; 2001. p. 75–107.
7. Grimwade D, Walker H, Harrison G, et al. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML 11 trial. Blood. 2001;98:1312–20.
8. Slovak ML, Kopecky KJ, Cassileth PA, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group study. Blood. 2000;96:4075–83.
9. Rowe JM, Liesveld JL. Treatment and prognostic factors in acute myeloid leukaemia. Baillieres Clin Haematol. 1996;9:87–105.
10. Schoch C, Kern W, Schnittger S, Buchner T, Hiddemann W, Haferlach T. The influence of age on prognosis of de novo acute myeloid leukemia differs according to cytogenetic subgroups. Haematologica. 2004;89:1082–90.
11. Arber DA, Stein AS, Carter NH, Ikle D, Forman SJ, Slovak ML. Prognostic impact of acute myeloid leukemia classification: