MRP3, BCRP, and P-Glycoprotein Activities are Prognostic Factors in Adult Acute Myeloid Leukemia

Zineb Benderra,[1] Anne Marie Faussat,[1] Lydia Sayada,[1] Jean-Yves Perrot,[2] Ruoping Tang,[1] Driss Chaoui,[1,3] Hamid Morjani,[4] Christophe Marzac,[1,2] Jean-Pierre Marie,[1,3] and Ollivier Legrand[1,3]

Abstract

Purpose: P-Glycoprotein (Pgp) is associated with poor outcome in acute myeloid leukemia (AML). We have investigated other ATP-binding cassette proteins such as BCRP, MRP1, MRP2, MRP3, and MRP5 for their potential implication in chemoresistance.

Experimental Design and Results: Eighty five AML patient samples were analyzed in this study. First, MRP3 function was higher in patients which had a high level of leukocytes ($P = 0.01$), a M5 FAB subtype ($P = 0.04$), and an intermediate or poor cytogenesis ($P = 0.05$). BCRP activity was not correlated with clinical or biological variables, but high Pgp activity was correlated with the following variables: CD34 expression ($P = 0.002$), FAB subtype ($P = 0.002$), intermediate or poor cytogenesis ($P = 0.02$), and elderly patients ($P = 0.03$). Second, Pgp, MRP3, and BCRP activities were correlated with complete remission ($P = 0.02$, $P = 0.04$, and $P = 0.04$, respectively), disease-free survival ($P = 0.02$, $P = 0.03$, and $P = 0.25$, respectively), and overall survival ($P = 0.04$, $P = 0.04$, and $P = 0.05$, respectively) in multivariate analysis. The patient samples expressing one or none of these Pgp, MRP3, or BCRP functional proteins have a better prognosis than the patients expressing two or three of these functional proteins (complete remission, $P = 0.02$; disease-free survival, $P = 0.01$; overall survival, $P < 0.001$).

Conclusions: BCRP and MRP3 may also be involved in chemoresistance in AML, especially MRP3 in patients with M5 FAB. Additional modulation of BCRP or MRP3 to Pgp modulation may be necessary in some patients in order to improve the treatment outcome.

Despite improvements accomplished in the last 30 years on the use of combination cytarabine (Ara-C) and intercalating agents, the overall prognosis for adult acute myeloid leukemia (AML) remains poor (1). One of the best-characterized resistance mechanisms in AML is the drug extrusion mediated by ABCB1 [P-glycoprotein (Pgp)], which has been shown to be associated with a poor outcome (2–8). Other ATP-binding cassette (ABC) proteins, such as ABCC1 (MRP1), also seem to contribute to the resistance in AML, especially in association with Pgp (6, 9).

A new ABC protein, ABCG2 (BCRP), was simultaneously characterized by different groups (10–12) from the cell lines resistant to anthracycline and mitoxantrone. The drug-resistance profile of BCRP-positive cells was done *in vitro* by Litman et al. (13). They were cross-resistant to mitoxantrone, daunorubicin, doxorubicin, bisantrene, and topotecan. Daunorubicin was also shown to be a BCRP substrate in AML cells (14), and Ara-C was not a BCRP substrate in infant acute lymphoblastic leukemia (15). The specificity of BCRP binding to its substrate could be modified by changes in its amino acid sequence (16). Amino acid 482 mutation in human cancers could affect the clinical application of ABCG2 antagonists. In a recent study by Ross et al., high expression of BCRP mRNA was sufficiently frequent to warrant more extensive investigations to determine the relation of disease and treatment outcome to BCRP expression (17). However, in another study, only 7% of the 40 AML samples tested contained BCRP mRNA levels within the range of their drug-resistant clone, although another 78% were higher than normal blood and bone marrow (18). In a clinical study, 59 childhood AML patients who expressed high levels of BCRP (the authors used the median of BCRP expression as the cutoff for high expression) had a worse prognosis (19). In a recent study, using mRNA expression, we showed that BCRP could be implicated in resistance to chemotherapies in adult AML (20). van den Heuvel-Eibrink et al. have shown that BCRP mRNA expression was significantly higher in refractory/relapsed AML than in *de novo* AML, suggesting that BCRP is associated with clinically resistant disease in AML (21), in contrast to the results of van der Kolk' study (22). Therefore, BCRP protein expression and functionality for prognosis in AML should be investigated.

Authors' Affiliations: [1]Laboratoire Institut National de la Sante et de la Recherche Medicale (INSERM U736), Universitaire Paris 6 (UMR 736), [2]Service d'Hématologie Biologique, [3]Département d'Hématologie Clinique, Hôpital Hôtel-Dieu, Assistance Publique-Hôpitaux de Paris, Paris, and [4]JE Onco-Pharmacologie, IFR53, UFR de Pharmacie, Reims Cedex, France

Received 9/16/04; revised 6/30/05; accepted 8/4/05.

Grant support: Association pour la Recherche sur le Cancer (no. ML/MLD/CM-P01/5).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Presented in part at the 45th Annual Meeting of the American Society of Hematology, San Diego, CA. December 6-9, 2003.

Requests for reprints: Ollivier Legrand, Département d'Hématologie, Hôpital Hôtel-Dieu, 1 Place du parvis, Notre Dame, 75004 Paris, France. Phone: 33-1-4234-8585; Fax: 33-1-4234-8406; E-mail: ollivier.legrand@htd.ap-hop-paris.fr.

doi:10.1158/1078-0432.CCR-04-1895

MRP1 homologues, such as ABCC2 (MRP2), ABCC3 (MRP3), and ABCC5 (MRP5), have been shown to be expressed at variable levels in AML patient cells. These proteins have been described to confer a resistance to chemotherapeutic agents, such as daunorubicin, mitoxantrone, and etoposide, which are used in the treatment of AML patients (23). However, the role of these proteins in drug resistance in adult AML has not been clarified. In recent studies, MRP3 and possibly MRP2, but not MRP4 and MRP5, have been shown to be involved in drug resistance in childhood AML and childhood T acute lymphoblastic leukemia (24, 25). Specific assays and protein expression studies are necessary to confirm these results. The different functional assays for these new ABC proteins are currently being analyzed, compared, and applied to the prognostic analysis.

Here, we report the protein expression of Pgp, MRP1, MRP2, MRP3, MRP5, and BCRP in six cell lines using flow cytometry. We compared three different probes and modulators to measure the functionality of those proteins. Finally, we have studied the prognostic effect of these six ABC proteins (expression and activity) in 85 adult AML samples.

Materials and Methods

Cell lines. The present study was carried out with six cell lines at different levels of six ABC proteins, Pgp, MRP1, MRP2, MRP3, MRP5, and BCRP (Table 1): K562, a human erythroleukemia (a gift from B Sickic, Pharmacology Department, Stanford University, Stanford, CA.) and its derivation, K562/HHT30 cell line, which was developed in our laboratory (26). HL60, human myeloid leukemia cells, and its derivation HL60/MRP, which is resistant to daunorubicin. PC13, a lung large cell carcinoma, and PC13 2-2, which are PC13 cells transfected with BCRP (a gift from Dr. H. Komatani, Banyu Tsukuba Research Institute, Ibaraki, Japan; ref. 27).

Patients. Between January 1998 and December 2002, 85 samples from *de novo* adult AML patients were successfully tested for the six ABC protein expressions. The diagnosis was based on French-American-British (FAB) criteria [11 M1 (13%), 40 M2 (47%), 17 M4 (20%), 16 M5 (19%), and 1 M6 (1%); ref. 28]. Immunophenotyping was done by flow cytometry. Acute promyelocytic leukemia patients were excluded from the study (due to retinoic acid treatment). Patients with t(9;22) were also excluded from the study. For each patient, several clinical and biological characteristics were analyzed (age, WHO performance status, WBC count at diagnosis, CD34 expression, FAB subtypes, and cytogenetics). Karyotypes were defined as previously reported (29).

All patient in this study had a history of prior therapy with anticancer drugs. All patients were treated with uniform chemotherapy and transplantation; having received a combination of Ara-C (100 mg/m^2/d) for 10 days, with daunorubicin (45 mg/m^2/d) for 3 days, and etoposide (100 or 50 mg/m^2/d) for 5 days. Those patients who achieved complete remission (CR) after one or two cycles of therapy received one cycle of consolidation therapy (Ara-C, 500 mg/m^2/12 hours for 6 days with daunorubicin, 45 mg/m^2/j for 3 days). Patients achieving CR were subsequently scheduled to proceed to allogeneic bone marrow transplantation if a matched sibling donor was available (10 patients); patients >50 years old or lacking a suitable donor received an autograft or chemotherapy (30). Patients receiving one allograft were censored at the time of transplant.

Table 1. Expression and function of six ABC proteins in six cell lines

	Cell lines					
	PC13	PC13 2-2	K562	HHT90	HL60	HL60/MRP
Protein* (ratio of fluorescence, MDR antibody/control antibody)						
Pgp	1.05 ± 0.9	1 ± 0.1	1 ± 0.1	7.1 ± 0.12	1 ± 0.01	1 ± 0.02
MRP1	5.87 ± 0.8	6 ± 0.7	1 ± 0.01	0	3.98 ± 0.01	12.2 ± 0.9
MRP2	1.02 ± 0.7	1 ± 0.2	0.98 ± 0.01	0	1 ± 0.01	1.02 ± 0.01
MRP3	7.8 ± 0.8	8.1 ± 0.7	1 ± 0.1	5.1 ± 0.8	1 ± 0.02	11.2 ± 1.2
MRP5	1.01 ± 0.7	0.98 ± 0.4	0.97 ± 0.1	1 ± 0.01	0.98 ± 0.01	1 ± 0.1
BCRP	1 ± 0.1	10.2 ± 0.8	1 ± 0.02	0.98 ± 0.01	1 ± 0.02	6.9 ± 1.1
Proteins expressed	MRP1, MRP3	MRP1, MRP3, BCRP	no	Pgp, MRP3	MRP1	MRP1, MRP3, BCRP
Function* (shift MFI)						
Mitoxantrone ± GG†	10 ± 3	350 ± 21	0 ± 2	320 ± 15	0 ± 6	310 ± 19
Mitoxantrone ± fumitremorgin C‡§	22 ± 5	320 ± 10	0 ± 2	0 ± 1	12 ± 2	210 ± 12
Mitoxantrone ± MK‡§	800 ± 40	920 ± 20	0 ± 4	450 ± 12	12 ± 3	1,450 ± 30
CAM ± GG‡§	21 ± 4	10 ± 2	9 ± 2	240 ± 23	10 ± 2	0 ± 8
CAM ± fumitremorgin C§	10 ± 1	21 ± 3	0 ± 3	0 ± 12	10 ± 6	12 ± 2
CAM ± MK‖	860 ± 42	870 ± 15	8 ± 2	380 ± 30	110 ± 3	1,220 ± 20
JC1 ± GG‡	31 ± 3	21 ± 4	0 ± 2	550 ± 28	14 ± 2	12 ± 5
JC1 ± fumitremorgin C§	18 ± 3	15 ± 8	0 ± 2	0 ± 12	20 ± 3	23 ± 12
JC1 ± MK§	24 ± 3	17 ± 7	7 ± 3	0 ± 14	12 ± 4	13 ± 17
JC1 ± CsA‡	12 ± 4	10 ± 2	4 ± 1	510 ± 40	14 ± 8	11 ± 2

*Each cell line was analyzed in triplicate.
†This functional assay was for both Pgp and BCRP proteins and therefore could not be used for one protein.
‡Each functional assay was specific for one protein.
§The functional assays were analyzed and any ABC proteins were studied.
‖This functional assay was for both MRP1 and MRP3 proteins and therefore it was not specific for any one protein.

Level of ABC protein expression in cell lines and acute myeloid leukemia samples. Pgp, MRP1, MRP2, MRP3, MRP5, and BCRP protein expression was measured by labeling fresh viable cells with the UIC2, QCRL3, M2I4, M3II9, M5I1, and BXP-34 monoclonal antibodies, respectively (concentration, 5×10^3 μg/mL), and phycoerythroin-labeled second antibody as described before (31). If necessary, cells were permeabilized in 15% (v/v) lysing solution G (Becton Dickinson, Le Pont de Claix, France) in water and incubated for 15 minutes in PBS/bovine serum albumin containing 1% (v/v) normal goat serum. The expression of ABC proteins was established only in blast cells selected by CD34 antibody ($HPCA_2$ clone, Becton Dickinson; two-color assays) or other markers (for example, CD33/CD7, CD33/CD2, CD33/CD19, or CD33/CD22 by three-color assays), or selected by physical characteristics when blast cells did not express characteristic markers. Fluorescence was analyzed on a EPICS Altra (Beckman Coulter, Brea, CA) flow cytometer. ABC-Protein expression was determined by the ratio of mean fluorescence intensity (MFI) of each specific antibody/control antibody (Pgp, MRP1, or MRP5/IgG_{2a}; MRP2, MRP3, or BCRP/IgG_1). For each sample, 5,000 events were collected.

Functional assays of ABC protein in cell lines and acute myeloid leukemia samples. Firstly, six cell lines with different levels of these five ABC proteins were used to assess the ability of three probes [calcein-AM (CAM; 10^{-6} mol/L), JC1 (7.5 μmol/L), and mitoxantrone (20×10^{-6} mol/L)] ± three modulators [MK571 (MK; 20×10^{-6} mol/L), GG918 (10^{-6} mol/L), and fumitremorgin C (10^{-5} mol/L)] to measure the functionality of these proteins by flow cytometry. Cell fluorescence was recorded using a Beckman Coulter (EPICS Altra) flow cytometer. The function of ABC proteins was established with blast cells selected by CD34 antibody (FL3 channel, $HPCA_2$ clone; Becton Dickinson), or by physical characteristics when blast cells did not express characteristic markers.

We used JC1 ± CsA (2×10^{-6} mol/L) to assess Pgp activity as previously described (8). CAM and JC1 functional assays were assessed as previously described (8, 31). For mitoxantrone staining, cells were washed twice and resuspended in PBS containing 20×10^{-6} mol/L of mitoxantrone at a concentration of 5×10^5 cells/mL and was incubated at 37°C for 20 minutes with or without modulator. Cells were washed twice in cold PBS, and then were analyzed. Mitoxantrone fluorescence was logarithmically emitted at 675 nm wavelength when excited by laser at 633 nm wavelength. The effect of the inhibitors was expressed as a shift of MFI of the dye accumulation. For each sample, 5,000 events were collected.

Statistical analysis. Correlations between multidrug resistance protein expression and multidrug resistance protein activity were estimated using Spearman's rank order correlation coefficient. The associations between variables were analyzed by Fisher's exact test for categorical variables and by Mann-Whitney *U* test or Kruskal-Wallis test for continuous variables. Clinical and biological factors were investigated for their influence on remission rate by Fisher's exact test for binary variables and by Mann-Whitney *U* test or Kruskal-Wallis test for continuous variables. The rates of (*a*) disease-free survival (DFS) were measured from the establishment of CR until relapse or death from any cause, with observation censored for patients last known alive without report of relapse; (*b*) overall survival (OS) was measured from diagnosis until death from any cause, with observations censored for time when patients were last reported to be alive. They were estimated with the method of Kaplan and Meier (31) and compared with a log-rank test. Analyses of prognostic factors for treatment outcomes were based on proportional hazards regression models for DFS and OS (32). Significance was defined as two-tailed $P \leq 0.05$. The Cox proportional model was used for the multivariate analyses (33). The median follow-up time for censored patients was 1,098 days. The time point used for the proportion of DFS and OS was December 31, 2003. We used StatView software (version 5.0) for statistical analysis (SAS Institute Inc., CA).

Results

Assessment of functional activity of ABC proteins in cell lines and acute myeloid leukemia samples

We have studied six cell lines: K562, K562/HHT30, HL60, HL60/MRP, PC13, and PC132.2. We found a good correlation between MRP3 expression and MRP3 functionality using mitoxantrone incorporation with or without modulator MK (mitoxantrone ± MK, $r = 0.93$; $P = 0.001$ using Spearman's rank correlation test), and also between BCRP expression and its functionality (mitoxantrone ± fumitremorgin C, $r = 0.98$; $P < 0.0001$; see data in Table 1). There was no correlation between MRP1 expression and its functionality (mitoxantrone ± MK). As previously published, Pgp functional assays using JC1 ± CsA were correlated with its expression. Any other functional assay was correlated with one protein expression.

In this study, we have also investigated 85 samples from AML patients. Five AML samples are shown in Fig. 1. We found good correlations between MRP3, BCRP, and Pgp expression with their respective functionalities (mitoxantrone ± MK, $r = 0.78$; $P < 0.001$; mitoxantrone ± fumitremorgin C, $r = 0.83$; $P < 0.001$; JC1 ± CsA, $r = 0.69$; $P < 0.001$; Fig. 2). Interestingly, nine M5 AML patients expressed a nonfunctional Pgp (Fig. 2). This dissociation between expression and function was not apparent for the other transporters. Other functional assays were not correlated with expression of ABC proteins. We failed to find a specific functional assay for MRP1. MRP1 expression correlated neither with its functionality (mitoxantrone ± MK; Fig. 2) nor with its functionality (CAM ± MK). CAM ± MK could detect both MRP1 and MRP3 activity at the same time. There was a good correlation between MRP1 expression and functionality only when the patient cells did not express MRP3 (CAM ± MK, $r = 0.65$; $P = 0.001$). Conversely, there was a good correlation between MRP3 expression and functionality when the patient cells did not express MRP1 (CAM ± MK, $r = 0.60$; $P = 0.002$; data not shown). Therefore, CAM ± MK might be able to assess MRP1 or MRP3 activity when one of two these proteins were absent. In Fig. 2, patients were considered to have high MRP3 activity when their mitoxantrone ± MK MFI levels were ≥200, and patients were considered to have high BCRP and Pgp activities when their mitoxantrone ± fumitremorgin C and JC1 ± CsA MFI levels were ≥150, respectively. Using this cutoff (Fig. 2), 50% and 40% of patients presented Pgp expression and activity, 39% and 35% for BCRP, and 32% and 31% for MRP3, respectively. In AML samples, MRP2 and MRP5 expression was very poor. Only two patients had an expression of MRP2 >1.2, and one patient expressed MRP5 at >1.2. According to the variables in Table 1 and Fig. 1, any assay could be used to evaluate MRP1 activity alone. CAM ± MK was available for MRP3 and MRP1 activities together (not to be used for one specific assay). Therefore, we have studied the correlation between MRP3, BCRP, and Pgp activities with clinical and biological variables.

Comparison of ABC protein activity and clinical as well as biological variables in acute myeloid leukemia

MRP3 activity (mitoxantrone ± MK) was higher in M5 AML than other FAB subtype AMLs (289 ± 90 versus 134 ± 45; P =

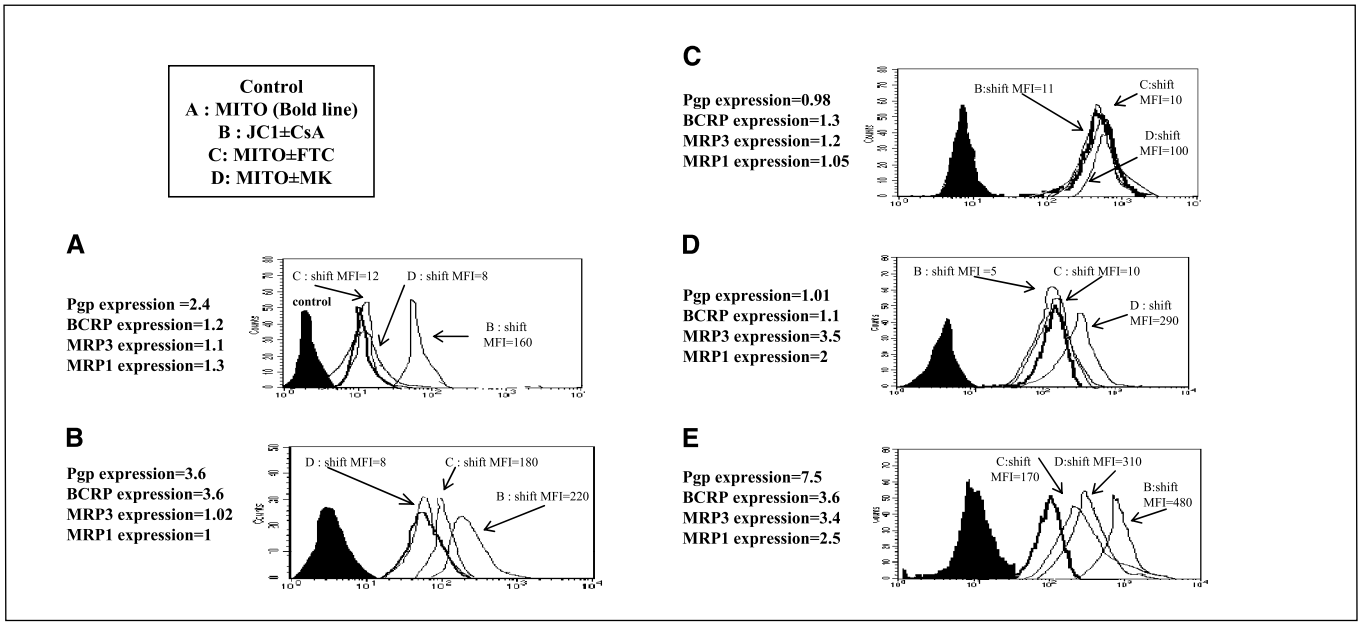

Fig. 1. Five fresh AML samples. *A,* sample with high Pgp expression alone. The only significant functional assay was the effect of CsA on JC1 (shift MFI = 160). *B,* sample with high activity for both Pgp and BCRP. The two significant functional assays were the effect of CsA on JC1 (shift MFI = 220), and the effect of fumitremorgin C on mitoxantrone (shift MFI = 180). *C,* example with activity for Pgp, BCRP, MRP3, and MRP1. *D,* sample with high activity for both MRP1 and MRP3. The significant functional assay was the effect of MK on mitoxantrone (shift MFI = 290). *E,* sample with high activity for Pgp, BCRP, MRP3, and MRP1. The significant functional assays were the effect of fumitremorgin C on mitoxantrone (shift MFI = 170), and MK on mitoxantrone (shift MFI = 310), and the effect of CsA on JC1 (shift MFI = 480).

0.04), as well as in patients with a hyperleucocytosis (275 ± 145 versus 89 ± 34; $P = 0.01$), and in patients with poor or intermediate cytogenetics than in patients with good cytogenetics (189 ± 103 versus 156 ± 45 versus 87 ± 39, respectively; $P = 0.05$; Table 2). BCRP activity (mitoxantrone ± fumitremorgin C) was not correlated with the clinical and biological variables.

Pgp activity (JC1 ± CsA) was higher in elderly patients than in younger patients (256 ± 60 versus 107 ± 96; $P = 0.03$), higher in CD34+ patients than in CD34− patients (254 ± 123 versus 58 ± 37; $P = 0.002$), higher in M1, M2, M4, and M6 FAB subtypes than in the M5 FAB subtype (287 ± 115 versus 48 ± 10; $P = 0.002$), higher in the poor or intermediate cytogenetic subgroups than in the good cytogenetic subgroup (231 ± 125 versus 182 ± 89 versus 68 ± 59, respectively; $P = 0.02$).

Treatment outcome

Fifty six of 85 patients achieved CR (66%). The percentage of DFS was 31 ± 5% at 4 years (median DFS, 371 days) and the percentage of OS was 27 ± 4% at 4 years (median OS, 341 days).

Univariate analysis. In this analysis, Pgp (JC1 ± CsA), MRP3 (mitoxantrone ± MK), and BCRP (mitoxantrone ± fumitremorgin C) activities, as continuous variables, were shown to correlate with prognosis. The Pgp mean value of MFI was 90 ± 45 in patients achieving CR versus 290 ± 78 in other patients ($P = 0.04$). This high activity was found in patients with poor DFS [$P = 0.04$; relative risk (RR), 1.90] and poor OS ($P = 0.01$; RR, 3.10). Elsewhere, MRP3 activity was very high in nonresponders with a MFI of 245 ± 89 versus 79 ± 34 in responders ($P = 0.009$). This high activity was also found in patients with poor DFS ($P = 0.01$; RR, 2.7) and poor OS ($P = 0.02$; RR, 2.01). BCRP activity was also higher in nonresponders with a MFI of 210 ± 78 than in responders with a MFI of 94 ± 34 ($P = 0.05$). This high activity was also found in patients with a poor DFS ($P = 0.03$; RR, 2), and poor OS ($P = 0.03$; RR, 2.12).

Similarly, the expressions of Pgp, MRP3, and BCRP were also correlated with treatment outcome (data not shown). Other significant variables in univariate analysis (CR, age, leukocytes, WHO performance status, and cytogenetics) are shown in Table 3.

Multivariate analysis. We evaluated several prognostic variables for CR, DFS, and OS in a multivariate Cox regression model (Table 3). ABC protein activity was analyzed as a continuous variable. We included all significant variables in univariate analysis in the models. Cytogenetics ($P = 0.01$; RR, 3.1), high Pgp activity ($P = 0.02$; RR, 2.89), high BCRP activity ($P = 0.04$; RR, 1.90), and high MRP3 activity ($P = 0.04$; RR, 2.1) had an independent adverse prognostic significance for CR. Cytogenetics ($P = 0.02$; RR, 3.1), high Pgp activity ($P = 0.02$; RR, 2.70), and high MRP3 activity ($P = 0.03$; RR, 2.12) had an independent adverse prognostic significance for duration of DFS. For duration of OS, the absence of CR ($P = 0.001$; RR, 5.1), cytogenetics ($P = 0.001$; RR, 4.01), high Pgp activity ($P = 0.04$; RR, 2.60), high MRP3 activity ($P = 0.04$; RR, 2.10), and high BCRP activity ($P = 0.05$; RR, 1.95) had an independent adverse prognostic significance.

Treatment outcome of patients with high activity of MRP3, BCRP, and P-Glycoprotein. In this analysis, ABC protein activity was analyzed as a dichotomized variable. Among 85 patients, 30 patients (35%) had high BCRP activities with a MFI of ≥150, 26 patients (31%) had high MRP3 activity with a MFI of ≥200, and 34 patients (40%) had high Pgp activity with a MFI of ≥150 (Fig. 2).

Patients with high BCRP activity had a poorer percentage of CR than other patients (50% versus 75%, respectively;

$P = 0.03$), as well as for DFS (21 ± 11% versus 39 ± 11% at 4 years; median DFS, 375 versus 600 days; $P = 0.12$; Fig. 3A); and for OS (11 ± 7% versus 41 ± 9% at 4 years; median OS, 230 versus 760 days; $P = 0.003$; Fig. 3B).

Independently, patients with a high MRP3 activity also had a poorer percentage of CR than other patients (46% versus 75%; $P = 0.01$) and for DFS (21 ± 9% versus 35 ± 7% at 4 years; median DFS, 156 versus 622 days, respectively; $P = 0.04$; Fig. 3 C), as well as for OS (15 ± 6% versus 38 ± 9% at 4 years; median OS, 230 versus 790 days, respectively; $P = 0.02$; Fig. 3D).

Patients with a high Pgp activity have also a poorer percentage of CR than other patients (52% versus 74%; $P = 0.04$), and for DFS (21 ± 5% versus 42 ± 8% at 4 years, median 350 versus 704 days, respectively; $P = 0.02$; Fig. 3E), as well as for OS (20 ± 5% versus 39 ± 3% at 4 years, median 198 versus 452 days, respectively; $P = 0.008$; Fig. 3F).

The patients who had only one or no (12 and 27 patients, respectively) high-activity Pgp, MRP3, or BCRP functional proteins had a better prognosis than the patients who had two or three (32 and 14 patients, respectively) high-activity Pgp, MRP3, or BCRP functional proteins. CR levels were 79% (31 patients) versus 54% (25 patients), respectively ($P = 0.02$); DFS was 43% versus 21% at 4 years, median 800 versus 370 days, respectively ($P = 0.01$; Fig. 3G); OS was 57 ± 11% versus 11 ± 5% at 4 years, median not reached versus 220 days, respectively ($P < 0.0001$; Fig. 3H).

Discussion

After discordant results during the past few years, several large studies confirmed the importance of functional Pgp in the clinical resistance of AML (2–8). However, the results of randomized trials, with the addition of potent Pgp inhibitors such as cyclosporine A, quinine, or PSC833 showed contradictory results. This gave revealed alternative or additional mechanisms of resistance (34–38). Other new ABC proteins, MRP2, MRP3, MRP5 BCRP, were described as potential chemotherapy resistance mechanisms in acute leukemia (17, 19, 20, 23, 24). However, the prognostic effect of these new ABC proteins is not well known.

In cell lines with different levels of resistance, we have analyzed several functional assays of these new ABC proteins. Some of those functional assays correlated with the protein

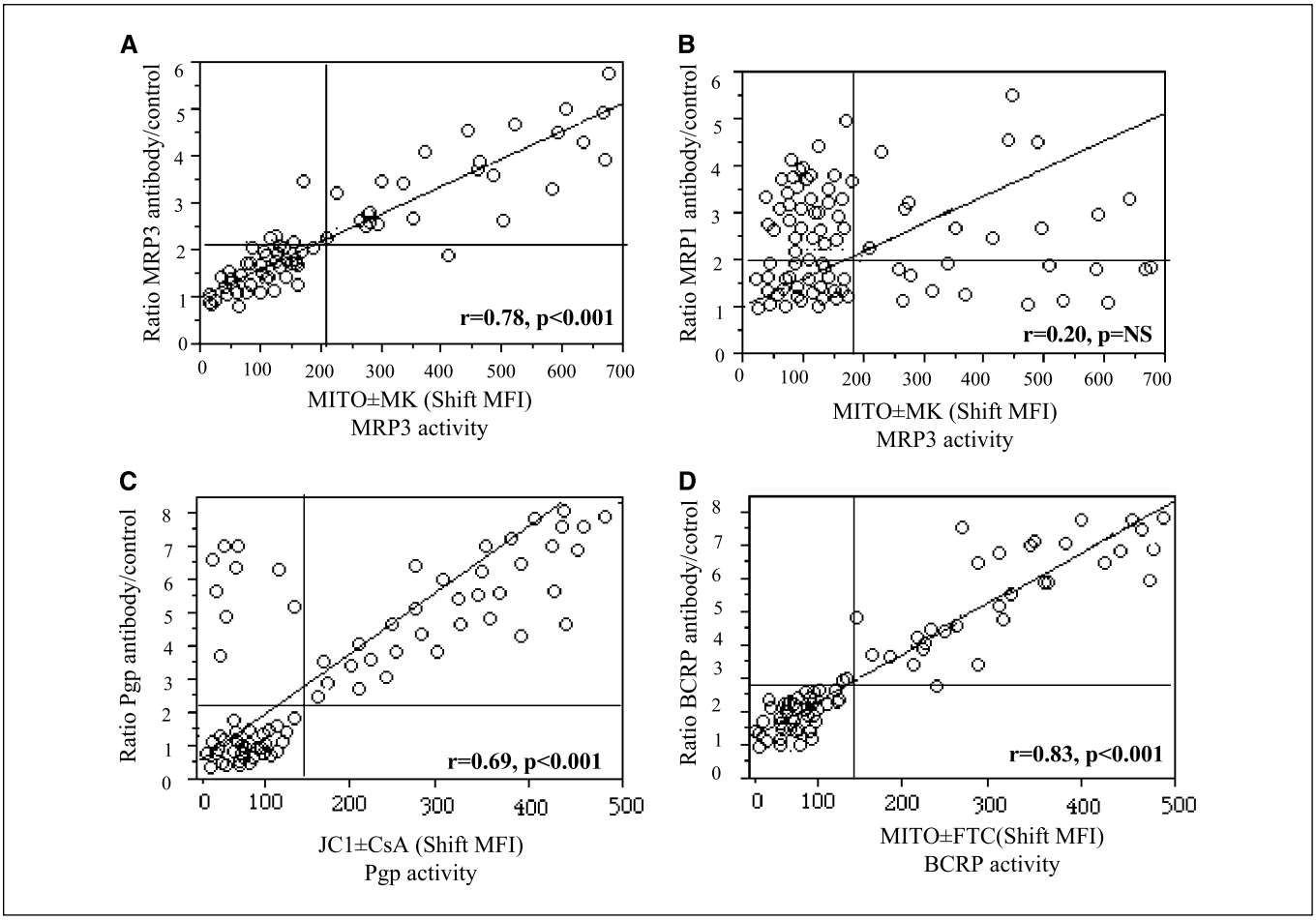

Fig. 2. Samples from 85 AML patients. *A,* correlation between MRP3 expression (ratio M3II9/control antibody) and mitoxantrone ± MK (shift MFI); *B,* correlation between MRP1 expression (QCRL3/control antibody) and mitoxantrone ± MK (shift MFI); *C,* correlation between Pgp expression (ratio of UIC2/control antibody) and JC1 ± CsA; *D,* correlation between BCRP expression (ratio of BXP-34/control antibody) and mitoxantrone ± fumitremorgin C (shift MFI). The data were obtained on blast cells selected by CD34 antibody, and in CD34-negative blast samples by other markers (see Materials and Methods).

Table 2. Correlations between clinical or biological variables and MRP3, BCRP, and Pgp activity

Characteristic	No. of patients	MRP3 activity*	*P*	BCRP activity†	*P*	Pgp activity‡	*P*
Age (y)			NS§		NS§		$P = 0.03$§
<55	47	155 ± 101		120 ± 87		256 ± 60	
≥55	41	168 ± 95		110 ± 67		107 ± 96	
WHO performance status			NS§		NS§		NS§
0 or 1	65	178 ± 89		130 ± 54		170 ± 67	
2, 3 or 4	20	134 ± 101		106 ± 89		168 ± 89	
Leukocytes ($\times 10^9$/L)			$P = 0.01$§		NS§		NS§
<20 × 10^9/L	50	89 ± 34		135 ± 89		187 ± 98	
≥20 × 10^9/L	35	275 ± 145		120 ± 34		164 ± 89	
FAB subtypes (%)			NS‖		NS‖		$P = 0.01$‖
M1	11	120 ± 78		110 ± 78		180 ± 90	
M2	40	134 ± 56		108 ± 89		290 ± 109	
M4	17	138 ± 54		120 ± 107		130 ± 78	
M5	16	289 ± 90		126 ± 90		48 ± 10	
M6	1	136 ± 67		178 ± 203		300 ± 178	
FAB subtypes			$P = 0.04$§		NS§		$P = 0.002$§
M5	16	289 ± 90		126 ± 90		48 ± 10	
Others	69	134 ± 45		107 ± 103		287 ± 115	
Karyotype			$P = 0.05$‖		NS‖		$P = 0.02$‖
good prognosis	13	87 ± 39		100 ± 80		68 ± 59	
Intermediate prognosis	51	156 ± 45		116 ± 78		182 ± 89	
poor prognosis	21	189 ± 103		125 ± 90		231 ± 125	
CD34 expression			NS§		NS§		$P = 0.002$§
negative	37	163 ± 90		102 ± 89		58 ± 37	
positive	48	132 ± 101		128 ± 93		254 ± 123	

*Using mitoxantrone ± MK. Effect of modulator was expressed as a shift of MFI of dye accumulation.
†Using mitoxantrone ± FTC. Effect of modulator was expressed as a shift of MFI of dye accumulation.
‡Using JC1 ± CsA. Effect of modulator was expressed as a shift of MFI of dye accumulation.
§Using Mann-Whitney test.
‖Using Kruskal-Wallis test.

expression. Thereafter, we selected these functional assays to assess the functionality of ABC proteins in AML samples.

In a cell line study, both mitoxantrone ± fumitremorgin C and mitoxantrone ± GG918 were correlated with BCRP expression. However, in AML samples, only the mitoxantrone ± fumitremorgin C assay could be correlated with BCRP expression as in some other studies (22, 39, 40), except for one study (41), because GG918 is also a good modulator of Pgp (39, 42). Therefore, we have selected the mitoxantrone ± fumitremorgin C assay rather than the mitoxantrone ± GG918 assay to assess BCRP function. In addition, BCRP substrate specificity could be modified by changes in its amino acid sequence. However, mitoxantrone is a substrate for both wild-type and mutant BCRP (16, 43, 44). Therefore, the modulation of mitoxantrone retention by the "BCRP-specific" modulator fumitremorgin C may serve as an assay for BCRP function for the cells expressing either wild-type or mutant protein. Despite numerous reports showing BCRP expression in AML, there is little evidence about the correlation between BCRP expression and any adverse clinical outcomes (17–19, 45). In our study, BCRP functionality was very strong in 35% of patients. BCRP function was not associated with clinical or biological characteristics. In this study, BCRP function was an independent prognostic factor, concordant with the mRNA expression results in childhood and adult AML (19, 20).

MRP3 activity was able to correlate with MRP3 expression only in mitoxantrone ± MK assay in patient samples. This activity was a prognostic factor as in other MRP3 mRNA expression studies in childhood AML and acute lymphoblastic leukemia (24, 25). MRP3 function was higher in patients with M5 AML (24), which expressed a nonfunctional Pgp (shown recently by us) but had a poor prognosis compared to other AMLs, except for AML with good cytogenetics (46). In another study, M5 and M4 AML also expressed low Pgp activity (5). Our study highlights the potential role of MRP3 in drug resistance in AML, especially in M5 AML.

The functions of BCRP, MRP3, and Pgp are independent prognosis factors. Previously, we have shown that the coexpression of Pgp and MRP1 was a poorer prognostic factor than the expression of only one of these proteins (9). In a recent study, childhood AML patients who expressed high levels of both *MRP2* and *MRP3* genes had a lower rate of survival than the patients who expressed only one of these two genes (24). In the same way, we have shown in this study that patients who coexpressed a high activity of two or three

ABC proteins among Pgp, BCRP, and MRP3 proteins had a poorer prognosis than patients who expressed a high activity of only one or none of these proteins. Therefore, these studies emphasized the importance of the simultaneous activity of ABC proteins in drug resistance. Hence, the modulation of only one of these proteins in clinical trials may not be enough. Some modulators could inhibit two ABC proteins. For example, GG918 could inhibit Pgp and BCRP activity in patients who coexpressed these two proteins (41, 42). Apart from anthracycline, Ara-C is a drug administered in the treatment of AML. The transfected cells with the cDNA of Pgp are not more resistant to Ara-C than nontransfected cells. Nevertheless, the sensitivity of blast cells to Ara-C is not known in cells transfected with MRP3 or BCRP. However, in one study, in infant acute lymphoblastic leukemia, despite the observed correlation between BCRP mRNA expression and Ara-C resistance, in a subline which expresses a high level of BCRP, Ko143, a specific BCRP inhibitor, did not sensitize the leukemic cell to Ara-C (14).

MRP2 protein expression was found only in a few patients, which is contrary to other studies. It is possible that the different results are due to the different methods used. We have

Table 3. Prognostic factors for CR, DFS, and OS in univariate and multivariate analysis

Variables	CR, 85 patients (RR, 95% CI)	DFS, 56 patients (RR, 95% CI)	OS, 85 patients (RR, 95% CI)
CR (yes vs. no)			
Univariate	—	—	$P = 0.001$* (RR, 4.1; 2-4.9)
Multivariate	—	—	$P = 0.001$† (RR, 5; 1.2-14)
Age (continuous variable)			
Univariate	$P = 0.001$‡ (RR, 4.5; 2.1-6.2)	$P = 0.004$* (RR, 3.2; 1.5-4.7)	$P = 0.001$* (RR, 6.1; 2-32.1)
Multivariate	NS§	NS†	NS†
Leukocytes (10^9/L; continuous variable)			
Univariate	$P = 0.03$ (RR, 2.3; 1.2-4.7)	NS	NS
Multivariate	NS	NS	NS
WHO performance status (0, 1 vs. 2, 3, 4)			
Univariate	$P = 0.03$‡ (RR, 2.3; 1.2-3.7)	NS	$P = 0.04$ (RR, 1.9; 1.1-2.9)
Multivariate	NS	NS	NS
Cytogenetics (G vs. I + P)			
Univariate	$P = 0.03$‡ (RR, 2.54; 1.1-5.9)	$P = 0.003$* (RR, 3.8; 1.6-4.5)	$P = 0.02$* (RR, 2.4; 1.3-3.9)
Multivariate	$P = 0.01$§ (RR, 3; 1.1-4.9)	$P = 0.02$† (RR, 3; 1.02-18)	$P = 0.001$† (RR, 4.01; 1.09-10.9)
Pgp function (continuous variable)			
Univariate	$P = 0.04$‡ (RR, 1.98; 1.01-3.75)	$P = 0.04$* (RR, 1.9; 1-5.01)	$P = 0.01$* (RR, 3.10; 1.5-4.01)
Multivariate	$P = 0.02$§ (RR, 2.89; 1.07-15)	$P = 0.02$† (RR, 2.70; 1.11-13)	$P = 0.04$† (RR, 2.60; 1.20-10.5)
MRP3 function (continuous variable)			
Univariate	$P = 0.01$‡ (RR, 3.8; 1.6-8.1)	$P = 0.01$* (RR, 2.7; 1.4-4.1)	$P = 0.02$* (RR, 2.12; 1.2-3.10)
Multivariate	$P = 0.04$§ (RR, 2.1; 1.2-6.8)	$P = 0.03$† (RR, 2.12; 1.03-10.1)	$P = 0.04$‡ (RR, 2.10; 1-5-5.80)
BCRP function (continuous variable)			
Univariate	$P = 0.04$‡ (RR, 2.12; 1.5-4.10)	$P = 0.03$* (RR, 2; 1.4-7.3)	$P = 0.03$* (RR, 2.12; 1.2-3.10)
Multivariate	$P = 0.04$§ (RR, 1.9; 1.3-4.8)	NS†	$P = 0.05$† (RR, 1.95; 1.11-6.01)

NOTE: 95% CI, 95% confidence intervals; NS, not significant; RR, relative risk.
*Using the log-rank test for binary variables and univariate Cox models for continuous variables.
†Using the Cox model.
‡Using Fisher's exact test for binary variables and a univariate logistic model for continuous variables.
§Using a multivariate logistic model.

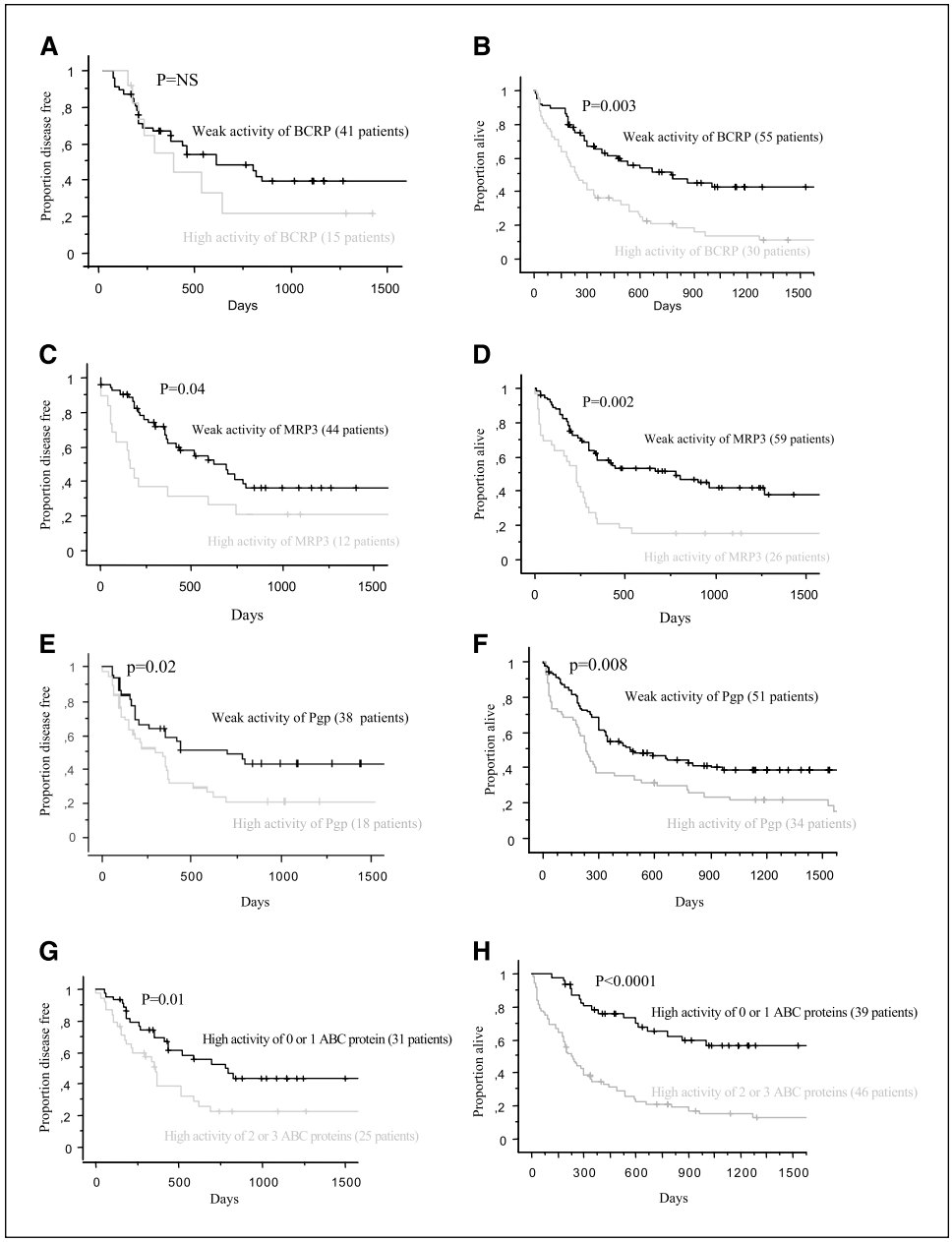

Fig. 3. DFS (*A*) and OS (*B*) of patients expressing high BCRP activity (*gray line*), or low BCRP activity (*black line*) assessed by mitoxantrone ± fumitremorgin C; DFS (*C*) and OS (*D*) of patients expressing high MRP3 activity (*gray line*), or low MRP3 activity (*black line*) assessed by mitoxantrone ± MK; DFS (*E*) and OS (*F*) of patients expressing high Pgp activity (*gray line*), or low Pgp activity (*black line*) assessed by JC-1 ± CsA; DFS (*G*) and OS (*H*) of patients with high activity of 0 or 1 of Pgp, MRP3, or BCRP protein (*black line*), or high activity of 2 or 3 of Pgp, MRP3, or BCRP proteins (*gray line*).

studied MRP2 protein expression by flow cytometry, but others have studied MRP2 mRNA expression (24, 47). MRP2 is expressed in normal lymphocytes (48), and even <10% of lymphocytes could increase the level of MRP2 in the blast samples (49). It is also possible that the affinity of our MRP2 antibody was not strong enough.

In conclusion, the ABC proteins, BCRP and MRP3, could be implicated in the resistance to chemotherapies in AML. Therefore, the modulation of BCRP and MRP3, as well as Pgp, could be essential.

Acknowledgment

We thank Dr. H. Komatani for generously providing the vector and BCRP PC13 cells.

References

1. Baudard M, Beauchamp-Nicoud A, Delmer A, et al. Has the prognosis of adult patients with acute myeloid leukemia improved over years? A single institution experience of 784 consecutive patients over a 16-year period. Leukemia 1999;13:1481–90.
2. Leith CP, Kopecky KJ, Godwin J, et al. Acute myeloid leukemia in the elderly: assessment of multidrug resistance (MDR1) and cytogenetics distinguishes biologic subgroups with remarkably distinct responses to standard chemotherapy. A Southwest Oncology Group Study. Blood 1997;89:3323–9.
3. Borg AG, Burgess R, Green LM, Scheper RJ, Liu Yin JA. Overexpression of lung resistance protein and increased P-glycoprotein function in acute myeloid leukaemia cells predict a poor response to chemotherapy and reduced patient survival. Br J Haematol 1998;103:1083–91.
4. Del Poeta G, Venditti A, Stasi R, et al. P-glycoprotein

and terminal transferase expression identify prognostic subsets within cytogenetic risk classes in acute myeloid leukemia. Leuk Res 1999;23:451–65.

5. Leith C, Kopecky K, Chen I, et al. Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia. A Southwest Oncology Group Study. Blood 1999;94: 1086–99.

6. van der Kolk DM, de Vries EG, van Putten WJ, et al. P-Glycoprotein and multidrug resistance protein activities in relation to treatment outcome in acute myeloid leukemia. Clin Cancer Res 2000;6:3205–14.

7. Wuchter C, Karawajew L, Ruppert V, et al. Clinical significance of P-glycoprotein expression and function for response to induction chemotherapy, relapse rate and overall survival in acute leukemia. Haematologica 2000;85:711–21.

8. Legrand O, Perrot JY, Simonin G, Baudard M, Marie JP. JC-1: a very sensitive fluorescent probe to test Pgp activity in adult acute myeloid leukemia. Blood 2001; 97:502–8.

9. Legrand O, Simonin G, Beauchamp-Nicoud A, Zittoun R, Marie J-P. Simultaneous activity of MRP1 and Pgp is correlated with *in vitro* resistance to daunorubicin and with *in vivo* resistance in adult acute myeloid leukemia. Blood 1999;94:1046–56.

10. Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, Dean M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res 1998;58:5337–9.

11. Doyle LA, Yang W, Abruzzo LV, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A 1998;95: 15665–70.

12. Ross DD, Yang W, Abruzzo LV, et al. Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. J Natl Cancer Inst 1999;91:429–33.

13. Litman T, Brangi M, Hudson E, et al. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J Cell Sci 2000;113:2011–21.

14. Sargent JM, Williamson CJ, Maliepaard M, Elgie AW, Scheper RJ, Taylor CG. Breast cancer resistance protein expression and resistance to daunorubicin in blast cells from patients with acute myeloid leukaemia. Br J Haematol 2001;11:257–62.

15. Stam RW, van den Heuvel-Eibrink MM, Den Boer ML, et al. Multidrug resistance genes in infant acute lymphoblastic leukemia: Ara-C is not a substrate for the breast cancer protein. Leukemia 2004;18:78–83.

16. Honjo Y, Hrycyna CA, Yan QW, et al. Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. Cancer Res 2001;61:6635–9.

17. Ross DD, Karp JE, Chen TT, Doyle LA. Expression of breast cancer resistance protein in blast cells from patients with acute leukemia. Blood 2000;96: 365–8.

18. Abbott BL, Colapietro AM, Barnes Y, Marini F, Andreff M, Sorrentino BP. Low levels of ABCG2 expression in adult AML blast samples. Blood 2002; 100:4594–601.

19. Steinbach D, Sell W, Voigt A, Hermann J, Zintl F, Sauerbrey A. BCRP gene expression is associated with a poor response to remission induction therapy in childhood acute myeloid leukemia. Leukemia 2002; 16:1443–7.

20. Benderra Z, Faussat AM, Sayada L, et al. Breast cancer resistance protein and P-glycoprotein in 149 adult acute myeloid leukemias. Clin Cancer Res 2004; 10:7896–902.

21. van den Heuvel-Eibrink MM, Wiemer EA, Prins A, et al. Increased expression of the breast cancer resistance protein (BCRP) in relapsed or refractory acute myeloid leukemia (AML). Leukemia 2002;16:833–9.

22. van der Kolk DM, Vellenga E, Scheffer GL, et al. Expression and activity of breast cancer resistance protein (BCRP) in *de novo* and relapsed acute myeloid leukemia. Blood 2002;99:3763–70.

23. van der Kolk DM, de Vries EG, Muller M, Vellenga E. The role of drug efflux pumps in acute myeloid leukemia. Leuk Lymphoma 2002;43:685–701.

24. Steinbach D, Lengemann J, Voigt A, Hermann J, Zintl F, Sauerbrey A. Response to chemotherapy and expression of the genes encoding the multidrug resistance-associated proteins MRP2, MRP3, MRP4, MRP5, and SMRP in childhood acute myeloid leukemia. Clin Cancer Res 2003;9:1083–6.

25. Steinbach D, Wittig S, Cario G, et al. The multidrug resistance-associated protein 3 (MRP3) is associated with a poor outcome in childhood ALL and may account for the worse prognosis in male patients and T-cell immunophenotype. Blood 2003;102:4493–8.

26. Zhou DC, Ramond S, Viguié F, Faussat AM, Zittoun R, Marie J-P. Progressive resistance to homoharringtonine in human myeloleukemia K562 cells: relationship to sequential emergence of MRP and MDR1 gene overexpression and MDR1 gene translation. Int J Cancer 1996;65:365–71.

27. Komatani H, Kotani H, Hara Y, et al. Identification of breast cancer resistant protein/mitoxantrone resistant/placenta-specific, ATP-binding cassette transporter as a transporter of NB-506 and J-107088, topoisomerase I inhibitors with an indolocarbazole structure. Cancer Res 2001;61:2827–32.

28. Bennett JM, Catovsky D, Daniel MT, et al. The morphological classification of acute lymphoblastic leukaemia: concordance among observers and clinical correlations. Br J Haematol 1981;47:553–61.

29. 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. The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood 1998; 92:2322–33.

30. Suciu S, Mandelli F, de Witte T, et al. EORTC and GIMEMA Leukemia Groups. Allogeneic compared with autologous stem cell transplantation in the treatment of patients younger than 46 years with acute myeloid leukemia (AML) in first complete remission (CR1): an intention-to-treat analysis of the EORTC/GIMEMA AML-10 trial. Blood 2003;102:1232–40.

31. Legrand O, Simonin G, Perrot JY, Zittoun R, Marie J-P. Pgp and MRP activities using calcein-AM are prognostic factors in adult acute myeloid leukemia patients. Blood 1998;91:4480–8.

32. Kaplan EI, Meier P. Non-parametric estimation from incomplete observations. J Am Stat Assoc 1958;53: 457–81.

33. Cox DR. Regression models and life tables (with discussion). J R Stat Soc B 1972;34:187–220.

34. Solary E, Witz B, Caillot D, et al. Combination of quinine as a potential reversing agent with mitoxantrone and cytarabine for the treatment of acute leukemias: a randomized multicenter study. Blood 1996;88: 1198–205.

35. Wattel E, Solary E, Hecquet B, et al. Quinine improves the results of intensive chemotherapy in myelodysplastic syndromes expressing P glycoprotein: results of a randomized study. Br J Haematol 1998;102:1015–24.

36. Liu Yin JA, Wheatley K, Rees JK, Burnett AK. UK MRC Adult Leukemia Working Party comparison of 'sequential' versus 'standard' chemotherapy as re-refractory/relapsed acute myeloid leukaemia (AML): results of the UK Medical Research Council AML-R trial. Br J Haematol 2001;113:713–26.

37. List AF, Kopecky KJ, Willman CL, et al. Benefit of cyclosporine modulation of drug resistance in patients with poor-risk acute myeloid leukemia: a Southwest Oncology Group study. Blood 2001;98:3212–20.

38. Baer MR, George SL, Dodge RK, et al. Phase 3 study of the multidrug resistance modulator PSC-833 in previously untreated patients 60 years of age and older with acute myeloid leukemia: Cancer and Leukemia Group B Study 9720. Blood 2002; 100:1224–32.

39. Plasschaert SL, van der Kolk DM, de Bont ES, et al. The role of breast cancer resistance protein in acute lymphoblastic leukemia. Clin Cancer Res 2003;9: 5171–7.

40. Rabindran SK, Ross DD, Doyle LA, Yang W, Greenberger LM. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. Cancer Res 2000;60: 47–50.

41. Suvannasankha A, Minderman H, O'Loughlin KL, et al. Breast cancer resistance protein (BCRP/MXR/ABCG2) in acute myeloid leukemia: discordance between expression and function. Clin Cancer Res 2004;18:1252–7.

42. Zhou DC, Simonin G, Faussat AM, Zittoun R, Marie JP. Effect of the multidrug inhibitor GG918 on drug sensitivity of human leukemic cells. Leukemia 1997;11: 1516–22.

43. Maliepaard M, van Gastelen MA, Tohgo A, et al. Circumvention of breast cancer resistance protein (BCRP)-mediated resistance to camptothecins *in vitro* using non-substrate drugs or the BCRP inhibitor GF120918. Clin Cancer Res 2001;7:935–41.

44. Robey RW, Honjo Y, Morisaki K, et al. Mutations at amino acid 482 in the ABCG2 gene affect substrate and antagonist specificity. Br J Cancer 2003;89: 1971–8.

45. Abbot BL. ABCG2 (BCRP) expression in normal and malignant hematopoietic cells. Hematol Oncol 2003;21:115–30.

46. Legrand O, Zompi S, Perrot JY, et al. High expression, but low functionality, of P-glycoprotein in both acute monocytic leukemia and acute myelo-monocytic leukemia. Haematologica 2004; 89:34–41.

47. van der Kolk DM, de Vries EG, Noordhoek L, et al. Activity and expression of the multidrug resistance proteins P-glycoprotein, MRP1, MRP2, MRP3 and MRP5 in *de novo* and relapsed acute myeloid leukemia. Leukemia 2001;15:1544–53.

48. Oselin K, Mrozikiewicz PM, Pahkla R, Roots I. Quantitative determination of the human MRP1 and MRP2 mRNA expression in FACS-sorted peripheral blood CD4+, CD8+, CD19+, and CD56+ cells. Eur J Haematol 2003;71:119–23.

49. Marie JP, Legrand O, Perrot JY, Chevillard S, Huet S, Robert J. Measuring multidrug resistance expression in human malignancies: elaboration of consensus recommendations. Semin Hematol 1997; 34:63–71.