The prognostic value of P-glycoprotein (ABCB) and breast cancer resistance protein (ABCG2) in adults with *de novo* acute myeloid leukemia with normal karyotype

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Multidrug resistance is a major cause of treatment failure in acute myeloid leukemia (AML). P-glycoprotein (PGP) over-expression has an unfavorable prognostic significance, while the role of breast cancer resistance protein (BCRP) is less clear, especially in AML patients with a normal karyotype. We studied 73 consecutive AML patients with a normal karyotype. BCRP was over-expressed in 24 patients (33%) and was significantly co-expressed with PGP (13/24 vs 11/49, p=0.006) and with CD56. Only PGP, along with age and CD34, affected the achievement of complete remission (p=0.02), while BCRP-positive cases showed an increased risk of relapse (p=0.005) and a shorter disease-free survival (p=0.027). BCRP over-expression did not influence the achievement of remission, but significantly affected the duration of complete remissions. BCRP may, therefore, be regarded as a prognostic factor in patients with normal karyotype AML, for the design of risk-adapted post-remission therapy.

Key words: PGP, BCRP, AML, normal karyotype, prognosis.

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Until now, the main obstacle to a successful cure of cancer has been the intrinsic or acquired resistance of the neoplastic cells to a variety of structurally and functionally heterogeneous anticancer agents, named multidrug resistance (MDR). In the past 10 years the molecular mechanisms responsible for this phenomenon have been intensively studied, particularly in leukemia patients. Among resistance factors, P-glycoprotein (PGP), the product of the *ABCB1* gene and part of a family of ATP-dependent membrane transporter proteins, has demonstrated a very high prognostic power. In fact, a negative correlation between PGP over-expression, remission rate and survival has been observed in different studies on patients with *de novo* or secondary acute myeloid leukemia (AML).[1-4] The prognostic role of PGP in leukemic patients is second only to that of cytogenetic abnormalities, and we have recently confirmed its negative impact also in patients with normal karyotype AML (*personal data, unpublished*). The *in vivo* role of the other proteins able to cause *in vitro* resistance is less clear. These proteins include multidrug resistance associated protein (MRP), a member of the same family of proteins as PGP and lung resistance-related protein (LRP), also known as *major vault protein*, which are involved in the physiological intracellular traffic of small molecules. Moreover, not all resistant cases can be explained by the presence of an increased membrane concentration of PGP. Functional studies have sometimes shown a discrepancy between the expression of MDR proteins and drug efflux, suggesting the presence of other drug transporters.[3] Recently a new ATP-binding cassette protein, the breast cancer resistance protein (BCRP), has been identified.[5] In cell line systems BCRP confers resistance to many different compounds and plays an important role in affecting drug disposition, although its effective role *in vivo* is much less well defined. In this study we compared the expression of BCRP and PGP in 73 consecutive cases of cytogenetically normal AML in an attempt to identify other prognostic factors potentially useful for designing risk-adapted therapy.

Design and Methods

Patients' characteristics and treatment and the definition of response

Seventy-three consecutive patients with a diagnosis of *de novo* AML with a normal karyotype admitted for therapy to the Division of Hematology of Udine University Hospital between 1997 and 2004 were included in this study. The diagnosis of AML was made from bone marrow smears according to the French-American-British (FAB) criteria. Normal and aberrant antigen expression of blast cells was evaluated by multiparametric flow cytometry using monoclonal antibodies to the following markers: CD34, CD38, CD33, CD13, CD14, CD68, CD117, HLA-DR, CD4, CD2, CD7, CD19, CD56 (BD, Milan Italy), Tdt and MPO (Dako, Milan, Italy). Cytogenetic analyses were carried out by a standard banding technique after incubation for 24-48 hours and metaphases were evaluated and named according to the International System for Human Cytogenetic Nomenclature.[6] A karyotype was considered normal if at least 20 metaphases without clonal aberrations were

seen. Patients with normal karyotype, but M3 morphology and/or molecular evidence of the *PML/RARA* rearrangement were excluded from the study. Forty-two patients were female, and the male/female ratio was 0.74. The median age was 53 years (range: 15-76) and 35 patients (48%) were older than 55 years. The median white cell count was 31×10^9/L and leukocytosis (defined as a white cell count $\geq30\times10^9$/L) was present in 37 cases (51%). The samples of AML, classified according to the FAB criteria, were M0 (n=7), M1 (n=17), M2 (n=17), M4 (n=13), and M5 (n=19). Patients were homogeneously treated with an induction regimen containing fludarabine, cytarabine and idarubicin, with or without etoposide (FLAI/FLAIE). A small number of patients (n=15) received induction therapy with ICE (idarubicin, cytarabine and etoposide) as they entered a randomized study comparing FLAI to ICE for induction treatment of AML. All patients received consolidation therapy with high-dose cytarabine and idarubicin. Remission status was determined after two courses of therapy, according to published criteria.[7] Resistant disease was defined by the presence of more than 5% of blast cells in the marrow or early death during induction (DDI) therapy or before hematologic recovery. Twenty-five patients considered at high risk of relapse (because of white cell count, MDR expression or resistance to first induction course) and with an identical donor underwent allogeneic stem cell transplantation (SCT).

PGP, MRP and LRP detection

Flow cytometric analysis of MDR-related proteins was performed on the mononuclear fraction after Ficoll sedimentation by an indirect staining technique using the MRK-16 antibody (for PGP), the MRPm1 antibody (for MRP1) and the LRP-56 antibody (for LRP), all from Kamiya Biochemicals (Seattle, WA, USA), according to our previously published protocol.[4] Only blast cells were considered in the analysis by gating out normal lymphocytes according to scatter parameters. In samples containing less than 90% of blast cells, the CD3-antibody was simultaneously incubated in each sample and only CD3 negative cells were analyzed to exclude all contaminating peripheral blood lymphocytes.

According to our previous report, results were expressed as the mean fluorescence index (MFI), i.e. the ratio between the mean fluorescence intensity of the labeled sample and that of the background fluorescence of their negative control. A MFI≥6 for PGP, ≥3 for MRP and ≥5 for LRP identified over-expressing cases. Cell lines overexpressing MDR proteins and their sensitive counterpart, as well as normal peripheral blood cells, were used as positive and negative references.[4] Only live cells (i.e. cells excluding propidium iodide added at 0.2% final concentration immediately before analysis) were considered.

BCRP evaluation

BCRP expression was tested on blast cells, obtained as above, by the BXP-34 antibody (Kamiya Biochemicals, Seattle, WA, USA), which binds to an internal epitope. In brief, $0.5\text{-}1\times10^6$ blast cells were incubated for 10 minutes at room temperature with FACS lysing solution (BD, Milan, Italy) to permeabilize the cell membrane, washed, and incubated with the primary unlabeled antibody at a final concentration of 0.5 μg/mL in a 0.02% saponin solution for 15 minutes. After two washes cells were stained with fluorescein-conjugated goat anti-mouse antibody for 15 minutes at room temperature, washed again and analyzed. Data are expressed as the MFI, calculated as above, considering as positive those cases with an MFI ≥5. The MCF-7 cell line and its BCPR overexpressing subclone (MCF-7 MX8),[8] as well as normal peripheral blood cells, were used as references.

Statistical analysis

Univariate and multivariate logistic regression analyses were used to evaluate the impact of each variable on complete remission. Survival curves were obtained by the Kaplan-Meier method and the different groups were compared using the log-rank test. Disease-free survival was defined as the interval from complete remission to relapse. Overall survival was defined as the interval from diagnosis to death, independently of the cause. The correlation between variables affecting survival was evaluated by multivariate Cox regression. p values <0.05 were considered statistically significant. Patients who underwent allogeneic SCT were censored at the time of their transplant.

Results and Discussion

BCRP was over-expressed in 24/73 (33%) patients. BCRP positive and negative patients did not differ for age, sex distribution, FAB subtype, white cell count at diagnosis or CD34 expression. However, the cases positive for BCRP showed a higher expression of CD56 antigen (11/24, 46%) compared to BCRP negative patients (10/47, 21%, χ^2=4.3, p=0.03). Similarly, PGP was more frequently over-expressed in patients with concomitant expression of BCRP (13/24, 54%) than in BCRP-negative cases (11/49, 22%, χ^2=7.3, p=0.006). LRP was over-expressed in 34 patients, without difference between BCRP-positive (12/34) and negative (22/49) cases. Nineteen cases were positive for MRP, of whom only two out of 24 (8%) of the BCRP-positive patients, compared to 17 out of 49 (35%) MRP-positive cases in the BCRP-negative subgroup (p=0.01).

MDR proteins and outcome

Complete remission was obtained in 55/73 (75%) patients. The impact of different clinical and biological parameters on the probability of achieving complete remission was evaluated by univariate and multivariate logistic regression, as shown in Table 1A. Only advanced age, high level of PGP and CD34 expression affected the remission rate in the univariate analysis. The first two factors also retained their statistical significance in the multivariate analysis, while $CD34^+$ showed a strong trend toward significance (p=0.06). In contrast, BCRP expression was not associated with the achievement of complete remission. However, the probability of relapse was significantly higher in patients with high BCRP expression: 14 out of 18 (78%) BCRP-positive patients relapsed, com-

Table 1A. Complete remission rate according to characteristics at diagnosis.

	Univariate logistic regression		*Multivariate logistic regression*	
	χ2	*p*	*χ2*	*p*
Age (>55)	9.56	0.001	4,42	0.05
WBC (>30×10⁹/L)	0.58	0.52	–	–
CD34⁺	4.95	0.02	3,8	0.06
CD56⁺	0.18	0.88	–	–
PGP+ (MFI ≥6)	7.93	0.006	4,51	0.02
MRP+ (MFI ≥3)	2.13	0.14	–	–
LRP+ (MFI ≥5)	0.03	0.85	–	–
BCRP+ (MFI ≥5)	1.27	0.25	–	–

Table 1B. Factors affecting overall survival.

	Univariate analysis		*Multivariate analysis*	
	χ2	*p*	*Z*	*p*
Complete remission	28.8	<0.0001	2.5	0.02
Age (>55)	10.4	0.001	–	–
WBC (>30×10⁹/L)	4.7	0.03	–	–
CD34⁺	2.7	0.09	–	–
CD56⁺	5.1	0.05	2.16	0.03
MDR proteins+*	4.1	0.04	1.98	0.05

**at least one among PGP, MRP, BCRP.*

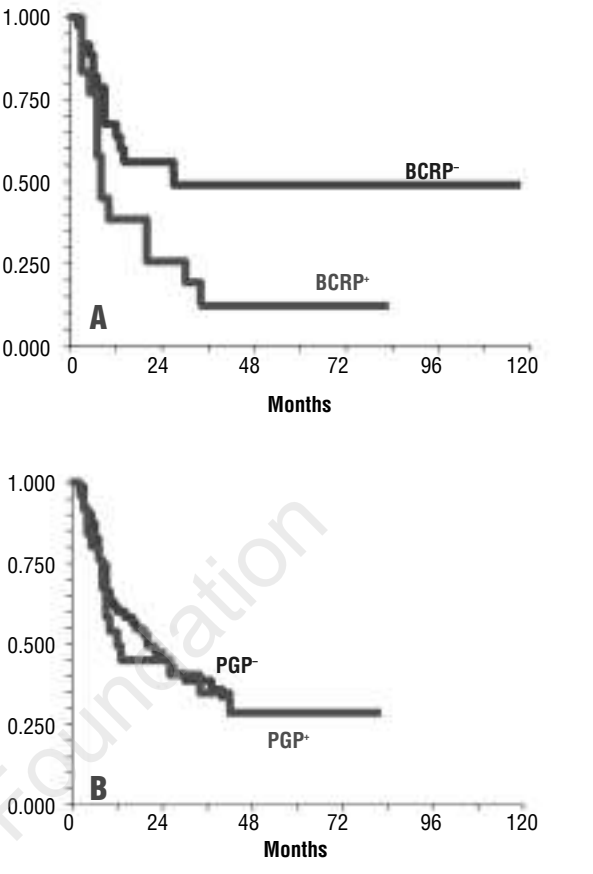

Figure 1. A. Disease-free survival according to BCRP expression. B. Disease-free survival according to PGP expression.

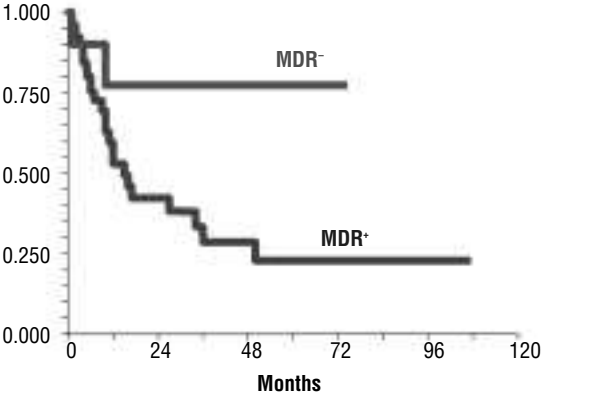

Figure 2. Overall survival according to the presence or absence of at least one MDR protein.

pared to only 14/37 (38%) in the BCRP-negative group (p=0.005). No other parameter was associated with an increased risk of relapse. A trend to a higher relapse rate was observed in the 13 double positive (PGP⁺/BCRP⁺) patients than in the 11 BCRP⁺/PGP⁻ cases (87% vs 66%), but this was not statistically significant due to the small number of cases. BCRP over-expression also affected disease-free survival: remission lasted a median of 8 months in BCRP-positive patients but 27 months in BCRP- negative patients (p=0.027) (Figure 1A). In contrast PGP expression, which is one of the strongest predictors of remission, did not influence disease-free survival (Figure 1B). Finally, a shorter survival was associated with response to induction therapy (complete remission or not, p<0.0001), older age (p=0.001), CD56 expression (p=0.05), high white cell count at diagnosis (p=0.03) and with the over-expression of at least one MDR-related protein (PGP, MRP or BCRP) (p=0.04) (Figure 2). In multivariate Cox regression analysis only achievement of complete remission (p=0.02), CD56 positivity (p=0.03) and expression of at least one MDR-associated protein (p=0.05) retained statistical significance (Table 1B).

We investigated BCRP expression in 73 patients with AML and a normal karyotype. Such patients are usually considered at intermediate risk of relapse but have a heterogeneous response to therapy. We found BCRP over-expression in 24 patients (33%). In previous papers the percentage of BCRP expression varied from 30 to 75%, but this expression was evaluated with different methods.[9,10] In our series we used the same method as that previously used for the detection of other MDR-related proteins.[4] This method allows good protein detection and easy identification of over-expressing cases.

Various studies have found a relatively higher expression of BCRP in cases with immature phenotype.[10,11] Moreover, one group described that BCRP was the only

protein expressed at a significantly higher level at relapse, also reporting a correlation between PGP and BCRP RNA levels at diagnosis.[12] In our patients BCRP was co-expressed in 54% of PGP-positive cases, without any association with FAB subtype, CD34 or MPO expression. Instead, we found a significant association between BCRP over-expression and aberrant expression of the CD56 antigen, previously reported as a negative independent prognostic factor in genetically heterogeneous acute leukemias.[13] Regarding the impact of BCRP expression on therapy outcome, in our cases BCRP expression did not influence achievement of complete remission, but did significantly influence remission duration. BCRP-positive patients had a significantly higher relapse rate compared to BCRP-negative cases. These data are only apparently in contrast with those of Benderra and co-workers, who found a strong association between BCRP expression and all the other parameters associated with outcome.[14] In fact, it should be highlighted that our cases represent a particular subset of patients with AML in whom the various prognostic factors may have different weights. Moreover, when the prognostic role of PGP and BCRP was analyzed according to the anthracycline used in the induction course, an association between BCRP, complete remission, disease-free survival and overall survival was evident only in patients receiving daunorubicin and mitoxantrone but not in those treated with idarubicin.[15] All the patients enrolled in our study received idarubicin during induction therapy. Nevertheless, the influence of BCRP on disease-free survival in our patients, in whom idarubicin was employed also during consolidation, suggests a potential role of this protein in the transport of this drug. On this basis, it can be hypothesized that the use of chemotherapeutic agents that are not substrates for the action of BCRP, such as gemtuzumab ozogamicin in CD33$^+$ cases, may be useful in this phase.

In conclusion, our data highlight the negative role of BCRP over-expression in patients with AML with normal karyotype, mainly because of an increased risk of relapse. BCRP may thus be regarded as an easily evaluable prognostic marker in a leukemia subgroup in which prognostic factors are still under debate. Recently, new molecular alterations, such as the *flt*-3 mutation,[16,17] the *CEPBA* mutation,[18] BAALC over-expression[19] or nucleophosmin gene mutation[20] are emerging as prognostic factors in acute leukemia. The study of the combined effect of MDR-related proteins and the different molecular features on disease outcome in larger series of AML patients with normal karyotype might define new prognostic markers in order to design risk-adapted therapy.

DD and MT: conception and design of the study, analysis of the data, drafting of the article; EC, AC, AM and MC: performed the experiments on MDR-related proteins and collected the data; MT, AG: collection of clinical data and clinical managment of the patients; DD and RF: critical revision of the manuscript and final approval of the version. The authors declare that they have no potential conflicts of interest. Manuscript received December 6, 2005. Accepted March 13, 2006.

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