

Semiquantitative RT-PCR evaluation of the *MDR1* gene expression in patients with acute myeloid leukemia

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Resistance to chemotherapy is one of the major obstacles to effective treatment in acute myeloid leukemia (AML). The most extensively studied protein involved in multidrug resistance (MDR) is the transmembrane glycoprotein P (P-gp), the product of the multidrug resistance gene 1 (*MDR1*). *MDR1*/P-gp overexpression is frequently observed in hematological malignancies, especially in acute leukemia, and has been reported to correlate with poor prognosis in acute myeloid leukemia (AML). The aim of this study was to evaluate the level of *MDR1* gene expression in bone marrow and/or peripheral blood samples in 92 AML patients in relation to their prognosis. The analyzed group was stratified according to presence or absence of prognostically favorable aberrations (PFAs), such as t(15;17) with *PML/RAR α* fusion gene, t(8;21) with *AML1/ETO* fusion gene or inv(16)/t(16;16) with *CBF β /MYH11* fusion gene. These prognostically favorable aberrations were detected by RT-PCR and/or standard cytogenetic techniques. *MDR1* expression was detected by semiquantitative comparative RT-PCR using software-based evaluation. The levels of *MDR1* expression in the bone marrow predicted induction of complete remission in the whole group of analyzed patients ($P = 0.032$). They were significantly lower in PFA negative patients who achieved complete remission compared to those who failed to achieve complete remission ($P = 0.008$). In PFA negative patients, *MDR1* expression was higher when compared to PFA positive patients ($P = 0.055$). No such difference was found when analyzing peripheral blood samples. Our experiments showed no impact of *MDR1* expression in bone marrow or peripheral blood cells on overall survival ($P = 1.000$ and $P = 0.903$ respectively). In summary, the present study shows the prognostic impact of *MDR1* expression on induction of complete remission in AML patients. We confirmed that *MDR1* overexpression is an unfavorable prognostic factor in AML, which may help to stratify the risk rate of PFA negative patients. In future studies, quantitative detection of *MDR1* expression might be a valuable tool to predict prognosis in this patient subset.

Key words: AML, *MDR1* gene, semiquantitative RT-PCR, cytogenetics, prognosis

Although the majority of patients with acute myeloid leukemia (AML) achieve complete hematological remission with conventional induction chemotherapy, only few patients are long term survivors and overall prognosis remains poor [1, 2]. Resistance to chemotherapy is one of the major obstacles to effective treatment in AML [3, 4]. One of the best characterized resistance mechanisms in AML is drug extrusion mediated by P-glycoprotein (product of *MDR1* gene), a 170 kDa member of ABC superfamily of transport proteins [5, 6]. P-gp is able to pump many xenobiotics out of the cell and contributes to resistance to a variety of anticancer drugs, including anthracyclines, mitoxantrone, taxanes, epipodophyllotoxins and vinca alkaloids [7, 8]. *MDR1* gene expression is an independent

unfavorable prognostic factor in AML and has been shown to be associated with poor treatment outcomes in AML patients, especially in elderly patients [9-15]. Whereas most studies revealed a significant impact of *MDR1*/P-gp expression on complete remission rates, its prognostic value for survival is still a matter of debate [16-19]. Many clinical studies dealing with *MDR1* expression have come up with discrepant results mainly due to using disparate methodologies [7, 8, 17, 18-20]. The consensual recommendations for *MDR1*/P-gp detection have been published by Marie et al. [4, 21]. As *MDR1*/P-gp is physiologically expressed in some defined subsets of blood cells, mainly in CD34+ cells [22-25], the ratio of *MDR1* and an internal control should be compared to a *MDR1* positive reference sample for proper estimation of *MDR1* expression [20, 21]. In PCR analysis, β -actin should not be used as an internal control

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gene as it is too variable [21]. It has been shown that *MDR1* expression is variable depending on FAB subtypes. Patients with low risk cytogenetics with prognostically favorable aberrations (PFAs), such as *PML/RAR α* , *AML1/ETO* or *CBF β /MYH11*, which result from t(15;17), t(8;21) and inv(16)/t(16;16) translocations, respectively, have been reported to have relatively low expression of P-gp compared to the other cytogenetically-defined risk groups of AML [4, 26]. Only in the case of promyelocytic leukemia, the low levels of P-gp and also low activity of this protein have been demonstrated [27]. This may provide the biological basis for the high sensitivity of this leukemia subtype to anthracyclines [27, 28]. AML with rearrangement of the core binding factor (CBF) α or β subunit gene (i.e., AML with t(8;21) with *AML1/ETO* fusion transcript or inv(16)/t(16;16) with *CBF β /MYH11* fusion transcript) have overall a favorable prognosis but relapses frequently occur even in these patients [29, 30]. *MDR1*/P-gp overexpression and hyperfunction were frequent in both subtypes of CBF-AML. However, treatment failures in *AML1/ETO*+ AML were associated with CD56 expression and they were not likely attributable to *MDR1*/P-gp coexpression [31].

In this report, an alternative method of *MDR1* gene quantitation is presented. A simple semiquantitative comparative RT-PCR evaluated by KODAK 1D software was employed in a cohort of 92 patients with AML, stratified according to presence or absence of prognostically favorable aberrations. We correlated the impact of the detected levels of *MDR1* with treatment outcome of these patients in terms of achieving complete remission (CR) induction and overall survival (OS). The results presented herein show that the level of *MDR1*/P-gp expression is predictive for further prognosis of AML patients, especially of those without prognostically favorable aberrations.

Patients and methods

Patients studied. A cohort of 92 pediatric and adult patients at diagnosis of AML according to WHO criteria [32] was prospectively studied; each patient had given a written informed consent to the study according to institutional guidelines. The majority of the patients was treated at the Institute of Hematology and Blood Transfusion (IHBT), Prague; the others came from 15 different hematology centers in the Czech Republic and Slovakia. The cohort comprised of 47 males and 45 females aged 0.45 – 78 (median 43.8) years. According to FAB classification of AML [33], 8 patients were diagnosed as M0, 10 as M1, 19 as M2, 21 as M3, 15 as M4, 6 as M5, 1 as M7, 2 were RAEB-T, 1 was classified as biphenotypic acute leukemia and 9 were not classified according to FAB classification. The patients were treated according to various protocols. The cooperating centers supplied data concerning complete remission induction and OS.

Cell separation, RNA extraction and reverse transcription (RT). Samples of 92 patients (24 from both the bone marrow and peripheral blood, 32 from peripheral blood only and 36 from the bone marrow only) were tested. RNA was isolated from Ficoll-Paque separated mononuclears using acid guanidium thiocyanate-phenol-chloroform extraction [34]. Reverse transcription was performed using random hexamers, the RT-mixture contained 1.75 μ l of deionized water, 2 μ l of 5x 1st Strand Buffer (Gibco BRL Life Technologies, Gaithersburg, MD, USA), 1 μ l of 100 mM dithiothreitol (Gibco BRL), 0.5 μ l of 10 mM dNTP (Promega, Madison, WI, USA), 0.25 μ l of RNasin (40 U/ μ l; Promega) and 0.5 μ l of RT Super Script II (200 U/ μ l; Gibco BRL).

Semiquantitative comparative RT-PCR detection of *MDR1* gene expression. PCR was performed with *MDR1* and *BCR* (breakpoint cluster region gene; an internal control gene) prim-

Table 1. Sequences of primers

Gene	Primer code*	Length (bp)	Primer sequence
<i>PML/RARα</i>	M2**	20	AgT gTA CgC CTT CTC CAT CA
	M4**	30	AgC TgC Tgg Agg CTg Tgg ACg CgC ggT ACC
	R8	25	CAG AAC TgC TgC TCT ggg TCT CAA T
<i>CBFβ/MYH11</i>	pr1	21	CAG gCA Agg TAT ATT TgA Agg
	2M	23	CTC CTC TTC TCC TCA TTC TgC TC
<i>AML1/ETO</i>	AM	19	ACC TCA ggT TTg TCg gTC g
	ET	24	gAA CTg gTT CTT ggA gCT CCT
<i>ABL</i> (control gene)	ABL-S	23	ggA CAG gCC CAT ggT ACC Agg Ag
	ABL-AS	20	CTC AgC CAG TAg CAT CTg AC
<i>MDR1</i>	MDR-S	21	TAC AgTggA ATT ggT gCT ggg
	MDR-AS	20	CCC AgT gAA AAA TgT TgC CA
<i>BCR</i> (control gene)	BCR-S	18	gAg Aag Agg gCg AAC AAg
	BCR-AS	20	CTC TgC TTA AAT CCA gTg gC

*For each gene (except for *PML/RAR α*), one forward (upper line) and one reverse (lower line) primer was used [35, 36, 39, 40].

**For *PML/RAR α* detection, 2 forward primers were used, M2 detecting transcript isoforms with bcr1 and bcr2 breakpoints of the *PML* gene, whereas M4 detected transcripts with the bcr3 breakpoint. R8 is the common reverse primer in both RT-PCR assays which employ the M2 and M4 forward primer, respectively [38].

ers in one test tube for each patient's sample. The master PCR mix for one reaction contained 25 μ l total volume (24 μ l of reagents and 1 μ l of sample cDNA). The PCR cycler Trio Thermoblock (Biometra, Goettingen, Germany) was used for DNA amplification. The sequences of primers for *MDR1* and *BCR* [35, 36] and amplification programs employed are shown in detail in Tables 1 and 2. The drug-sensitive K-562 cell line and its resistant subline with the *MDR1* gene overexpression (kindly provided by Dr. J. Jelínek, IHBT Prague, Czechia) were used for PCR reaction adjustment and as negative and positive controls. 25 μ l of each PCR product was evaluated by 2% agarose gel electrophoresis stained with ethidium bromide and then analyzed by gel electrophoresis on a Power PAC 300 (Biorad, CA, USA) and visualized on Mini-Transluminator (Biorad). The Kodak Digital Science™ 1D Image Analysing Software and Electrophoresis Documentation and Analysis System (Kodak EDAS; Eastman Kodak, Scientific Imaging Systems, NY, USA) was used for the semiquantitative detection and calculation of *MDR1/BCR* expression. A digital photograph of ethidium bromide stained agarose gel containing DNA mass standards (100bp DNA Ladder [Promega]) was taken by Kodak Digital Science™ DC120 Zoom Digital Camera (Eastman Kodak) and then evaluated by Kodak EDAS. PCR product bands of *MDR1* and *BCR* genes were software-evaluated, compared to a DNA mass standard and subsequently quantified using isomolecular weight lines and special mathematic functions [37]. The results are given as "relative *MDR1* expression", i.e. as a ratio of *MDR1* and *BCR* expressions.

Molecular detection of the AML fusion genes. In all patients, RT-PCR was used to detect presence of the prognostically favorable aberrations, i.e. *PML/RAR α* [38], *AML1/ETO* [39] or *CBF β /MYH11* [40] fusion transcripts. All of them are regarded as prognostically favorable aberrations. cDNA resulting from RT (see above) was used for single-step PCR detecting the respective genes of interest, using the *ABL* gene as quality control. The total volume of PCR master mix for one reaction was 25 μ l (24 μ l of reagents and 1 μ l of sample cDNA). The PCR cycler Trio Thermoblock (Biometra) was used for DNA amplification. The sequences of primers and amplification programs employed are shown in detail in Tables 1 and 2. PCR products were evaluated by gel electrophoresis as described above.

Cytogenetic studies. 77 bone marrow samples were analyzed by standard cytogenetic techniques (G-banding). In 7 cases, no mitoses were obtained. Fluorescence *in situ* hybridization (FISH) was employed in addition to verify RT-PCR results in 17 cases. The t(8;21) fusion was detected by the whole chromosomal painting (WCP) probe (Cambio, Cambridge, UK); the two-color labelled translocation probe LSI (Abott Vysis, Downers Grove, IL) was used for detection of t(15;17) and inv(16)/t(16;16) fusions. Prognostic relevance of the cytogenetic aberrations was evaluated according to Grimwade et al. [41] within PFA negative patients. It allowed us to discriminate between intermediate and unfavorable cytogenetic risk groups.

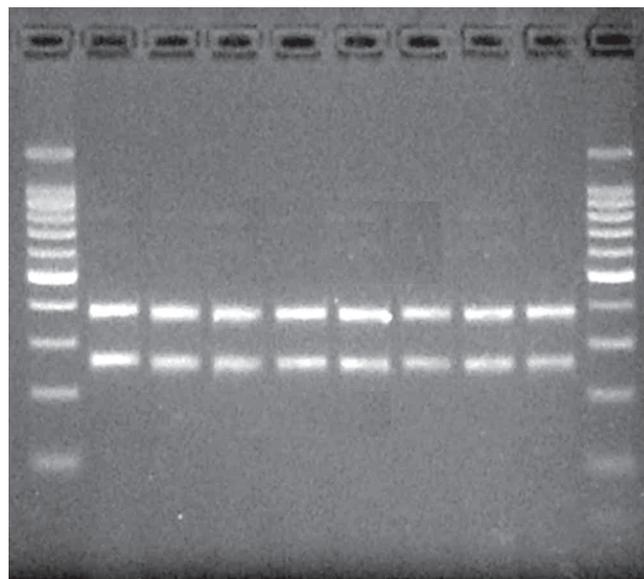


Figure 1. A digital photograph of gel electrophoresis taken by Kodak Digital Science DC120 Zoom Digital Camera. Samples of 8 randomly chosen patients tested (columns 1-8). The upper row of bands represents the control gene *BCR* (the breakpoint cluster gene) used as an internal standard; the lower one represents *MDR1* gene expression. A DNA mass standard (100 bp DNA Ladder) is depicted at both sides of gel to enable the evaluation by Kodak EDAS software.

Statistical analysis. For analysis of quantitative data, medians were detected and a non-parametric two-tailed Mann-Whitney test was performed. Overall survival was analyzed using the Kaplan-Mayer regression method and the statistical significance was calculated using the Mantel-Haenschel log-rank test. All analyses were performed at the 95% confidence interval and the P values were found using the GraphPad Prism version 3.03 software (GraphPad Software, San Diego, CA, USA).

Results

Detection of prognostically favorable aberrations. Of 92 patients tested, 25 patients carried a prognostically favorable

Table 2. Amplification programs

Temperature (°C)	Time	Number of cycles			
		<i>PML/RARα</i>	<i>ABL</i>	<i>CBFβ/MYH11</i> and <i>AML1/ETO</i>	<i>MDR1/BCR</i>
95	9 min			Initiation	
95	45 s				
57	45 s	40	33	40	28
72	1 min				
72	9 min			Final extension	
4				Cooling	

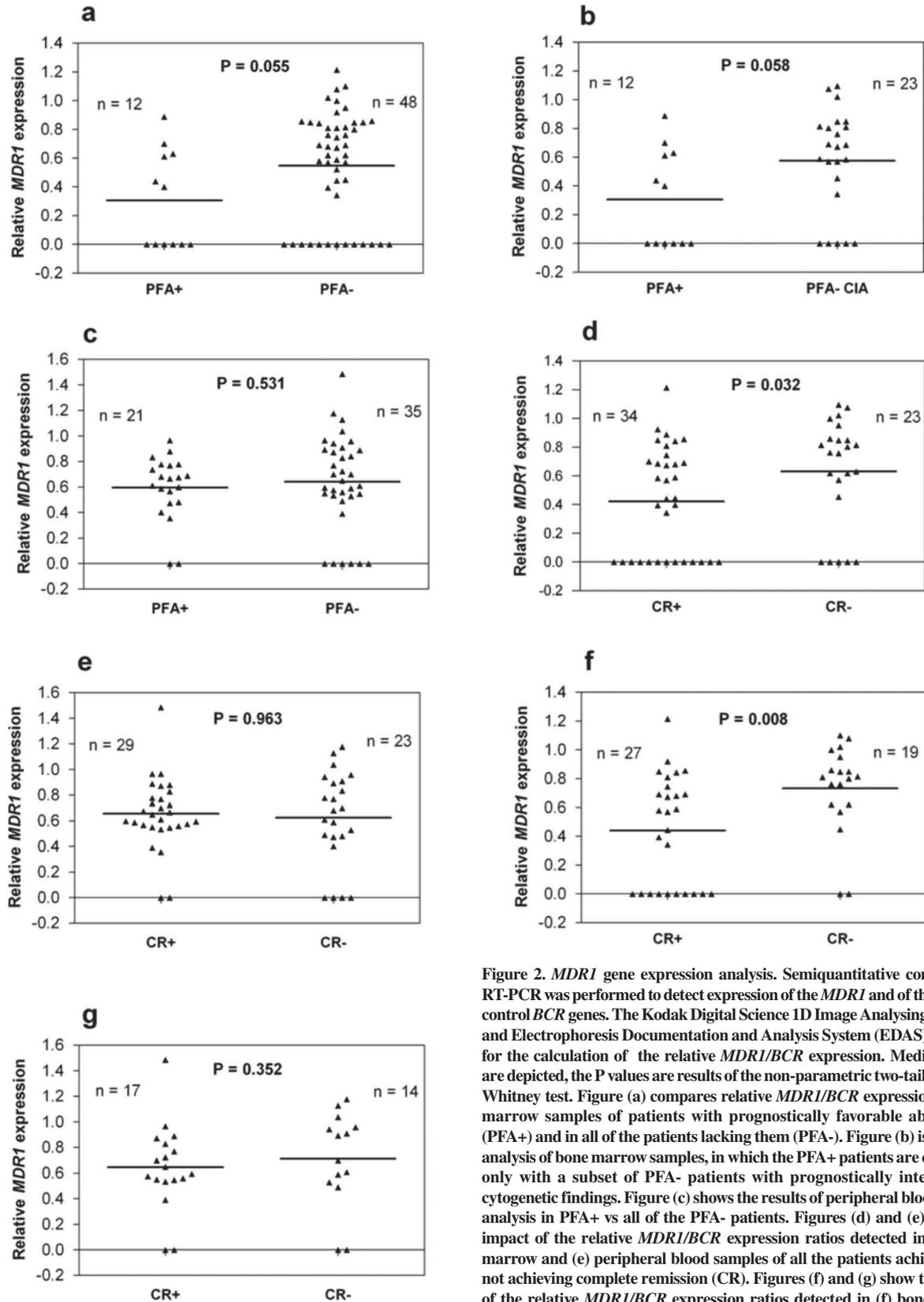


Figure 2. *MDR1* gene expression analysis. Semiquantitative comparative RT-PCR was performed to detect expression of the *MDR1* and of the internal control *BCR* genes. The Kodak Digital Science 1D Image Analysing Software and Electrophoresis Documentation and Analysis System (EDAS) was used for the calculation of the relative *MDR1/BCR* expression. Median values are depicted, the P values are results of the non-parametric two-tailed Mann-Whitney test. Figure (a) compares relative *MDR1/BCR* expression in bone marrow samples of patients with prognostically favorable aberrations (PFA+) and in all of the patients lacking them (PFA-). Figure (b) is a similar analysis of bone marrow samples, in which the PFA+ patients are compared only with a subset of PFA- patients with prognostically intermediate cytogenetic findings. Figure (c) shows the results of peripheral blood sample analysis in PFA+ vs all of the PFA- patients. Figures (d) and (e) show the impact of the relative *MDR1/BCR* expression ratios detected in (d) bone marrow and (e) peripheral blood samples of all the patients achieving and not achieving complete remission (CR). Figures (f) and (g) show the impact of the relative *MDR1/BCR* expression ratios detected in (f) bone marrow and (g) peripheral blood samples of PFA- patients achieving and not achieving complete remission (CR).

aberration. The majority of them – 21 patients out of 92 tested – were *PML/RAR α* positive in RT-PCR. Seven of them carried the *bcr1* transcript subtype, 2 of them *bcr2*, 9 of them *bcr3* and 3 were not tested for the *PML/RAR α* transcript subtype. Of 92 patients, 88 were tested for the presence of *AML1/ETO* and *CBF β /MYH11* fusion genes using RT-PCR. In one patient, the *AML1/ETO* gene fusion was detected and 3 patients were *CBF β /MYH11* positive. Of 79 patients with available molecular and/or cytogenetic and FISH data to assign prognosis according to Grimwade et al. [41], 33 were classified as prognostically intermediate and 19 as prognostically unfavorable.

MDR1 gene expression in bone marrow and peripheral blood samples. The results of *MDR1* analysis were expressed as “relative *MDR1* expression” (see above), calculated by Kodak EDAS Software. An example of gel electrophoresis as taken by the Kodak EDAS camera (which was subsequently evaluated by the Kodak EDAS Software) is given in Figure 1. Due to differences in *MDR1* physiological expression in peripheral blood and bone marrow, both groups were analyzed separately. 60 bone marrow samples were tested. Their mean value of *MDR1* expression was 0.50 (median 0.60, range 0 – 1.21). 56 peripheral blood samples were tested. The mean value of their *MDR1* expression was 0.62 (median 0.66, range 0 – 1.49).

MDR1 expression analysis. The relative *MDR1* expression (i.e. the ratio of *MDR1* and control *BCR* gene expressions) in the bone marrow was lower in patients with prognostically favorable aberrations when compared to all of the patients lacking these aberrations ($P = 0.055$; Figure 2a) and also when compared to a subset of patients lacking the prognostically favorable aberrations but having prognostically intermediate cytogenetics ($P = 0.058$; Figure 2b). When the same comparison of *MDR1* expression was performed in peripheral blood samples of patients with a prognostically favorable aberration vs in all of the patients lacking them, no significant difference was revealed ($P = 0.531$; Figure 2c). The only *AML1/ETO* positive case and all 3 *CBF β /MYH11* positive cases showed increased expression of *MDR1* gene above average (data not shown). Of 83 patients with available clinical data, 45 achieved CR. The level of *MDR1* expression (i.e. the ratio of *MDR1* and control *BCR* gene expressions) in the bone marrow significantly influenced the induction of complete remission in all AML patients tested ($P = 0.032$; Figure 2d). No difference in *MDR1* expression was found when analyzing the peripheral blood samples of AML patients who achieved complete remission and who did not ($P = 0.963$; Figure 2e). In the subgroup of patients without prognostically favorable aberrations, *MDR1* expression in the bone marrow was significantly lower in patients who achieved complete remission compared to those who did not ($P = 0.008$; Figure 2f). In the same group of patients, *MDR1* expression in peripheral blood was not significantly different when patients achieving and failing to achieve complete remission were compared ($P = 0.352$; Figure 2g).

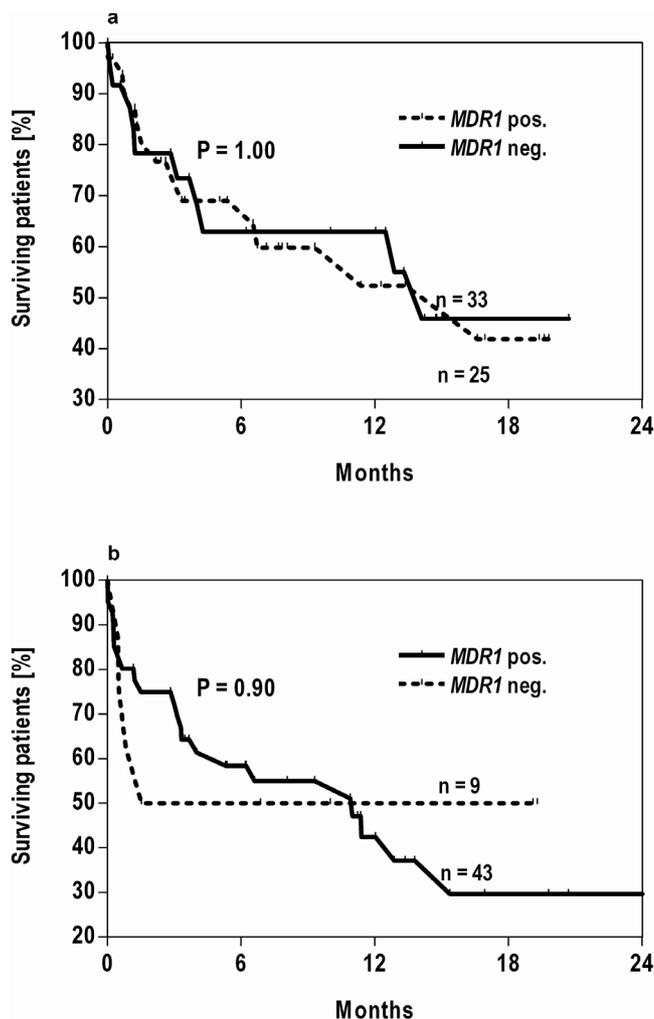


Figure 3. The impact of *MDR1* gene expression in the bone marrow (a) and in peripheral blood (b) cells on overall survival. The cut-off value of the *MDR1/BCR* ratio for discriminating *MDR1* positive and *MDR1* negative patients was set at ≥ 0.5 . Survival curves were analyzed according to the Kaplan-Meier regression method and the P values given are results of the Mantel-Haenschel log-rank test.

Overall survival and MDR1 expression. By 31st December 2000, overall survival data were available for 58 patients and/or for 52 patients whose bone marrow and/or peripheral blood samples, respectively, were analyzed for *MDR1* expression. Of these patients, 34 and 24, respectively, were still alive. When evaluating overall survival in all AML patients, we have not revealed any impact of *MDR1* expression. This applies for bone marrow ($P = 1.000$; Figure 3a), as well as for peripheral blood sample analysis ($P = 0.903$; Figure 3b). No correlation has been found between *MDR1* expression and overall survival when analyzing bone marrow or peripheral blood samples of patients lacking prognostically favorable aberrations (data not shown).

Discussion

The present study has demonstrated that semiquantitative comparative detection of *MDR1* expression is fully efficient for prognostic evaluations. The methodology presented herein adds to the already wide spectrum of methods that can be used for this purpose [7, 8, 16, 18–20]. The semiquantitative comparative method can prove to be of value especially in laboratories not equipped by real-time cyclers enabling fully quantitative estimations. Our results confirm the prognostic impact of *MDR1/P-gp* expression with regard to induction of complete remission in patients with AML. In agreement with hitherto published information about multidrug resistance, it can be concluded that in patients with low level of *MDR1* expression at diagnosis, induction of complete remission is easier as their cells better respond to chemotherapy [9, 10, 12]. Also the results concerning overall survival (no significant impact of *MDR1* expression) are consistent with some of the previously published data [4, 16, 17]. We confirmed that patients with a prognostically favorable aberration have on average lower *MDR1* expression and a more favorable treatment outcome. The vast majority of the PFA+ patients in our cohort had acute promyelocytic leukemia with the *PML/RAR α* fusion gene. These patients are known to express only very low levels of P-gp and respond well to anthracycline chemotherapy [27]. Quite surprisingly, the other patients with a prognostically favorable aberrations, i.e. the four patients with CBF-AML, showed *MDR1* hyperexpression. Nevertheless, 3 out of 4 of them achieved CR. This observation may be in accord with the recent publication [31] showing that in some CBF-AML patients, P-gp overexpression need not necessarily be linked with inverse prognosis. The relative scarcity of CBF-AML cases in our group of patients (4 of 92; 4.3%) probably reflects the situation in relatively unselected patients. We have previously found only 12 *CBF β /MYH11+* cases among 256 Czech patients analyzed (4.5%), which is nearly 3 times less than in the British AML studies. The higher percentage in the clinical trial may have represented patient selection [44]. Similarly low percentages of *AML1/ETO+* cases (approximately 4%) are found among unselected Czech patients (unpublished data).

P-gp expression and cytogenetic aberrations have been reported to be the only independent predictors of treatment outcome in multivariate analysis [9–15, 26]. Within the major group of AML patients with a prognostically intermediate cytogenetic result, prognosis may be highly variable. In this subset of patients, prognostic stratification based on *MDR1* expression might be of value. Indeed, the results of this study confirmed that our method of *MDR1* evaluation allowed us to further stratify the risk rate of these patients lacking prognostically favorable aberrations according to their level of *MDR1* expression. Besides *MDR1/P-gp* expression, there are many other molecular markers which may be helpful in refining prognosis of the intermediate-risk AML patients. In the recent years, internal tandem duplications of the *FLT3*

gene, partial tandem duplications of the *MLL* gene, *CEBP- α* gene mutations or the *BAALC* gene overexpression were found to subclassify prognosis in patients with normal karyotypes [42]. Other genes involved in MDR, such as those encoding lung resistance protein (LRP), breast cancer resistance protein (BCRP), multidrug resistance associated protein-1 (MRP1) and Wilms tumor gene (*WT1*) can serve as independent predictors of treatment outcome in patients with AML. Co-expression of these genes and *MDR1* has also been published [7, 11, 17, 18, 43].

As mentioned above in the “Results” section, only the levels of *MDR1* in the bone marrow, but not in peripheral blood, showed prognostic impact on induction of CR. This fact can be explained, at least in part, by higher levels of *MDR1/P-gp* basal expression in peripheral blood cells compared to the bone marrow cells, probably due to the presence of some well-defined subsets of normal peripheral blood cells physiologically expressing relatively high amounts of *MDR1/P-gp* (e.g. lymphocytes and NK cells) [22]. This may be the reason why many investigators use to study *MDR1/P-gp* expression only in isolated CD34+ progenitor cells. Alternatively, it has also been suggested to remove the “*MDR1* naturally positive cells” before evaluating *MDR1/P-gp* expression [22–25]. Controversion exists as to *MDR1/P-gp* expression in relapse of AML. Some authors [45] have demonstrated increased P-gp expression in relapsed cases. On the other hand, previous studies did not demonstrate increased *MDR1* RNA levels in peripheral blasts of relapsed AML patients [46].

Taken together, our results confirmed that semiquantitative detection of *MDR1* gene expression may be used to predict prognosis, which may be an advantage especially in patients without prognostically favorable aberrations. We envision that fully quantitative methods of evaluating *MDR1* expression will be preferentially used for this purpose.

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