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

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ABSTRACT

Purpose: The family of multidrug resistance-associated proteins (MRPs) belongs to the ATP-binding cassette superfamily of transporters, which have the ability to function as outward pumps for chemotherapeutic drugs. Their structure, function, and substrate specificity have been studied intensively, but little is known about their clinical relevance in malignant diseases.

Experimental Design: In this study, the expression of the MRP2, MRP3, MRP4, MRP5, and SMRP genes was measured using TaqMan real-time PCR in 53 children with de novo acute myeloid leukemia. Nine patients were also analyzed in relapse.

Results: MRP3 gene expression was higher in patients who did not achieve remission (P = 0.023). Expression of MRP2 (P = 0.09) or MRP3 (P = 0.041) was associated with a lower rate of survival, and patients who expressed high levels of both genes had a particularly poor prognosis (P < 0.01). No significant association was found for overall survival or remission rate and the expression of MRP4, MRP5, and SMRP.

Conclusions: This study provides first data on the clinical relevance of five MRPs in acute myeloid leukemia patients. The results strongly suggest that MRP3 and possibly also MRP2 are involved in drug resistance in this disease. Those two proteins therefore represent interesting markers for risk-adapted therapy and possible targets for the development of specific drugs to overcome multidrug resistance.

INTRODUCTION

A major issue in the treatment of AML is resistance to chemotherapeutic drugs. Many patients fail to respond to chemotherapy, and others relapse with resistant disease. Even with aggressive therapy, the survival rate in children with AML is only ~50% (1, 2).

Several mechanisms of drug resistance have been identified. One of these is the overexpression of ATP-dependent membrane proteins that function as drug efflux pumps. The best characterized drug efflux pump is the P-gp, which is encoded by the MDR1. The expression of P-gp/MDR1 has been identified as an independent adverse prognostic factor for complete remission and survival in adult patients with AML (3, 4). Recent studies suggested that the results in the treatment of AML can be improved by combining chemotherapy with drugs that inhibit the function of P-gp (5, 6). However, the clinical relevance of P-gp seems to be much smaller in childhood than in adult AML (7, 8), and some adult patients too show multidrug resistance in the absence of P-gp expression.

The family of MRPs also belongs to the ATP-binding cassette superfamily of transporters (9). Their structure, function, and substrate specificity have been studied intensively (10, 11), but little is known about their clinical relevance. Thus far, only MRP activity and the expression of MRP1 have been studied in larger groups of AML patients. It was found that MRP activity (12, 13), but not MRP1 expression (14–16), is associated with a poor response to chemotherapy. Therefore, it seems possible that other members of the MRP family contribute to MRP activity and cause drug resistance in AML.

Van der Kolk et al. (17) studied the expression of MRP1, MRP2, MRP3, and MRP5 in 30 paired samples of de novo and relapsed AML in adult patients. They found that none of the genes was consistently elevated at relapse.

The aims of our study were to find out whether the more recently discovered members of the MRP family (MRP2, MRP3, MRP4, MRP5, and SMRP) are expressed in childhood AML and whether they are associated with a poor response to chemotherapy.

PATIENTS AND METHODS

Patients and Therapy. All 53 patients were diagnosed with AML untreated previously. The main patient characteristics are summarized in Table 1. The patients were treated...
Expression of MRP Genes in Childhood AML

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Measurable amounts of MRP2, MRP4, MRP5, and SMRP were found in all patients. MRPs3 and MRP5 were not detectable in 8 patients. The variation from the 10th percentile to the 90th percentile was 5-fold for MRP2, 8-fold for MRP4, 10-fold for MRP5, and 13-fold for SMRP.

The final concentration of the primers was 900 nM (300 nM for MRP3 and SMRP); the final concentration of the TaqMan probes was 300 nM (200 nM for MRP3 and SMRP). All TaqMan probes were labeled with FAM and TAMRA.

The expression of the resistance genes was standardized for the expression of β-2-microglobulin, which was measured using Pre-Developed Assay Reagents (Applied Biosystems).

The final volume for each PCR was 30 μl, including 1.5 μl of the investigated sample. Universal PCR Master Mix (Applied Biosystems) was used according to the manufacturer’s instructions.

Serial dilutions of cDNA of reference cell lines were used to generate standard curves. The reference cell lines were: MCF7/CH1000 (MRP2 and MRP3) and K562 (MRP4, MRP5, and SMRP). The expression of each gene in each sample was analyzed in duplicate. The regression coefficients of the standard curves ranged between 0.995 and 0.998.

Statistical Methods. Kaplan-Mayer statistics and Log-rank tests were calculated to estimate the significance of differences between survival curves. To prevent a bias because of the different outcomes in the four studies, all tests were recalculated with the number of the study as a stratification variable. For all genes, we used the median as cutoff between high and low expression. The Mann-Whitney test, the Kruskal-Wallis test, and the Spearman’s correlation coefficient were used to investigate the association between gene expression and other findings. All Ps are given for two-sided tests.

RESULTS

Expression of MRPs in Childhood AML. Measurable amounts of MRP2, MRP4, MRP5, and SMRP were found in all patients. MRPs3 and MRP5 were not detectable in 8 patients. The variation from the 10th percentile to the 90th percentile was 5-fold for MRP2, 8-fold for MRP4, 10-fold for MRP5, and 13-fold for SMRP.

Table 1 Initial patient data and distribution of FAB subtypes

<table>
<thead>
<tr>
<th>FAB type (number of patients)</th>
<th>No. of patients</th>
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<tbody>
<tr>
<td>M1</td>
<td>10</td>
</tr>
<tr>
<td>M2</td>
<td>9</td>
</tr>
<tr>
<td>M3</td>
<td>14</td>
</tr>
<tr>
<td>M4</td>
<td>16</td>
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<td>M5</td>
<td>2</td>
</tr>
<tr>
<td>M6</td>
<td>2</td>
</tr>
<tr>
<td>M7</td>
<td></td>
</tr>
<tr>
<td>n.a.</td>
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a n.a. = not available.

Peripheral blood was only used to analyze MRP expression in patients with >60% of leukemic cells before and >80% after Ficoll-Hypaque density gradient centrifugation.

according to four multicenter studies in Germany: (a) AML-I/82 (13 patients; Ref. 18); (b) AML-II/87 (17 patients; Ref. 18); (c) AML-BFM-93 (11 patients; Ref. 1); and (d) AML-BFM-98 (12 patients). All studies included induction therapies with cytosine-arabinoside, anthracycline, and thioguanine or etoposide, followed by bone marrow transplantation or intensive consolidation protocols with prophylactic central nervous system irradiation and maintenance therapy. Most (68%) of the patients (n = 36) achieved remission, and the overall survival was 38% after 5 years.

Sample Collection and Processing. Before the beginning of chemotherapy, leukemic cells were isolated from bone marrow or peripheral blood by Ficoll-Hypaque density gradient centrifugation. After this procedure, the percentage of leukemic cells was >80% in all patient samples as determined by May-Gruenwald-Giems-stained smears.

All samples were cryopreserved in liquid nitrogen. Total RNA was isolated using RNeasy Mini Kit, including DNase digestion (Qiagen, Hilden, Germany). The amount of RNA was measured by photometry, and a stock solution of 1 μg of RNA in 40 μl was prepared. RNA was transcribed into cDNA using Omniscript (Qiagen).

Quantitative Real-time PCR. Quantitative PCR was performed using the ABI Prism 7700 Sequence Detector (Applied Biosystems, Weiterstadt, Germany). Primers and TaqMan probes for MRP2, MRP5, and SMRP were used as described previously (19). Primers and TaqMan probes for MRP3 and MRP4 were:

- MRP3: Forward 5'-GCACCATTTGCTGCTGCTACA-3'; Reverse 5'-CGACGAGACCCCG GACCATT-3'; TaqMan probe 5'-CATCTCCCACCCACCTGTCACACT-3'
- MRP4: Forward 5'-TGATATGTCGGCTTTGAAAACC-3'; Reverse 5'-AGCCCAAATGAG CTGCAA-3'; TaqMan probe 5'-CGTACGGCTATGCCACGGTGCTA-3'

The expression of the resistance genes was standardized for the expression of β-2-microglobulin, which was measured using Pre-Developed Assay Reagents (Applied Biosystems).

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SMRP. The variation from the lowest measurable value for MRP3 to the 90th percentile was 27-fold.

The strongest correlation was found between the expression of MRP5 and SMRP, which is a splicing variant of MRP5 and encoded by the same gene (Ref. 19; Spearman’s correlation coefficient = 0.87, P < 0.001). A significant correlation was also found between all other members of the MRP family but with smaller Spearman’s correlation coefficients (range from 0.27 to 0.53). None of the MRP genes was associated with the expression of the Breast Cancer Resistance Protein or MDR1, which were analyzed previously in the same group of patients (6, 20). All genes were investigated for their association with sex, age, FAB type, initial WBC, presence of Auer rods, and the chromosomal aberrations t(8;21), t(9;11), and inv (16). Higher levels for MRP5 and SMRP were found in patients with high initial WBC (Spearman’s correlation coefficients: 0.34 and 0.29; P: 0.015 and 0.041). The expression of MRP3 was higher in patients with the FAB types M4 and M5 (Kruskal-Wallis test: P = 0.002). All other correlations were not significant.

**MRP Expression and Remission.** The median expression of MRP3 was more than two times higher in patients who did not achieve remission (P = 0.023; Fig. 1). This finding was consistent throughout all four studies. MRP2, MRP4, MRP5, and SMRP were not significantly associated with a failure to achieve remission.

**MRP Expression and Survival.** As shown in Fig. 2, patients with high levels of MRP2 or MRP3 had a lower rate of survival. The Ps were 0.02 for MRP2 and 0.034 for MRP3. When using the number of the study as a stratification variable, the Ps were 0.09 for MRP2 and 0.041 for MRP3. Patients who expressed high levels of both genes (MRP2 and MRP3) had an even lower rate of survival than patients who expressed high levels for only one of the two genes (Fig. 2; P < 0.01 with or without stratification). The prognostic impact of MRP3 remained statistically significant when relapse-free survival was calculated instead of overall survival (P = 0.034). This was not the case for MRP2 (P > 0.1). The impact on overall survival of both genes remained significant (P < 0.05) when the analysis was restricted to patient samples with a purity of leukemic cells of >90% (n = 42).

No significant association was found for overall survival or relapse-free survival and the expression of MRP4, MRP5, and SMRP. When the expression levels of all five genes were analyzed as continuous variables in univariate Cox-regression analyses, only MRP3 was found to be associated with a poor prognosis (P < 0.001).

**MRP Expression at the Time of Diagnosis and at Relapse.** In 9 patients, the expression of the five MRP genes was measured at the time of diagnosis, as well as in first relapse. The expression of all five genes was more often and to a higher degree elevated than reduced at the time of relapse. However, this trend was not statistically significant.

**DISCUSSION**

To our knowledge, this study provides first data on the association between the response to chemotherapy and the expression of MRP2, MRP3, MRP4, MRP5, and SMRP in AML patients. Our results strongly suggest that MRP3 is involved in drug resistance in this disease. It therefore represents an interesting marker for risk-adapted therapy and a possible target for the development of specific drugs to overcome multidrug resistance. Sirotnak et al. (21) found that the function of MRPs can be inhibited by probenecid and that the efficacy of chemotherapeutics in mice could be enhanced by the coadministration of probenecid.

Additionally in our study, MRP2 was associated with a poor prognosis, but the results were less convincing. It was not associated with a poor remission rate, and the impact on relapse-free survival was much smaller than the impact on overall survival. Patients who expressed high levels of both genes, MRP2 and MRP3, had a particularly poor prognosis.

Studies, including specific functional assays and the analysis of protein expression, are necessary to confirm our results. In a study on lung cancer cell lines, Young et al. (22) could demonstrate a good correlation between mRNA and protein levels for MRP2 and MRP3.

Our results do not indicate a clinical relevance of MRP4, MRP5, or SMRP in childhood AML. Either the expression of these genes in the leukemic cells is too small to cause a signif-
iant efflux of chemotherapeutic drugs or our group of patients was too small and heterogeneous to detect their clinical relevance. Another possible explanation is that the level of mRNA of these genes does not strongly correlate with the amount of functional protein in the cell membrane.

The expression levels of MRP5 and SMRP were closely correlated (Spearman’s correlation coefficient = 0.87, \( P < 0.001 \)). This correlation does not support the hypothesis that the grades of transcription of these two splicing variants are regulated differently (19).

ACKNOWLEDGMENTS

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REFERENCES

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