ABCA3 as a Possible Cause of Drug Resistance in Childhood Acute Myeloid Leukemia

Daniel Steinbach, Jean-Pierre Gillet, Axel Sauerbrey, Bernd Gruhn, Kristin Dawczynski, Vincent Bertholet, Françoise de Longueville, Felix Zintl, Jose Remacle, and Thomas Efferth

Abstract

Background: A major issue in the treatment of acute myeloid leukemia (AML) is resistance to chemotherapeutic drugs. Multidrug resistance can be caused by ATP-binding cassette (ABC) transporters that function as drug efflux pumps. The majority of these proteins have not yet been examined in malignant diseases.

Experimental Design: A newly developed microarray for the simultaneous quantification of 38 ABC transporter genes and Taqman real-time PCR was used to analyze the expression of ABC transporters in pediatric AML and healthy bone marrow. Small interfering RNA was used to verify the role of ABCA3 in drug resistance.

Results: Using the microarray, we identified four new ABC transporters, which were overexpressed in many AML samples compared with healthy bone marrow: ABCA2, ABCA3, ABCB2, and ABCC10. The overexpression of these four genes was verified by real-time PCR in 42 samples from children with AML and 18 samples of healthy bone marrow. The median expression of ABCA3 was three times higher in 21 patients who had failed to achieve remission after the first course of chemotherapy than in a well-matched group of 21 patients who had achieved remission at this stage ($P = 0.023$). Incubation of cell lines with a number of different cytostatic drugs induced an up-regulation of ABCA3. Down-regulation of ABCA3 by small interfering RNA sensitized cells to doxorubicin.

Conclusion: Our results show that ABCA2, ABCA3, ABCB2, and ABCC10 are overexpressed in childhood AML compared with healthy bone marrow. ABCA3 is the most likely transporter to cause drug resistance.

A major issue in the treatment of acute myeloid leukemia (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 about 50% (1–3).

Several mechanisms of drug resistance have been identified. One of these is the overexpression of ATP-binding cassette (ABC) transporters that function as drug efflux pumps (4–6). The family of ABC transporters consists of >40 different proteins. The function of many members of this family has not yet been investigated (7). The best-characterized ABC transporter is the permeability P-glycoprotein, which is encoded by the multidrug resistance gene 1 ($MDR1$ or $ABCB1$). Expression of P-glycoprotein/MDR1 has been identified as an independent adverse prognostic factor for complete remission and survival in adult patients with AML (4–6). Some studies suggested that it might be possible to improve the treatment of adults with AML by combining chemotherapy with drugs that inhibit the function of P-glycoprotein (8, 9). However, the clinical relevance of P-glycoprotein is much smaller in children with AML (10, 11).

It becomes more and more evident that a number of transporters act in concert to cause drug resistance. We could recently show that the expression of the breast cancer resistance protein (BCRP or $ABCG2$) and the expression of the MDR-associated protein 3 (MRP3 or $ABCC3$) are associated with a poor response (PR) to chemotherapy in children with AML (12, 13). Benderra et al. found the same results in adult patients with AML (14, 15). MRP3 is also associated with drug resistance in childhood ALL (16, 17).

The aim of this study was to investigate whether there are additional ABC transporters that are expressed in AML and involved in drug resistance. We could recently show that the expression of the breast cancer resistance protein (BCRP or $ABCG2$) and the expression of the MDR-associated protein 3 (MRP3 or $ABCC3$) are associated with a poor response (PR) to chemotherapy in children with AML (12, 13). Benderra et al. found the same results in adult patients with AML (14, 15). MRP3 is also associated with drug resistance in childhood ALL (16, 17).

The aim of this study was to investigate whether there are additional ABC transporters that are expressed in AML and involved in drug resistance. We were particularly interested in those ABC transporters that are overexpressed in AML samples compared with normal bone marrow because this might be an important prerequisite for using such proteins as therapeutic targets.
Patients and Methods

Patients and diagnosis. Pretreatment samples from 42 selected children with primary AML were analyzed. All of these patients had received full remission induction chemotherapy, and the remission status of all patients was analyzed before the next phase of therapy. Half of the patients showed a good response (GR); that is, they had achieved remission at this stage. The other 21 patients showed a PR; that is, they had not achieved remission after induction therapy. The patient characteristics of both groups are summarized in Table 1.

We used all samples from PR patients that were available. The 21 GR patients were selected from a total group of 35 GR patients. The selection criterion was to find the subgroup of GR patients that was the best possible match for the PR group with respect to the patient characteristics (Table 1). In doing so, we were able to compare two groups of patients with similar clinical features but a clearly defined difference in response to chemotherapy (i.e., in vivo drug resistance).

The patients were treated according to four multicenter studies: AML-I/82 (12 patients: 7 PR and 5 GR), AML-II/87 (13 patients: 7 PR and 6 GR), AML-BFM-93 (7 patients: 4 PR and 3 GR), and AML-BFM-98 (10 patients: 3 PR and 7 GR). All studies included induction therapies based on cytosine-arabinoside and anthracyclines (18). The cumulative dosages in the induction therapies were AML-I/82 (100 mg/m² daunorubicin, 400 mg/m² cytarabine, 6 mg/m² vincristine), AML-II/87 (120 mg/m² daunorubicin, 1,050 mg/m² cytarabine, 450 mg/m² etoposide), AML-BFM-93 (180 mg/m² daunorubicin, 1,400 mg/m² cytarabine, 450 mg/m² etoposide), and AML-BFM-98 (36 mg/m² daunorubicin, 1,400 mg/m² cytarabine, 450 mg/m² etoposide).

The initial diagnosis of AML was determined by standard methods (1, 18). Written consent was given for the use of all patient samples for this study.

All patients were treated and diagnosed at the University Children's Hospital Jena. The samples were taken to the lab immediately.

Healthy donors. Eighty samples of bone marrow were obtained from healthy adults (ages 19-41 years; 11 female and 8 male) who donated for bone marrow transplantation. Written consent was given for the use of these samples for this study. The samples were processed exactly in the same way as the patient samples.

Processing of samples. Ficoll-Hypaque density gradient centrifugation was done in all peripheral blood and bone marrow samples from patients and healthy controls. After this procedure, the percentage of leukemic cells in the AML samples was >90% as determined by May-Gruenwald-Giemsa–stained cytoptins. Isolation of total RNA and transcription into cDNA were done as described (12, 13).

Expression profiling of ABC transporters with the DualChip human ABC. A detailed description of the DualChip human ABC, its validation, the procedure protocols, and the statistical analysis was given recently (19). In short, the chip is composed of single-strand DNA probes attached to a glass support by a covalent link. Each DNA probe is present in triplicates. The chip contains probes for 38 ABC transporters, eight housekeeping genes, and a number of positive and resistance genes was standardized for the expression of two housekeeping genes, \( \beta-2 \)-microglobulin and \( \text{Abelson gene 1} \). Both genes were shown to be expressed with little variability in healthy hematologic cells and AML (20). The geometric mean of both standardizations was used for the calculation of the expression of the resistance genes.

Serial dilutions of cDNA of reference cell lines were used to generate standard curves. The reference cell lines were MCF7/CH1000 (ABCA2, ABCA3, and ABCG10) and KG-1 (ABC2). The expression of each gene in each sample was analyzed in duplicate. The regression coefficients of the standard curves ranged between 0.994 and 0.999. The variation of the duplicate measurements was extremely small compared with the variation between different samples. In the few cases where there was a substantial difference between the two values, the sample was reanalyzed.

Cell culture. Human leukemic Jurkat and CCRF-CEM cell lines were seeded in RPMI 1640 supplemented with 10% FCS. Human osteosarcoma 143B cells were grown in DHG medium (DMEM supplemented with 4.5 g/L glucose; Invitrogen) and 10% FCS (Life Technologies). All of the cells were incubated under standard culture condition (5% CO₂ and 37°C).

Drug treatment. To analyze the changes in the expression of ABCA3 after incubation with cytostatic drugs, Jurkat and CCRF-CEM were treated with doxorubicin, cytarabine, vinblastine, vincristine, and epirubicin. The concentrations used with Jurkat cells were 0.1, 1, 0.1, 0.003, 0.1 µg/mL, respectively, and with CCRF-CEM cells were 1.0, 0.01, 0.003, and 1 µg/mL, respectively. These concentrations were selected by determining the LD₅₀ for each drug in both cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays.

Transfection of small interfering RNA. Small interfering RNA (siRNA) transfection experiments were done using double-stranded RNA synthesized by Dharmacont (Lafayette, CO). A nontargeting siRNA (Scramble, Eurogentec, Searing, Belgium) was used as control.

Table 1. Initial patient data in 42 children with AML: 21 with GR to therapy and 21 with PR to therapy

<table>
<thead>
<tr>
<th></th>
<th>GR</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. patients</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>10/11</td>
<td>10/11</td>
</tr>
<tr>
<td>Age (25th/50th/75th percentile)</td>
<td>3/10/15</td>
<td>4/11/15</td>
</tr>
<tr>
<td>WBC count in 10⁷/L</td>
<td>15/43/153</td>
<td>26/52/80</td>
</tr>
<tr>
<td>(25th/50th/75th percentile)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of leukemic cells in WBC</td>
<td>55/66/83</td>
<td>49/80/87</td>
</tr>
<tr>
<td>(25th/50th/75th percentile)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inversion inv(16) (positive/negative)</td>
<td>5/16</td>
<td>1/20</td>
</tr>
<tr>
<td>Translocation t(8/21) (positive/negative)</td>
<td>2/19</td>
<td>3/18</td>
</tr>
<tr>
<td>Translocation t(9/11) (positive/negative)</td>
<td>3/18</td>
<td>2/19</td>
</tr>
<tr>
<td>Liver ≥3cm below costal margin (yes/no)</td>
<td>11/10</td>
<td>10/11</td>
</tr>
<tr>
<td>Spleen ≥3cm below costal margin (yes/no)</td>
<td>7/14</td>
<td>8/13</td>
</tr>
<tr>
<td>Auer rods (positive/negative)</td>
<td>2/19</td>
<td>3/18</td>
</tr>
<tr>
<td>FAB type (no. patients)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>4</td>
<td>3</td>
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<td>4</td>
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<td>M4</td>
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<td>7</td>
</tr>
<tr>
<td>M5</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>
were transfected with Dharmafect 1 (Dharmacon) according to the manufacturer's instructions. Transfection efficiency in cells plated on coverslips was determined using FITC-labeled siRNA and evaluated to 85% to 90% after 24 and 48 hours by cell counting using a confocal microscope (Leica). siRNA efficiency on ABCA3 expression was determined by quantitative reverse transcription-PCR.

The effect of ABCA3 expression disruption in chemotherapy-induced resistance was analyzed as follows: 143B cells were seeded in 12-well plates at 100,000 per well 24 hours before being transfected with Dharmafect 1 for 24 hours with 50 nmol/L ABCA3 siRNA or equivalent treatment with nontargeting siRNA (Scramble, Eurogentec). Twenty-four hours after transfection, media were refreshed, and cells were incubated with 0.3 μg/mL doxorubicin. This concentration was selected by determining the LD50 for doxorubicin in 143B cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. ABCA3 mRNA level and the effect of drug treatment and silencing combination on cell viability were measured by quantitative reverse transcription-PCR and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 48 hours after transfection.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were seeded on 24-well plates and treated with doxorubicin (0.3 μg/mL) for 24 hours at 37°C. After drug treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Sigma, Gillingham, United Kingdom) was added to each well and incubated for 2 hours at 37°C before medium removal. DMSO was then added and mixed for 2 hours at 37°C. Absorbance was measured at 570 nm using a spectrophotometer (Ultramark, Bio-Rad, Richmond, CA).

Statistical methods. Because the levels of gene expression did not follow normal distributions, the correlation between the ABC transporters and other clinical features was investigated by means of Spearman's correlation coefficients. Gene expression in different groups of patients was compared using the Mann-Whitney test for two groups and the Kruskal-Wallis test for more than two groups. All calculations were done using the SPSS 11.0 program (SPSS, Inc., Chicago, IL).

Cluster analysis. To investigate relationships between samples, we used the cluster program called EpClust (Expression Profiler, European Bioinformatics Institute, Cambridge, United Kingdom; http://ep.ebi.ac.uk/EP). We worked following the variables: hierarchical clustering, Manhattan distance, complete linkage (maximum distance).

Institutional review. This study was approved by the institutional review board of the University of Jena (#1545-05/05).

Results

Detection of overexpressed ABC transporters by expression profiling. Using microarrays, we aimed to identify ABC transporters that are overexpressed in AML samples compared with
healthy bone marrow. DualChip human ABC microarrays were done in samples from 33 patients (15 GR and 18 PR) and two samples of healthy bone marrow.

Many genes were overexpressed in a number of patient samples compared with healthy bone marrow. The five most consistently overexpressed genes were ABCA2/ABCA3 (ABCA2 and ABCA3 were detected by the same capture probe), ABCB2, ABCC1, and ABCC10.

In 22 patients, ABCA2/A3 was overexpressed >2-fold compared with both samples of healthy bone marrow. This was the case for ABCB2 in 24 patients, for ABCC1 in 24 patients, and for ABCC10 in 17 patients. The expression of all genes in all patient samples compared with one sample of healthy bone marrow is given in Fig. 1.

ABCC1 encodes the MRP1. Its expression in AML has already been studied intensively (4–6). The other four genes were selected for further analysis.

We also did an unsupervised hierarchical clustering with all genes and all patients. This analysis did not cluster the patients into the PR and the GR group, suggesting that the majority of ABC transporters do not have a major effect on drug resistance.

Analysis of overexpressed ABC transporters by real-time PCR. The expression of the selected genes was then analyzed by real-time PCR in all 42 patients and in 18 samples of healthy bone marrow. In accordance with the results of the microarrays, all four genes were overexpressed in many of the leukemic samples compared with the healthy controls. The median expression in the patient samples was two to four times higher, and the differences were highly significant (Fig. 2). Nevertheless, among the patients, there was a large variability in the expression of all four genes. The variation from the 25th percentile to the 75th percentile was 3-fold for ABCA2, 12-fold for ABCA3, 2-fold for ABCB2, and 5-fold for ABCC10.

Correlation with clinical features of AML. The four genes were investigated for their association with sex, age, FAB type, initial WBC, initial percentage of leukemic cells in WBC, and the presence of Auer rods and chromosomal aberrations t(8;21), t(9;11), and inv(16). The only associations that were statistically significant were a trend for higher levels of ABCA2 in patients with a higher percentage of leukemic cells in the initial WBC count ($P = 0.005$; Spearman’s correlation coefficient = 0.47) a trend for lower levels of ABCA3 in older patients ($P = 0.041$; Spearman’s correlation coefficient = −0.29), and a trend for higher levels of ABCC10 in patients with a higher initial WBC count ($P = 0.034$; Spearman’s correlation coefficient = 0.31).

Correlation with response to remission induction therapy. An important question was whether any of the overexpressed ABC transporters were associated with PR to therapy because this would suggest that they are involved in drug resistance. The median expression of ABCA3 was about three times higher in the group of patients who did not achieve remission after
induction chemotherapy ($P = 0.023$; Fig. 3). When we compared PR and GR patients from each study separately, the expression of ABCA3 was always higher in the PR group. Therefore, this finding was not biased by the fact that the patients were treated according to four different multicenter studies with different induction therapies.

The PR and GR groups were well matched for the main clinical features of AML. The only important difference was a higher number of patients with the chromosomal aberration inv(16) in the GR group (Table 1). However, the expression of ABCA3 was not associated with this inversion.

None of the other three genes was associated with response to therapy.

**Correlation of ABCA3 with previously analyzed ABC transporters.** In the same group of patients who were analyzed in this study, we could recently show that ABCC3 (MRP3) and ABCG2 (BCRP) were associated with PR to therapy (12, 13). Neither ABCC3 nor ABCG2 were significantly correlated to the expression of ABCA3. In addition, the expression of ABCB1 (MDR1) was not significantly associated with the expression of ABCA3. In a partial correlation analysis with multivariate controlling for all of these three transporters, the association between ABCA3 and achievement of remission was still statistically significant ($P = 0.046$). Thus, ABCA3 was independently associated with response to therapy and not just coexpressed with other drug resistance genes.

**Up-regulation of ABCA3 after treatment with cytostatic drugs.** We first determined the LD$_{50}$ for each drug studied. The leukemic cell lines Jurkat and CCRF-CEM were then incubated with five different cytostatic drugs. Each drug induced an up-regulation of the expression of ABCA3 (Fig. 4). This finding too suggests that ABCA3 might be involved in drug resistance. Either the cells show a higher expression as a reaction to the drugs or those cells, which already showed a high expression before the treatment, were more resistant and therefore survived the treatment.

**Silencing of ABCA3 sensitizes cells to doxorubicin.** Transfection in leukemic cell lines was done unsuccessfully. siRNA was then used to silence the expression of ABCA3 in the osteosarcoma cell line 143B. The level of the ABCA3 mRNA was quantified by real-time PCR. We found a down-regulation of 79% in the siRNA-transfected cells compared with the untransfected one (Fig. 5A). Anthracyclines are equally used in the treatment of osteosarcoma and AML. We, therefore, used doxorubicin to test whether the down-regulation of ABCA3 had a sensitizing effect on 143B cells. As shown in Fig. 5B, this was the case.

**Discussion**

Many studies have been carried out on the clinical relevance of ABC transporters. Thus far, all of these studies analyzed one or a small group of ABC transporters. For the most members of this family, the clinical relevance has still not been examined.

In this study, we used a newly developed low density DNA microarray for the simultaneous expression analysis of 38 ABC transporters. The advantage of a low-density microarray over
high-density microarrays, which cover almost all known genes, is the good reproducibility and the specific validation that was done for the group of genes on the chip (19). This validation is particularly important in a group of genes that shows as much homology as the family of ABC transporters (21).

The aim of this study was to help to identify genes that cause drug resistance and hence can be used as therapeutic targets to sensitize resistant leukemic cells. In order not to sensitize normal hematopoietic cells at the same time, it is important to find those transporters that are expressed in leukemic cells but not or to a much lower degree in healthy bone marrow.

Using the microarrays, we could identify four new ABC transporters that were overexpressed in many AML patients compared with two samples of healthy bone marrow: ABCA2, ABCA3, ABCB2, and ABCC10. These findings were confirmed by real-time PCR in a larger group of patients and a much larger group of healthy controls (Fig. 2).

By using only two healthy controls for the microarrays and reserving the majority for the independent validation, we were at a slightly higher risk of missing interesting genes, but we could perform a more solid statistical analysis of the selected genes (Fig. 2).

We did not perform any purification of putative leukemic stem cells in the leukemic samples. Therefore, as healthy controls, we used bone marrow samples that were processed exactly in the same way as the leukemic samples with Ficoll-Hypaque density gradient centrifugation but no other selection process.

Of the four new ABC transporters that we found overexpressed in childhood AML, only ABCA3 was associated with PR to therapy ($P = 0.023$; Fig. 3). It is, therefore, the most likely candidate to cause drug resistance, but the prognostic effect of ABCA3 needs validation in a larger, nonselected, and homogeneously treated cohort of patients.

Furthermore, we could show that the incubation of leukemic cells with a number of different cytostatic drugs leads to a higher expression of ABCA3 (Fig. 4). Although this might also be an unspecified stress reaction, this finding too points towards a role of ABCA3 in drug resistance.

143B cells could be sensitized to doxorubicin by silencing the expression of ABCA3 (Fig. 5B). The effect of silencing the expression of ABCA3 on cell survival was only 23% (Fig. 5B).

This is not a major effect but, a down-regulation of mRNA does not lead to a full loss of the protein and its function. Therefore, the difference in cell survival is not a measure for the amount of drug resistance, which is caused by ABCA3. This amount is difficult to measure, but our clinical data suggest that it is relevant.

Recent results from other groups also suggested that ABCA3 could be involved in drug resistance. Yasui et al. (22) found that a number of drug-resistant cancer cell lines showed higher copy numbers of the ABCA3 gene and a stronger expression of the gene compared with the drug-sensitive parental cell lines. Hirschmann-Jax et al. showed that ABCA3 and BCRP are expressed in various malignant stem cells, and that the two genes together might be involved in the resistance of these cells against mitoxantrone (23).

Norwood et al. (24) described the expression of ABCA3 in an in vivo propagated human AML cell line. The same group could show that the protein ABCA3 was expressed in a panel of AML samples, and that the expression of the protein was strongly correlated to the expression of the gene (25). The latter finding is particularly important in combination with our data, showing that the expression of the gene is associated with response to therapy.

ABCA3 is located at intracellular membranes (26). It does not confer a “classic” drug efflux but rather seems to be involved in the intracellular sequestration and the vesicular transport of its physiologic substrates as well as chemotherapeutic agents, such as daunorubicin (25, 26).

Our results suggest that ABCA3 might be involved in drug resistance in AML. This finding warrants further studies on its physiologic role, the transport capacity, the clinical relevance, and the potential use as a therapeutic target to overcome drug resistance.

References


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