

Expression of some ATP-binding cassette transporters in acute myeloid leukemia

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Abstract

Hematopoietic cells express ATP binding cassette (ABC) transporters in relation to different degrees of differentiation. One of the known multidrug resistance mechanisms in acute myeloid leukemia (AML) is the overexpression of efflux pumps belonging to the superfamily of ABC transporters such as *ABCB1*, *ABCG2* and *ABCC1*. Although several studies were carried out to correlate ABC transporters expression with drug resistance, little is known about their role as markers of diagnosis and progression of the disease. For this purpose we investigated the expression, by real-time PCR, of some ABC genes in bone marrow samples of AML patients at diagnosis and after induction therapy. At diagnosis, *ABCG2* was always down-regulated, while an up regulated trend for *ABCC1* was observed. After therapy the examined genes showed a different expression trend and approached the values of healthy subjects suggesting that this event could be considered as a marker of AML regression. The expression levels of some ABC transporters such as *ABCC6*, seems to be related to gender, age and to the presence of *FLT3/ITD* gene mutation.

Introduction

The ATP Binding Cassette (ABC) is the largest transporter proteins superfamily which translocate a variety of substrates across the plasma membrane as well as across intracellular membranes of some organelles. The 49 human ABC genes are expressed in both normal and malignant cells and, among them, at least 14 are involved in transport of drugs and xenobi-

otics.¹ High expression of specific ABC transporter proteins contributes to multidrug resistance via the ATP-dependent drug efflux, a process that serves to protect cells from cytotoxic drugs causing failure of chemotherapy. *ABCB1*, *ABCG2* along with *ABCC1* represent the three principal multidrug resistance genes that have been identified in tumor cells.²

ABC transporters besides being involved in drug resistance may contribute to the protection of undifferentiated human stem cells and of cells during initial tissue differentiation. Leukemia stem cells are characterized by a high expression of several ABC transporters³ and their down-regulation occurs upon differentiation.⁴ In addition, over-expression of certain ABC transporters in leukemic cells has been associated with major complication in the treatment of acute leukemia.⁵ In acute myeloid leukemia (AML) patients, multidrug resistance can be present intrinsically at diagnosis or can arise during chemotherapy as well as at relapse.⁶ Cancer treatment failure can be caused by MDR1 up-regulation, which was often associated with poor prognosis of AML.^{7,8} The *ABCG2* gene was found overexpressed in patients with AML and it was associated with resistance to therapy.^{9,10} Furthermore, the expression of *ABCG2* and *ABCB1* genes was detected in patients with AML with poor prognosis;¹¹ in particular it was observed that *ABCB1* and the expression of *ABCG2* are prognostic factors in patients treated with daunorubicin and mitoxantrone but not with idarubicin.¹²

The involvement of the protein MRP1 (*ABCC1*) in chemoresistance of patients with AML is controversial.¹³ However, it was reported that, at the level of mRNA, MRP1 is overexpressed in patients with relapsed AML or who have not responded to treatment, compared with healthy controls or patients who have achieved complete remission. Moreover, the expression of this gene was correlated with some clinical characteristics, such as cytogenetic and FAB classifications.¹⁴

Therefore if these ABC transporters could play a role in drug resistance in AML, any correlation between MRP protein expression and clinical response in AML patients has been detected.

Few studies have been carried out on the involvement of the multidrug-resistance protein 6 (MRP6/*ABCC6*) in drug resistance. Among the molecules transported *in vitro* by MRP6, there are some drugs widely used in chemotherapy such as certain anthracyclines.¹⁵ In order to investigate the possible correlation of ABC transporters expression in AML diagnosis and response to drug treatments we studied mRNA

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Contributions: AMS performed real time PCR experiments, FC analyzed data, SC, RN and SPP prepared cell samples, IA and MP selected patients and discussed data, FB and AO designed the research and wrote the paper.

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expression levels of *ABCC6* gene along with the expression of the well-known *ABCB1*, *ABCC1* and *ABCG2* genes in bone marrow samples from 11 AML patients and from 7 healthy donors. The interest for *ABCC6* gene derives from the observation, reported in recent studies, that *ABCC6* is involved in the control of the cell cycle¹⁶ and regulates genes involved in the immune response.¹⁷ MRP6 is structurally and functionally poorly characterized;¹⁸⁻²¹ mutations in this gene cause the Pseudoxanthoma elasticum, but its function and physiological substrate(s) are not known.²²

There are some evidences about the possible involvement of epigenetic mechanisms regulating the expression of some ABC genes;²³ it is known that histone deacetylase inhibitors (HDACis) and methyltransferase inhibitors cause upregulation of *ABCB1* and *ABCG2*.²⁴⁻²⁶ The effect of histone deacetylase and methyltransferase inhibitors on *ABCB1* and *ABCC6* expression was evaluated in AML cell line HL-60.

Materials and Methods

Study setting

In this retrospective study we included a total of 26 bone marrow samples, taken, after informed consent, from 11 adult AML

patients (for eight of them there were both samples at the onset of the disease and after treatment) and 7 subjects without leukemia, who were seen between 2008 and 2012 at the Division of Hematology of the San Carlo Hospital, Potenza, Italy. We have excluded patients with acute promyelocytic leukemia. The study was performed in accordance with the Declaration of Helsinki. Patients were stratified by risk categories according to the criteria of the European LeukemiaNet.²⁷ The percent of blasts in the bone marrow was detected with morphological examination by optical microscope. Cytogenetic investigations were carried out using conventional analysis and FISH; molecular analysis was done by qualitative PCR and real-time PCR.

Patients under 60 years of age received induction treatment with continuous infusion cytarabine (Ara-C) 100 mg/m² for 7 days plus daunoblastin 60 mg/m²/day for 3 days in infusion for 30 minutes; patients over 60 years of age received induction treatment in infusion with Ara-C 3 g/m² for 2 hours for 5 days plus idarubicin 45 mg/m² in infusion for 1 hour on the fourth day.

Complete remission (CR) status was defined as normocellular marrow with marrow blasts <5%, absence of extramedullary disease, no blasts with Auer rods, normal peripheral blood counts and, independence from transfusion of red cells. Patients who did not fulfill the aforementioned criteria were defined as primary resistant (PR).

Subjects without AML were used as controls and compared to patients at diagnosis.

Cell culture and in vitro treatment with Trichostatin A and 5-Aza-2'deoxyctidine

The HL60 cell line used in this study was purchased from DSMZ (www.dsmz.de) and maintained in RPMI-1640 (Sigma-Aldrich) culture medium, supplemented with 10% fetal bovine serum (EuroClone), 1% glutamine (EuroClone) and 1% of the antibiotics penicillin and streptomycin (EuroClone) in polystyrene flasks at 37°C and 5% CO₂.

Cells were treated with 50 ng/mL or 500 ng/mL of Trichostatin A (TSA, Sigma – Aldrich) or with 5 μM or 50 μM of 5-Aza-2'deoxyctidine (Aza, Sigma - Aldrich) for 24 hours. Cells were harvested by centrifugation at 1200 rpm for 5 minutes at 22°C.

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from about 1x10⁷ marrow cells or from 1x10⁶ cultured cells using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) accord-

ing to the manufacturer's instructions and transcribed to cDNA using random primers and High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). The following primers designed to span exon-exon junctions to eliminate any undesirable genomic DNA amplification, were used: *ABCC6*, forward 5'-TCGCTGTCTTCCTGAGCCTC-3' and reverse 5'-CATTTGCTCCTCCTGATGGT-3'; *ABCG2*, forward 5'-ATCACTGATCCTTCCATCTTG-3' and reverse 5'-GCTTAGACATCCTTTTCAGG-3'; *ABCB1*, forward 5'-CCTTCAGGGTTTCACATTTGG-3' and reverse 5'-ACTCACATCCTGTCTGAGCA-3'; *ABCC1*, forward 5'-GATCATGCTCACTTTCTGGC-3' and reverse 5'-TGGGCATCCTCTTTTAAAGGC-3'; *GAPDH*, forward 5'-GATCATCAGCAATGCCTCCT-3' and reverse 5'-ACTGTGGTCATGAGTCCTC-3'.

Level of gene transcripts was measured by real-time PCR amplification performed on a 7500 Fast Real Time PCR System (Applied Biosystems) using the Power SYBR Green PCR Master Mix (Applied Biosystems) for detection of PCR products. Analysis were performed in triplicate. The specificity of amplifications was confirmed by analyzing the melting curves. To evaluate the difference in the expression of target

genes, the C_t (cycle threshold) of the targets was normalized with the C_t of the endogenous control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). From obtained ΔC_t values, the relative expression level for each target PCR was calculated using the ΔΔC_t method.

Statistical analysis

Data were expressed as the mean ± standard error (SE). Differences between any two groups were determined by Student's t test; differences among more than two groups were determined by ANOVA. A Pearson product-moment correlation coefficient was computed to assess the relationship between gene expression and age, WBC count and % BM blasts and between expression of drug resistance genes among themselves. A P≤0.05 was considered statistically significant. The analysis was carried out using the statistical package R (version 3.1.3).

Results

To investigate the involvement of *ABCB1*, *ABCC1*, *ABCG2* and *ABCC6* transporters in the diagnosis and prognosis of AML, gene expression at the mRNA level has been determined in bone marrow sam-

Table 1. Demographic, clinical and hematological data of patients with acute myeloid leukemia.

Variables	Acute myeloid leukemia patients (n=11)
Age (years)	Median: 61 (range 37-81)
Gender	
Males	6
Females	5
WBC count (×10 ³ cells/mm ³)	Median: 46.1 (range 3.56-223.7)
PLT count (×10 ³ cells/mm ³)	Median: 36 (range 5.0-295)
% Bone marrow blasts	Median: 80 (range 54-100)
FAB morphology	
M0	2
M2	1
M4	4
Not evaluable	4
Karyotype	
Favorable	1
Intermediate (I e II)	7
Adverse	1
Not evaluable	2
FLT3 Status	
WT	4
Internal tandem duplication	7
NPM1 Status	
WT	5
MUT	6
CR Status* (n=8)	4 (50%)

*Complete remission evaluated after induction chemotherapy.

ples obtained at diagnosis of the disease and after chemotherapy. We analyzed samples from 11 patients with AML (six males and five females; median age=61 years, range=37-81), treated with cytarabine and anthracyclines, and samples from 7 healthy subjects (four males and three females; median age=54 years, range=23-84).

Clinical, laboratory and disease-specific characteristics of the 11 patients with AML are shown in Table 1. Among patients who received induction chemotherapy, 4 patients achieved a complete remission, 4 patients were primary resistant, two were non-evaluable due to death during induction and one patient has not undergone chemotherapy due to age.

Expression level of genes in AML and healthy samples are presented in Figure 1. In healthy controls, *ABCC6* gene showed expression levels lower than the other three genes (in terms of C_t values were comprised between 9,66 and 18,2) while *ABCC1* has shown higher levels of expression than the others; *ABCB1* and *ABCG2* showed similar and low expression levels. In AML patients at diagnosis, expression levels of *ABCC6* and *ABCB1* were similar to those found in the controls (in terms of C_t values resulted between 5.83 and 15.88 and between 6.74 and 13.88, respectively). Conversely, *ABCC1* showed higher levels compared to the controls: it was significantly overexpressed in 73% of patients ($P=0.019$). Instead, *ABCG2* was always down-regulated in AML patients at diagnosis ($P=0.0004$), compared with controls.

Analysis to evaluate the potential inter-relationship of these genes with clinical variables (such as some prognostic factors) of patients with AML has been performed (Table 2). We have observed a correlation between expression levels of genes and the age of patients: older patients presented lower expression of *ABCB1*, *ABCC1* and *ABCC6* than younger ones ($P<0.05$). *ABCC6* and *ABCC1* expression levels resulted also related to gender ($P<0.05$), in particular we have observed that female patients presented expression levels higher than males. The expression levels of *ABCC6* and *ABCC1* were also higher in AML samples positive for *FLT3/ITD* gene mutation ($P<0.05$) than in the negative ones, while the level of *ABCG2* was higher in samples negative for the same mutation ($P<0.01$) than in the positive ones. No significant association was observed between gene expression levels and other clinical variables.

mRNA levels of genes have been also analyzed after drug treatment (induction therapy) of 8 patients and compared to those of healthy subjects and of patients at

diagnosis of the pathology (Figure 2). After the chemotherapy treatment *ABCG2* showed expression levels higher than at diagnosis ($P=0.012$). *ABCC1* ($P=0.47$) and *ABCB1* ($P=0.48$) gene expressions showed a downward trend in most of the patients after treatment compared to the diagnosis. Instead, no variation was observed in *ABCC6* gene expression ($P=0.77$).

It has been reported that some ABC genes are transcriptionally controlled by epigenetic mechanisms. To evaluate the effect of Trichostatin A (an inhibitor of histone deacetylase) and 5-Aza-2'-deoxycytidine (an inhibitor of DNA methyltransferase) on the *ABCC6* gene expression, real-time PCR analysis was performed in the AML cell line HL-60. As a control, we eval-

uated the expression level of *ABCB1* gene as it is known that its expression is controlled by epigenetic modification.²⁴⁻²⁶ Data obtained are shown in Figure 3: in HL-60 both Aza and TSA, at two different concentrations, induced a significant up-regulation of *ABCC6* as well as of *ABCB1*.

Discussion

It is known that ABC transporters are the major causes of drug resistance in both solid and hematological cancers. Leukemia stem cells are characterized by a high expression of several ABC transporters,³ above all P-glycoprotein (*ABCB1*), MRP1 (*ABCC1*) and BCRP (*ABCG2*) whose role in multidrug resistance has often been con-

Table 2. Correlation between expression of drug resistance genes with clinical variables of patients with acute myeloid leukemia.

Clinical and demographic variables	ABCB1	ABCG2	ABCC1	ABCC6
Age	0.03*	0.94	0.05*	0.047*
Gender	0.87	0.66	0.04*	0.03*
WBC ($\times 10^3$ cells/mm ³)	0.95	0.36	0.14	0.69
% BM blasts	0.82	0.73	0.23	0.43
Karyotype	0.31	0.15	0.91	0.71
FLT3 Status	0.51	0.009*	0.03*	0.03*
NPM1 Status	0.55	0.25	0.39	0.57

*The values in the table indicate the P-values obtained as described in the Statistical Analysis section.

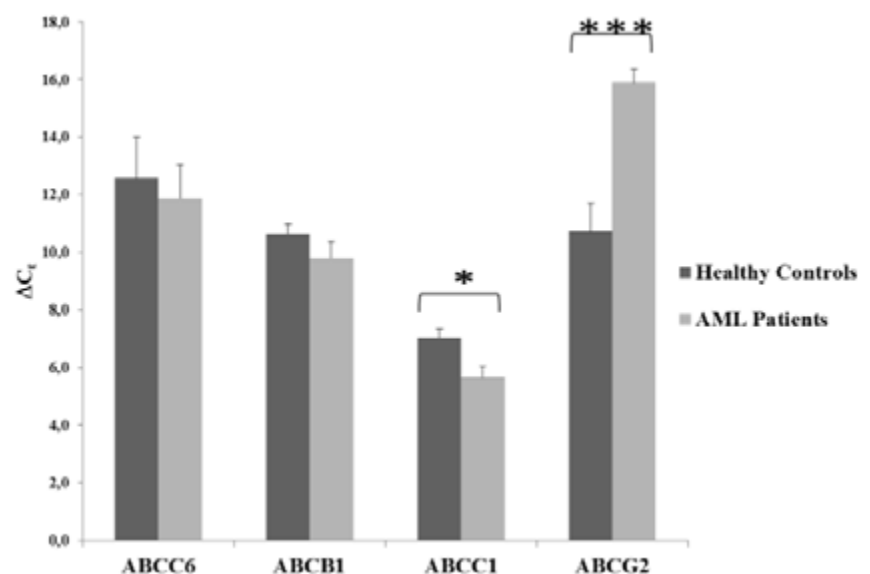


Figure 1. Expression level of *ABCC6*, *ABCB1*, *ABCC1* and *ABCG2* genes at diagnosis in subjects with (AML Patients, n=11) and without (Healthy Controls, n=7) AML. ΔC_t values were obtained by [C_t target gene - C_t GAPDH]. Results are represented as the mean \pm S.E., * $P<0.05$; * $P<0.001$.**

tradictory.⁹⁻¹³ Only few studies have addressed the role of other ABC transporters and no data have been reported for possible involvement in AML of MRP6 (*ABCC6*) which has been proven to affect the cell cycle and genes expression.^{16,17} In this study we investigate the correlation of mRNA levels of some ABC transporters in bone marrow samples obtained from healthy donors and AML patients, at diagnosis and after treatment with a combination of Ara-C and either daunoblastin or idarubicin.

Results showed that at diagnosis *ABCG2* gene is always down-regulated and *ABCC1* is always up-regulated compared to healthy donors, while no correlation was observed between *ABCB1*, *ABCC6* and AML. mRNA analysis of samples from AML patients after drug treatment indicated that *ABCB1* and *ABCC1* tend to be down-regulated and *ABCG2* is almost always overexpressed. Therefore, *ABCG2* increases and *ABCC1* tends to decrease, bringing their expression values averagely closer to those of healthy subjects, showing that

these transporters, above all *ABCG2*, are closely correlated with AML or rather that their expression may be correlate to the disease and may represent markers of therapeutic response.

We have observed a correlation between mRNA levels of some genes and the age of AML patients: older patients presented lower expression of *ABCB1*, *ABCC1* and *ABCC6* than younger ones. *ABCC1* and *ABCC6* mRNA levels showed a correlation with gender (female patients presented expression levels higher than males) and with the *FLT3/ITD* gene mutation (patients positive for this mutation showed overexpression of these genes).

The 5-Aza-2'deoxyctidine and Trichostatin A experiments on cell culture confirm the epigenetic control for these genes.

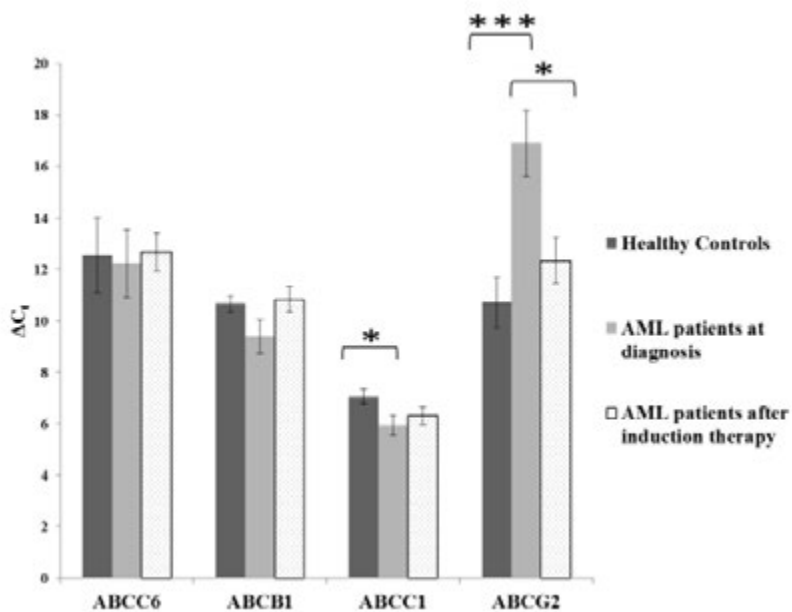


Figure 2. Expression level of *ABCC6*, *ABCB1*, *ABCC1* and *ABCG2* genes in AML patients (n=8), at the diagnosis of the pathology and after induction therapy, and in healthy controls. ΔC_t values were obtained by $[C_{t \text{ target gene}} - C_{t \text{ GAPDH}}]$. Results are represented as the mean \pm S.E., * $P < 0.05$.

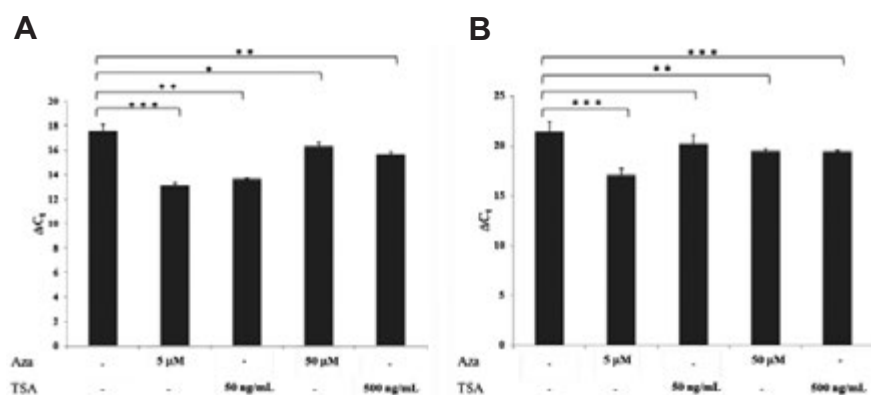


Figure 3. Expression level of *ABCC6* (A) and *ABCB1* (B) genes in HL-60 cell line treated with 5-Aza-2'deoxyctidine (5 μ M or 50 μ M) or with Trichostatin A (50 ng/mL or 500 ng/mL). ΔC_t values were obtained by $[C_{t \text{ target gene}} - C_{t \text{ GAPDH}}]$. Error bars represent the Standard Error (S.E.) of the mean for three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Conclusions

Although our study was conducted on a small set of patients, it has shown that a transcription analysis of a small set of differentially expressed ABC genes might be useful not only for AML diagnosis (*ABCG2* was always down-regulated, while an up regulated trend for *ABCC1* was observed) but also to follow the progression of the disease (both transcripts tend to approach the values of healthy subjects after induction therapy). Further studies on a larger cohort of patients should be performed to confirm the results reported here and might provide further information on the possible role of *ABCC6* in drug resistance induced by epigenetic drugs used in the treatment of patients with AML and on its correlation with gender, age and presence of *FLT3/ITD* gene mutation.

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