# ATP Binding Cassette transporters associated with chemoresistance: transcriptional profiling in extreme cohorts and their prognostic impact in a cohort of 281 acute myeloid leukemia patients

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*The online version of this article has a Supplementary Appendix.*

## ABSTRACT

### Background

A major issue in the treatment of acute myeloid leukemia is resistance to chemotherapeutic drugs. An increasing number of ATP-Binding-Cassette transporters have been demonstrated to cause resistance to cancer drugs. The aim of this study was to highlight the putative role of other ATP-Binding-Cassette transporters in primary chemoresistant acute myeloid leukemia.

### Design and Methods

In the first part of this study, using taqman custom arrays, we analyzed the relative expression levels of 49 ATP-Binding-Cassette genes in 51 patients divided into two extreme cohorts, one very sensitive and one very resistant to chemotherapy. In the second part of this study, we evaluated the prognostic impact, in a cohort of 281 patients, of ATP-Binding-Cassette genes selected in the first part of the study.

### Results

In the first part of the study, six genes (*ATP-Binding-CassetteA2, ATP-Binding-CassetteB1, ATP-Binding-CassetteB6, ATP-Binding-CassettC13, ATP-Binding-CassetteG1, and ATP-Binding-CassetteG2*) were significantly over-expressed in the resistant group compared with the sensitive group. In the second cohort, overexpression of 5 of these 6 ATP-Binding-Cassette genes was correlated with outcome in univariate analysis, and only the well-known *ATP-Binding-CassetteB1* and *G2*, and the new *ATP-Binding-CassetteG1* in multivariate analysis. Prognosis decreased remarkably with the number of these over-expressed *ABC* genes. Complete remission was achieved in 71%, 59%, 54%, and 0%, ($P$=0.0011) and resistance disease in 21%, 37%, 43%, and 100% ($P$<0.0001) of patients over-expressing 0, 1, 2, or 3, *ABC* genes, respectively. The number of ATP-Binding-Cassette genes expressed, among *ATP-Binding-CassetteB1*, *G1*, and *G2*, was the strongest prognostic factor correlated, in multivariate analysis, with achievement of complete remission ($P$=0.01), resistant disease ($P$=0.01), and overall survival ($P$=0.02).

### Conclusions

Using expression profiling, we have emphasized the diversity of ATP-Binding-Cassette transporters that cooperate to promote chemoresistance rather than overexpression of single transporters and the putative role of new ATP-Binding-Cassette tranporters, such as *ATP-Binding-CassetteG1*. Modulation of these multiple transporters might be required to eradicate leukemic cells.

Key words: acute myeloid leukemia, AML, adult, ABC genes, MDR.

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## Introduction

A major issue in the treatment of acute myeloid leukemia (AML) is resistance to chemotherapeutic drugs.[1] The best characterized resistance mechanism is mediated by the ATP Binding Cassette B1 (*ABCB1*; MDR1/Pgp) gene.[2-4] Concomitant administration of chemotherapy and modulators of ABC proteins has been used to try to overcome clinical multidrug resistance. Several large multicenter phase III trials testing different modulators have been completed in relapsed and *de novo* AML, but no striking differences between the two arms have been shown. This has dampened enthusiasm about the use of ABCB1 inhibitors in drug resistant leukemias.[5-9] Nevertheless, when patients were stratified according to ABCB1 functionality, and in case of high ABCB1 activity, Solary *et al*. showed a clear advantage over use of a specific modulator (quinine).[8] Future clinical trials using such inhibitors should, therefore, be restricted only to adult patients who express a functional ABCB1 protein. Furthermore, improved clinical outcome has been observed in AML in a trial using cyclosporine A (CsA), a non-specific ABC protein inhibitor, in contrast to the poor results of studies using a selective ABCB1 modulator, such as PSC833, on long-term disease outcome.[10] These results support the assumption that, *in vitro*, CsA is an ABC-transporter inhibitor, with effects on at least ABCB1, ABCC1, and ABCG2.[11]

An increasing number of ABC transporters have indeed been shown to cause resistance to cancer chemotherapeutic agents.[12] Overexpression of *ABCA3*, *ABCB1*, *ABCC1*, *ABCC3*, and *ABCG2* genes has been shown to be a prognostic factor in AML.[13-16] These data also emphasize that ABC transporter-mediated protection against xenobiotics and other toxic substrates may be linked to simultaneous activity of many redundant family members, such as the well-known ABCB1, ABCC1, ABCG2, or others.[13,14, 17,18]

In a recent study identifying the expression profile of 45 ABC transporter genes in human hematopoietic stem cell fraction, 36 out of 45 ABC transporters had higher expression levels in $CD34^+/CD38^-$ cells than in committed $CD34^+38^+$ cells.[19] The highly conserved homology between the 49 known ABC transporters predicts that additional members might be involved in extrusion of xenobiotics and chemotherapeutic compounds. However, many of them have not yet been studied in AML.

In order to test the clinical relevance of this hypothesis, we first studied the differential expression of 49 ABC transporters in two extreme cohorts of adult AML patients treated with conventional chemotherapy, one with very poor disease free survival, and the other with very good outcome. The aim of this first part of the study was to highlight the putative role of other ABC transporters in primary chemoresistance in AML. In the second part of the study, we evaluated the prognostic impact of the ABC genes that emerged from this work in a large cohort of 281 patients treated homogeneously in EORTC protocols.

## Design and Methods

### Patients and samples

We selected 51 consecutive adult AML patients from January 2002 to December 2006 in our department, homogeneously treated by conventional chemotherapy in AML12 and 13 EORTC protocols. These patients were divided into one good prognosis cohort (n=31) and one very poor prognosis cohort (n=20). Study subjects were selected from among 68 AML patients treated during the same period under the same protocols in our department, in accordance with local ethics committee approval (APHP, formulary EORTC study, n. 06991 and n. 06954). Patients' characteristics are shown in Table 1. Median follow-up time of alive patients was 2,060 days. These 51 patients were representative of 281 patients analyzed in the second part of our study. The good prognosis cohort was composed of patients still in first complete remission with only one induction treatment after a follow up of two years or more. The poor prognosis cohort was composed of patients who failed to reach complete remission after one or two cycles of chemotherapy, or of patients in whom complete remission lasted less than six months. The group representing primary resistant disease was pooled with that representing early relapse seeing that both these groups had a similar overall survival. Bone marrow or peripheral blood samples from patients were taken after obtaining informed consent.

In the second part of the study, 281 AML patients were analyzed after giving their informed consent (in accordance with local ethics committee APHP, Formulary EORTC study, n. 06991 and n. 06954). Bone marrow or peripheral blood samples were collected between January 2000 and March 2008. Young patients had been included in the EORTC AML 12 trial and older patients in the AML 13 trial. Treatments used in the EORTC AML12 and AML13 protocols have been described in detail elsewhere.[20,21] Patients who underwent allogeneic transplantation were censured at the date of transplant. Patients with acute promyelocytic leukemia were excluded from the study due to retinoic acid treatment. Median follow-up time of alive patients was 1,432 days.

Bone marrow or peripheral blood samples were obtained from our cell bank ("Tumorothèque Leucémies" Hôtel-Dieu n. 579).

### Expression profiling of ABC transporter family in extreme cohorts[22,23]

Transcriptional profile of the human ABC transporter family in extreme cohorts was performed with real-time PCR assays using Taqman® Low Density Array technology (TLDA, Applied Biosystems). Gene expression level (49 ABC transporters and 20 housekeeping genes) were determined with different sets of Taqman® Human Endogenous Control Arrays and Taqman® custom Arrays, pre-loaded with Taqman® Gene Expression Assay ordered from Applied Biosystems (see list of qPCR assays ID). The qPCR experiments were all performed on a 7900HT Fast Real-Time PCR system with automation accessory. Details of techniques are available in the *Online Supplementary Appendix*.

To determine whether contaminating mononuclear cells influenced ABC gene expression, we compared percentage of blasts in samples of the sensitive and the resistant cohorts (80±10% in the sensitive cohort *vs.* 83±9% in the resistant cohort, *P*=NS). There were 32% (10 patients) of blood samples in the sensitive cohort, and 35% (7 patients) in the resistant cohort. The percentage of blast cells was also similar in blood (85±8% and 87±10%, respectively) and marrow samples (76±5% and 80±8%, respectively). ABC gene expression was similar in blood and marrow in the sensitive and also in the resistant cohorts. We also analyzed the *ABC* gene expression in patients with 60% to 70% of blasts, 70% to 80%, 80% to 90%, and 90% to 100% in the first cohort. There was no statistical difference between these different groups.

### ABC gene expression study in a cohort of 281 patients

As endogenous internal control for each sample, *ABCA2*, *ABCB1*, *ABCB6*, *ABCC13*, *ABCG1*, *ABCG2* and *ABL* were analyzed in duplicate in the same MicroAmp optical 96-well plates using a 7900HT Real-Time PCR System (Applied Biosystems). A

third test was performed in cases in which a more than 1.0 discrepancy of threshold cycle (Ct) value between the two tests was observed. Details of techniques are available in the *Online Supplementary Appendix*. The comparative deltaCt method was used to determine the relative expression levels of ABC genes. For each transcript, the 30$^{th}$ percentile was selected as a cut-off value to define positive and negative samples. In fact, in our previous studies of expression of several ABC proteins, the 30$^{th}$ percentile had been used as cut-off value to define positive patients.[13,24] In all other analyses, ABC gene expression was exclusively expressed as a continuous variable. To determine whether *ABC* gene expression was influenced by sample origin (blood or bone marrow), it was seen that levels of *ABC* gene expression obtained from blood (62 samples) and bone marrow (219 samples) were concordant for all *ABC* genes studied: *ABCA2* (1.58±2.18 *vs.* 1.29±1.52, respectively; *P*=NS), *ABCB1* (1.61±3.24 *vs.* 1.78±5.2, respectively; *P*=NS), *ABCB6* (0.43±0.24 *vs.* 0.48±0.7, respectively; *P*=NS), *ABCC13* (0.42±1.27 *vs.* 0.408±0.82, respectively; *P*=NS), *ABCG1* (3.16±5.28 *vs.* 2.9±2.59, respectively; *P*=NS), and *ABCG2* (0.79±2.12 *vs.* 0.87±2.23, respectively; *P*=NS).

### *FLT3-ITD and NPM1 mutations*

DNA was extracted from frozen bone marrow or peripheral blood samples using Nucleon kits (GE Healthcare) according to the manufacturer's protocol. *FLT3*-ITD and *NPM1* mutations were identified as previously described.[25] Details of techniques are available in the *Online Supplementary Appendix*.

### *Statistical analysis*

Comparisons of ABC gene expression between the resistant and the sensitive groups were performed by Mann-Whitney test. Associations between ABC gene expression groups and baseline clinical and biological features were analyzed by Fisher's exact test using categorical variables, and Mann Whitney or Kruskall Wallis tests using continuous variables.

Complete remission (CR) was defined as recovery of bone marrow morphology with less than 5% blasts, neutrophil count 1×10$^9$/L or more, platelet count 100×10$^9$/L or more, and no evidence of extramedullary leukemia. Resistant disease (RD) was defined as treatment resistance when evaluation did not meet the criteria of complete remission. Early death was defined as death before completion of the induction therapy cycle. These latter patients were not included in evaluation of resistant disease. Disease free survival (DFS) was measured from the date of complete remission until the date of relapse or death from any cause, with observation censored for patients last known to be alive without report of relapse. Overall survival (OS) was measured from the date of diagnosis until the date of death from any cause, with observation censored for patients last known to be alive.

Estimated probabilities of disease free survival and overall survival were calculated using the Kaplan-Meier method and differences between survival distributions were evaluated by the log rank test. Proportional hazards models were constructed to determine whether *ABC* gene expression was associated with outcome when adjusting for other prognostic variables. A full model used the significant variables in univariate analysis with a *P* value less than 0.1. Logistic regression was used to determine the impact of *ABC* gene expression on resistant disease and achievement of complete remission. We used StatView software (version 5.0) for statistical analysis (SAS Institute, Inc., San Diego, CA, USA). Hierarchical clustering was performed by complete linkage analysis using Genesis software.[26]

**Table 1.** Patients' characteristics in two extreme cohorts, and in cohort of 281 patients.

| Characteristics | Extreme cohorts (51 patients) | | Cohort of 281 patients |
|---|---|---|---|
| | Sensitive patients (n=31) | Resistant patients (n=20) | |
| Age year: median (range) | 48 (17-78) | 52 (19-73) | 52 (16-80) |
| Gender | | | |
| Male | 20 | 10 | 140 |
| Female | 11 | 10 | 14 |
| WBC (×10$^9$/L): median (range) | 27 (1-284) | 20 (0.5-300) | 30 (0.52-285) |
| Disease status | | | |
| Secondary | 3 | 3 | 31 |
| *De novo* | 28 | 17 | 250 |
| Cytogenetics* | | | |
| Good prognosis | 11 | 0 | 25 |
| Intermediate prognosis | 14 | 12 | 195 |
| Poor prognosis | 6 | 8 | 46 |
| Failure or not done | 0 | 0 | 15 |
| Molecular mutation status | | | |
| *NPM1* | | | |
| Mutated | 12 | 3 | 70 |
| Not mutated | 19 | 17 | 211 |
| *FLT3* | | | |
| ITD | 4 | 8 | 50 |
| No ITD | 27 | 12 | 231 |

**Good karyotypes included t(8 ;21) and inv(16); poor karyotypes included, 3q26 rearangements, t(6 ;9), del(7)/-7, del(5)/-5 and three or more than three abnormalities.*

## Results

### *Expression profiles of ABC transporter genes in extreme cohorts*

In hierarchical clustering, ABC genes can be divided in five groups (Figure 1A). In groups 1 and 2, ABC genes were highly expressed in AML cells of both resistant and sensitive patients; homogeneously in group 1 (5 genes: *ABCB2*, *ABCB7*, *ABCB10*, *ABCE1*, and *ABCF2*), but in a more variable way in group 2 (11 genes: *ABCA2*, *ABCA7*, *ABCB3*, *ABCB8*, *ABCC1*, *ABCC4*, *ABCD1*, *ABCD3*, *ABCD4*, *ABCF1*, and *ABCF3*). In contrast, group 5 consisted of 17 ABC genes with no detectable or very weak expression in both the sensitive and the resistant groups (*ABCA4*, *ABCA8*, *ABCA9*, *ABCA10*, *ABCA12*, *ABCB4*, *ABCB5*, *ABCB11*, *ABCC3*, *ABCC7*, *ABCC8*, *ABCC9*, *ABCC11*, *ABCC12*, *ABCG4*, *ABCG5*, and *ABCG8*). In group 3, a weak expression was detected in many samples, both in the sensitive and the resistant groups (5 genes: *ABCA3*, *ABCA6*, *ABCC2*, *ABCC6*, and *ABCD2*). Group 4 consisted of 11 genes with intermediate but variable expression levels (11 genes: *ABCA1*, *ABCA5*, *ABCA13*, *ABCB1*, *ABCC13*, *ABCB6*, *ABCB9*, *ABCC5*, *ABCC10*, *ABCG1*, and *ABCG2*). The most relevant genes were thus in groups 2 and 4.

### Differentially expressed genes between the sensitive and the resistant groups

We compared the mean expression level of all these genes between sensitive and resistant patients. Thirty-nine ABC transporters could have been analyzed whereas 10 had no detectable expression. As shown in Figure 1B and C, higher expression levels were found in the resistant group for most genes (29 of 39). Seven of these 29 genes, belonging to group 4 (5 genes), group 2 (1 gene) and group 5 (1 gene), had differential expression (DE=mean expression of resistance group divided by mean expression of sensitive group) ratios above or equal to 2, suggesting significant overexpression (Mann-Whitney test: *ABCA2*, DE=2, *P*=0.01; *ABCB1*, DE=3.32, *P*=0.03; *ABCB5*, DE=2.17, *P*=0.007; *ABCB6*, DE=2.23, *P*=0.06; *ABCC13*, DE=3.1, *P*=0.03; *ABCG1*, DE=2.11, *P*=0.001; *ABCG2*, DE=3.82, *P*=0.003). Remarkably, no significant overexpression of ABC genes could be observed in the sensitive group. As *ABCB5* in group 5 had a very weak and infrequent expression in the samples (4 samples) when compared to the other 6 selected ABC genes (Figure 1A), it was not retained for the second part of our study.

### ABCA2, ABCB1, ABCB6, ABCC13, ABCG1, and ABCG2 expression in a cohort of 281 AML patients and correlation with clinical and biological characteristics

Two hundred and eighty-one patients were included in this cohort. Two hundred and forty-nine were tested for *ABCA2*, 278 for *ABCB1*, 264 for *ABCB6*, 261 for *ABCC13*, 264 for *ABCG1*, and 261 for *ABCG2*. All these 6 ABC genes were analyzed in 242 patients. In those samples, 61 patients had no overexpression of any of the 6 ABC genes, while 54 patients had overexpression of only one of the 6 genes, 38 patients had concomitant overexpression of 2 of 6, 35 patients of 3 of 6, 32 patients of 4 of 6, 16 patients of 5 of 6, and 6 patients of 6 of 6 genes, using the 30$^{th}$ percentile as a cut-off value to define positive and negative samples.

Using an adjustment for multiple comparisons, there was a significant correlation between *ABCG1* and *ABCB1* expression (*P*<0.0001), *ABCG2* and *ABCB6* (*P*<0.0001), *ABCC13* and *ABCB6* (*P*=0.01), and between *ABCB1* and *ABCA2* (*P*=0.04).

Correlation between ABC genes and clinical and biological characteristics are shown in Table 2.

**Table 2.** Correlations between ABC gene expression, and clinical and biological characteristics.

| | ABCA2 | *P* Value | ABCB1 | *P* Value | ABCB6 | *P* Value | ABCC13 | *P* Value | ABCG1 | *P* Value | ABCG2 | *P* Value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age (years) | | | | | | | | | | | | |
| <50 | 1.03±1.35 | *P*=0.0004 | 0.97±1.51 | *P*=0.10 | 0.36±0.64 | *P*=0.68 | 0.36±0.84 | *P*=0.99 | 2.27±1.83 | *P*=0.41 | 0.76±3.22 | *P*=0.88 |
| ≥50 | 1.54±1.82 | | 2.10±5.77 | | 0.40±0.57 | | 0.36±0.75 | | 3.26±3.86 | | 0.71±1.22 | |
| WBC (×10$^9$/L) | | | | | | | | | | | | |
| <30 | 1.51±1.82 | *P*=0.02 | 1.88±5.35 | *P*=0.40 | 0.40±0.52 | *P*=0.0011 | 0.39±0.80 | *P*=0.01 | 3.17±3.63 | *P*=0.03 | 0.69±1.14 | *P*=0.006 |
| ≥30 | 1.12±1.41 | | 1.11±2.49 | | 0.10±0.09 | | 0.04±0.07 | | 1.78±1.88 | | 0.14±0.21 | |
| CD34 | | | | | | | | | | | | |
| Positive | 1.57±1.95 | *P*=0.23 | 2.44±5.8 | *P*=0.03 | 0.43±0.56 | *P*=0.002 | 0.42±0.9 | *P*=0.01 | 3.7±3.9 | *P*=0.001 | 0.79±1.3 | *P*=0.0007 |
| Negative | 1.14±1.26 | | 0.79±4.22 | | 0.20±0.36 | | 0.12±0.27 | | 1.9±2.7 | | 0.21±0.05 | |
| CD33 | | | | | | | | | | | | |
| Positive | 1.39±1.71 | *P*=0.24 | 1.83±5.48 | *P*=0.82 | 0.34±0.49 | *P*=0.36 | 0.30±0.62 | *P*=0.04 | 2.95±3.49 | *P*=0.16 | 0.53±0.87 | *P*=0.002 |
| Negative | 0.77±0.41 | | 2.15±2.27 | | 0.46±0.53 | | 0.70±1.72 | | 4.24±4.63 | | 1.39±2.58 | |
| Cytogenetics* | | | | | | | | | | | | |
| Good | 0.79±0.83 | *P*=0.05 | 0.65±0.55 | *P*=0.04 | 0.18±0.25 | *P*=0.003 | 0.18±0.38 | *P*=0.09 | 1.85±1.63 | *P*=0.01 | 0.27±0.63 | *P*=0.004 |
| Intermediate | 1.46±1.89 | | 1.68±4.62 | | 0.40±0.62 | | 0.39±0.90 | | 2.95±3.69 | | 0.79±2.35 | |
| Poor | 1.88±0.93 | | 2.42±6.77 | | 0.48±0.59 | | 0.32±0.48 | | 3.56±2.82 | | 0.74±0.78 | |
| NPM1 | | | | | | | | | | | | |
| Not mutated | 1.41±1.81 | *P*=0.74 | 2.07±5.12 | *P*=0.13 | 0.45±0.64 | *P*=0.08 | 0.48±1.07 | *P*=0.09 | 3.24±3.73 | *P*=0.01 | 0.96±2.37 | *P*=0.04 |
| Mutated | 1.12±1.18 | | 0.90±4.80 | | 0.28±0.53 | | 0.22±0.42 | | 1.94±1.99 | | 0.28±0.51 | |
| FLT3 | | | | | | | | | | | | |
| No ITD | 1.39±1.74 | *P*=0.41 | 1.83±4.77 | *P*=0.65 | 0.45±0.65 | *P*=0.002 | 0.47±1.01 | *P*=0.02 | 3.01±3.54 | *P*=0.79 | 0.90±2.21 | *P*=0.03 |
| ITD | 1.05±1.11 | | 1.45±5.63 | | 0.11±0.10 | | 0.07±0.15 | | 2.85±2.45 | | 0.12±0.14 | |
| FAB subtypes | | | | | | | | | | | | |
| M | 1.31±1.10 | | 2.64±6 | | 0.67±0.82 | | 0.60±1.28 | | 2.93±2.13 | | 1.2±1.93 | |
| M0 | 1.36±1.00 | | 1.54±1.6 | | 0.38±0.23 | | 0.19±0.20 | | 7.41±10.6 | | 0.99±1.22 | |
| M1 | 1.34±1.14 | | 2.90±8.36 | | 0.21±0.31 | | 0.32±0.83 | | 3.42±3.95 | | 0.37±0.58 | |
| M2 | 1.56±2.16 | *P*=0.70 | 2.16±4.09 | *P*<0.0001 | 0.42±0.44 | *P*<0.0001 | 0.44±0.57 | *P*<0.0001 | 2.95±2.45 | *P*=0.02 | 0.66±0.65 | *P*<0.0001 |
| M4 | 1.03±0.97 | | 0.58±0.86 | | 0.26±0.35 | | 0.17±0.36 | | 2.16±1.35 | | 0.33±0.51 | |
| M5 | 1.46±5.71 | | 0.33±0.53 | | 0.24±0.56 | | 0.20±0.67 | | 2.01±1.95 | | 0.80±4.05 | |
| M6 | 1.13±1.26 | | 1.31±2.25 | | 1.47±1.21 | | 1.21±1.3 | | 3.69±2.24 | | 2.52±5.65 | |
| M7 | | | / | | | | | | | | | |
| Disease status | | | | | | | | | | | | |
| Secondary | 1.75±2.30 | *P*=0.24 | 2.70±6.48 | *P*=0.01 | 0.63±0.81 | *P*=0.001 | 0.49±1.33 | *P*=0.33 | 3.46±2.08 | *P*=0.01 | 1.49±2.37 | *P*=0.001 |
| *De novo* | 1.33±1.64 | | 1.64±4.80 | | 0.31±0.45 | | 0.32±0.66 | | 2.89±3.57 | | 0.48±0.70 | |

**Supplementary data by particular cytogenetic abnormalities are shown in the Online Supplementary Table S1.*

**Table 3.** Prognostic value of ABC gene expression in univariate and multivariate analysis.

| | *ABC A2* *P* value HR 5%CI | *ABC B1* *P* value HR 95%CI | *ABC B6* *P* value HR 95%CI | *ABC G1* *P* value HR 95%CI | *ABC G2* *P* value HR 95%CI | *ABC C13* *P* value HR 95%CI |
|---|---|---|---|---|---|---|
| **RD** | | | | | | |
| Univariate analysis | NS<br>1.035<br>(95% CI, 0.882-1.215) | *P*=0.02<br>1.868<br>(95%CI, 1.071-3.257) | *P*=0.05<br>1.443<br>(95%CI, 0.955-2.178) | *P*=0.0002<br>1.239<br>(95%CI, 1.105-1.389) | *P*<0.0001<br>3.439<br>(95%CI, 1.931-6.126) | NS<br>1.188<br>(95%CI, 0.910-1.550) |
| Multivariate analysis* | NI | *P*=0.04<br>1.521<br>(95%CI, 1.023-2.058) | NS<br>1.031<br>(95%CI, 0.874-1.203) | *P*=0.018<br>1.427<br>(95%CI, 1.025-1.876) | *P*=0.0004<br>1.095<br>(95%CI, 1.020-1.101) | NI |
| **CR** | | | | | | |
| Univariate analysis | NS<br>0.984<br>(95%CI, 0.845-1.145) | *P*=0.04<br>0.590<br>(95%CI, 0.352-0.988) | NS<br>0.808<br>(95%CI, 0.550-1.186) | *P*=0.0006<br>0.835<br>(95%CI, 0.752-0.926) | *P*=0.0002<br>0.368<br>(95%CI, 0.216-0.627) | NS<br>0.887<br>(95%CI, 0.683-1.1151) |
| Multivariate analysis* | NI | *P*=0.04<br>0.610<br>(95%CI, 0.370-0.991) | NI | *P*=0.03<br>0.374<br>(95%CI, 0.271-0.701) | *P*=0.0007<br>0.874<br>(95%CI, 0.801-0.974) | NI |
| **OS** | | | | | | |
| Univariate analysis | *P*=0.009<br>1.569<br>(95%CI, 1.117-2.183) | *P*=0.02<br>1.046<br>(95%CI, 1.008-1.086) | *P*=0.05<br>1.43<br>(95%CI, 1.003-2.054) | *P*=0.006<br>1.063<br>(95%CI, 1.018.-1.110) | *P*=0.03<br>1.71<br>(95%CI, 1.054-2.782) | NS<br>0.930<br>(95%CI, 0.731.-1.183) |
| Multivariate analysis* | NS<br>1.120<br>(0.887-1.412) | *P*=0.01<br>1.125<br>(95%CI, 1.025-1.258) | NS<br>1.287<br>(0.774-1.587) | *P*=0.04<br>1.82<br>(95%CI, 1.125-2.987) | *P*=0.05<br>1.48<br>(95%CI, 1.112-2.312) | NI |

*RD: resistant disease; CR: complete remission; OS: overall survival; NI: not included; NS: not significant. ABC gene expression was included in multivariate analysis, significant in univariate analysis (*i.e. *ABCB1, B6, G1 and G2 for RD, ABCB1, G1, and G2 for CR, and ABCA2, B1, B6, G1 and G2 for OS. For DFS only ABCA2 [1.107 (95% CI : 1.0007-1.218), P=0.02] was a prognostic factor in univariate analysis, and we did not achieve a multivariate analysis.*

### *ABC gene expression and clinical outcome*

Complete remission (CR) after induction chemotherapy was achieved in 65% of 281 patients. Eighteen patients died before completion of the induction therapy cycle. Five-year disease free survival rate was 38±4% (median=512 days), and 5-year overall survival rate was 35±3.3% (median=503 days).

In univariate analysis, resistant disease and achievement of complete remission after induction chemotherapy were correlated with expression of 3 of these 6 ABC genes: *ABCB1*, *P*=0.02, and *P*=0.04, respectively; *ABCG1*, *P*=0.0002 and *P*=0.0006, respectively, and *ABCG2*, *P*=0.006, and *P*=0.003, respectively. *ABCB6* expression was correlated with resistant disease (*P*=0.05). Disease free survival was correlated only with ABCA2 (*P*=0.03). Overall survival was correlated with 5 of 6 these genes: *ABCA2*, *P*=0.009; *ABCB1*, *P*=0.02; *ABCG1*, *P*=0.006; *ABCB6*, *P*=0.05; *ABCG2*, *P*=0.03. *ABCC13* was not a prognostic factor for achievement of complete remission, resistant disease, disease free survival, or overall survival.

Multivariate analysis was performed with ABC gene expression (Table 3). Only significant ABC gene expression significantly associated with prognosis in univariate analysis was included in the multivariate model. Among ABC B1, B6, G1 and G2, only ABCG2 (*P*=0.0004), ABCG1 (*P*=0.018), and ABCB1 (*P*=0.04) were associated with resistant disease in a multivariate model. Among ABCB1, G1, and G2, ABCG2 (*P*=0.0007), ABCG1 (*P*=0.03), and ABCB1 (*P*=0.04) were associated with achievement of complete remission in multivariate analysis. Among ABCA2, B1, B6, G1 and G2, only ABCB1 (*P*=0.01), ABCG1 (*P*=0.04), and ABCG2 (*P*=0.05) were associated with overall survival in multivariate analysis.

Using significant ABC gene expression in multivariate analysis (ABCB1, G1 and G2), prognosis decreased remarkably with the number of these over-expressed ABC genes. Complete remission was achieved in 71%, 59%, 54%, and 0% (*P*=0.0011) of patients over-expressing 0, 1, 2, or 3, ABC genes, respectively. Twenty-one percent, 37%, 43%, and 100% (*P*<0.0001) of patients with resistant disease expressed 0, 1, 2, or 3 ABC genes, respectively. The number of over-expressed ABC genes also correlated with overall survival (*P*=0.001). In multivariate analysis including ABCB1, G1, and G2 expression, and the number of over-expressed ABC genes, only these latter data remained statistically significant (RD, *P*=0.009; CR, *P*=0.02; OS, *P*=0.03). Therefore, only the number of over-expressed ABC genes was included in the multivariate model in the presence of other clinical and biological prognostic factors.

### *Other prognostic factors and multivariate analysis*

In addition to ABC gene expression, age, white blood cell (WBC) count, *de novo* or secondary leukemia, cytogenetics, mutated *NPM1*, *FLT3*-ITD, and use of different treatment protocols, were also evaluated as prognostic factors. Only significant parameters in univariate analysis were included in the multivariate analysis model (Table 4).

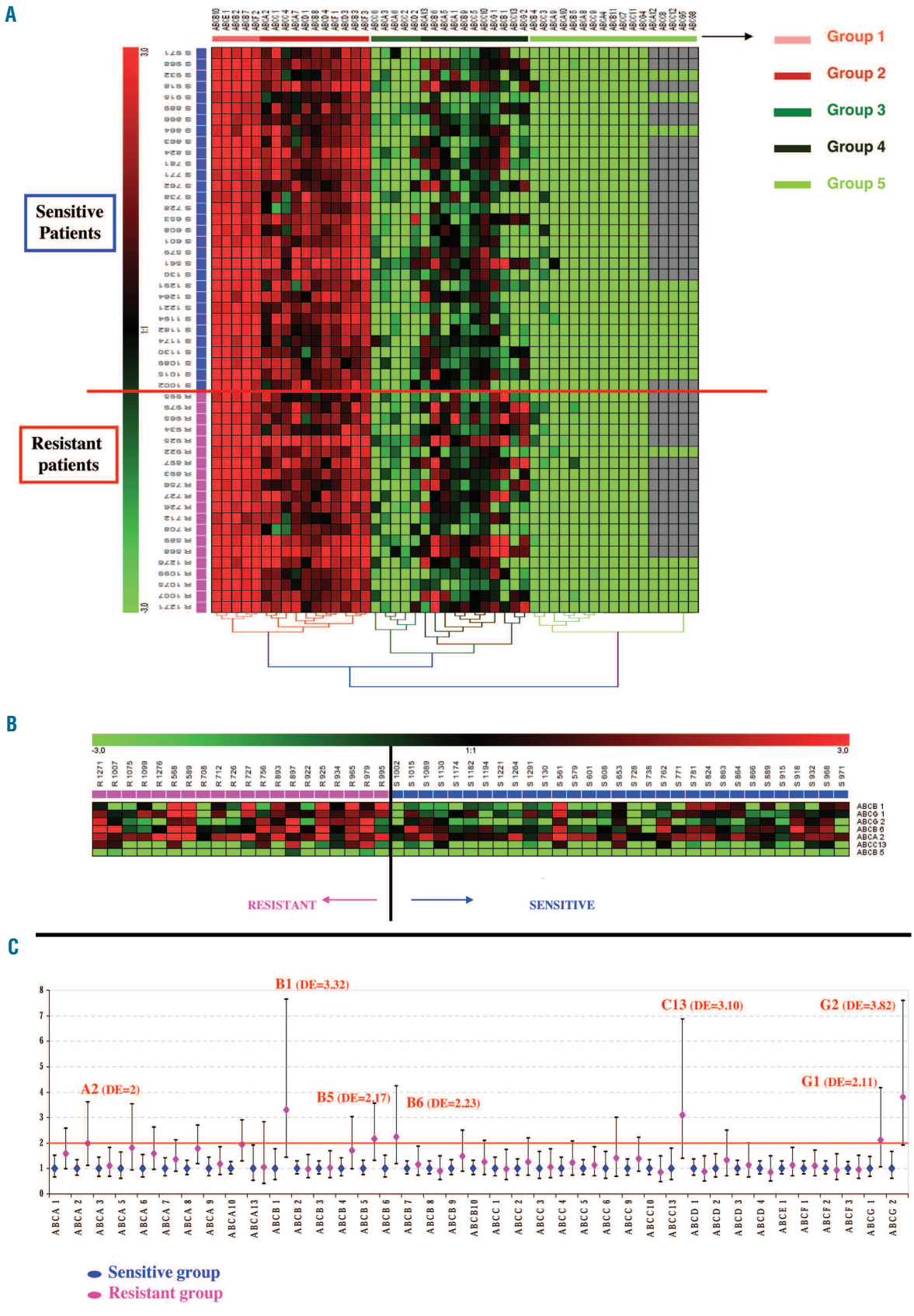


Figure 1. (A) ABC gene expression profiling in AML samples. In hierarchical clustering, the ABC genes were divided in five groups. (See *Results section*). (B) Gene expression profiling of 6 *ABC* genes with RR≥2 between sensitive group (31 patients) and resistant group (20 patients). (C) Differential expression (DE) of 39 *ABC* genes in resistant patient group (pink points) compared to sensitive patients (blue points). Ten *ABC* genes showed inconstant or very low expression levels and were not represented in the figure.

In multivariate analysis, including the number of ABC gene expression, pre-leukemic phase (*P*=0.02, *P*=0.02), *NPM1* mutated (*P*=0.05, *P*=0.04), cytogenetics (*P*=0.043, *P*=0.03) and the number of ABC genes expressed (*P*=0.01, *P*=0.03) were independent prognostic factors for resistant disease and achievement of complete remission, respectively. The number of ABC genes expressed, age (*P*=0.01), *NPM1* mutated (*P*=0.05), *FLT3*-ITD (*P*=0.02), cytogenetics (*P*=0.035), and ABC genes (*P*=0.01) were independent prognostic factors for overall survival.

*ABCA2* was the only *ABC* gene correlated with disease free survival in univariate analysis, but it was not an independent prognostic factor for disease free survival in multivariate analysis when other prognostic factors were included in the model.

**Table 4.** Multivariate analysis including the number of over-expressed ABC genes, in the presence of other prognostic factors. Only significant parameters in univariate analysis were included in the analysis

| | CR *P* value HR 95%CI | RD *P* value HR 95%CI | OS *P* value HR 95%CI |
|---|---|---|---|
| Age (continuous variable) | NS 1.214 (95%CI, 0.850-1.587) | NS 1.09 (95%CI, 0.974-1.110) | *P*=0.01 1.031 (95%CI, 1.012-1.123) |
| Protocols of treatment (AML12 *vs.* AML13) | NS | NS | NS |
| Preleukemic phase (yes *vs.* no) | *P*=0.02 2.781 (95%CI, 1.512-4.21) | *P*=0.02 3.784 (95%CI, 2.478-5.14) | NS 0.987 (95%CI, 0.521-1.841) |
| NPM1 (mutated *vs.* not mutated) | *P*=0.04 1.754 (95%CI, 1.048-2.789) | *P*=0.05 0.178 (95%CI, 0.07-0.657) | *P*=0.05 1.648 (95%CI, 1.12-2.741) |
| FLT3 (ITD *vs.* no ITD) | NS* in univariate analysis | NS* in univariate analysis | *P*=0.02 0.784 (95%CI, 0.421-0.987) |
| Cytogenetic (good *vs.* intermediate *vs.* poor) | *P*=0.03 1.789 (1.214-2.994) | *P*=0.043 1.547 (95%CI, 1.287-3.574) | *P*=0.035 1.689 (95%CI, 1.271-3.412) |
| Number of ABC genes over-expressed* (continuous variable)*** | *P*=0.01 1.321 (95%CI, 1.031-1.412) | *P*=0.01 1.486 (95%CI, 1.04-1.83) | *P*=0.02 1.621 (95%CI, 1.10-1.995) |

*NS: not significant; *not included in multivariate analysis; **ABC genes over-expressed among ABCB1, G1, and G2; ***When you included in the multivariate model the clinical and biological prognostic factors, the number of over-expressed ABC genes, and also ABCB1, G1, and G2 expression, only the number of over-expressed ABC genes remained significantly associated to CR achievement, RD and OS, in addition to other clinical and biological prognostic factors, but not ABCB1, G1, and G2 expression.*

## Discussion

Many studies have discussed the clinical relevance of ABC transporters' expression and functionality but most of them have analyzed only one ABC protein or sometimes a small number of them. For most members of this family, clinical relevance has still not been examined in a large cohort.

Two studies, using a similar technology, have compared either expression of 38 ABC gene from AML samples and healthy bone marrow,[15] or expression of 45 ABC genes from normal and leukemic $CD34^+/CD38^-$ cells *versus* more committed $CD34^+/38^+$ progenitor cells.[19] In the latter study, 36 of 45 ABC transporters analyzed were detectable both in normal and in leukemic $CD34^+/CD38^-$ cells. In the other study, *ABCA2*, *ABCA3*, *ABCB2*, and *ABCC10* were over-expressed in childhood AML compared to healthy bone marrow.

In our study, 22 ABC genes (groups 3 and 5) had a very weak or no detectable expression in AML samples. In contrast, 5 ABC genes (group 1) were highly but similarly expressed in both sensitive and resistant groups. Of interest, 22 ABC genes had more variable expression levels between the sensitive and the resistant groups, including 7 that were increased more than 2-fold in the resistant group (ABC A2, B1, B5, B6, C13, G1, and G2). None was over-expressed in the sensitive group. In addition to well known expected transporters (ABCB1 and ABCG2) ABCA2, a candidate regulator of neural transmembrane lipid transport, was relevant for drug resistance in AML and ALL,[15,27] and in a small cell lung cancer cell line resistant to mitoxantrone.[28,29]

ABCG1 is actually, like ABCA2, a cholesterol transporter, but has not yet been demonstrated to be directly involved in resistance to chemotherapy.[30] Nevertheless, cholesterol alterations can influence other ABC proteins' localization and function, such as ABCB1, which might contribute to chemoresistance.[31,32] Furthermore, some AML patients potentially demonstrate hyperactive cholesterol metabolism.[30] In addition, a recent study identified specific ABC transporter expression signature in hematopoietic stem cells (HSCs) compared to non-HSCs. The mean expression values of ABCG1, as ABCB1, was more than 1,000-fold higher in HSCs while the other genes were approximately 10-fold higher.[33]

Frank *et al.* identified ABCB5 as a novel drug transporter and chemoresistance mediator in human malignant melanoma. In their study, ABCB5 correlated significantly with tumor resistance to anthracyclin, a family of drugs used in the treatment of AML.[34] But in our study, ABCB5 expression was very weak, except for one patient, and was not suitable for further analysis.

ABCB6 has been identified as a prognostic marker in breast cancer that affects clinical response to neoadjuvant chemotherapy,[35] and Yasui *et al.* have shown that ABCB6 was amplified in 19 resistant cell lines.[36] Otherwise, ABCB6 was shown to be involved in resistance of tumor cells towards artesunate[37] and to be significantly over-expressed in melanoma cells, when compared to normal melanocytes.[38]

ABCC13 has been cloned by Yabuuchi *et al.*[39] and is actually an unusual truncated ABC transporter. The amino acid sequence corresponding to putative membrane-spanning domains is remarkably similar to *ABCC1*, *C2*, *C3*, and *ABCC6*. *ABCC13* expression is high in fetal liver, and is decreasing in K562 cells during cell differentiation, suggesting a link to hematopoiesis. These data were suggested by de Grouw *et al.*, comparing $CD34^+/CD38^-$ cells to more differentiated $CD34^+/CD38^+$ progenitors.[19] Like *ABCB6*, *ABCC13* affects clinical response to neoadjuvant chemotherapy in breast cancer.[37] In the present study their expressions were strongly correlated.

In the second part of our study, overexpression of 5 of the 6 selected genes (*ABCA2*, *ABCB1*, *ABCB6*, *ABCG1*, *ABCG2*) was correlated with poor prognostic factors such as cytogenetics, NPM1 wild type, and secondary AML,

and with outcome in univariate analysis, suggesting implication in chemoresistance in AML. But in multivariate analysis, including these 5 ABC genes, only overexpression of ABCB1, ABCG1, and ABCG2 were correlated with outcome suggesting importance in chemoresistance of these 3 genes in AML. But the strongest prognostic factor was the number of ABC genes expressed among ABCB1, ABCG1, and ABCG2. Therefore, ABC transporters may cooperate to promote chemoresistance rather than overexpression of single transporters, and modulation of multiple transporters might be required to increase intracellular drug accumulation and to induce chemotherapeutic eradication of leukemic cells in AML. This might provide an explanation to the poor results observed in clinical trials with specific ABCB1 modulation by PSC833.[7,9] In the same way, broad-spectrum modulation by CsA or quinine might be effective in AML in which multiple ABC proteins are co-expressed.[8,10] In addition to positive clinical results in AML[8,10] and myelodysplasia,[40] clinical trials using CsA in combination with chemotherapy regimens in solid tumors have also generated encouraging results.[41-43] A potential disadvantage of broad-spectrum modulators is a lesser degree of efficacy against individual ABC proteins. Another strategy to overcome ABC transporter induced chemoresistance would require the development of drugs that are not substrates of ABC proteins, such as amonafide L-malate.[44,45]

We have selected and analyzed ABC genes with a 2-fold cut off to determine overexpression. We cannot rule out the implication of other ABC genes, for example *ABCA5* or *ABCB9* (group 4), whose expression was increased by 1.5 fold in the resistant group.

Interestingly, the presence of *FLT3*-ITD was correlated with low expression of ABC genes. Although the FLT3 ITD/WT ratio has been proved to be a prognostic factor,[46] ABCB1 expression or activity retained an independent influence on treatment outcome.[24] We previously showed a mutual exclusion between *FLT3*-ITD and high ABCB1 functionality, possibly because of a loss of the *ABCB1* phenotype under increased proliferative capacity, as reported by Smeets *et al.* and others.[47,48] The authors concluded that non-cycling progenitors, both normal and leukemic, have a relatively high MDR functionality. Relationships between *FLT3*-ITD tyrosine kinase activity, and expression of other ABC genes are not known. In our study, patients with high expression of ABC genes (*ABCB6*, *ABCC13*, *ABCG1*, *ABCG2*) had lower white blood cell count than other patients with low expression of ABC gene. Further studies will be required to examine relationships between tyrosine kinase pathways and expression of ABC genes.

In conclusion, using expression profiling, we have emphasized the diversity of ABC transporters that cooperate to promote chemoresistance rather than overexpression of single transporters and the putative role of new ABC transporters, such as ABCG1, in adult AML. More data are needed to confirm the role of these transporters.

## Authorship and Disclosures

*The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.*

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