Prognostic impact of high ABC transporter activity in 111 adult acute myeloid leukemia patients with normal cytogenetics when compared to *FLT3*, *NPM1*, *CEBPA* and *BAALC*

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ABSTRACT

ATP-binding cassette transporter (and specially P-glycoprotein) activity is a well known prognostic factor in acute myeloid leukemia, but when compared to other molecular markers its prognostic value has not been well studied. Here we study relationships between this activity, fms-like tyrosine kinase 3(*FLT3/ITD*), nucleophosmin(*NPM1*), CAAT-enhancer binding protein alpha(*CEBPα*), and brain and acute leukemia cytoplasmic protein (*BAALC*), in 111 patients with normal cytogenetics who underwent the same treatment, and evaluate its prognostic impact.

Independent factors for survival were age (P=0.0126), ATP-binding cassette transporter activity (P=0.018) and duplications in the fms-like tyrosine kinase 3 (P=0.0273). In the 66 patients without fms-like tyrosine kinase 3 duplication and without nucleophosmin mutation, independent prognostic factors for complete remission achievement and survival were age and ATP-binding cassette transporter activity.

In conclusion, ATP-binding cassette transporter activity remains an independent prognostic factor, and could assist treatment decisions in patients with no nucleophosmin mutation and no fms-like tyrosine kinase 3 duplication.

Key words: acute myeloid leukemia, ABC transporter, *FLT3*, *NPM1*, *CEBPA*. *BAALC*.

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Introduction

The overall prognosis for adult acute myeloid leukemia (AML) remains poor. Cytogenetic analysis provides the major prognostic information,[1] but more than 40% of patients have normal cytogenetics (CN-AML). In these cases, many molecular alterations with prognostic significance have been described to guide treatment. Mutations involving *nucleophosmin* (*NPM1*) and the *CCAAT/enhancer binding protein alpha* (*CEBPA*) have been included as references in the World Health Organization classification of AML, and examination for mutations of *FLT3* is also strongly recommended.[2] Moreover, many other molecular alterations and/or deregulation of gene expression have been identified and analyzed, such as *brain and acute leukemia cytoplasmic protein* (*BAALC*) expression.[3-5] Unfortunately, their prognostic value has not been shown in all studies.[6]

In addition to these markers, the prognostic role of ABC proteins has been well characterized.[7] Among them, the best known is ABCB1 (MDR1/Pgp) whose expression and activity[8] have been associated with poor outcome. However, the role of other members of the ABC protein family has also been described.[9,17]

To our knowledge, relationships between ABC proteins and those molecular markers have not yet been explored, except for *FLT3/ITD*.[10] Whether ABC protein activity at the time of diagnosis remains an independent prognosis factor in adult AML, despite the use of these new molecular markers, should be evaluated.

Here we explore the relationships between ABC protein activity, *FLT3/ITD*, *NPM1*, *CEBPA*, and *BAALC* expression, and evaluate whether ABC protein activity remains a prognostic factor and can be helpful for therapeutic decisions in 111 CN-AML patients who underwent the same treatment according to EORTC protocols.

Design and Methods

Patients and treatments

Bone marrow or blood samples from the time of diagnosis were

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obtained from 111 CN-AML patients after receiving their informed consent, and in accordance with local ethics committee approval (APHP, Formulary EORTC study n. 06931, n. 06991 and n. 06954). All patients treated in our institution between 1996 and 2008, included in either the EORTC AML-10 or AML-12 trials (for patients younger than 60 years) or in AML-13 trial (for patients older than 60 years) with available material were included. These treatments have already been described in detail.[11-13] In the AML-10 and AML-12 protocols, patients with prior myelodysplasia or myeloproliferative disease were not included. The median follow-up time of patients who were still alive was five years. The availability of an HLA-matched donor was recorded for all young patients. Forty-two patients underwent allogeneic stem cell transplantation in first CR. For these patients, survival data have been censored at the time of transplantation.

FLT3/ITD, NPM1 mutations, CEBPA mutations and BAALC expression analyses

FLT3/ITD, *NPM1* mutations and *BAALC* expression analyses have been previously described in detail[14] and are available in the *Online Supplementary Appendix*.

Relative *BAALC* expression values were calculated with the comparative cycle threshold method using *ABL* as endogenous internal control, and one patient sample as positive control, whose expression was set at 1.

Concerning *CEBPA* mutations, all samples were first analyzed with genescan technology. All samples were analyzed a second time using High Resolution Melting technology and sequencing. Detailed methods are available in the *Online Supplementary Appendix*. As single *CEBPA* mutations had no prognostic impact in our patients, we only considered double *CEBPA* mutations in further statistical analyses, as already described.[15]

ABC transporter functional assay with JC1 probe

JC1 assay has already been described in detail.[16] Briefly, cells were incubated with JC1 monomer with or without cyclosporine (CsA) then washed. Cell fluorescence was recorded using a FACSORT flow cytometer (Becton-Dickinson). Results were established in the whole blast cell population selected by CD45 antibody weak expression. JC1 uptake was expressed as D value ranging from 0 (no difference) to 1 (no overlap) generated by the Kolmogorov-Smirnov test, which was used to determine the differential distribution in the presence and in the absence of CsA. D of 0.6 or over was considered as high functionality.[16] This assay was initially described to test ABCB1 activity, but it seems that it could actually evaluate the pooled activity of the whole ABC protein family *(personal data, unpublished)*.

Statistical analysis

Associations between ABC protein functional assay and patients' baseline characteristics were analyzed using Fisher, Mann-Whitney or Kruskall-Wallis tests. Complete remission (CR) was defined as recovery of morphologically normal bone marrow and normal blood count (i.e. neutrophil count ≥ 1×10^9/L and platelet count ≥ 100×10^9/L), with no evidence of extramedullary disease. Disease free survival (DFS) was measured from the date of CR until the date of relapse or death from any cause. Overall survival (OS) was measured from the date of diagnosis until the date of death from any cause. Estimated probabilities of DFS and OS 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 high ABC protein functional assay was associated with outcome, when adjusting for other prognostic variables. Full models used variables with a *P* value of less than 0.2 in univariate analysis. For all analyses, results were considered as significant when *P* value was 0.05 or less. StatView software (version 5.0) was used for statistical analysis (SAS Institute, Inc., San Diego, CA, USA).

Results and Discussion

One hundred and eleven patients were enrolled. Main clinical and biological characteristics are summarized in Table 1. Twenty-one patients (19%) had high ABC protein activity. High ABC protein activity was associated with CD34 expression (*P*=0.01). There was also a trend in patients with high ABC protein activity to have higher *BAALC* expression (*P*=0.08) and *NPM1* WT (*P*=0.12), but there was no difference in other variables (Table 1).

In the whole population, 79% of patients (88 of 111) achieved CR, 40%±5% achieved DFS, and 42%±5% achieved OS at five years. Survival curves for DFS and OS according to ABC protein activity are available in the *Online Supplementary Appendix* (*Online Supplementary Figure S1*). We used univariate analysis to evaluate the following parameters for CR, DFS and OS: age, leukocyte count, existence of a pre-leukemic phase, *FLT3/ITD* status, *NPM1*

Table 1. Comparison of clinical and biological variables according to ABC protein activity.

Characteristics	Patients with normal karyotype N= 111	High ABC activity N= 21	Low ABC activity N= 90	*P* value
Age, years: median (range)	56 (16-81)	56 (17-78)	55.5 (16-81)	*P*=0.75
Median leukocyte, 10^9/L (range)	16 (0.52-211)	9.2 (1-163)	19.4 (0.52-211)	*P*=0.64
FAB subtypes,[1] n. (%)				*P*=0.31
Not available	8 (7)	2 (10)	6 (7)	
M0	6 (5)	1 (5)	5 (6)	
M1	31 (28)	6 (28)	25 (28)	
M2	25 (23)	7 (33)	18 (20)	
M4	17(15)	0 (0)	17 (19)	
M5	19 (17)	5 (24)	14 (16)	
M6	4 (4)	0 (0)	4 (4)	
CD 34 expression,[2] n. (%)				*P*=0.01
CD 34 +	52 (58)	14 (87)	38 (52)	
CD 34 -	37 (42)	2 (13)	35 (48)	
Pre-leukemic phase,[3] n. (%)				*P*>0.99
Yes	8 (7)	1 (5)	7 (9)	
No	96 (92)	19 (95)	77 (91)	
NPM1 mutation status, n. (%)				*P*=0.12
NPM1 +	38 (34)	4 (19)	34 (38)	
NPM1 -	73 (66)	17 (81)	56 (62)	
FLT3/ITD status, n. (%)				*P*=0.51
FLT3/ITD+	18 (16)	2 (10)	16 (18)	
FLT3/ITD-	93 (84)	19 (90)	74 (82)	
CEBPA mutation status, n. (%)				*P*=0.75
CEBPA +	19 (21)	4 (19)	15 (17)	
CEBPA -	92 (79)	17 (81)	75 (83)	
CEBPA double mutations, n. (%)				*P*=0.99
CEBPA dm +	12 (11)	2 (10)	10 (11)	
CEBPA dm -	99 (89)	19 (90)	80 (89)	
BAALC expression, median (range)[4]	0.197 (0.002-16.79)	0.280 (0.01-13.56)	0.134 (0.002-16.79)	*P*=0.08

[1]not reported in one patient; [2]not reported in 22 patients; [3]not reported in 7 patients; [4]not reported in 2 patients.

mutational status, *CEBPA* mutational status, *BAALC* expression, ABC protein activity and type of anthracycline used. A multivariate model for CR, DFS and OS was used for all variables with a *P* value less than 0.2 in univariate analysis. Age was the only prognostic factor we found for CR (*P*=0.0009 univariate analysis; *P*=0.0056 multivariate analysis; *data not shown*).

For DFS, in univariate analysis, age (*P*=0.0423), leukocyte count (*P*=0.0064), and ABC protein activity (*P*=0.0256) had significant prognostic value, but other variables did not. In our multivariate model, leukocyte count (*P*=0.0031), age (*P*=0.0273), and ABC protein activity (*P*=0.0153) were independent prognostic factors (Table 2).

Table 2. Univariate and multivariate analyses for disease free survival and overall survival in the whole population.

	Univariate HR (range) *P* value	Multivariate[1] HR (range) *P* value
DFS		
Age	1.020 (1.001-1.04) *P*=0.0423	1.032 (1.003-1.061) *P*=0.0273
WBC count	1.007 (1.002-1.013) *P*=0.0064	1.009 (1.003-1.015) *P*=0.0031
Absence of pre-leukemic phase	0.451 (0.161-1.265) *P*=0.1301	0.285 (0.077-1.053) *P*=0.0598
NPM1 mutated *vs.* *NPM1* WT	0.901 (0.515-1.574) *P*=0.71	NI
FLT3 WT *vs.* *FLT3/ITD*	0.563 (0.281-1.127) *P*=0.1048	0.593 (0.274-1.286) *P*=0.1857
No *CEBPA* double mutations *vs.* *CEBPA* double mutations	2.769 (0.861-8.899) *P*=0.0873	1.540 (0.463-5.125) *P*=0.4815
BAALC expression	1.019 (0.903-1.150) *P*=0.7601	NI
Low ABC activity *vs.* high ABC activity	0.487 (0.259-0.916) *P*=0.0256	0.414 (0.203-0.844) *P*=0.0153
Anthracycline type	0.602 (0.321-1.247) *P*=0.0605	1.256 (0.544-2.901) *P*=0.2695
OS		
Age	1.033 (1.014-1.053) *P*=0.0006	1.030 (1.006-1.054) *P*=0.0126
WBC count	1.005 (1-1.009) *P*=0.0382	1.004 (0.999-1.009) *P*=0.1273
Absence of pre-leukemic phase	0.782 (0.313-1.954) *P*=0.5986	NI
NPM1 mutated *vs.* *NPM1* WT	1.4251 (1.002-1.053) *P*=0.0147	1.244 (0.699-2.211) *P*=0.4578
FLT3 WT *vs.* *FLT3/ITD*	0.475 (0.262-0.862) *P*=0.0143	0.477 (0.247-0.921) *P*=0.0273
No *CEBPA* double mutations *vs.* *CEBPA* double mutations	4.590 (1.122-18.783) *P*=0.03	3.252 (0.757-13.967) *P*=0.1127
BAALC expression	1.041 (0.961-1.127) *P*=0.3238	NI
Low ABC activity *vs.* high ABC activity	0.441 (0.254-0.766) *P*=0.0036	0.475 (0.256-0.880) *P*=0.0180
Anthracycline type	0.587 (0.291-1.158) *P*=0.08	1.897(0.492-7.314) *P*=0.6210

NI: not included. [1]Multivariate models included all variables with P *value < 0.2 in univariate analysis.*

For OS, in univariate analysis, age (*P*=0.0006), leukocyte count (*P*=0.0382), *NPM1* mutation (*P*=0.0147), *FLT3/ITD* (*P*=0.0143), double *CEBPA* mutations (*P*=0.03) and ABC protein activity (*P*=0.0036) had significant prognostic value, but *BAALC* expression (*P*=0.32) did not (Table 2). In

Table 3. Statistical analyses for CR, DFS and OS in patients with both *NPM1* WT and no *FLT3*/ITD.

	Univariate HR (range) *P* value	Multivariate[1] HR (range) *P* value
CR		
Age	0.88 (0.82-0.95) *P*=0.002	0.869 (0.789-0.958) *P*=0.0046
WBC count	0.996 (0.984-1.009) *P*=0.57	NI
Absence of pre-leukemic phase	0.325 (0.071-1.487) *P*=0.14	0.599 (0.107-3.356) *P*=0.5603
No *CEBPA* double mutations *vs.* *CEBPA* double mutations	1.86 (0.53-6.741) *P*=0.36	NI
BAALC expression	0.90 (0.77-1.06) *P*=0.21	NI
Low ABC activity *vs.* high ABC activity	0.45 (0.133-1.521) *P*=0.1988	0.157 (0.027-0.904) *P*=0.0382
Anthracycline type	1.125 (0.111-11.367) *P*=0.92	NI
DFS		
Age	1.022 (0.996-1.048) *P*=0.0974	1.033 (1.000-1.067) *P*=0.05
WBC count	1.007 (0.999-1.015) *P*=0.0856	1.007 (0.998-1.016) *P*=0.1157
Absence of pre-leukemic phase	0.406 (0.138-1.200) *P*=0.1031	0.540 (0.163-1.786) *P*=0.3128
No *CEBPA* double mutations *vs.* *CEBPA* double mutations	2.740 (0.821-9.141) *P*=0.1011	1.777 (0.502-6.285) *P*=0.3725
BAALC expression	1.068 (0.944-1.208) *P*=0.2967	NI
Low ABC activity *vs.* high ABC activity	0.518 (0.217-1.237) *P*=0.1386	0.410 (0.150-0.995) *P*=0.0498
Anthracycline type	0.725 (0.258-1.874) *P*=0.511	NI
OS		
Age	1.045 (1.017-1.073) *P*=0.0012	1.047 (1.012-1.083) *P*=0.0076
WBC count	1.003 (0.997-1.009) *P*=0.3546	NI
Absence of pre-leukemic phase	0.732 (0.283-1.898) *P*=0.5214	NI
No *CEBPA* double mutations *vs.* *CEBPA* double mutations	9.296 (1.271-67.975) *P*=0.028	6.177 (0.800-47.708) *P*=0.0808
BAALC expression	1.063 (0.979-1.154) *P*=0.1478	1.032 (0.948-1.125) *P*=0.4651
Low ABC activity *vs.* high ABC activity	0.369 (0.185-0.736) *P*=0.0046	0.426 (0.202-0.895) *P*=0.0243
Anthracycline type	0.744 (0.321-1.102) *P*=0.09	2.373 (0.400-14.090) *P*=0.3418

NI: not included. [1]Multivariate models included all variables with P *value < 0.2 in univariate analysis.*

multivariate analysis, independent prognostic factors for OS were age (P=0.0126), ABC protein activity (P=0.0180) and *FLT3/ITD* (P=0.0273) (Table 2).

As treatment decisions in patients with both *NPM1* WT and no *FLT3/ITD* are one of the major problems in CN-AML, we performed a second analysis in this subgroup (n=66). In these patients, 13 had high ABC protein activity. We evaluated the same variables as previously in univariate analysis. Results are shown in Table 3.

In multivariate models, including all parameters with P value less than 0.2 under univariate analysis, the only significant prognostic factors for achievement of CR, DFS and OS were age (P=0.0046, P=0.05 and P=0.0076, respectively) and ABC protein activity (P=0.0382, P=0.0498 and P=0.0243, respectively).

Many molecular alterations have been described and can be used to guide treatments in CN-AML, but identification of powerful markers is still needed to refine treatment strategy. Here, we evaluated the impact of the well-known ABC proteins and compared them to currently used molecular markers. ABC protein activity was evaluated with JC1 probe and cyclosporine. Using flow cytometry, this test, which is easily performed as part of daily routine, is highly reproducible and is more sensitive than the rhodamine-123 assay.[16,18] JC1 assay was initially developed to test ABCB1 activity, but even though until now, it has not been fully studied it seems that it actually evaluates the pooled activity of the whole ABC protein family. Indeed, a recent study performed by our team has shown that the "D value" of JC1 assay was correlated to the number of ABC proteins expressed in blast cells. This might be linked to a lack of specifity of JC1, and to the broad spectrum of ABC proteins inhibited by CsA *(personal data, unpublished)*. Consequently, it is likely that our results reflect the activity of at least a part of the ABC protein family, and not only ABCB1.

Patients with high ABC protein activity had higher CD34 expression and tended to have higher *BAALC* expression. When we studied a larger sample of 206 AML patients, including patients with abnormal cytogenetics, high ABC protein activity was significantly associated with *NPM1* WT and higher *BAALC* expression (*Online Supplementary Table S1*). It is already known that CD34$^+$ cells have a higher physiological ABC protein activity[19] and in a gene expression study, high *BAALC* expression was associated with higher *CD34* and *ABCB1* expression.[4] Our data confirm this association. Surprisingly, *BAALC* expression had no prognostic value in our study. This could be partially explained by the small size of our sample, but the high P value (P>0.2) associated with BAALC expression in all univariate analyses, when other variables are highly significant, seems to go against this hypothesis. The association between *NPM1* WT and high ABC proteins' activity has never been described to our knowledge, and might contribute to the poorer prognosis associated with *NPM1* WT. Interestingly, in our patients, *NPM1* mutational status had significant impact on OS in univariate analysis, but not in multivariate analysis, which could be partially explained by this association. Relationships between *NPM1* and ABC proteins and between *BAALC* and ABC proteins should be investigated more deeply.

Around 20% of patients in the whole study population had high ABC protein activity; this includes patients with *NPM1* WT and no *FLT3/ITD*. The independent prognostic factors we found for OS in the whole population were age, *FLT3/ITD* and ABC protein activity. In cases of *NPM1* WT and no *FLT3/ITD*, ABC protein activity was the only independent prognostic factor we found, in addition to age. Therefore, we think that ABC protein activity should be assessed in patients with normal cytogenetics at the time of diagnosis, and could be extremely useful in guiding therapeutic decisions, at least in cases of *NPM1* WT and no *FLT3/ITD*, due to the high frequency of high ABC protein activity, and to its high prognostic value.

In all our analyses, we found no prognostic significance for single *CEPBA* mutations. When considering only double *CEBPA* mutations, as recently described,[15] we found a significant prognostic value for OS in univariate analysis, but only a trend in our multivariate models. Although these results could be explained in part by the relatively small number of patients included, it underlines again that ABC protein activity seems to be a more powerful marker than the currently used molecular markers.

In conclusion, we showed that evaluating ABC protein activity at the time of diagnosis in CN-AML remains a valuable prognostic factor, especially in cases of *NPM1* WT and no *FLT3/ITD*. Furthermore, ABC protein activity was the only independent prognostic factor in addition to age and to *FLT3/ITD*. We think that the JC1 test, which can easily be performed as part of daily routine, should be carried out in every adult patient with CN-AML, and could be helpful in refining treatment strategy. This has to be confirmed in larger prospective studies.

Authorship and Disclosures

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