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*

Pierre Hirsch,[1,2] Ruoping Tang,[1,2] Christophe Marzac,[3] Jean-Yves Perrot,[3] Fanny Fava,[1,2] Chantal Bernard,[3] Dorota Jeziorowska,[4] Jean Pierre Marie,[1,2] and Ollivier Legrand[1,2]

[1]Département d'Hématologie, Hôpital Saint-Antoine, Assistance Publique - Hôpitaux de Paris (APHP), 184 Rue du Faubourg Saint-Antoine, Paris; [2]Université Pierre et Marie Curie, INSERM UMRs 872, Equipe 18, Hôpital Saint-Antoine; [3]Laboratoire d'Immunologie et Hématologie Biologique, Hôpital Saint–Antoine, Assistance Publique - Hôpitaux de Paris (APHP) Paris; [4]Laboratoire Commun de Biologie et Génétique Moléculaires, Hôpital Saint-Antoine, Assistance Publique-Hôpitaux de Paris (AP-HP), Paris, France

Citation: Hirsch P, Tang R, Marzac C, Perrot J-Y, Fava F, Bernard C, Jeziorowska D, Marie JP, and Legrand O. 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. *Haematologica 2012;97(2):241-245. doi:10.3324/haematol.2010.034447*

Design and Methods

FLT3/ITD *detection*

FLT3 DNA was amplified by PCR. Amplicons were migrated in agarose gels, allowing the detection of a 240-bp fragment for wild-type alleles and/or longer fragments for *FLT3/ITD* alleles. Samples showing less than 10% of the mutated allele were considered negative (*FLT3/ITD-*).

Primers used for FLT3 DNA amplification:

Forward : 5'-TGGTGTTTGTCTCTTCTTCATTGT-3'

Reverse : 5'-GTTGCGTTCATCACTTTTCCAA-3'

NPM1 mutation detection

Absolute RT-PCR quantification assays were performed using forward and reverse primers for exon 12 of *NPM1* and three specific probes for detecting type A, type B and type D mutations.

Primers and probes used for NPM1 mutation detection:

Forward : 5'-GGATGACTGACCAAGAGGCTATTC-3'

Reverse: 5'-CAGAAATGAAATAAGACGGAAAATTTTT-TAACAAATTGT-3'

WT probe : FAM-5'-CTCCACTGCCAGAGAT-NFQ-3'

Mutation AProbe: FAM-5'-CACTGCCAGACAGAGAT-NFQ-3'

Mutation B Probe: FAM-5'-ACTGCCATGCAGAGAT-NFQ-3'

Mutation D Probe: FAM-5'-CTGCCAGGCAGAGAT-NFQ-3'

BAALC *expression*

Comparative RT-PCR assays (7900HT RT-PCR System) were performed using *BAALC* primers and probes (Applied Biosystems). The comparative cycle threshold (Ct) method was used to determine the relative expression level of *BAALC*, and *ABL* was used as endogenous internal control for each sample. One patient sample was used as positive control, and set at 1, and was included in each run for standardization. Relative *BAALC* expression values were calculated as:

$2^{-\mu\Delta\Delta Ct}$ with $\mu\Delta\Delta Ct = \mu\Delta Ct_{\text{x patient}} - \mu\Delta Ct_{\text{positive control}}$.

Forward and reverse primers and probes were designed and provided by Applied Biosystems.

CEBPA mutations detection

Genescan assay:

TAD1, TAD2 and bZIP domains were amplified by PCR and labeled using 50ng DNA, 1X_PCR buffer containing 1.5mM MgCl2, 5% dimethylsulfoxide, 0.20mM of each dNTP, 0.4U of Taq-Gold DNA polymerase (Applied Biosystems) and 500nM of specific primers. The mixture was heated at 95°C for 10 min and subjected to 30 PCR cycles (60 s at 95°C, 40 s at 55°C and 90 s at 72°C) with a final extension of 5 min at 72°C.

After amplification, 2 μL of each product were added to 10 μL formamide and 1 μL of GeneScan 500 size marker (Applied Biosystems) followed by a denaturation step (5 min at 95°C) and immediate cooling on ice. Capillary electrophoresis was performed on ABI 3130xl genetic analyzer (Applied Biosystems) using POP7 polymer. Data were analyzed with GeneMapper software v.4.0.

List of primers for GeneScan assay:

TAD1

Forward: 5'-TCGGCCGACTTCTACGAGGC-3'

Reverse: 5'-ACTTTGACTACCCGGGCGC-3'

TAD2

Forward: 5'-TACCTGGACGGCAGGCTGGA-3'

Reverse: 5'-AGACCACCATGCACCTGCA-3'

bZIP

Forward: 5'-AGAAGTCGGTGGACAAGAACAGCAA-3'

Reverse: 5'-GTCAAGGCCATGGGCAACT-3'

HRM analysis on Light Cycler 480 (Roche Applied Sciences™) and sequencing:

Four fragments covering the whole *CEBPA* coding sequence were amplified using 15μg/mL DNA, 0.5 μM of each primer, SensiMix™ HRM kit (BIOLINE) containing EvaGreen™ DNA binding dye, and 0.5M GC-rich resolution solution (Roche Applied Sciences™). Amplification was performed by 50 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by High Resolution Melting program, consisting in 95°C for 1 min, 40°C for 1 min, and 25 acquisitions per 1°C during the melting phase (from 75°C to 99°C).

All patient samples were analyzed and compared to 2 wild-

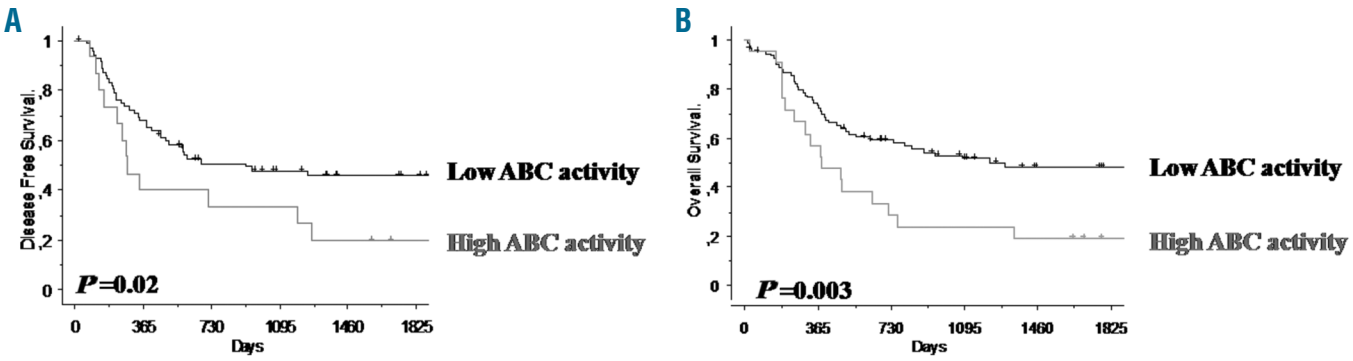
type samples used as reference. All samples with abnormal melting profiles were directly sequenced using a 3130XL-DNA sequencing system (Applied Biosystems ™). Sequences were analyzed with SeqScape software (Applied Biosystems™).

List of primers for HRM assay:
FRAGMENT 1 (*nt* 120–414)
Forward: TCGCCATGCCGGGAGAACTCTAAC
Reverse: CCTGCTGCCGGCTGTGCTGGAAC
FRAGMENT 2 (*nt* 365–664)
Forward: CTTCAACGACGAGTTCCTGGCCGA
Reverse: AGCTGCTTGGCTTCATCCTCCT
FRAGMENT 3 (*nt* 615–1004)
Forward: CCGCTGGTGATCAAGCAGGA
Reverse: CCGGTACTCGTTGCTGTTCT
FRAGMENT 4 (*nt* 962–1317)
Forward: GGGCAAGGCCAAGAAGTC
Reverse: GGCTGGCCCAGGGCGGT

Online Supplementary Table S1. Comparison of clinical and biological variables of 206 AML patients with all karyotypes according to ABC protein activity.

Characteristics	All patients n=206	High ABC activity n= 42	Low ABC activity n=164	*P* value
Age, years: median (range)	54.5 (16-81)	57.5 (17-78)	53 (16-81)	*P*=0.15
Median leukocyte, 10^9/L (range)	15 (0.52-284)	7.1 (0.6-230)	19.4(0.52-284)	*P*=0.01
FAB subtypes- n. (%)***				*P*=0.09
Not available	24 (12)	9 (22)	15 (9)	
M0	10 (5)	2 (5)	8 (5)	
M1	43 (21)	10 (24)	33 (21)	
M2	51 (25)	13 (30)	38 (24)	
M4	29 (14)	1 (2)	28 (17)	
M5	40 (20)	6 (15)	34 (22)	
M6	5 (2)	1 (2)	4 (2)	
CD 34 expression**				*P*=0.004
CD 34 +	115 (67)	29 (87)	86 (62)	
CD 34 -	56 (33)	4 (13)	52 (38)	
Karyotype,[1] n (%)				*P*=0.21
Good	17 (8)	2 (5)	15 (9)	
Intermediate	155 (75)	30 (71)	125 (76)	
Normal	111 (54)	21 (50)	90 (55)	
Other	44 (21)	9 (21)	35 (21)	
Poor	32 (16)	10 (24)	22 (13)	
Not done or not assessable	2 (1)	0 (0)	2 (1)	
Pre-leukemic phase*, n(%)				*P*=0.55
Yes	19 (10)	5 (12)	14 (9)	
No	170 (90)	35 (88)	135 (91)	
NPM1 mutation status, n(%)				*P*=0.03
NPM1 +	44 (21)	4 (10)	40 (24)	
NPM1 -	162 (79)	38 (90)	124 (76)	
FLT3/ITD status, n(%)				*P*=0.11
FLT3/ITD+	26 (13)	2 (5)	24 (14)	
FLT3/ITD-	180 (87)	40 (95)	140 (86)	
CEBPA mutation status, n(%)				*P*=0.8
CEBPA +	29 (14)	5 (12)	24 (15)	
CEBPA -	177 (86)	37 (88)	140 (85)	
CEBPA double mutations, n(%)				*P*=0.53
CEBPA dm+	16 (8)	2 (5)	14 (9)	
CEBPA dm-	190 (92)	40 (95)	150 (91)	
BAALC expression median (range)****	0.287(0.002-16.79)	0.564 (0.01-16.58)	0.225 (0.002-16.79)	*P*=0.0079

not reported in 17 patients; ** not reported in 35 patients; * not reported in 4 patients; **** not reported in 5 patients. [1]Good karyotypes included t(8;21) and inv(16); poor karyotypes included 3q26 rearrangements, t(6;9), del(7)/-7, del(5)/-5 and 3 or more than 3 abnormalities.*



Online Supplementary Figure S1. (A) Disease free survival and (B) overall survival in the 111 CN-AML patients according to ABC protein activity.