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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Online Supplementary Appendix

Design and Methods

Expression profiling of ABC transporters family in extreme cohorts

For these experiments, mononuclear cells were separated by Ficoll Hypaque® gradient centrifugation, and total RNAs were extracted using Trizol Reagent (Invitrogen). RNA concentrations were measured on a NanoDrop® spectrophotometer (Thermo Scientific) and RNA preparations were stored at –80°C. High-capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems) was used to synthesize cDNA, starting from 2 μg of total purified RNA in a final volume of 50 μL. The reverse transcription reaction was performed with random primers and incubated for 10 min at 25°C then 120 min at 37°C according to the manufacturer's recommendations. cDNAs were used to perform real-time PCR experiments.

Reference genes were first selected using TaqMan® Human Endogenous Control Arrays, allowing the testing of 16 housekeeping genes in triplicate for each sample; 150 ng of cDNA and 50 μL of TaqMan® Universal PCR Master Mix were mixed in a final volume of 100 μL. Each sample was used to load one pore of the microfluidic card to generate 48 qPCR reactions. Thirty-two cDNA were studied and 8 samples were loaded per card. Real-time qPCR amplifications were carried out (10 min 94.5°C, 40 cycles of 30 sec at 97°C and 1 min at 59.7°C) and Ct values were analyzed. GeNorm and Normfinder functions in Genex 4.3.8 (MultiD, Göteborg, Sweden) were used to evaluate the housekeeping gene stability in samples and the most stable genes were selected for the next step of qPCR experiments.

Gene expression quantification of ABC transporters in patients' samples was determined using Taqman® custom Arrays, pre-loaded with Taqman® Gene Expression Assay for the 49 human ABC genes and the previously selected housekeeping genes.

Then 600 ng of cDNA and 200 μL of TaqMan® Universal PCR Master Mix were mixed in a final volume of 400 μL. Each sample was used to load 4 pores of the Taqman® custom Array, allowing 96 qPCR reactions with technical duplicates for each biological sample (1.5 ng cDNA per reaction). Real-time PCR amplification was performed with the following parameters: 10 min at 94.5°C, 40 cycles of 30 sec at 97°C and 1 min at 59.7°C. Thermal cycling and fluorescence detection were carried out with SDS 2.3 and RQ manager ABI softwares. Thresholds were set manually and optimal Ct value cut off was limited to 35 cycles. Determined Ct values were then used for further analysis. Genex 4.3.8 (MultiD, Göteborg, Sweden) was used to evaluate the housekeeping gene stability and the 3 most stable genes (GAPDH, PPIA and 18S) were selected to normalize the ABC transporter gene expression in our samples. The comparative delta Ct method was used to determine the relative expression levels of ABC transporter genes and a relative quantification (RQ) value was calculated for each gene with the control group as reference.

ABC gene expression study in a cohort of 281 patients

Comparative real-time RT-PCR assays were performed following the manufacturer's instructions in a final reaction volume of 20 μL, containing10 μL of 2 x TaqMan Universal PCR Master Mix with AmpErase UNG, 50 ng of cDNA, and l μL of 20 x forward, reverse primers and probes (Applied Biosystems). Each specific probe was labeled with FAM and with non-fluorescent quencher (NFQ). Amplification was carried out at 50°C for 2 min, at 95° for 10 min, and followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. Without detectable amplification within 40 cycles, expression values were set at 0. Different cell lines or patient samples were used as calibrators between runs.

FLT3/ITD and NPM1 mutations

DNA extraction and FLT3/ITD detection

DNA was extracted from frozen bone marrow or peripheral blood samples using Nucleon kits (Amersham Biosciences) according to the manufacturer's protocol.

Flt3 DNA (one part of exon 13, exon 15 and full exon 14) was amplified with 35 PCR cycles (94°C for 30 sec, 60°C for 1 min,

and 72°C for 1 min) using 250ng genomic DNA, 0.3µmol/L each of primers ITD-f (TGGTGTTTGTCTCTTCTTCATTGT) and ITD-r (GTTGCGTTCATCACTTTTCCAA), 750 µmol/L of deoxynucleotide triphosphate, 5mmol/L of $MgCl_2$, 1.25 units of Taq GOLD polymerase, and 1 x buffer GOLD 1 (Perkin-Elmer, Norwalk, CT, USA) in a total of 50 µL. Amplicons were migrated in 2% agarose gels, allowing the detection of a 240-bp fragment for wild-type alleles and/or longer fragments for Flt3-ITD alleles. Two categories were identified: samples showing an absence or a weak detection of the mutated allele were considered negative (ITD-) for this study, and samples with marked or predominantly mutated bands were considered positive (ITD^+).[1]

Analysis of type A, type B and type D mutations of NPM1

Specific forward and reverse primers for exon 12 of NPM1, and three specific probes for detecting type A, type B and type D mutations of NPM1 were designed by Applied Biosystems. NPM1-Forward (5'-GGATGACTGACCAAGAGGCTATTC-'3) and NPM1-reverse (5'-CAGAAATGAAATAAGACG-GAAAATTTTTTAACAAATTGT-3') primers are common, wild-type probe is FAM-5'-CTCCACTGCCAGAGAT-NFQ-3', and specific type A probe is FAM-5'-CACTGCCAGACAGA-GAT-NFQ-3', specific type B probe is FAM-5'- ACTGCCAT-GCAGAGAT-NFQ-3', specific type D probe is FAM-5'-CTGCCAGGCAGAGAT-NFQ-3'. Absolute real-time RT-PCR quantification assays were performed following the Applied Biosystems manufacturer's instructions in a final reaction volume of 20 µL, containing10 µL of 2 x TaqMan genotyping Master Mix, 10ng cDNA, and l µL of 20 x forward, reverse primers and probes (Applied Biosystems). Amplification conditions were as described above (TaqMan 7900HT, Applied Biosystems).

References

1. Marzac C, Teyssandier I, Calendini O, Perrot JY, Faussat AM, Tang R, et al. Flt3 internal tandem duplication and P-glycoprotein functionality in 171 patients with acute myeloid leukemia. Clin Cancer Res. 2006;12(23):7018-24.

Online Supplementary Table S1. Supplementary data by particular cytogenetic abnormalities and ABC gene expression.

	ABCA2	*ABCB1*	*ABCB6*	*ABCC13*	*ABCG1*	*ABCG2*
Good	0.79±0.83	0.65±0.55	0.18±0.25	0.18±0.38	1.85±1.63	0.27±0.63
t(8 ;21)	0.73±0.45	0.51±0.10	0.12±0.1	0.23±0.02	1.74±0.6	0.25±0.14
inv(16)	0.80±0.32	0.75±0.16	0.23±0.14	0.15±0.10	1.98±1.02	0.30±0.21
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
Normal	1.23±0.56	1.70±0.23	0.41±0.10	0.34±0.14	3.02±2.01	0.82±0.15
+8	1.53±0.57	1.65±1.2	0.42±0.18	0.43±0.21	2.94±1.20	0.80±0.20
11q2.3	1.55±0.69	1.62±0.27	0.36±0.14	0.38±0.1	2.68±1.03	0.75±0.13
Other	1.44±0.89	1.72±1.25	0.39±0.14	0.40±0.10	3.01±1.27	0.76±0.19
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
3q2.6	1.92±1.21	2.58±1.25	0.44±0.2	0.30±0.19	3.78±1.24	0.89±0.23
del(7)/-7	1.85±0.65	2.36±1.2	0.58±0.14	0.34±0.18	3.45±1.27	0.70±0.14
del(5)/-5	1.98±0.99	2.41±1.3	0.50±0.17	0.38±0.30	3.49±2.7	0.71±0.18
complex	1.86±0.46	2.46±1.3	0.46±0.2	0.27±0.15	3.60±1.24	0.72±0.32