# Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling

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## ABSTRACT

We examined the gene expression profiles of two independent cohorts of patients with acute myeloid leukemia [n=247 and n=214 (younger than or equal to 60 years)] to study the applicability of gene expression profiling as a single assay in prediction of acute myeloid leukemia-specific molecular subtypes. The favorable cytogenetic acute myeloid leukemia subtypes, i.e., acute myeloid leukemia with t(8;21), t(15;17) or inv(16), were predicted with maximum accuracy (positive and negative predictive value: 100%). Mutations in *NPM1* and *CEBPA* were predicted less accurately (positive predictive value: 66% and 100%, and negative predictive value: 99% and 97% respectively). Various other characteristic molecular acute myeloid leukemia subtypes, i.e., mutant *FLT3* and *RAS*, abnormalities involving 11q23, -5/5q-, -7/7q-, abnormalities involving 3q (abn3q) and t(9;22), could not be correctly predicted using gene expression profiling. In conclusion, gene expression profiling allows accurate prediction of certain acute myeloid leukemia subtypes, e.g. those characterized by expression of chimeric transcription factors. However, detection of mutations affecting signaling molecules and numerical abnormalities still requires alternative molecular methods.

Key words: acute myeloid leukemia, gene expression profiling, prediction.

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## Introduction

Acute myeloid leukemia (AML) is not a single disease but a group of neoplasms with various genetic abnormalities and variable responses to treatment. The pre-treatment karyotype is still essential in therapy decision-making in AML.[1-3] In recent years, a number of novel molecular markers have been associated with AML prognostics.[2,3] Several attempts have been made to investigate whether genome-wide *GEP* could be valuable for prediction of certain subtypes of AML.[4-12] Although there was concordance in predictive signatures in the various studies, none of those studies validated the derived signatures to predict the recurrent molecular markers using independent representative AML cohorts. The question, therefore, remains whether *GEP* could substitute current diagnostic techniques and could be applied as a reliable single test to simultaneously detect known cytogenetic and molecular abnormalities. The aim of this study was to validate *GEP* as preferred single assay to predict prognostically relevant AML subtypes using two large independent cohorts of young adults with AML.

## Design and Methods

Bone marrow aspirates or peripheral blood samples of two independent representative cohorts of *de novo* AML patients (lower or equal than 60 years), consisting of 247 and 214 patients, were collected (Table 1). The first cohort represents a subset of 285 patients previously studied,[8] while the second cohort has not yet been described.

Blast cell purification and RNA isolation were carried out as previously described.[8] All samples were analyzed using Affymetrix Human Genome U133Plus2.0 GeneChips (Affymetrix, Santa Clara, CA, USA). Labeling, hybridization, scanning and data normalization were performed as previously described.[8] The variation between the scaling/normalization factors of the GeneChips in both cohorts was less than 3-fold [cohort1: 0.53(±0.15); cohort2: 0.73(±0.20)]. Also, the percentage of genes present [cohort1: 39.1(±3.1); cohort2: 40.6(±3.7)], GAPDH 3'/5' ratio [cohort1: 1.07(±0,13); cohort2: 1.08(±0.16)] and actin 3'/5' ratio [cohort1: 1.26(±0.21); cohort2: 1.33(±0.29)] were indicative for high overall quality and consistency

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between both AML sample populations. Mutational analyses to detect recurrent mutations in AML were performed as previously described.[13-16] All supervised class prediction analyses were performed with Prediction Analysis for Microarrays (PAM) software version 1.28 in R version 2.1.0.[17]

Clinical, cytogenetic and molecular information as well as the gene expression profiles of all primary AML cases is available at the Gene Expression Omnibus (*www.ncbi.nlm.nih.gov/geo, accession number GSE6891*).

## Results and Discussion

In this study of 461 clinically and molecularly well-characterized cases of AML (Table 1), we were able to comprehensively validate the application of GEP to predict therapeutically relevant molecular subtypes in AML.

We applied PAM to investigate whether karyotypic and mutational abnormalities with prognostic or therapeutic value in AML were accurately predictable based on GEP. PAM allows the selection of the minimal number of genes required for optimal prediction, which may be beneficial in a diagnostic setting. The AML cohort1 (n=247) was used as training set to derive predictive signatures that were subsequently validated on AML cohort2 (n=214). The deduced expression signatures are available in the *Online Supplementary Tables S1-18*.

The cytogenetic status of all AML patients with favorable risk, i.e. those with t(8;21), t(15;17) or inv(16) abnormalities, was predicted with 100 percent accuracy (Table 2). In fact, among these predicted AML cases, there were cases with favorable cytogenetics that had previously been missed by routine cytogenetics (4 out of 37 inv(16) and 4 out of 25 t(15;17)). The presence of the translocation-related fusion transcripts in these specific cases was confirmed by real-time quantitative PCR. Thus, GEP is a reliable alternative to discriminate these three AML subtypes,[2,3] which represent approximately 20% of all cases.[2,3] Prediction of t(15;17) and inv(16) required only few genes, as seen previously.[8] For the t(8;21) cases, 76 probe sets were needed to correctly classify all samples. However, as few as two probe sets, including one associated with the *RUNX1T1* (*ETO*) gene, were sufficient to accurately classify all but one t(8;21) cases, which is also consistent with earlier studies[8] (*Online Supplementary Figure S3*).

AML cases with mutations in the transcription factor CCAAT/enhancer binding protein α (*CEBPA*), which are associated with a relatively favorable treatment outcome, were predicted with positive and negative predictive values of 100% and 97% respectively. Six out of 15 *CEBPA* mutant cases were missed in the validation set (sensitivity 60%; Table 2). Of note, the misclassified cases all carried a single heterozygous *CEBPA* mutation, whereas samples with biallelic mutations (either homo- or heterozygous) were all correctly recognized (*data not shown*). In the training cohort, all but two (14/16) samples carried biallelic mutations[14,18] and in cross-validation in the training cohort the two heterozygous mutants were the only misclassified samples as well.

Previous work has shown that mutations in nucleophosmin (*NPM1*) are strongly associated with a discriminative *HOX*- and *TALE* gene-specific signature.[16,19] In this study, AML cases carrying a *NPM1* mutation were indeed recognized with high accuracy based on such a signature (Table 2 and *Online Supplementary Table S5*). However, a relatively high number of AML cases without *NPM1* mutations was incorrectly predicted positive (32 out of 151), suggesting the presence of genetic alterations resulting in a similar upregulation of the *HOX*- and TALE genes in those cases. Among these false positives were several AMLs carrying 11q23 abnormalities, which is in line with the role of the mixed lineage leukemia (MLL) protein as an important regulator of *HOX* gene expression.[16] Of note, all t(6;9) AML

**Table 1. Clinical and molecular data.**

| | AML cohort 1 (n=247) | AML cohort 2 (n=214) |
|---|---|---|
| Gender | | |
| Male | 119 | 113 |
| Female | 128 | 101 |
| Age median (range) | 43 (15-60) | 46 (17-60) |
| White blood cell count (×10⁹/L) | 30 (0-278) | 29 (1-349) |
| Bone marrow blast count | 68 (0-98) | 64 (0-96) |
| Platelet count | 49 (3-931) | 59 (5-998) |
| FAB | | |
| M0 | 6 | 10 |
| M1 | 55 | 41 |
| M2 | 54 | 52 |
| M3 | 17 | 7 |
| M4 | 43 | 41 |
| M5 | 62 | 42 |
| M6 | 3 | 3 |
| not determined | 7 | 18 |
| Cytogenetics[1] | | |
| normal | 99 (41%) | 95 (46%) |
| inv(16) | 21 (9%) | 16 (7%) |
| t(15;17)[2] | 18 (7%) | 7 (3%) |
| t(8;21) | 21 (9%) | 14 (7%) |
| t(6;9) | 4 (2%) | 2 (1%) |
| abn3q | 7 (3%) | 9 (4%) |
| del5(q) | 3 (1%) | 12 (6%) |
| del7(q) | 17 (7%) | 14 (7%) |
| 11q23 | 13 (5%) | 8 (4%) |
| +8 | 22 (9%) | 11 (5%) |
| t(9;22) | 4 (2%) | 1 (<1%) |
| complex | 13 (5%) | 21 (10%) |
| other | 63 (26%) | 45 (22%) |
| Mutations[1] | | |
| CEBPA | 16 (6%) | 15 (7%) |
| *NPM1* | 77 (31%) | 63 (29%) |
| FLT3-ITD | 65 (26%) | 61 (29%) |
| *FLT3-TKD* | 30 (12%) | 19 (9%) |
| KRAS | 4 (2%) | 0 (0%) |
| *NRAS* | 23 (9%) | 22 (10%) |

*[1]All patients with a specific abnormality were considered, irrespective of the presence of additional abnormalities. Percentages were calculated based on the total number of cases investigated for the particular abnormality, as also indicated in Table 2. [2]The overall frequencies of the AML-specific recurrent (cyto)genetic abnormalities in both cohorts is similar, except for the number of AML-M3 cases carrying t(15;17), which is lower in cohort 2. In recent studies these AML t(15;17) patients were enrolled into appropriate alternative clinical protocols.*

**Table 2.** Class prediction using prediction analysis for microarrays.

| Molecular abnormality | Cross-validation error[1] | | Error validation set[2] | | Probe sets | Supp. data | Sensitivity[3] | Specificity[3] | Predictive value[3] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Neg | Pos | Neg | Pos | | | | | Neg | Pos |
| inv(16) | 0/226 | 0/21 | 0/198 | 0/16 | 2 | F1 / T1 | 100 | 100 | 100 | 100 |
| t(15;17) | 0/229 | 0/18 | 0/207 | 0/7 | 7 | F2 / T2 | 100 | 100 | 100 | 100 |
| t(8;21) | 0/226 | 0/21 | 0/200 | 0/14 | 76 | F3 / T3 | 100 | 100 | 100 | 100 |
| *CEBPA* | 3/231 | 2/16 | 0/197 | 6/15 | 15 | F4 / T4 | 60 | 100 | 97 | 100 |
| *NPM1* | 30/170 | 0/77 | 32/151 | 1/63 | 68 | F5 / T5 | 98 | 79 | 99 | 66 |
| *FLT3*-ITD | 14/182 | 17/65 | 12/153 | 20/61 | 64 | F6 / T6 | 67 | 92 | 88 | 77 |
| *FLT3*-TKD | 89/216 | 14/30 | 72/194 | 5/19 | 2307 | F7 / T7 | 74 | 63 | 96 | 16 |
| *FLT3*-ITD and/or -TKD | 34/155 | 25/91 | 24/135 | 20/78 | 407 | F8 / T8 | 74 | 82 | 85 | 71 |
| *NPM1*-/FLT3-ITD+ | 35/219 | 7/28 | 30/189 | 8/25 | 194 | F9 / T9 | 68 | 84 | 95 | 36 |
| *NPM1*+/FLT3-ITD- | 56/207 | 3/40 | 59/187 | 1/27 | 86 | F10 / T10 | 96 | 68 | 99 | 31 |
| *NPM1*+/FLT3-ITD+ | 54/210 | 1/37 | 39/178 | 1/36 | 50 | F11 / T11 | 97 | 78 | 99 | 47 |
| *KRAS* | 33/241 | 2/4 | 32/214 | 0/0 | 173 | F12 / T12 | N/A | 85 | N/A | N/A |
| *NRAS* | 65/223 | 7/23 | 55/192 | 9/22 | 225 | F13 / T13 | 59 | 71 | 94 | 19 |
| t(6;9) | 1/240 | 0/4 | 0/203 | 1/2 | 25 | F14 / T14 | 50 | 100 | 100 | 100 |
| 3q | 29/237 | 1/7 | 20/196 | 3/9 | 51 | F15 / T15 | 67 | 90 | 98 | 23 |
| -5(q) | 9/240 | 2/3 | 7/193 | 11/12 | 66 | F16 / T16 | 8 | 96 | 94 | 13 |
| -7(q) | 16/226 | 3/17 | 16/190 | 1/14 | 96 | F17 / T17 | 93 | 92 | 99 | 45 |
| 11q23 | 7/231 | 4/13 | 8/197 | 1/8 | 40 | F18 / T18 | 88 | 96 | 100 | 47 |

*[1]The prediction error was calculated by 10-fold cross validation within the training set (cohort 1) (Cross-validation error; indicated in Supplementary Figures S1 to S19). [2]The deduced gene expression signature was tested on the independent validation set (cohort 2) (Error validation set). [3]The following calculations were used for evaluation measures: positive predictive value = true positives/(true positives + false positives), negative predictive value = true negatives/(true negatives + false negatives), sensitivity = true positives/(true positives + false negatives), and specificity = true negatives/(true negatives + false positives). Values are given as percentages. The overall number of cases in cohort 1 and cohort 2 vary slightly because in rare instances the molecular abnormality was unknown. Number of probe sets: Number of probe sets used for prediction. Supp. Data: Number Online Supplementary Figure (F) and Table (T) . For identities of the probe sets and genes see Online Supplementary Tables.*



cases in the training and validation cohort (n=6) were predicted to also carry an *NPM1* mutation, raising the possibility that the DEK-CAN fusion protein also induces *HOX*-related gene expression. Interestingly, prediction of t(6;9) translocation was partly feasible using a unique signature (Table2 and *Online Supplementary Table S14*), although these results are based on a relatively low number of cases.

*NPM1* mutations are associated with relatively favorable survival parameters in patients with a normal karyotype and standard risk AML.[16,20-22] The favorable risk is particularly associated with AMLs lacking internal tandem duplications (*ITD*) in the fms-related tyrosine kinase (*FLT3*) gene.[16,20-22] Analyses of AML subsets defined by combined presence or absence of *NPM1* and *FLT3* ITD abnormalities demonstrated that only patients carrying both mutations could be moderately predicted, whereas the remaining subtypes could not be discriminated (Table 2). Restriction of these analyses to normal karyotype cases only did not result in a significant improvement in prediction accuracy (*Online Supplementary Table S19*). Of note, prediction of *NPM1* mutation in preselected normal karyotype samples led to a slightly increased positive predictive value (83 *vs.* 66%), which may be consistent with the lack of interfering 11q23 positive samples. The remaining cytogenetic and molecular subgroups we studied were not associated with strong predictive signatures. Whereas the positive predictive value for *FLT3* ITD aberrations was relatively high (77%), the high number of false predictions eliminates GEP, with the currently available analyses tools, as a reliable test to determine the *FLT3* ITD status. Restriction to the normal karyotype group did not lead to a marked improvement (*Online Supplementary Table S19*). Likewise, the low positive predictive values for FLT3 tyrosine kinase domain (TKD) or RAS mutations, abnormalities involving 11q23, -5/5q-, -7/7q- and abn3q, and the translocation t(9;22), disqualify GEP as single detection method for these abnormalities. Similarly, 3q aberrations were not readily predictable. Nevertheless, the most discriminative gene for abn3q abnormalities was the oncogenic transcription factor ecotropic viral integration site1 (*EVI1*) (*Online Supplementary Table S15*), which is frequently involved in 3q26 abnormalities. Of note, in these predictions we included the cases carrying a cryptic abn3q recently identified by gene expression analyses and fluorescence *in situ* hybridization.[23]

Classifiers were also deduced using a number of other approaches, i.e. compound covariate predictor, linear discriminant analysis, 1-nearest neighbor and 3-nearest neighbors, nearest centroid and support vector machines (probe set selection at 0.001 significance level). These alternative analyses were carried out in BRB-ArrayTools, version 3.7.0 β2 release, developed by Dr. Richard Simon and Amy Peng Lam. Overall, this comparative analysis yielded highly similar results, i.e. the favorable cytogenetic subclasses were predictable with (close to) 100% accuracy, whereas other subtypes showed a similar prediction pattern as depicted in Table 2 (*data not shown*). One exception was *NPM1* mutation status, for which prediction accuracy was better using an approach based on support vector machines (positive predictive value 91% with a negative predictive value of 99%). Several general causes

for the inability to predict specific recurrent abnormalities could apply: (i) if different recurrent genetic aberrations affect similar pathways, their GEP signatures may overlap; (ii) mutations affecting signaling pathways may not result in strong discriminative mRNA expression signatures; (iii) the expression of differentiation-related genes may affect accurate prediction; (iv) secondary mutations, or bi-allelic versus monoallelic mutations as in the case of *CEBPA*, may prohibit reliable prediction. More specifically, (v) the various partners of the MLL gene may affect reliable prediction of 11q23 abnormalities, and (vi) the numerical changes in (part of) the chromosomes 5 and 7 may only result in minor changes in gene expression that are insufficient for GEP prediction. Of note, still almost all discriminative genes with decreased expression in the deduced signature for 7(q) abnormalities were located on chromosome 7, including *FASTK, GSTK1, LSM8* and *ZNF746* (*Online Supplementary Table S17*).

Altogether, we conclude that AML cases with favorable cytogenetics are predictable with high accuracy with the currently available genome-wide gene expression technology and analyses tools. All other prognostically and therapeutically known abnormalities in AML still require additional molecular methods for detection.

## Authorship and Disclosures

RGWV: performed research, analyses and wrote manuscript; BJW: performed research, analyses and wrote manuscript; CAJE: performed research; SA: performed research; HBB: performed research; SL: performed research; BC: designed research and wrote manuscript; RD: designed research and wrote manuscript; PJMV: performed research and analyses, designed research and wrote manuscript. The authors reported no potential conflicts of interest.



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