Brief report

Molecular stratification model for prognosis in cytogenetically normal acute myeloid leukemia

Carlos M. Santamaría,[1,2] María C. Chillón,[1] Ramón García-Sanz,[1,2] Cristina Pérez,[3] María D. Caballero,[1] Fernando Ramos,[4] Alfonso García de Coca,[5] José M. Alonso,[6] Pilar Giraldo,[7] Teresa Bernal,[8] José A. Queizán,[9] Juan N. Rodriguez,[10] Pascual Fernández-Abellán,[11] Abelardo Bárez,[12] María J. Peñarrubia,[13] Ana Balanzategui,[1] María B. Vidriales,[1] María E. Sarasquete,[1] Miguel Alcoceba,[1] Joaquín Díaz-Mediavilla,[3] Jesús F. San Miguel,[1,2] and Marcos Gonzalez[1,2]

[1]Hospital Universitario, Salamanca; [2]Centro de Investigación del Cáncer-Instituto de Biologia Molecular y Celular del Cancer (Universidad de Salamanca-Consejo Superior de Investigaciones Cientificas), Salamanca; [3]Hospital Clínico San Carlos, Madrid; [4]Complejo Hospital de León and Ibiomed, Universidad de León, León; [5]Hospital Clínico de Valladolid, Valladolid; [6]Hospital Río Carrión de Palencia, Palencia; [7]Hospital Miguel Servet, Zaragoza; [8]Hospital Central de Asturias, Oviedo; [9]Hospital General de Segovia, Segovia; [10]Hospital Juan Ramón Jiménez, Huelva; [11]Hospital Universitario de Alicante, Alicante; [12]Hospital Nuestra Señora de Sonsoles, Ávila; and [13]Hospital Río Hortega, Valladolid, Spain

We have evaluated 9 new molecular markers (*ERG, EVI1, MLL-PTD, MN1, PRAME, RHAMM,* and *WT1* gene-expression levels plus *FLT3* and *NPM1* mutations) in 121 de novo cytogenetically normal acute myeloblastic leukemias. In the multivariate analysis, high *ERG* or *EVI1* and low *PRAME* expressions were associated with a shorter relapse-free survival (RFS) and overall survival (OS). A 0 to 3 score was given by assigning a value of 0 to favorable parameters (low *ERG*, low *EVI1*, and high *PRAME*) and 1 to adverse parameters. This model distinguished 4 subsets of patients with different OS (2-year OS of 79%, 65%, 46%, and 27%; *P* = .001) and RFS (2-year RFS of 92%, 65%, 49%, and 43%; *P* = .005). Furthermore, this score identified patients with different OS (*P* = .001) and RFS (*P* = .013), even within the *FLT3/NPM1* intermediate-risk/high-risk subgroups. Here we propose a new molecular score for cytogenetically normal acute myeloblastic leukemias, which could improve patient risk-stratification. (Blood. 2009;114:148-152)

Introduction

Patients with acute myeloid leukemia and normal cytogenetics (CN-AML) are usually categorized as an intermediate-risk group, with a 5-year survival rate varying between 24% and 42%. It is probable that differences in outcome reflect the molecular heterogeneity of CN-AML whose prognosis is influenced by several gene mutations or aberrant gene expression.[1,2] *FLT3* internal tandem duplications (*FLT3*-ITD),[3-5] *MLL* partial tandem duplication (*MLL*-PTD),[6] and overexpression of *ERG*,[7] *WT1*,[8] and *MN1*[9] have been associated with a poor prognosis in CN-AML, whereas *NPM1* gene mutations are associated with a favorable outcome.[10-12] Furthermore, in the intermediate- and high-risk karyotypic groups, *EVI1* overexpression is associated with an adverse prognosis,[13] whereas a high *PRAME* expression defines a good prognosis in several AML subtypes, especially those with favorable cytogenetic translocations.[14-16]

Although most studies in CN-AML patients have focused on one or 2 molecular markers, there is increasing evidence suggesting that possible outcomes based on single-gene abnormalities are hard to predict, and a more accurate prediction can be obtained by identifying risk categories based on the information provided by 2 or more parameters.[1] For this reason, we have simultaneously evaluated 9 new molecular markers in 121 CN-AML patients, showing that *ERG, EVI1,* and *PRAME* afford independent prognostic information and allow us to establish a simple score system for risk stratification.

Methods

We have analyzed pretreatment bone marrow samples from 121 adults diagnosed as novo CN-AML. All patients were treated according to the Spanish Program for the Study and Treatment of Malignant Hemopathies (PETHEMA) LAM-99 protocols.[17] Ten patients (8.3%) died before they had reached complete remission (CR), 91 (75.2%) achieved CR with induction therapy, and 20 (16.5%) were refractory to the standard induction treatment. Nine patients from this latter group achieved CR after salvage therapy. Finally, 43 of the 100 patients who achieved CR eventually relapsed during the evaluation period. The median follow-up for censored patients was 26 months (range, 10-72 months). In addition, 10 bone marrow samples from healthy donors were processed as controls for gene-expression analysis. Informed consent to use biologic samples and clinical data were obtained in all cases in accordance with the Declaration of Helsinki. This study was approved by the Institutional Review Board of the University Hospital of Salamanca and the Scientific and Ethics Committee of the PETHEMA group.

Total RNA from diagnostic bone marrow and subsequent reverse transcription were performed according to the protocols approved by the Europe against Cancer Group program.[18] All samples were analyzed for *FLT3*-ITD,[5] mutations in NPM1,[11] and relative expression of the following genes: *ABL1* (as control gene), *ERG, EVI1, MLL*-PTD, *MN1, PRAME, RHAMM,* and *WT1*, using the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Relative quantification was calculated using the equation $2^{-\Delta\Delta Ct}$, as previously described.[16] The prognostic impact of the gene expression was evaluated using quartiles as cutoff points and selecting the one with the lowest *P* value.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Table 1. Influence of clinical-biologic characteristics and gene expression profile at diagnosis in 121 CN-AML patients regarding their OS and RFS

	OS (n = 121)					RFS (n = 100)				
	n	2-year, %	Univariate	Multivariate*	HR (95% CI)†	n	2-year, %	Univariate	Multivariate*	HR (95% CI)†
Clinical-biologic feature										
WBC at diagnosis, × 10⁹/L‡			.001	< .001				.018	.031	
Less than or equal to 50.0	88	62			2.8 (1.6-4.8)	76	64			2.1 (1.0-5.2)
More than 50.0	33	35				24	33			
Age, y‡§			.003	.004				.008	.006	
Less than or equal to 65	97	59			2.5 (1.3-4.5)	84	62			2.7 (1.4-5.6)
More than 65	24	36				16	28			
FLT3/NPM1 phenotype			.070	.055				.030	.037	
*FLT3*wt/*NPM1*mutated	38	61			ND	34	70			2.1 (1.1-4.0)
Other phenotypes	83	50				66	49			
Sex			.080	> .1				.056	.074	
Male	61	47			ND	49	48			ND
Female	60	60				51	63			
Platelet at diagnosis, × 10⁹/L‖			> .1	ND				> .1	ND	
Less than or equal to 60	61	49			ND	51	50			ND
More than 60	60	59				49	62			
Hemoglobin, g/dL‖			> .1	ND				> .1	ND	
Less than or equal to 9.1	61	56			ND	51	60			ND
More than 9.1	60	52				49	51			
PB blasts at diagnosis, %‖			> .1	ND				> .1	ND	
Less than or equal to 44	61	55			ND	52	56			ND
More than 44	60	53				48	54			
BM blasts at diagnosis, %‖			> .1	ND				> .1	ND	
Less than or equal to 67	61	53			ND	61	56			ND
More than 67	60	54				60	55			
Gene expression (quartiles)¶										
ERG (median)			.020	.024				.010	.014	
Less than or equal to 0.54	61	66			1.9 (1.1-3.3)	54	67			2.2 (1.1-3.8)
More than 0.54	60	42				46	44			
PRAME (75th percentile)			.035	.066				.017	.026	
Less than or equal to 150	91	51			ND	74	48			0.4 (0.2-0.9)
More than 150	30	63				26	79			
EVI-1 (75th percentile)				.030					.050	
Less than or equal to 0.2	91	59	.042		1.9 (1.0-3.3)	77	60	.051		2.0 (1.0-3.8)
More than 0.2	30	42				23	46			
MLL-PTD (75th percentile)			> .1					.061	> .1	
Less than or equal to 0.3	91	56		ND	ND	73	59			ND
More than 0.3	30	46				27	46			
WT1 (75th percentile)			> .1	ND				> .1	ND	
Less than or equal to 374	91	56			ND	75	59			ND
More than 374	30	46				25	47			
MN1 (median)			> .1	ND					ND	
Less than or equal to 50	61	59			ND	54	58	> .1		ND
More than 50	60	47				46	53			
RHAMM (75th percentile)			> .1	ND					ND	
Less than or equal to 1.3	91	55			ND	75	57	> .1		ND
More than 1.3	30	47				25	52			

HR indicates hazard ration; and ND, not done.

*Multivariate analysis was performed by including those features with a *P* value < .1 in the univariate analysis. Only variables with a *P* value less than .05 in the Cox regression model were considered as having an independent prognostic value.

†Hazard ratio (HR) was calculated for WBC > 50 × 10⁹/L, age > 65 years, non-*FLT3*wt/*NPM1*mutated phenotype, and high *ERG, EVI1*, or *PRAME* expression.

‡Variables were dichotomized based on high-risk criteria.

§Fifty-two of 84 (62%) patients younger than 65 years in complete remission underwent an autologous stem cell transplantation (SCT), whereas 19 (23%) received an allogeneic-SCT.

‖Variables were dichotomized based on median value.

¶For each gene, the quartile providing the best separation of survival curves (lowest *P* value) is shown.
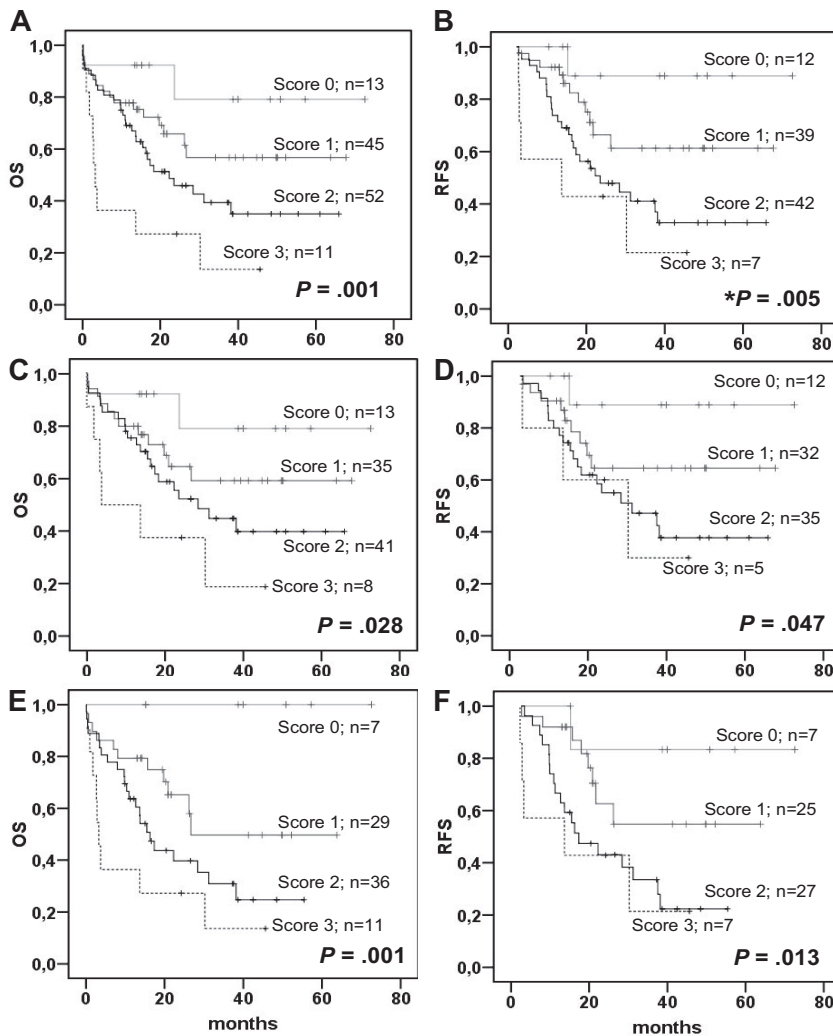
Figure 1. OS and RFS of CN-AML patients based on ERG/EVI1/PRAME score. CN-AML patients were grouped according to favorable (low *ERG*, low *EVI1*, or high *PRAME*) or adverse (high *ERG*, high *EVI1*, or low *PRAME*) expression profile, according to the selected quartile in the individual analysis. Therefore, the proposed score was drawn up by assigning a value of 0 for each gene expressing a favorable RNA level and a value of 1 for each gene with an adverse expression profile. (A-B) OS and RFS for the 121 CN-AML patients, respectively. (C-D) Survival analyses for 97 CN-AML patients younger than 65. (E-F) Survival analyses for 83 CN-AML patients included within the *FLT3/NPM1* intermediate-risk/high-risk subgroups [*FLT3*wt/*NPM1*wt (n = 47), *FLT3*-ITD/*NPM1* mutated (n = 20), or *FLT3*-ITD/*NPM1*wt (n = 16)]. A 2-year OS of 100%, 64%, 39%, and 27% was observed for scores 0, 1, 2, and 3, respectively (P = .001; E), whereas the 2-year RFS for the same subgroups was 86%, 62%, 43%, and 43% (P = .013; F). Similar results were observed for the other combinations of *FLT3* and *NPM1*, although statistically significant differences were achieved only in the *FLT3*wt/*NPM1*wt (OS, P = .016; and RFS, P = .019) because of the sample size in these subgroups. *P < .001 if the analysis is restricted to nonrefractory patients.

All tests were carried out using the SPSS 15.0 program (SPSS). For univariate analyses, the Student *t* test was performed to evaluate refractoriness to treatment and gene-expression levels. The relapse-free survival (RFS) and overall survival (OS) were calculated using the Kaplan-Meier method. The impact of multiple predictor variables on RFS and OS was assessed by multivariate analysis according to the Cox regression model (forward conditional method), as described elsewhere.[16]

Results and discussion

Patients with clinically adverse features, such as white blood cell (WBC) counts more than 50×10^9/L and an age greater than 65 years, were associated with a poorer OS and RFS, whereas patients harboring a *FLT3* wild-type (wt) and *NPM1*-mutated phenotype were associated with a better prognosis (Table 1). In addition, molecular markers with a clinical impact on OS were: *ERG* (50th percentile, P = .020), *PRAME* (75th percentile, P = .035), and *EVI1* (75th percentile, P = .042). Similarly, the genes that showed significant influence on RFS were: *ERG* (P = .010), *PRAME* (P = .017), and *EVI1* (P = .051). Interestingly, patients who were refractory to induction therapy showed higher *ERG* (1.0 ± 0.8 vs 0.6 ± 0.6; P = .01) and lower *PRAME* (29 ± 53 vs 1641 ± 6102; P = .01) levels compared with patients who achieved CR after the induction therapy.

Features selected in the multivariate analysis as having an independent prognostic value for a shorter OS were: WBC more

than 50×10^9/L (P < .001), age more than 65 years (P = .004), high *ERG* expression (P = .024), and high *EVI1* expression (P = .030). In addition, patients with no *FLT3*wt/*NPM1*-mutated phenotype (P = .055) and a low *PRAME* expression (P = .066) showed a trend toward a poorer OS. Parameters with an independent prognostic value for a shorter RFS were: age more than 65 years (P = .006), high *ERG* expression (P = .014), low *PRAME* expression (P = .026), WBC more than 50×10^9/L (P = .031), no *FLT3*wt/*NPM1*-mutated phenotype (P = .037), and high *EVI1* expression (P = .050). Our data confirm the adverse prognostic influence that has been shown for *ERG*[7,19] and *EVI1*[13] genes. Preliminary studies have suggested that *PRAME* overexpression is associated with a good prognosis in childhood AML, although this effect might be the result of its correlation with favorable cytogenetics, ie, t(8;21).[15] Here we show, for the first time, that the prognostic value of *PRAME* up-regulation is independent of other karyotypic abnormalities because *PRAME* overexpression was associated with a better response to induction therapy and longer survival in our series, in which all patients had a normal cytogenetics.

Based on the results described, we investigated whether the combination of the *ERG*, *EVI1*, and *PRAME* markers could improve their individual prognostic significance. Thus, we drew up a molecular score by assigning a value of 1 point per gene expression associated with an adverse prognosis (high *ERG*, high *EVI1*, and low *PRAME* RNA levels). By contrast, a value of 0 was

assigned to a favorable expression profile (low *ERG* or low *EVI1* or high *PRAME*). This score allowed us to discriminate 4 different risk categories for both OS and RFS analysis, independently of other clinical-biologic features. The 2-year OS for scores 0, 1, 2, and 3 was 79%, 65%, 46%, and 27%, respectively ($P = .001$; Figure 1A). Moreover, the 2-year RFS for the same subgroups was 92%, 65%, 49%, and 43%, respectively ($P = .005$; Figure 1B). Similar results were observed when the analysis was restricted to the 97 patients younger than 65 years (Figure 1C-D). Multivariate analysis confirmed the findings in the complete series because the features selected as having an independent prognostic value for either a shorter OS or RFS were: the proposed molecular score ($P < .001$ and $P < .001$), WBC counts more than 50×10^9/L ($P = .002$ and $P = .04$), and age more than 65 years ($P = .007$ and $P = .005$). Furthermore, the *FLT3*wt/*NPM1*-mutated phenotype displayed an independent prognostic value in the multivariate analysis for better RFS ($P = .05$) and a trend toward longer OS ($P = .09$).

A further benefit of the proposed score was the discrimination between different prognostic categories within those patients considered as having an intermediate-risk/high-risk based on the *FLT3/NPM1* classification.[10-12] Thus, patients harboring *FLT3*wt/*NPM1*wt (n = 47) or *FLT3*-ITD/*NPM1*-mutated (n = 20) or *FLT3*-ITD/*NPM1*wt (n = 16) phenotype displayed differentiated OS ($P = .001$; Figure 1E) and RFS ($P = .013$; Figure 1F) according to score subgroup. It is worth noting that scores 0 and 1 showed that 43% of patients (36 of 83) had a good prognosis, which could be considered as redefining their risk category.

Our score system integrates 3 prognostic markers that could provide a more accurate stratification than single marker analysis[7,9,13]; and, unlike wide gene-expression profiling,[14,20] it could be easily implemented in the context of routine clinical laboratories. Nevertheless, because a molecular score based on gene-expression levels could be less objective than mutation assessment,[2,21] this score system needs to be validated in an independent series of patients before it is incorporated into clinical practice.[20,22]

In conclusion, we propose a score based on *ERG*, *EVI1*, and *PRAME* gene expression that allows a greater distinction between CN-AML patients with significantly different outcomes.

Authorship

Contribution: C.M.S. and M.C.C. carried out all molecular studies and prepared the database for the final analysis; C.M.S. performed the statistical analysis and prepared the initial version of the paper; R.G.-S. helped in the design of the work, reviewed the database, contributed toward the statistical analysis, and provided the preapproval of the final version; A. Balanzategui, M.E.S., and M.A. participated in the generation of the molecular results; C.P., M.D.C., F.R., A.G.d.C., J.M.A., P.G., T.B., J.A.Q., J.N.R., P.F.-A., A. Bárez, M.J.P., M.B.V., and J.D.-M. were the clinicians responsible for the patients and who ensured the application of protocol, sampling, and the collection of clinical data; J.F.S.M. and M.G. initially promoted the study, were responsible for securing financial support, were responsible for the group, and were responsible for the most important revision of the draft; and M.G. approved the final version to be sent to the editor.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Ramón García-Sanz, Department of Hematology, University Hospital of Salamanca, Paseo de San Vicente, 58-182, Salamanca, 37007 Spain; e-mail: rgarcias@usal.es.

References

1. Mrozek K, Marcucci G, Paschka P, Whitman SP, Bloomfield CD. Clinical relevance of mutations and gene-expression changes in adult myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification? *Blood.* 2007;109:431-448.

2. Schlenk RF, Dohner K, Krauter J, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med.* 2008;358:1909-1918.

3. Kottaridis PD, Gale RE, Frew ME, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood.* 2001;98:1752-1759.

4. Schnittger S, Schoch C, Dugas M, et al. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood.* 2002;100:59-66.

5. Thiede C, Steudel C, Mohr B, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood.* 2002;99:4326-4335.

6. Whitman SP, Liu S, Vukosavljevic T, et al. The MLL partial tandem duplication: evidence for recessive gain-of-function in acute myeloid leukemia identifies a novel patient subgroup for molecular-targeted therapy. *Blood.* 2005;106:345-352.

7. Marcucci G, Baldus CD, Ruppert AS, et al. Overexpression of the ETS-related gene, ERG, predicts a worse outcome in acute myeloid leukemia with normal karyotype: a Cancer and Leukemia Group B study. *J Clin Oncol.* 2005;23:9234-9242.

8. Barragán E, Cervera J, Bolufer P, et al. Prognostic implications of Wilms' tumor gene (WT1) expression in patients with de novo acute myeloid leukemia. *Haematologica.* 2004;89:926-933.

9. Heuser M, Beutel G, Krauter J, et al. High meningioma 1 (MN1) expression as a predictor for poor outcome in acute myeloid leukemia with normal cytogenetics. *Blood.* 2006;108:3898-3905.

10. Dohner K, Schlenk RF, Habdank M, et al. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. *Blood.* 2005;106:3740-3746.

11. Schnittger S, Schoch C, Kern W, et al. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. *Blood.* 2005;106:3733-3739.

12. Thiede C, Koch S, Creutzig E, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood.* 2005;107:4011-4020.

13. Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, van Putten WL, et al. High EVI1 expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. *Blood.* 2003;101:837-845.

14. Bullinger L, Dohner K, Bair E, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med.* 2004;350:1605-1616.

15. Greiner J, Schmitt M, Li L, et al. Expression of tumor-associated antigens in acute myeloid leukemia: implications for specific immunotherapeutic approaches. *Blood.* 2006;108:4109-4117.

16. Santamaria C, Chillon MC, Garcia-Sanz R, et al. The relevance of preferentially expressed antigen of melanoma (PRAME) as a marker of disease activity and prognosis in acute promyelocytic leukemia. *Haematologica.* 2008;93:1797-1805.

17. Suarez L, Vidriales MB, Moreno MJ, et al. Differences in anti-apoptotic and multidrug resistance phenotypes in elderly and young acute myeloid leukemia patients are related to the maturation of blast cells. *Haematologica.* 2005; 90:54-59.

18. Gabert J, Beillard E, van der Velden V, et al. Standardization and quality control studies of "real-time" quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia: a Europe Against Cancer program. *Leukemia.* 2003;17: 2318-2357.

19. Marcucci G, Maharry K, Whitman SP, et al. High expression levels of the ETS-related gene, ERG, predict adverse outcome and improve molecular risk-based classification of cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B Study. *J Clin Oncol.* 2007;25:3337-3343.

20. Metzeler KH, Hummel M, Bloomfield CD, et al. An 86-probe-set gene-expression signature predicts survival in cytogenetically normal acute myeloid leukemia. *Blood.* 2008;112:4193-4201.

21. Paschka P, Marcucci G, Ruppert AS, et al. Wilms tumor 1 gene mutations independently predict poor outcome in adults with cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B Study. *J Clin Oncol.* 2008;26: 4595-4602.

22. Radmacher MD, Marcucci G, Ruppert AS, et al. Independent confirmation of a prognostic gene-expression signature in adult acute myeloid leukemia with a normal karyotype: a Cancer and Leukemia Group B study. *Blood.* 2006;108:1677-1683.