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## Epigenetics Meets Genetics in Acute Myeloid Leukemia: Clinical Impact of a Novel Seven-Gene Score

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### ABSTRACT

#### Purpose

Molecular risk stratification of acute myeloid leukemia (AML) is largely based on genetic markers. However, epigenetic changes, including DNA methylation, deregulate gene expression and may also have prognostic impact. We evaluated the clinical relevance of integrating DNA methylation and genetic information in AML.

#### Methods

Next-generation sequencing analysis of methylated DNA identified differentially methylated regions (DMRs) associated with prognostic mutations in older ( $\geq 60$  years) cytogenetically normal (CN) patients with AML ( $n = 134$ ). Genes with promoter DMRs and expression levels significantly associated with outcome were used to compute a prognostic gene expression weighted summary score that was tested and validated in four independent patient sets ( $n = 355$ ).

#### Results

In the training set, we identified seven genes (*CD34*, *RHOC*, *SCRN1*, *F2RL1*, *FAM92A1*, *MIR155HG*, and *VWA8*) with promoter DMRs and expression associated with overall survival (OS;  $P \leq .001$ ). Each gene had high DMR methylation and lower expression, which were associated with better outcome. A weighted summary expression score of the seven gene expression levels was computed. A low score was associated with a higher complete remission (CR) rate and longer disease-free survival and OS ( $P < .001$  for all end points). This was validated in multivariable models and in two younger ( $< 60$  years) and two older independent sets of patients with CN-AML. Considering the seven genes individually, the fewer the genes with high expression, the better the outcome. Younger and older patients with no genes or one gene with high expression had the best outcomes (CR rate, 94% and 87%, respectively; 3-year OS, 80% and 42%, respectively).

#### Conclusion

A seven-gene score encompassing epigenetic and genetic prognostic information identifies novel AML subsets that are meaningful for treatment guidance.

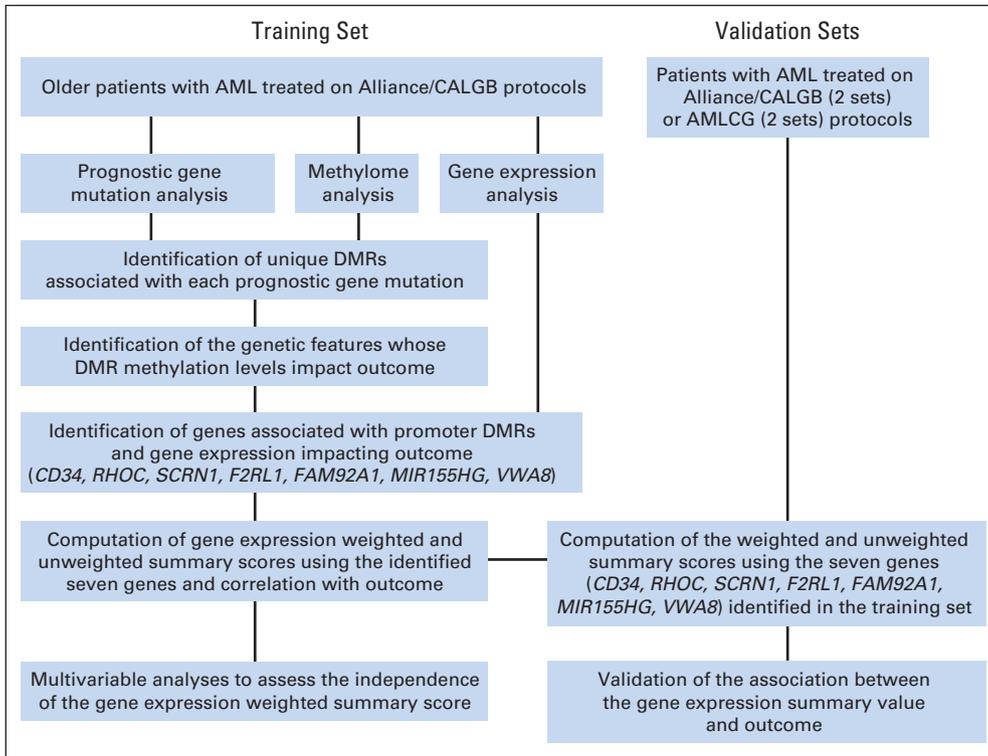
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### INTRODUCTION

Most adults with acute myeloid leukemia (AML) are not cured with current therapies, with only approximately 40% of younger (age  $< 60$  years) and approximately 10% of older (age  $\geq 60$  years) patients achieving long-term survival.<sup>1,2</sup> Therefore, novel strategies are needed to improve outcome and identify new therapeutic targets. To achieve this goal, recent studies have focused on identifying molecular features associated with clinical outcome and have applied them to patients' risk stratification and treatment guidance.<sup>3</sup> To date, disease classification

and prognostication for patients with AML have been largely based on cytogenetic and genetic testing, whereas other factors, including epigenetic changes, have not been considered.<sup>3,4</sup>

Epigenetics describes modifications of gene expression without alteration of DNA sequences.<sup>5,6</sup> Aberrant DNA methylation is among the best characterized and therapeutically targetable of the epigenetic changes occurring in leukemia.<sup>5,6</sup> However, most studies of aberrant DNA methylation in AML have focused on changes occurring only in subsets of genes, and few reports have analyzed whole-genome DNA methylation changes.<sup>7,8</sup> Prognostic signatures



**Fig 1.** Overview of the experimental strategy applied to a training set and validation sets of patients with cytogenetically normal acute myeloid leukemia (AML). Established AML prognostic mutations were studied for differentially methylated regions (DMRs) associated with each of the mutations. The association of the DMR methylation levels of distinct genomic features with outcome (ie, overall survival) was analyzed. Promoter DMRs were associated with outcome. Genes with DMRs and expression significantly associated with outcome at  $P \leq .001$  were identified. Using the expression levels of these genes, a weighted summary score was computed and its clinical impact was tested in the training set and validated in four independent sets of patients. AMLCG, German Acute Myeloid Leukemia Cooperative Group; CALGB, Cancer and Leukemia Group B.

based solely on DNA methylation have been reported,<sup>7,9</sup> but to our knowledge, an integrated and clinically applicable biomarker in AML that encompasses both genetic and epigenetic information for prognostication and treatment response prediction is not available. Therefore, we developed a novel approach that used the prognostic methylated DNA profiles associated with known prognostic gene mutations to compute a clinically meaningful gene expression summary score. This score was studied in cytogenetically normal (CN) patients with AML, the largest cytogenetic AML subgroup.

**METHODS**

For detailed methods see the Data Supplement. For study strategy and glossary, see Figure 1 and Table 1, respectively.

**Patients and Treatment**

A training set of older ( $\geq 60$  years) patients ( $n = 134$ ) with untreated primary CN-AML who received first-line cytarabine-daunorubicin-based therapy on Cancer and Leukemia Group B (CALGB)/Alliance first-line protocols were sequenced for methylated DNA using a high-throughput approach.<sup>10</sup> Of 134 patients, 126 also had gene expression data measured.<sup>11,12</sup>

**Table 1.** Glossary

Term	Description
Prognostic mutations	<i>NPM1</i> , <i>FLT3</i> -ITD, <i>DNMT3A</i> ( <i>DNMT3A</i> R882, <i>DNMT3A</i> non-R882), <i>IDH1</i> , <i>IDH2</i> ( <i>IDH2</i> R140, <i>IDH2</i> R172), <i>TET2</i> , <i>RUNX1</i> , <i>CEBPA</i> ( <i>CEBPA</i> -2mut, <i>CEBPA</i> -1mut), <i>FLT3</i> -TKD, <i>AXSL1</i> , <i>WT1</i> , <i>MLL</i> -PTD.
DMRs	Regions with significant changes in the levels of DNA methylation when patients with mutations v wild-type patients were compared. The analysis to identify the DMRs was repeated for each of the prognostic mutations.
Unique DMRs	DMRs associated with only one of the prognostic gene mutations.
High-methylation DMRs	DMRs with methylation level higher in the mutated v wild-type patients.
Low-methylation DMRs	DMRs with methylation level lower in the mutated v wild-type patients.
Genomic features	Large genomic regions with distinct putative functions. They host DMRs.
Weighted summary score	A linear combination of the median dichotomized values of the expressions of seven genes whose promoter DMR methylation and expression levels were simultaneously predictive of clinical outcome. The weights for the linear combination were the overall survival Cox regression coefficients of the genes. The median value of the score was used to dichotomize patients into those with high and low scores for prognostic analyses.
Unweighted summary score	A linear combination of the median dichotomized values of the expressions of seven genes whose promoter DMR methylation and expression levels were both predictive of clinical outcome. This score indicated the number of genes that were highly expressed in each patient.

Abbreviation: DMR, differentially methylated region.

Four independent sets comprising 355 patients were used for validation. Two sets comprised older and younger (< 60 years) adults with untreated, primary CN-AML receiving intensive cytarabine-daunorubicin-based therapy on CALGB/Alliance first-line protocols (Data Supplement). Two other sets comprised older and younger adult patients with CN-AML treated on the German AML Cooperative Group (AMLCG) 1999 trial.<sup>11</sup>

All patients provided written informed consent. Study protocols were in accordance with the Declaration of Helsinki and approved by institutional review boards at each center.

### Genetic Molecular Analyses

For the CALGB/Alliance training and validation sets, prognostic mutations (*NPM1*, *FLT3-ITD*, *DNMT3A* [*DNMT3A* R882, *DNMT3A* non-R882], *IDH1*, *IDH2* [*IDH2* R140, *IDH2* R172], *TET2*, *RUNX1*, *CEBPA* [*CEBPA*-2mut, *CEBPA*-1mut], *FLT3-TKD*, *AXSL1*, *WT1*, *MLL-PTD*; Fig 1; Data Supplement) and gene expression microarray data were analyzed centrally.<sup>11,12</sup> For patients in the AMLCG validation sets, molecular status, gene expression, and outcome were previously reported.<sup>11</sup>

### DNA Methylation Analysis

For the training set, high-throughput next-generation sequencing of methylated DNA fragments (MethylCap-seq; Diagenode, Denville, NJ; 150 to 200 base pairs) enriched by MBD2 protein (MethylMiner Methylated DNA Enrichment Kit; Invitrogen, Grand Island, NY) was performed on pretreatment bone marrow, as previously reported.<sup>10</sup> Reduced representation bisulfite sequencing<sup>13</sup> and MassArray<sup>14</sup> were used to validate the MethylCap-seq findings (Data Supplement).

### Differentially Methylated Regions

In the training set, DNA methylation patterns associated with each of the prognostic mutations were identified by comparing patients mutated for each mutation with patients wild-type for that mutation. Differentially methylated regions (DMRs) were defined as DNA sequences with significantly different DNA methylation levels between mutated and wild-type patients (Wilcoxon rank sum test). *P* values were adjusted for multiple comparisons using false discovery rate correction. Only regions with a false discovery rate cutoff of 0.05 were deemed DMRs. DMRs were considered unique if they were associated with only one prognostic mutation. If the same DMR was identified for two or more mutations, it was not included in further analyses. High- and low-methylation DMRs were DMRs with median methylation levels higher or lower, respectively, in the mutated than in the wild-type patients.

### Definition of Genomic Features

Distribution of all unique DMRs among genomic features, large genomic regions with putative distinct functions, was evaluated. For this analysis, seven genomic features (Data Supplement) were selected for their potential impact on gene expression.<sup>10,15-22</sup> The association of the summed DMR methylation levels with outcome was tested for each genomic feature. We found that the summed DMR methylation levels of the RefSeq gene promoter (hereafter called promoter) genomic features impacted on outcome (*P* ≤ .05).

### Association of DMRs With Gene Expression

The promoter genomic features are part of genes. This enabled us to identify in the training set genes whose promoter DMR methylation levels and expression levels were associated with outcome (see Statistical Analyses). The genes with both methylation and expression levels associated with overall survival (OS) at *P* ≤ .001 were used to compute a gene expression weighted summary score.

### Statistical Analyses

Definition of outcome end points and detailed statistical methods, including the derivation of the seven-gene expression weighted summary score, are reported in the Data Supplement (see also the footnote marked with a dagger symbol (†) in Table 4). The associations of the weighted summary score (low/high) with baseline clinical, demographic, and molecular features were compared using the Wilcoxon rank sum and Fisher's exact tests for continuous and categorical variables, respectively. For time-to-event analyses, survival

estimates were calculated using the Kaplan-Meier method, and groups were compared using the log-rank test.

We compared the weighted summary score with other molecular prognosticators and previously reported profiles by computing the Akaike information criterion (AIC).<sup>23</sup> Because the weights in the expression weighted summary score were nearly identical for all genes, we also tested an unweighted summary score that simply uses the number of individual genes with high expression. Additional detailed methodology on multivariable analyses and statistical methods are provided in the Data Supplement.

## RESULTS

### DMRs Associated With Established Prognostic Gene Mutations in CN-AML

To develop a molecular risk score that incorporated both epigenetic and genetic prognostic information (Fig 1), we postulated that these mutations in CN-AML are characterized by distinct DNA methylation patterns that complement their prognostic impact. Furthermore, because both mutations and DNA methylation changes may impact on gene expression, we also postulated that it is possible to derive a gene expression profile that depends on and encompasses the prognostic significance of both mutations and their corresponding DMRs.

To test these hypotheses, we analyzed a training set of older patients with CN-AML (*n* = 134) who were molecularly characterized for the most common prognostic mutations in AML (Data Supplement). The comparisons of mutated versus wild-type patients for each of the 15 mutations considered revealed 32,681 unique DMRs (Fig 2; Data Supplement). Comparative methylation analyses using alternative techniques (ie, reduced representation bisulfite sequencing and MassArray assays) validated our DMR calls (Data Supplement).

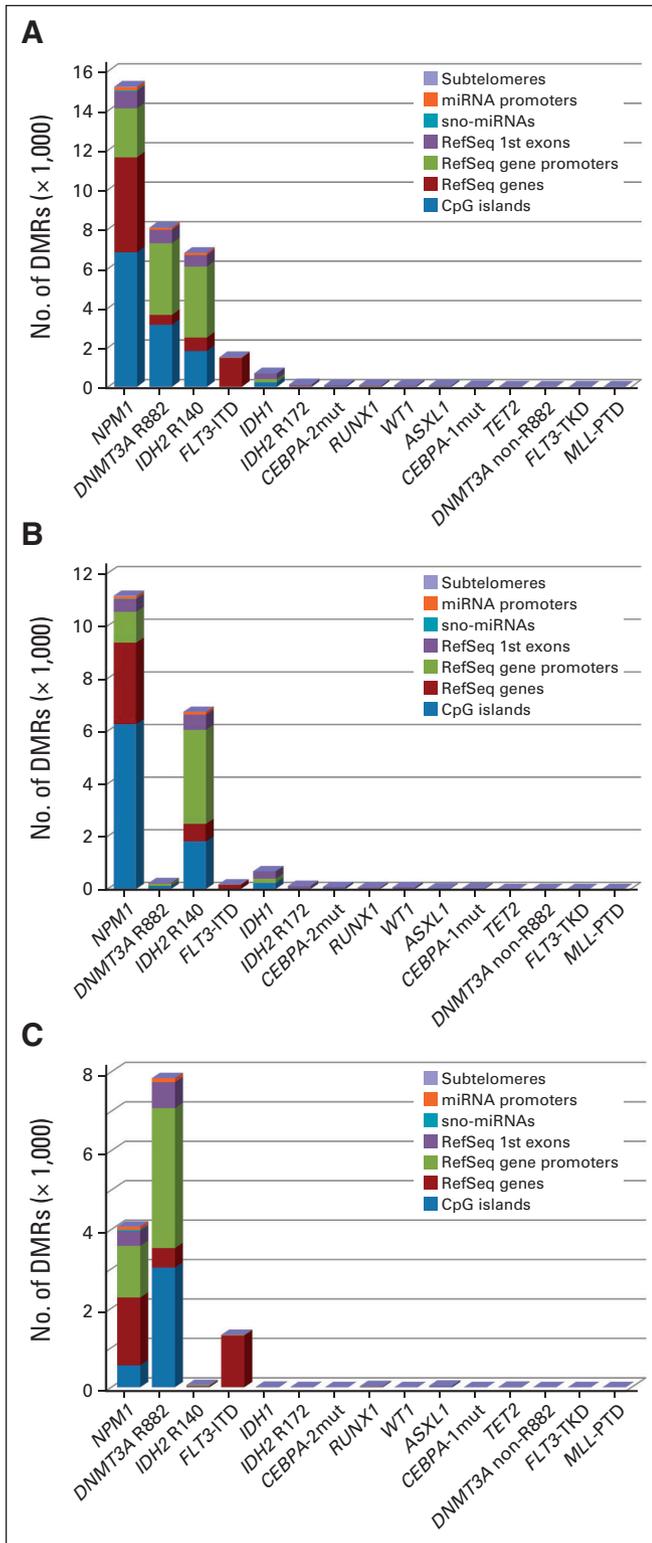
Nearly all DMRs (99.0%) were associated with *NPM1* (46.5%), *DNMT3A*-R882 (24.7%), *IDH* mutations (23.2%), or *FLT3-ITD* (4.6%; Data Supplement). Of the 32,681 DMRs, 59% were high-methylation DMRs. *IDH* and *NPM1* mutations were significantly associated with high-methylation DMRs (*P* < .001 for both), and *DNMT3A*-R882 mutation and *FLT3-ITD* were significantly associated with low-methylation DMRs (*P* < .001 for both). The findings were validated using permutation and equal patient number sampling tests (Data Supplement).

### Integrating Epigenetic and Genetic Information in the Training Set

Distribution of the DMRs among the genomic features is shown in the Data Supplement. The summed methylation levels of DMRs in the promoter features impacted on outcome (data not shown). Summed high-methylation levels of promoter DMRs were associated with longer OS than low-methylation levels (*P* = .02; 3-year OS, 28% v 9%, respectively).

The promoter genomic feature is associated with gene coding sequences. We identified 82 individual genes whose promoter DMRs were associated with OS (*P* ≤ .001; Data Supplement). For 80 of them, higher DMR methylation was associated with longer OS (*P* ≤ .001).

Seven of the 82 genes (*CD34*, *RHOC*, *SCRN1*, *F2RL1*, *FAM92A1*, *MIR155HG*, and *VWA8*; Table 2; Data Supplement) had not only DMRs but also expression levels that were associated with outcome (*P* ≤ .001 for OS). For each of these seven genes, lower expression and higher DNA methylation were associated with longer OS. With the



**Fig 2.** Unique differentially methylated regions (DMRs) in distinct genomic features identified by comparing patients with cytogenetically normal acute myeloid leukemia with each of the prognostic gene mutations with wild-type patients for that gene mutation. The height of each color block depicts the number of DMRs identified in the respective genomic feature. Data are shown for (A) all DMRs, (B) high-methylation DMRs, and (C) low-methylation DMRs. Genomic regions were selected based on their reported function (Data Supplement), as follows: subtelomeres; microRNA (miRNA) promoters; sno-miRNA; RefSeq first exon; RefSeq gene promoters; RefSeq genes; and CpG islands.

exception of *VWA8*, all of these genes had previously been associated with leukemia (*CD34* and *MIR155HG*) or other cancer types (*MIR155HG*, *RHOC*, *SCRN1*, *F2RL1*, and *FAM92A1*).<sup>24-30</sup>

**Computing a Prognostic Weighted Summary Score**

A weighted summary score of the expression levels of the seven genes was computed and tested for its prognostic significance in the training set. Patients were dichotomized into low- and high-score groups using as a cutoff the median of the patients’ score values. Low-score patients presented more frequently with prognostically favorable molecular markers (ie, *NPM1* mutations and low *BAALC*, *ERG*, and *MN1* expression) and less frequently with unfavorable markers (ie, *FLT3-ITD*, *ASXL1*, and *RUNX1* mutations) compared with high-score patients (Data Supplement). Low-score patients had higher complete remission (CR) rates ( $P < .001$ ) and longer disease-free survival (DFS;  $P < .001$ ) and OS ( $P < .001$ ) than high-score patients (Table 3, Fig 3A). When we compared the weighted summary score with previously reported prognostic molecular markers, the AIC value of the weighted summary score was lower than that of all other prognostic markers, suggesting that the weighted summary score provided the best prognostic information for all three clinical end points (Data Supplement).

In multivariable analyses, the weighted summary score was independently associated with higher CR rates ( $P = .01$ ) and longer DFS ( $P < .001$ ) and OS ( $P < .001$ ), after adjusting for other clinical and molecular prognosticators (Table 4). Patients with low scores had a more than 80% increase in the odds of achieving CR and approximately 3.5-fold decrease in the risk of disease relapse or death compared with patients with high scores.

**Validation of the Weighted Summary Score**

The clinical impact of the weighted summary score was validated in four independent CN-AML patient sets (n = 355). Validation set 1 comprised older CALGB/Alliance patients with primary AML; patients with low scores had a trend for higher CR rates ( $P = .12$ ) and longer DFS ( $P = .04$ ) and OS ( $P = .02$ ) than those with high scores (Table 3; Fig 3B). Validation set 2 comprised younger CALGB/Alliance patients with primary AML; patients with low scores had a higher CR rate ( $P = .004$ ) and longer DFS ( $P < .001$ ) and OS ( $P < .001$ ) than those with high scores (Table 3; Fig 3C). Validation sets 3 and 4 comprised older and younger AMLCG patients with CN-AML, respectively.<sup>11</sup> In validation set 3, a low score was associated with longer DFS ( $P < .001$ ) and OS ( $P = .01$ ) but not a significantly better CR rate ( $P = .20$ ; Table 3; Fig 3D). In validation set 4, a low score was associated with a better CR rate ( $P = .007$ ) and longer DFS ( $P = .003$ ) and OS ( $P < .001$ ; Table 3; Fig 3E).

When we considered all patients together (training and validation sets; n = 481), those with low scores, compared with those with high scores, had a higher CR rate (85% v 58%, respectively;  $P < .001$ ) and longer DFS (3-year DFS, 40% v 15%, respectively;  $P < .001$ ) and OS (3-year OS, 48% v 15%, respectively;  $P < .001$ ; Data Supplement). In multivariable models, the weighted summary score remained an independent predictor for CR ( $P < .001$ ), DFS ( $P < .001$ ), and OS ( $P < .001$ ), after adjusting for other clinical and molecular variables (Data Supplement).

When we compared the weighted summary score with previously reported prognostic gene expression profiles, the AIC values of the weighted summary score were lower than the AIC values for the

**Table 2.** Seven Genes With Promoter DMRs and Gene Expression Associated With Overall Survival

Gene*	Chromosome Location†	DMR Methylation			Gene Expression			Role in Cancer
		HR	95% CI	P	HR	95% CI	P	
<i>CD34</i>	1q32	0.98	0.97 to 0.99	< .001	2.05	1.41 to 2.99	< .001	High expression associated with poor prognosis in AML <sup>24</sup>
<i>RHOC</i>	1p13.1	0.96	0.93 to 0.98	< .001	1.96	1.34 to 2.86	< .001	Involved in solid tumor growth and invasion <sup>25</sup>
<i>SCRN1</i>	7p14.3-p14.1	0.93	0.88 to 0.97	.001	1.95	1.34 to 2.85	< .001	High expression associated with poor prognosis in colon cancer <sup>26</sup>
<i>F2RL1</i>	5q13	0.95	0.93 to 0.98	< .001	1.93	1.33 to 2.82	< .001	High expression associated with tumor cell proliferation <sup>27</sup>
<i>FAM92A1</i>	8q22.1	0.94	0.91 to 0.97	< .001	1.93	1.32 to 2.81	< .001	Promotes cell growth in renal carcinogenesis <sup>28</sup>
<i>MIR155HG</i>	21q21.3	0.97	0.95 to 0.99	.001	1.86	1.27 to 2.71	.001	Upregulated in aggressive types of CN-AML <sup>29</sup>
<i>VWA8</i>	13q14.11	0.94	0.90 to 0.98	.001	1.85	1.27 to 2.70	.001	Role in cancer unknown <sup>30</sup>

NOTE. For DMR methylation, HR < 1 indicates better survival with increasing methylation values. For gene expression, HR > 1 indicates worse survival with increasing gene expression values.

Abbreviations: AML, acute myeloid leukemia; CN, cytogenetically normal; DMR, differentially methylated region; HR, hazard ratio.

\*Genes are ordered according to decreasing gene expression HRs.

†Methylation regions were as follows: chr1:206150306-206152306 for *CD34*, chr1:113050201-113052201 for *RHOC*, chr7:29995430-29997430 for *SCRN1*, chr5:76149588-76151588 for *F2RL1*, chr8:94780948-94782948 for *FAM92A1*, chr21:25855327-25857327 for *MIR155HG*, and chr13:41432221-41434221 for *VWA8*.

leukemia stem-cell profile of Eppert et al<sup>31</sup> and the AML profile of Li et al<sup>32</sup> for all clinical end points, suggesting that the score provides a better prognostic model for CN-AML than other previously reported gene expression profiles (Data Supplement).

### Clinical Applicability of the Unweighted Summary Score

Although the weighted summary score allowed for the concurrent assessment of the prognostic impact of expression of all seven genes, for prospective evaluation and risk stratification of individual patients, it is simpler to compute an unweighted summary score reporting the number of the genes with high expression. Thus, we computed and tested the unweighted summary score for the seven genes. Patients in the training set with no highly expressed genes or one highly expressed gene had 96% CR, 32% 3-year DFS, and 39% 3-year OS rates, compared with rates of 25%, 0%, and 4%, respectively, in patients with six or seven highly expressed genes (Table 3). The unweighted summary score also proved to be a better model compared with all other prognostic markers (Data Supplement).

Furthermore, patients with no highly expressed genes or only one highly expressed gene had an excellent outcome compared with patients with six or seven highly expressed genes in all four validation sets (Table 3). Older patients in validation sets 1 and 3 with no highly expressed genes or one highly expressed gene had CR rates of 89% and 69%, respectively, compared with 50% for patients with six or seven highly expressed genes, and had 3-year OS rates of 44% and 46%, respectively, compared with 10% and 12%, respectively, for patients with six or seven highly expressed genes. Younger patients in validation sets 2 and 4 with no highly expressed genes or one highly expressed gene had CR rates of 91% and 100%, respectively, compared with 71% and 53%, respectively, for patients with six or seven highly expressed genes, and had 3-year OS rates of 82% and 76%, respectively, compared with 24% and 7%, respectively, for patients with six or seven highly expressed genes. The unweighted summary score's AIC values were also better than the AIC values of the other previously reported gene expression profiles (Data Supplement).<sup>31,32</sup>

## DISCUSSION

We report here a gene expression score that incorporates prognostic genetic and epigenetic information. The score was derived from a training set of older patients with CN-AML and validated in four independent older and younger patient sets.

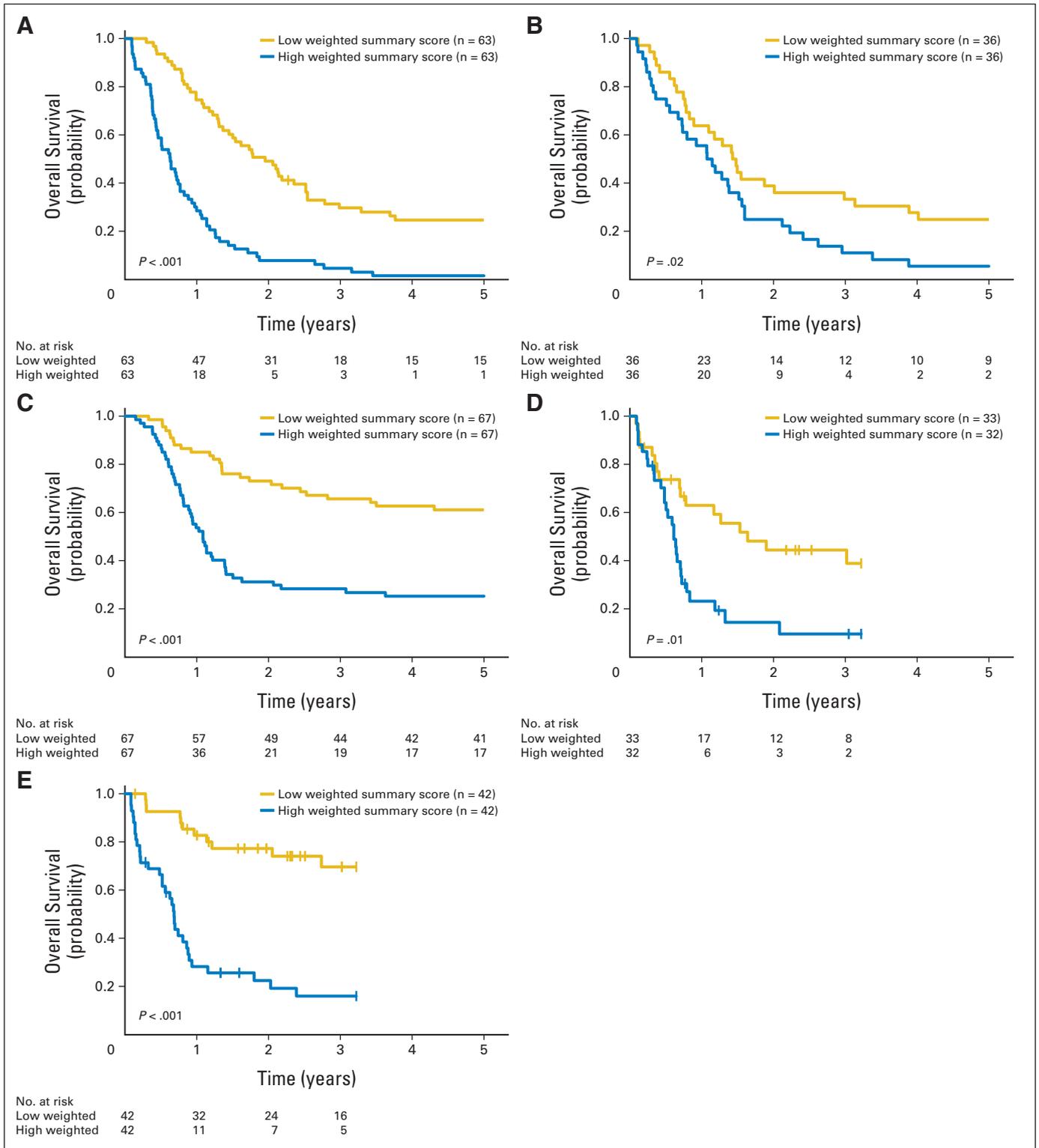
Our approach is novel and relatively straightforward and provides a strategy to integrate epigenetic and genetic information. Different from studies that have been mainly descriptive of the concurrent presence of aberrant genetic and epigenetic patterns in AML,<sup>8</sup> our work provides insight into the clinical relevance of prognostic mutations and the mutation-associated gene DNA methylation promoter and expression patterns. In fact, we postulated that each prognostic gene mutation had a defined methylation profile that distinguished mutated from wild-type patients. The methylation profile comprised DMRs, regions with significantly different methylated levels. The DMRs associated with prognostic gene mutations were distributed among different genomic features. DMRs in the promoter feature had a prognostic impact; high methylation levels of these DMRs were associated favorably with OS. Considering individual DMRs, we found seven genes whose promoter DMRs and expression levels were both significantly associated with outcome. These genes could not have been identified without the initial steps of relating prognostic mutations with their corresponding DNA methylation patterns. In fact, none of the seven genes were previously reported in other prognostic studies based exclusively on gene methylation status.<sup>7,9</sup> However, it should be noted that methodologic differences prevented a meaningful, direct comparison of our integrated genetic-epigenetic score with previously reported epigenetic signatures.<sup>7,9</sup>

For all seven genes, high methylation and low expression levels were associated with better outcome, thereby supporting an inverse functional relationship between hypermethylated promoters, usually resulting in epigenetic gene silencing, and low gene expression. Notably, of the seven genes in the score, six (*CD34*, *RHOC*, *SCRN1*, *F2RL1*, *FAM92A1*, and *MIR155HG*) have been associated with an aggressive tumor phenotype, supporting not only a prognostic but also a biologic

**Table 3. Clinical Outcome Based on the Gene Expression Summary Scores in Five Independent Sets of Patients With CN-AML**

Score	Training Cohort CALGB/Alliance, ≥ 60 Years (n = 126)				VS1 CALGB/Alliance, ≥ 60 Years (n = 72)				VS2 CALGB/Alliance, < 60 Years (n = 134)				VS3 AMLCG, ≥ 60 Years (n = 65)				VS4 AMLCG, < 60 Years (n = 84)							
	3-Year DFS (%)		3-Year OS (%)		3-Year DFS (%)		3-Year OS (%)		3-Year DFS (%)		3-Year OS (%)		3-Year DFS (%)		3-Year OS (%)		3-Year DFS (%)		3-Year OS (%)					
	Rate	95% CI	Rate	95% CI	Rate	95% CI	Rate	95% CI	Rate	95% CI	Rate	95% CI	Rate	95% CI	Rate	95% CI	Rate	95% CI	Rate	95% CI				
CR (%)	< .001	< .001	< .001	< .001	.12	.04	.03	.004	< .001	< .001	< .001	< .001	< .001	< .001	< .001	< .001	< .001	< .001	< .001	< .001				
Low score	86	26	15 to 38	30	19 to 54	81	8 to 37	33	19 to 49	94	54	41 to 65	66	53 to 76	70	31	14 to 51	42	24 to 58	85	58	39 to 73	70	51 to 82
High score	43	4	0 to 16	5	1 to 12	61	5	0 to 19	11	4 to 24	75	24	13 to 36	28	18 to 39	52	0	10	2 to 27	57	26	10 to 45	16	6 to 30
CR (%)	< .001	< .001	< .001	< .001	.01	.05	.007	.02	< .001	< .001	< .001	< .001	< .001	< .001	< .001	.20	< .001	.03	.01	.007	.003	.003	.003	< .001
Weighted summary score <sup>§</sup>	< .001	< .001	< .001	< .001	.12	.04	.03	.004	< .001	< .001	< .001	< .001	< .001	< .001	< .001	.20	< .001	.03	.01	.007	.003	.003	.003	< .001
Unweighted summary scores <sup>¶</sup>	< .001	< .001	< .001	< .001	.01	.05	.007	.02	< .001	< .001	< .001	< .001	< .001	< .001	< .001	.20	< .001	.03	.01	.007	.003	.003	.003	< .001
No. of genes and % <sup>  </sup>	96	32	14 to 51	39	20 to 58	89	NE	44	14 to 72	91	65	40 to 82	82	59 to 93	69	42	11 to 71	46	19 to 70	100	60	25 to 83	76	30 to 94
2-3, 40%	80	22	10 to 37	24	14 to 39	77	NE	26	12 to 42	89	44	31 to 56	50	37 to 61	64	23	6 to 47	33	15 to 54	80	51	31 to 68	63	43 to 77
4-5, 27%	54	5	0 to 20	5	1 to 15	64	NE	14	3 to 31	77	25	10 to 43	29	15 to 45	60	0	0	10	1 to 36	55	42	15 to 67	18	5 to 38
6-7, 17%	25	0	0 to 17	4	0 to 17	50	NE	10	1 to 36	71	17	3 to 41	24	7 to 45	50	0	0	12	1 to 38	53	13	1 to 42	7	0 to 28

NOTE. Median ages in the patient sets were as follows: training cohort, 69 years (range, 60 to 83 years); VS1, 69 years (range, 60 to 81 years); VS2, 46 years (range, 18 to 69 years); VS3, 67 years (range, 60 to 83 years); and VS4, 47 years (range, 17 to 59 years).  
Abbreviations: AMLCG, German Acute Myeloid Leukemia Cooperative Group; CALGB, Cancer and Leukemia Group B; CN-AML, cytogenetically normal acute myeloid leukemia; CR, complete remission; DFS, disease-free survival; NE, data not evaluable as a result of small sample sizes (ie, if any of the categories have five or fewer patients for a given end point); OS, overall survival; VS, validation set.  
<sup>†</sup>Data from Metzeler et al.<sup>11</sup> Data set accession number GSE12417. Patients with induction death were excluded.  
<sup>‡</sup>The weighted summary score is the Cox regression coefficient weighted sum of the median dichotomized values for the seven genes [weighted summary score = 0.71900(CD34) + 0.61927(MIR155H3) + 0.67258(RHOC) + 0.66829(SCRN1) + 0.65925(F2RL1) + 0.65777(FAM92A1) + 0.61491(VW48)]. For each gene, high and low expression was based on the cohort-specific median cutoff. The weighted summary score was then dichotomized using a cohort-specific median cut to determine the low and high groups.  
<sup>§</sup>For CR, the *P* value is from the Fisher's exact test; for DFS and OS, the *P* value is from the log-rank test.  
<sup>¶</sup>The summary score is the unweighted sum of the median dichotomized values for the seven genes (CD34, MIR155H3, RHOC, SCRN1, F2RL1, FAM92A1, and VW48). For each gene, high and low expression was based on the cohort-specific median cutoff. The value for the score can also be interpreted as the number of genes in the summary score with high expression.  
<sup>||</sup>*P* values are calculated using the continuous unweighted summary score. For CR, the *P* value is from the logistic regression model; for DFS and OS, the *P* value is from the Cox proportional hazards model.  
<sup>¶¶</sup>Percentage of patients in each category out of the total set of patients analyzed (N = 481). See the Data Supplement for additional detail.



**Fig 3.** Overall survival of patients with cytogenetically normal acute myeloid leukemia (CN-AML). (A) Training set. Validation sets (VSs) were as follows: (B) older (VS1) and (C) younger (VS2) Cancer and Leukemia Group B/Alliance patients with CN-AML; and (D) older (VS3) and (E) younger (VS4) German Acute Myeloid Leukemia Cooperative Group patients with CN-AML.<sup>9</sup>

relevance of the expression levels of these genes that could be regarded as putative oncogenes.<sup>24-30</sup>

The expression levels of the seven genes were used to compute a weighted summary score. A low score corresponding to low expres-

sion levels of the seven genes was associated with better outcome in the training set of older patients with CN-AML. The weighted summary score not only predicted CR rates and DFS and OS duration, but also provided better prognostic information than other molecular

**Table 4.** Multivariable Models for Clinical Outcome in the Training Set of Patients With Cytogenetically Normal Acute Myeloid Leukemia

Variables in Final Model*	OR/HR	95% CI	P
<b>CR</b>			
Weighted summary score,† high v low	0.17	0.07 to 0.41	.01
<i>NPM1</i> , mutated v wild-type	2.37	1.00 to 5.59	.05
<b>DFS</b>			
Weighted summary score,† high v low	3.59	1.83 to 7.03	< .001
<i>ERG</i> expression, high v low	1.93	1.04 to 3.55	.04
<b>OS</b>			
Weighted summary score,† high v low	3.41	2.13 to 5.47	< .001
<i>DNMT3A</i> , R882 v wild-type or non-R882	2.35	1.38 to 4.00	.002
<i>BAALC</i> expression, high v low	1.73	1.10 to 2.71	.02
Platelets, continuous, 50-unit increase	1.16	1.02 to 1.33	.02

Abbreviations: CR, complete remission; DFS, disease-free survival; HR, hazard ratio; OR, odds ratio; OS, overall survival.

\*Variables that were significant at  $P < .20$  in univariable models were considered for multivariable model inclusion and were as follows: for CR, weighted summary score (high v low), *NPM1* (mutated v wild-type), *IDH2* (R140 or R172 mutated v wild-type), *ASXL1* (mutated v wild-type), *RUNX1* (mutated v wild-type), *BAALC* expression (high v low), platelets (continuous, 50-unit increase), and WBC count (continuous, 50-unit increase); for DFS, weighted summary score (high v low), *FLT3-ITD* (present v absent), *FLT3-TKD* (present v absent), *ASXL1* (mutated v wild-type), *DNMT3A* (R882 mutated v non-R882 mutated or wild-type), *RUNX1* (mutated v wild-type), *ERG* expression (high v low), *BAALC* expression (high v low), age (continuous, 10-year increase), and sex (male v female); for OS, weighted summary score (high v low), *NPM1* (mutated v wild-type), *FLT3-ITD* (present v absent), *CEBPA* (mutated v wild-type), *ASXL1* (mutated v wild-type), *DNMT3A* (R882 mutated v non-R882 mutated or wild-type), *RUNX1* (mutated v wild-type), *ERG* expression (high v low), *BAALC* expression (high v low), and platelets (continuous, 50-unit increase).

†The weighted summary score is the Cox regression coefficient weighted sum of the median dichotomized values for the seven genes [weighted summary score =  $0.71900(CD34) + 0.61927(MIR155HG) + 0.67258(RHOC) + 0.66929(SCFN1) + 0.65925(F2RL1) + 0.65777(FAM92A1) + 0.61491(VWA3)$ ]. For each gene, high and low expression was based on the cohort-specific median cutoff. The weighted summary score was then dichotomized using a cohort-specific median cut to determine the low and high groups. An OR > 1 (< 1) corresponds to a higher (lower) odds of achieving a CR for the first level listed of a dichotomous variable. An HR > 1 corresponds to worse outcome for higher values of continuous variables and the first level listed of a dichotomous variable.

prognosticators, including *NPM1* mutation, one of the strongest prognosticators in older patients with CN-AML.<sup>33</sup> The prognostic value of the weighted summary score was validated in four independent sets of older and younger patients with CN-AML. Furthermore, the excellent prognostic value of the weighted summary score was also supported by the comparison of the score with other recently reported prognostic gene expression profiles, including a stem-cell profile.<sup>30,31</sup> The lowest AIC value of the weighted summary score among these gene expression profiles indicated that it provided the best prognostic model.

Importantly, all seven genes in the weighted summary score had a similar prognostic impact. This enabled us to compute an unweighted summary score to report simply the number of the seven highly expressed genes for each patient. In all five sets of patients analyzed, we observed a similar trend, where patients with no highly expressed genes or only one highly expressed gene, representing approximately 15% of patients with CN-AML, had an excellent outcome (Data Supplement). These results support the usefulness for treatment guidance of the unweighted summary score for identifying a relatively small, but clinically significant subset of patients who respond better to chemotherapy. This could be particularly valuable for older patients

with AML. These patients have an unfavorable prognosis and often are treated with reduced-intensity treatment or best supportive care to spare them the toxicity of intense, but frequently ineffective, chemotherapy. In our study, older patients with no or only one highly expressed gene had excellent CR rates (range, 69% to 96%) and 3-year OS rates (range, 39% to 46%), suggesting that the seven-gene score can select for chemotherapy-responsive older patients. Furthermore, low-score patients may also be excluded from hypomethylating treatments. In fact, a low score indicated low expression of genes that are highly methylated and partially repressed. Thus, DNA hypomethylating agents could harmfully reactivate one or more of the seven genes, thereby unleashing their leukemogenic potential.

From a biologic standpoint, we also observed that *FLT3-ITD* and *DNMT3A-R882* mutations were preferentially associated with low-methylation DMRs, and *NPM1* and *IDH* mutations were associated with high-methylation DMRs. Our results are consistent with those reported by other groups.<sup>7,8,33-35</sup> However, concerning *DNMT3A* mutations,<sup>8,36</sup> we noted that only the *DNMT3A-R882* mutations, and not the *DNMT3A* non-R882 mutations, were associated with low-methylation DMRs, supporting the notion that loss of function may be restricted to the former. Because we showed that low methylation of the promoter features was associated with worse outcome, these results are consistent with our previous report showing that the *DNMT3A-R882* mutation, which may lead to a decrease of DNA methylating activity of the encoded protein, also has a worse prognostic impact in older patients with CN-AML.<sup>37</sup>

In summary, integration of epigenetic and genetic prognostic information is feasible and revealed a novel seven-gene summary score that identified clinically meaningful groups of patients with AML who responded differently to chemotherapy regardless of age. Considering the relatively small number of genes making up the score, it should be feasible to rapidly develop and implement a simple quantitative assay that allows identification of the number of the seven genes with high expression and, in turn, prospective risk stratification of individual patients. Gene expression is indeed already used in other types of cancer (ie, breast).<sup>38</sup> Moreover, except for *MIR155HG* and *CD34*, the remaining five genes in the score have not been previously associated with AML, suggesting that they may be involved in novel leukemogenic pathways and thus represent therapeutic targets that warrant future investigation.

#### AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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