Molecular Cancer Therapeutics

# Prognostic and Biologic Relevance of Clinically Applicable Long Noncoding RNA Profiling in Older Patients with Cytogenetically Normal Acute Myeloid Leukemia

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## Abstract

We have previously shown that expression levels of 48 long noncoding RNAs (lncRNA) can generate a prognostic lncRNA score that independently associates with outcome of older patients with cytogenetically normal acute myeloid leukemia (CN-AML). However, the techniques used to identify and measure prognostic lncRNAs (i.e., RNA sequencing and microarrays) are not tailored for clinical testing. Herein, we report on an assay (based on the nCounter platform) that is designed to produce targeted measurements of prognostic lncRNAs in a clinically applicable manner. We analyzed a new cohort of 76 older patients with CN-AML and found that the nCounter assay yielded reproducible measurements and that the lncRNA score retained its prognostic value; patients with high lncRNA scores had lower complete remission (CR) rates ($P = 0.009$; 58% vs. 87%), shorter disease-free ($P = 0.05$; 3-year rates: 0% vs. 21%), overall (OS; $P = 0.02$, 3-year rates: 10% vs. 29%), and event-free survival (EFS; $P = 0.002$, 3-year rates: 0% vs. 18%) than patients with low lncRNA scores. In multivariable analyses, the lncRNA score independently associated with CR rates ($P = 0.02$), OS ($P = 0.02$), and EFS ($P = 0.02$). To gain biological insights, we examined our initial cohort of 71 older patients with CN-AML, previously analyzed with RNA sequencing. Genes involved in immune response and B-cell receptor signaling were enriched in patients with high lncRNA scores. We conclude that clinically applicable lncRNA profiling is feasible and potentially useful for risk stratification of older patients with CN-AML. Furthermore, we identify potentially targetable molecular pathways that are active in the high-risk patients with high lncRNA scores.

## Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease with regard to the underlying molecular abnormalities and its clinical course (1, 2). The outcome of patients with AML is generally poor (2), especially in the case of patients who are 60 years of age or older. Only 10% of older patients with AML who are fit to receive induction chemotherapy will remain alive and leukemia-free 5 years after their diagnosis (2). Thus, it is important to identify molecular markers that could distinguish between the patients who will respond to standard treatment from those who will not and who could benefit from experimental therapeutic approaches. Currently, chromosomal alterations, which are detected in approximately 55% to 60% of all AML cases, are used in the clinic to guide treatment decisions (2–5). In patients who lack microscopically detectable chromosomal abnormalities and thus have cytogenetically normal AML (CN-AML), recurrent gene mutations that associate with clinical outcome have been identified and are currently used to risk-stratify the treatment of patients with CN-AML (6–8).

Long noncoding RNAs (lncRNA) comprise a novel class of noncoding RNA molecules, which are equal to or longer than 200 nucleotides (9). lncRNAs have been shown to regulate many key cellular functions (10–12) and have been implicated in cancer pathogenesis (13–15). Our group has previously shown that expression levels of lncRNAs have prognostic significance in older patients with CN-AML (16). Specifically, we have demonstrated that a weighted summary expression score of 48 lncRNAs (called

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lncRNA score) provides independent prognostic information in this patient population.

Although 2 independent techniques of transcriptome interrogation (i.e., a microarray platform and total RNA sequencing, RNA-seq) were used to generate and validate the prognostic lncRNA score in the aforementioned study (16), these techniques are not suitable for patient testing in the clinic. Consequently, there is a need for development of a fast, reproducible and clinically applicable assay that would enable the translation of lncRNA profiling from the bench to the bedside. To address this need, we designed an assay allowing targeted measurements of prognostic lncRNAs using the nCounter analysis system (NanoString Technologies, Inc.). The nCounter platform has been developed to provide RNA measurements in a single reaction without amplification and is compatible with real-life clinical testing. This technology is currently used as the basis of an FDA-approved assay that measures the expression of RNA molecules for risk stratification of patients with breast cancer (17, 18). Herein, we analyzed a cohort of 76 older patients with CN-AML, and report on the prognostic value of the lncRNA score, as measured by the nCounter lncRNA assay. In addition, to identify potentially targetable molecular pathways in the subset of patients with high lncRNA scores, we performed transcriptome analyses in our initial cohort of older patients with CN-AML who had been previously analyzed with total RNA-seq (16).

## Materials and Methods

### Patients and treatment

In this study, we analyzed pretreatment bone marrow (BM) or blood of older patients (aged $\geq$60 years) with *de novo* CN-AML, who received intensive cytarabine/anthracycline-based therapy on Cancer and Leukemia Group B (CALGB)/Alliance for Clinical Trials in Oncology (Alliance) frontline clinical trials. We studied only patients who were alive 30 days after initiation of induction chemotherapy. Per protocol, no patient received allogeneic stem cell transplantation in first complete remission (CR). Details regarding treatment protocols are provided in the Supplementary Data. There were no patients who were selected for the study but not included in the final analyses because of poor RNA quality or failure of the profiling experiments. All patients provided written informed consent for the analyses of their samples and data. All study protocols were in accordance with the Declaration of Helsinki and approved by institutional review boards at each center.

### Cytogenetic and molecular analyses

Cytogenetic analyses were performed in CALGB/Alliance-approved institutional laboratories and the results were confirmed by central karyotype review (19). The diagnosis of normal karyotype was based on at least 20 metaphase cells analyzed in BM specimens subjected to short-term (24- or 48-hour) unstimulated cultures.

Targeted amplicon sequencing using the Miseq platform (Illumina) was used to analyze DNA samples for the presence of gene mutations that have been reported to associate with clinical outcome of patients with CN-AML [i.e., mutations in the *ASXL1*, *DNMT3A* (R882 and non-R882), *IDH1*, *IDH2* (R140 and R172), *NPM1*, *RUNX1*, *TET2* and *WT1* genes, and *FLT3*-tyrosine kinase domain (*FLT3*-TKD) mutations], as described previously (16, 20). A variant allele frequency of $\geq$10% was used as the cut-off to distinguish between mutated versus wild-type alleles of these genes. The presence of mutations in the *CEBPA* gene and *FLT3*-internal tandem duplications (*FLT3*-ITD) were evaluated using Sanger sequencing (21) and fragment analysis (22), respectively as described previously. As recent publications (2, 23, 24) indicate that only biallelic *CEBPA* mutations confer prognostic significance, we considered patients with this genotype as mutated.

### Transcriptome analyses

The nCounter analysis system from NanoString allows direct profiling of individual RNA molecules in a single reaction without amplification. The custom assay we designed includes 46 of the 48 lncRNAs that are used to generate the prognostic lncRNA score, as well as selected mRNAs and miRNAs whose expression were previously reported as associated with clinical outcome of patients with CN-AML. These include *BAALC* (25), *ERG* (26), *MN1* (27), miR155 (28), miR3151 (29), and miR181a (30), and the 7 genes that comprise the integrated genetic-epigenetic score (*CD34*, *MIR155HG*, *RHOC*, *SCRN1*, *F2RL1*, *FAM92A1*, and *VWA8*; ref. 31). Internal controls for normalization (e.g., *GAPDH*, *ABL*) were also included per NanoString guidelines. These probes were designed and synthesized by NanoString Technologies and the experiments were performed at The Ohio State University using the nCounter Diagnostic analysis system. Total RNA extracted with TRIzol reagent was used as input material for the assay. For the details concerning calculation of the prognostic lncRNA score with the nCounter assay measurements, see the Supplementary Data. The nCounter profiling experiments have been submitted to the GEO repository under the accession number GSE130923.

The expression status (i.e., high or low expresser) of each of the aforementioned prognostic mRNA and miR transcripts (e.g., *BAALC*, miR155) was determined for each patient using the median expression value measured by the nCounter assay as the cut-off.

### Statistical analyses

Clinical endpoint definitions are given in the Supplementary Data. Baseline demographic, clinical, and molecular features were compared between patients with high and those with low lncRNA scores using the Wilcoxon rank sum and Fisher exact tests for continuous and categorical variables, respectively (32). The estimated probabilities of disease-free (DFS), overall (OS), and event-free survival (EFS) were calculated using the Kaplan–Meier method, and the log-rank test evaluated differences between survival distributions (33). Cox proportional hazards models were used to calculate hazard ratios for DFS, OS, and EFS (34). Multivariable Cox proportional hazards models were constructed using a forward selection procedure. All statistical analyses were performed by the Alliance Statistics and Data Center. The researchers who conducted the laboratory profiling experiments were blinded to patient outcome results.

## Results

### Design of nCounter assay and reproducibility of the measurements

nCounter probes capable of interrogating the lncRNA expression levels could be generated for 46 of the 48 prognostic lncRNAs described previously (Supplementary Table S1; ref. 16). The sequences of the designed probes are provided in the

Supplementary Data section (Supplementary Table S2). The assay also included mRNA and miRNA transcripts that have been previously reported to be prognostic in AML and reference genes for quality control of the analyzed material (Supplementary Table S3). Samples of 76 older patients with *de novo* CN-AML were measured with the nCounter platform in 5 independent experiments. To evaluate the reproducibility of lncRNA measurements across time and measured batches, we measured 16 of the 76 samples repeatedly. Eleven samples were measured twice in separate batches, 3 samples were measured 3 times in the same batch, and 2 samples measured 4 times in the same batch, yielding a total of 39 replicate runs. Analyses of samples that were measured repeatedly showed satisfactory reproducibility within and across measured batches in all but one case. The median of the Pearson *r*-squared values for the correlation of the nCounter measurements for each pair of replicate runs was 0.94 (range: 0.44–0.98; Supplementary Figs. S1 and S2). The samples of 3 patients that were measured twice in the same or separate batches (Fig. 1A–C) and of 1 patient, which were measured 3 times in the same batch (Fig. 1D–F) are depicted in Fig. 1 as examples.

To study whether the separation of the measurements in different batches impacted on the nCounter results, we generated multidimensional scaling plots. These plots display the pairwise Euclidean distance between the samples in 2 dimensions, and the physical distance between samples represents sample similarity (i.e., the shorter the distance between 2 annotated measurements, the higher the similarity between them). We observed that when all analyzed samples were depicted simultaneously, there was no segregation of the nCounter measurements by batches (Fig. 1G).

### Association of lncRNA score with pretreatment characteristics of patients

To examine whether the lncRNA score retained its prognostic value when determined by nCounter assay measurements, we performed outcome analysis in our new cohort of 76 older patients with CN-AML. Specifically, we used the median value of the lncRNA score, as measured with the nCounter assay, to divide our dataset into patients with high and patients with low lncRNA scores. With regard to pretreatment characteristics, patients with high lncRNA scores were older ($P = 0.04$), and

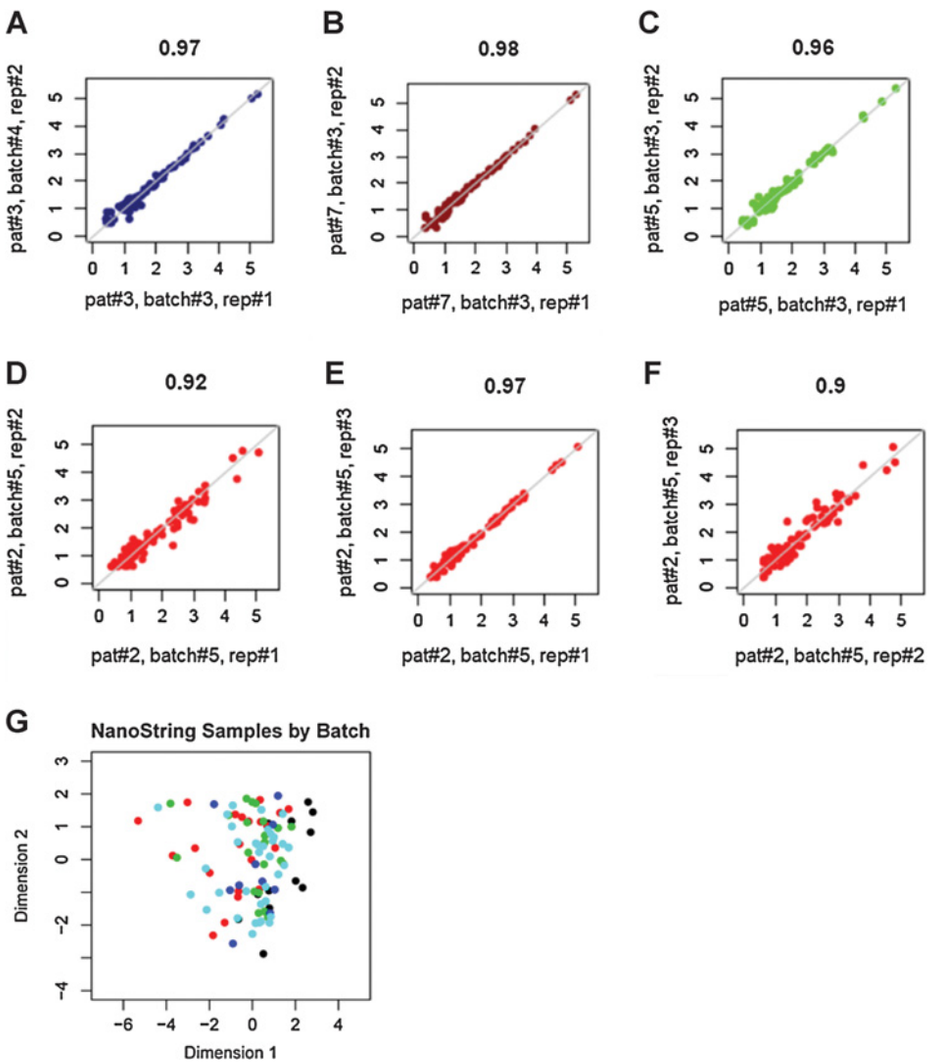



**Figure 1.**
Scatterplots depicting the correlation of nCounter results in repeated measurement of samples. **A–C**, Measurements of 3 samples that were analyzed twice in separate batches. **D–F,** Measurements of 1 sample that were analyzed three times in one batch. The Pearson *r*-squared value is annotated on the top of each plot. **G,** Multidimensional scaling plots showing relationships between the total measurements of the 76 samples analyzed with the nCounter assay. Physical distance between samples indicates similarity: the shorter the distance, the higher the similarity of the measurements. Each dot represents the combined measurements of an individual sample. Measurements are colored by batch: Black indicates batch 1; red, batch 2; green, batch 3; deep blue, batch 4; and light blue, batch 5.

**Table 1.** Comparison of clinical and molecular characteristics by low and high lncRNA score in the cohort of 76 older patients (ages ≥60 years) with CN-AML, who were analyzed with the nCounter assay

| Characteristic | Low lncRNA score ($n = 38$) | High lncRNA score ($n = 38$) | *P* |
|---|---|---|---|
| Age, years | | | 0.04 |
| Median | 66 | 71 | |
| Range | 60–81 | 60–82 | |
| Sex, *n* (%) of females | 21 (55) | 19 (50) | 0.82 |
| Race, *n* (%) | | | 1.00 |
| White | 35 (95) | 34 (92) | |
| Non-white | 2 (5) | 3 (8) | |
| Hemoglobin, g/dL | | | 0.15 |
| Median | 9 | 9.4 | |
| Range | 6.5–11.9 | 6.8–11.3 | |
| Platelet count, x$10^9$/L | | | 0.67 |
| Median | 70 | 70 | |
| Range | 18–507 | 5–592 | |
| WBC count, x$10^9$/L | | | 0.54 |
| Median | 29.7 | 28.5 | |
| Range | 1.4–343.6 | 1.0–173.1 | |
| Blood blasts, % | | | 0.18 |
| Median | 36 | 58 | |
| Range | 0–90 | 0–95 | |
| BM blasts, % | | | 0.56 |
| Median | 68 | 64 | |
| Range | 6–99 | 0–95 | |
| Extramedullary involvement, *n* (%) | 7 (19) | 4 (13) | 0.53 |
| *NPM1, n* (%) | | | 0.003 |
| Mutated | 28 (80) | 15 (44) | |
| Wild type | 7 (20) | 19 (56) | |
| *FLT3*-ITD, *n* (%) | | | 0.005 |
| Present | 8 (26) | 20 (63) | |
| *FLT3*-ITD allelic raio ≥0.50 | 4 | 7 | |
| *FLT3*-ITD allelic ratio <0.50 | 4 | 13 | |
| Absent | 23 (74) | 12 (38) | |
| *CEBPA, n* (%) | | | 1.00 |
| Biallelic mutations | 1 (4) | 0 (0) | |
| Wild type or monoallelic mutations | 27 (96) | 25 (100) | |
| *FLT3*-TKD, *n* (%) | | | 1.00 |
| Present | 2 (6) | 2 (6) | |
| Absent | 32 (94) | 31 (94) | |
| *WT1, n* (%) | | | 0.71 |
| Mutated | 3 (9) | 4 (12) | |
| Wild type | 31 (91) | 29 (88) | |
| *TET2, n* (%) | | | 0.59 |
| Mutated | 11 (32) | 8 (24) | |
| Wild type | 23 (68) | 25 (76) | |
| *IDH1, n* (%) | | | 0.75 |
| Mutated | 5 (15) | 6 (18) | |
| Wild type | 29 (85) | 27 (82) | |
| *IDH2, n* (%) | | | 0.51 |
| Mutated | 4 (12) | 6 (18) | |
| Wild type | 30 (88) | 27 (82) | |
| *ASXL1, n* (%) | | | 0.43 |
| Mutated | 2 (6) | 4 (12) | |
| Wild type | 32 (94) | 29 (88) | |
| *DNMT3A, n* (%) | | | 0.12 |
| Mutated | 15 (44) | 8 (24) | |
| Wild type | 19 (56) | 25 (76) | |
| *RUNX1, n* (%) | | | 0.03 |
| Mutated | 1 (3) | 7 (21) | |
| Wild type | 33 (97) | 26 (79) | |
| ELN risk category[a], *n* (%) | | | <0.001 |
| Favorable | 22 (79) | 6 (23) | |
| Intermediate | 5 (18) | 9 (35) | |
| Adverse | 1 (4) | 11 (42) | |

*(Continued on the following column)*

**Table 1.** Comparison of clinical and molecular characteristics by low and high lncRNA score in the cohort of 76 older patients (ages ≥60 years) with CN-AML, who were analyzed with the nCounter assay (Cont'd)

| Characteristic | Low lncRNA score ($n = 38$) | High lncRNA score ($n = 38$) | *P* |
|---|---|---|---|
| *ERG* expression group[b], *n* (%) | | | <0.001 |
| High | 11 (29) | 27 (71) | |
| Low | 27 (71) | 11 (29) | |
| *BAALC* expression group[b], *n* (%) | | | 0.003 |
| High | 12 (32) | 26 (68) | |
| Low | 26 (68) | 12 (32) | |
| *MN1* expression group[b], *n* (%) | | | 0.04 |
| High | 24 (63) | 14 (37) | |
| Low | 14 (37) | 24 (63) | |
| miR181a expression group[b], *n* (%) | | | 0.82 |
| High | 20 (53) | 18 (47) | |
| Low | 18 (47) | 20 (53) | |
| miR3151[b], *n* (%) | | | 0.82 |
| High | 20 (53) | 18 (47) | |
| Low | 18 (47) | 20 (53) | |
| miR155 expression group[b], *n* (%) | | | 0.04 |
| High | 14 (37) | 24 (63) | |
| Low | 24 (63) | 14 (37) | |

Abbreviations: ELN, European LeukemiaNet; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutation in the *FLT3* gene.

[a]Among patients with CN-AML, the ELN Favorable Risk Category comprises patients with biallelic mutations in *CEBPA* and patients with mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{low}$. The ELN Intermediate Risk Category includes patients with wild-type *CEBPA* and either wild-type *NPM1* without *FLT3*-ITD, wild-type *NPM1* and *FLT3*-ITD$^{low}$ or mutated *NPM1* and *FLT3*-ITD$^{high}$. The ELN Adverse Risk Category comprises patients with wild-type *CEBPA* and wild-type *NPM1* with *FLT3*-ITD$^{high}$, patients with mutated *TP53*, and patients with mutated *RUNX1* and/or mutated *ASXL1* (if these mutations do not co-occur with Favorable-Risk AML subtype). *FLT3*-ITD$^{low}$ is defined by an *FLT3*-ITD/*FLT3* wild-type allelic ratio of less than 0.5, and *FLT3*-ITD$^{high}$ is defined by an *FLT3*-ITD/*FLT3* wild-type allelic ratio of equal to or more than 0.5.

[b]The median expression value was used as the cut point.

harbored mutated *NPM1* ($P = 0.003$) less frequently and mutated *RUNX1* ($P = 0.03$) and *FLT3*-ITD ($P = 0.005$) more frequently than patients with low lncRNA scores. Patients with high lncRNA scores were also more frequently classified in the Intermediate or Adverse Risk Groups of the ELN Classification ($P < 0.001$; ref. 2), and were high expressers of *ERG* ($P < 0.001$), *BAALC* ($P = 0.003$), and miR155 ($P = 0.04$), and low expressers of *MN1* ($P = 0.04$) more often than patients with low lncRNA scores (Table 1).

### Association of lncRNA score with clinical outcome

Survival analyses in the new cohort of older patients with CN-AML showed that patients with high lncRNA scores were less likely to achieve a CR than those with low lncRNA scores (58% vs. 87%, $P = 0.009$). High lncRNA score status associated with shorter DFS ($P = 0.05$; Fig. 2A). None of the patients with high lncRNA scores were alive and leukemia-free 3 years after diagnosis in contrast to 21% of the patients with low lncRNA scores who were. Patients with high lncRNA scores also had shorter OS ($P = 0.02$, 3-year rates: 10% vs. 29%; Fig. 2B). In addition, high lncRNA scores associated with shorter EFS ($P = 0.02$; Fig. 2C, Table 2). Three years after diagnosis, 18% of the patients with low lncRNA scores were alive and had not experienced an event in comparison to none of the patients with high lncRNA scores.

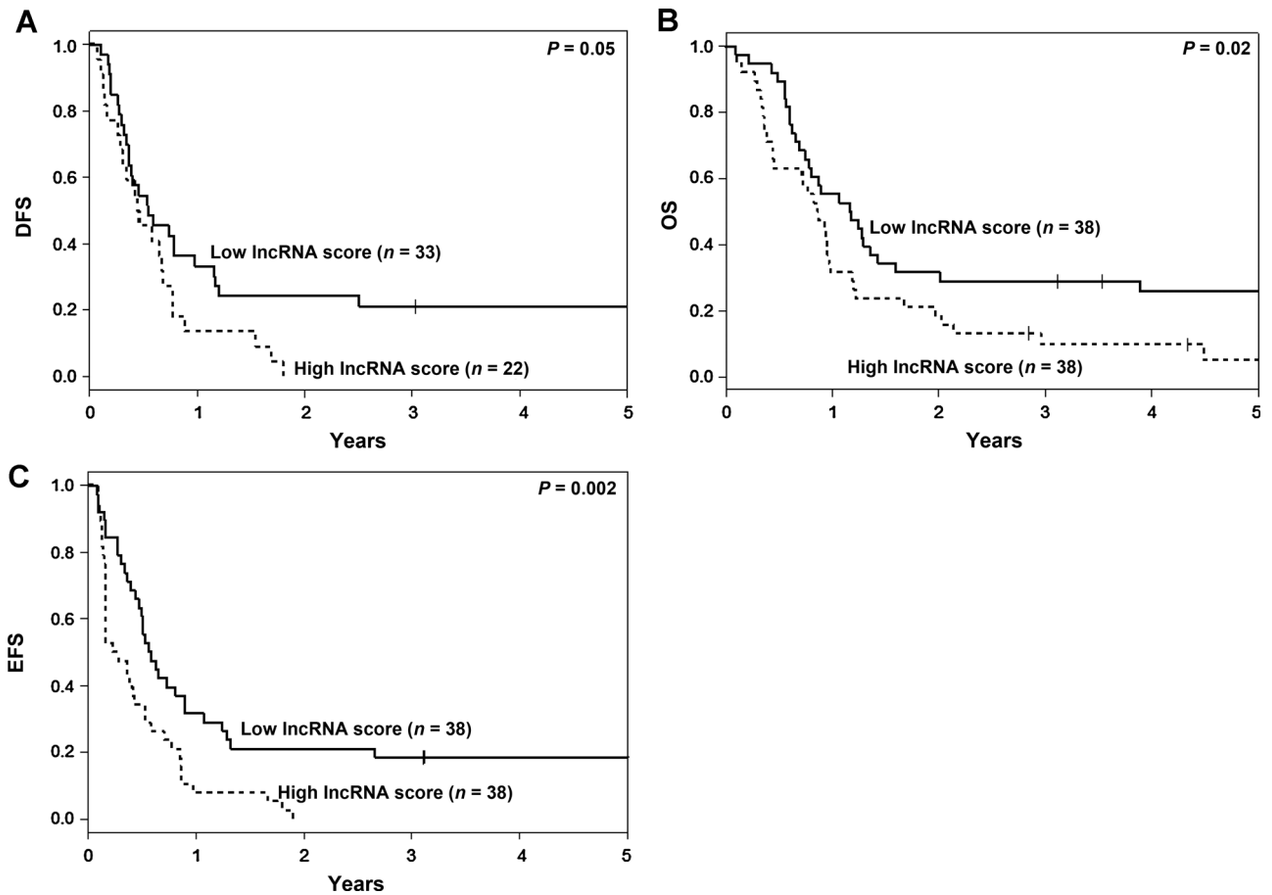



**Figure 2.**

Outcomes of older patients (ages ≥60 years) with CN-AML with low and high lncRNA scores. DFS (**A**), OS (**B**), and EFS (**C**). The lncRNA score of each individual patient was computed as a weighted score, based on the nCounter assay measurements of 46 prognostic lncRNAs.

### Multivariable analyses

To assess whether the prognostic lncRNA score provides independent prognostic information in the context of other established prognostic markers, we constructed multivariable proportional hazards models. High lncRNA scores independently associated with a lower CR rate ($P = 0.02$), after adjusting for *BAALC* expression status ($P = 0.02$). High lncRNA score status also independently associated with shorter OS ($P = 0.02$), after adjusting for white blood cell (WBC) counts ($P = 0.005$) and the sex of patients ($P = 0.01$). Finally, high lncRNA score was an independent marker of shorter EFS ($P = 0.02$); patients with high lncRNA scores had approximately a 2-fold increase in their risk of experiencing an event than those with low lncRNA score, after adjusting for WBC counts ($P = 0.002$) and *BAALC* expression status ($P = 0.02$, Table 3). The lncRNA score status did not remain significantly associated with DFS duration in multivariable analysis.

**Table 2.** Outcome of older patients (ages ≥60 years) with CN-AML by low and high lncRNA score status

| End point | Low lncRNA score ($n = 38$) | High lncRNA score ($n = 38$) | *P* |
|---|---|---|---|
| CR | | | 0.009 |
| *n*, (%) | 33 (87) | 22 (58) | |
| DFS | | | 0.05 |
| Median, years | 0.5 | 0.5 | |
| Disease-free at 3 years, % (95% CI) | 21 (9–36) | 0 | |
| Disease-free at 5 years, % (95% CI) | 21 (9–36) | 0 | |
| OS | | | 0.02 |
| Median, years | 1.2 | 0.9 | |
| Alive at 3 years, % (95% CI) | 29 (16–44) | 10 (3–22) | |
| Alive at 5 years, % (95% CI) | 26 (13–40) | 5 (0–18) | |
| EFS | | | 0.002 |
| Median, years | 0.6 | 0.3 | |
| Event-free at 3 years, % (95% CI) | 18 (8–32) | 0 | |
| Event-free at 5 years, % (95% CI) | 18 (8–32) | 0 | |

Abbreviation: CI, confidence interval.

### Biological insights regarding the molecular pathways that associate with the lncRNA score

Using the nCounter assay, we could demonstrate that lncRNA score profiling could distinguish between the patients who were more likely to respond to standard chemotherapy from those who were not. However, older patients often have comorbidities that preclude intensification of chemotherapy and allogeneic stem cell transplantation as therapeutic options. It is therefore important to identify targetable molecular pathways, in particular in those

**Table 3.** Multivariable analyses for outcome in the dataset of older (ages ≥ 60 years) patients with CN-AML

| | CR | | OS | | EFS | |
|---|---|---|---|---|---|---|
| **Variables in final models[a]** | **OR (95% CI)** | ***P*** | **HR (95% CI)** | ***P*** | **HR (95% CI)** | ***P*** |
| lncRNA score, high versus low | 0.18 (0.04, 0.75) | 0.02 | 1.84 (1.09, 3.10) | 0.02 | 1.88 (1.08, 3.27) | 0.02 |
| *BAALC*, high versus low | 0.17 (0.04, 0.74) | 0.02 | — | — | 1.88 (1.10, 3.21) | 0.02 |
| WBC counts, continuous | — | — | 1.34 (1.09, 1.64) | 0.005 | 1.38 (1.13, 1.68) | 0.002 |
| Sex, male versus female | — | — | 1.90 (1.14, 3.17) | 0.01 | — | — |

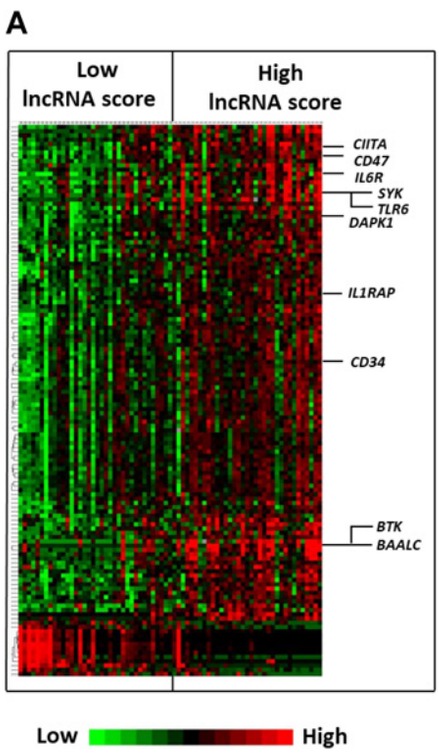
NOTE: ORs less than 1 indicate lower chances for achieving CR. HRs greater than 1 indicate higher risk for failure to achieve CR or death (OS) or for failure to achieve CR, relapse, or death (EFS) for the first category listed. Variables considered for model inclusion were as follows: lncRNA score status (high vs. low), age (as a continuous variable, in 10-year increments), sex (male vs. female), race (white vs. non-white), WBC count (as a continuous variable, in 50-unit increments), hemoglobin (as a continuous variable, in 1-unit increments), platelet count (as a continuous variable, in 50-unit increments), extramedullary involvement (present vs. absent), *ASXL1* mutations (mutated vs. wild-type), *CEBPA* mutations (biallelic mutations vs. monoallelic mutations or wild-type), *DNMT3A* mutations (mutated vs. wild-type), *FLT3*-ITD (present vs. absent), *FLT3*-TKD (present vs. absent), *IDH1* mutations (mutated vs. wild-type), *IDH2* mutations (mutated vs. wild-type), *NPM1* mutations (mutated vs. wild-type), *RUNX1* mutations (mutated vs. wild-type), *TET2* mutations (mutated vs. wild-type), *WT1* mutations (mutated vs. wild-type), *BAALC* expression levels (high vs. low), *ERG* expression levels (high vs. low), *MN1* expression levels (high vs. low), miR181a expression levels (high vs. low), miR3151 (expressed vs. not expressed), and miR155 expression levels (high vs. low). For *BAALC, ERG, MN1*, miR181a, and miR155 the median expression value, as calculated by the nCounter assay, was used as the cut point to divide patients into high and low expressers.

Abbreviation: CI, confidence interval.

[a]The lncRNA score status did not remain significantly associated with DFS in multivariable analysis.

patients that are predicted not to benefit from standard therapy. To this end, we performed correlation analysis in our initial cohort of 71 older patients with CN-AML, who were analyzed with total RNA-seq (16). This sequencing technique provides comprehensive information of both coding and noncoding fractions of the transcriptome. We applied stringent criteria (*P* value of <0.001 and FDR of <0.05) and identified 115 transcripts that were upregulated and 3 transcripts which were downregulated in patients with high lncRNA scores (Fig. 3A; Supplementary Table S4).

Among transcripts overexpressed in patients with high lncRNA scores, we identified genes that are key regulators of the immune system, such as *CD74* and *CIITA* that are implicated in peptide processing and presentation in antigen-presenting cells; *BTK* and




B

| Gene Ontology functional groups that positively correlated with high lncRNA scores | | | |
|---|---|---|---|
| Biologic process | *P* | Fold enrichment | *n* of genes |
| Leukocyte migration | $3.2x10^{-4}$ | 15 | 5 |
| Inflammatory response | $5.5x10^{-4}$ | 4.75 | 9 |
| Mitochondrial function | $1.5x10^{-2}$ | 3.48 | 7 |
| Apoptosis | $7.6x10^{-3}$ | 2.84 | 10 |
| Immune response | $5.5x10^{-4}$ | 2.23 | 13 |

**Figure 3.**

Messenger RNA (mRNA) transcripts that associate with the prognostic lncRNA score in older patients (ages ≥60 years) with CN-AML. **A,** Heat map of the gene-expression signature associated with the lncRNA score. Rows represent protein-coding genes and columns represent patients. Patients are grouped by lncRNA score: low on the left and high on the right. The lncRNA score of each individual patient was computed as a weighted score of 46 prognostic lncRNAs. Expression values of the mRNA transcripts are represented by color: green, expression less than median value; red, expression greater than median value. **B,** Gene Ontology functional groups that positively correlate with high lncRNA scores in older patients with CN-AML. Gene Ontology functional groups are ranked according to fold enrichment.

*SYK* implicated in B-cell receptor signaling; and *IL1RAP*, *IL6R*, and *TLR6*, which are important cytokine receptors. We also found aberrant overexpression of *CD34* in patients with high lncRNA scores, a surface marker of leukemic stem cells whose expression has been related to chemotherapy resistance and poor outcome in AML (35). In addition, genes implicated in leukemogenesis such as *DAPK1* (36) and *IDH1* (37) were also upregulated in patients with high lncRNA scores. Finally, in keeping with the adverse prognostic impact of high lncRNA score, we found mRNAs, which are established markers of poor outcome in CN-AML, such as *BAALC* (25) to be enriched in the subset of patients with high lncRNA scores.

To further characterize and classify the genes and molecular pathways that are active in patients with high lncRNA scores, we performed Gene Ontology analysis (38). Gene Ontology analysis revealed enrichment for genes involved in leukocyte migration, inflammatory response, mitochondrial function, apoptosis, and immune response in patients with high lncRNA scores (Fig. 3B).

## Discussion

lncRNAs are gaining increasing recognition as key regulators of important cellular functions including imprinting, cell-cycle regulation and apoptosis (10–12, 39–40). Over the past years, it has become evident that lncRNAs are functionally associated with malignant diseases (40–42) and that they affect clinical outcome of patients with cancer. In CN-AML, we reported a prognostic score, which is based on the expression levels of 48 lncRNAs and provides independent prognostic information in older patients with CN-AML (16). Importantly, we found that the prognostic lncRNA score showed no association with recurrent prognostic gene mutations that are currently used for risk stratification of patients with CN-AML, such as biallelic *CEBPA* mutations, *NPM1* mutations, or *FLT3*-ITD. For this reason, we hypothesized that the lncRNA score could further refine risk stratification of older patients with CN-AML. However, the techniques that were used to identify and measure the prognostic lncRNA molecules are not clinically applicable. To acquire fast and reproducible transcriptome measurements, which would facilitate translation of lncRNA profiling to the clinic, we designed a prognostic lncRNA-measuring assay using the nCounter technology. The nCounter platform has been specifically developed to serve as the basis of clinically applicable tests, and is currently used in FDA-approved transcriptome profiling assays (17, 18).

To test the efficacy and reproducibility of our nCounter lncRNA assay, we analyzed a new cohort of 76 older patients with CN-AML, who were treated on frontline CALGB/Alliance studies. We performed a total of 5 experiments using standard RNA extraction techniques and methods. We sought to evaluate the performance of the assay in real-life conditions and, therefore, did not discard any samples on the basis of RNA yield or quality. We performed multiple measurements of individual samples, so as to evaluate the robustness and reproducibility of our assay. We found a satisfactory correlation of the repeated measurements when these were conducted within the same run of the assay or in independent experiments in all but one case.

To examine whether the nCounter-based lncRNA score retained its prognostic value, we performed outcome analyses in our new cohort of older patients with CN-AML. We found that the lncRNA score was significantly associated with outcome and that patients with high lncRNA scores were less likely to achieve a CR and had shorter DFS, OS, and EFS than patients with low lncRNA scores. We also detected associations of the lncRNA score status with prognostic mutations, such as those in the *RUNX1* gene and *FLT3*-ITD. Despite these associations, in multivariable analyses, the nCounter assay lncRNA score was shown to be an independent prognosticator for achievement of CR, as well as OS and EFS duration after adjusting for other covariates.

Our current study was conducted in a cohort of older patients with CN-AML of relatively small size and was designed to evaluate the feasibility and utility of lncRNA profiling by the use of a nCounter assay in the clinical setting. lncRNA score-based risk assessment in this study was dependent on profiling of a group of patients to establish the median lncRNA score value for this group that was then used to distinguish low- from high-risk patients. Nevertheless, because the nCounter platform allows individualized transcriptomic measurements, it could be potentially used for risk-assignment of individual patients in the future. To achieve this goal, a larger number of older patients with CN-AML should be analyzed to establish the optimal lncRNA score value that should be used as a widely accepted cut-off between patients with a low and those with a high lncRNA score in the clinic.

Although it is important to identify those older patients with CN-AML that will respond to conventional therapeutic modalities, those who will not represent a therapeutic challenge. Confounding comorbidities often preclude the use of such options as intensification of chemotherapy or allogeneic stem cell transplantation that have been proven to be efficacious in younger adult AML patients. To gain biological insights and identify potentially targetable pathways active in patients with high lncRNA scores, we examined which mRNA transcripts correlate with unfavorable lncRNA profiles and performed gene ontology analyses in our initial cohort of 71 older patients with CN-AML, previously analyzed with RNA seq (16). We found genes involved in the regulation of the immune response and B-cell receptor signaling, such as *BTK* and *SYK*, to be overexpressed in patients with high lncRNA scores. High expression levels of immune response-related genes are reminiscent of the mRNA expression signature associated with *RUNX1* mutations in patients with CN-AML (43). The relatively small number of patients with *RUNX1* mutations in our initial cohort ($n = 8$) renders it unlikely that these mutations are the sole drivers of the detected lncRNA score-related gene expression signature. It could be hypothesized instead that high expression of the prognostically unfavorable lncRNAs has a similar impact on the transcriptome to *RUNX1* mutations.

In recent years, targeting BTK with inhibitory molecules has proven to be a successful therapeutic approach for certain lymphoid malignancies (44, 45) and BTK inhibitors are currently included among the standard-of-care therapeutic agents for these diseases. Use of BTK inhibitors has also yielded encouraging preclinical results in AML (46). The high expression of *BTK* in patients with high lncRNA score that we detected could provide the rationale for exploring the efficacy of BTK-targeting agents in these patients. Thus, lncRNA profiling could be potentially used not only to risk-stratify treatment of older patients with CN-AML but also to guide novel therapeutic approaches in patients who are at high risk of treatment failure.

In summary, we demonstrate the technical feasibility of using the nCounter assay for prognostic lncRNA profiling in

a clinically applicable manner. We have also validated the prognostic value of lncRNA expression in older patients with CN-AML, in our new cohort of patients analyzed using a different profiling method than the ones used previously. We believe that the value of the nCounter assay for improving risk stratification of patients with AML warrants evaluation in future prospective clinical trials.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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