Expression and prognostic impact of IncRNAs in acute myeloid leukemia


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Long noncoding RNAs (IncRNAs) are transcripts longer than 200 nucleotides, located within the intergenic stretches or overlapping antisense transcripts of protein coding genes. IncRNAs are involved in numerous biological roles including imprinting, epigenetic regulation, apoptosis, and cell cycle. To determine whether IncRNAs are associated with clinical features and recurrent mutations in older patients (aged ≥ 60 y) with cytogenetically normal (CN) acute myeloid leukemia (AML), we evaluated IncRNA expression in 148 untreated older CN-AML cases using a custom microarray platform. An independent set of 71 untreated older patients with CN-AML was used to validate the outcome scores using RNA sequencing. Distinctive IncRNA profiles were found associated with selected mutations, such as internal tandem duplications in the FLT3 gene (FLT3-ITD) and mutations in the NPM1, CEBPA, IDH1, and ASXL1 genes. Using the IncRNAs most associated with event-free survival in a training cohort of 148 older patients with CN-AML, we derived an IncRNA score composed of 48 IncRNAs. Patients with an unfavorable compared with favorable IncRNA score had a lower complete response (CR) rate (P = 0.001, odds ratio = 0.14, 54% vs. 89%), shorter disease-free survival (DFS) (P < 0.001, hazard ratio (HR) = 2.88) and overall survival (OS) (P = 0.001, HR = 2.95). The validation set analyses confirmed these results (CR, P = 0.03; DFS, P = 0.009; OS, P = 0.009). Multivariable analyses for CR, DFS, and OS identified the IncRNA score as an independent marker for outcome. In conclusion, IncRNA expression in AML is closely associated with recurrent mutations. A small subset of IncRNAs is correlated strongly with treatment response and survival.

IncRNAs | acute myeloid leukemia | outcome

Acute myeloid leukemia (AML) is diagnosed most often in older adults (age ≥ 60 y) who often have a worse prognosis, with longer-term overall survival (OS) rates of only 5–16% (1–3). The reasons for the poor outcome of older patients relate to higher frequencies of secondary disease (e.g., AML diagnosed after hematologic disorders and/or therapy-related disease), adverse cytogenetics (e.g., complex karyotype), comorbid conditions, and poor performance status (3–7). Nonrandom chromosomal abnormalities (e.g., deletions, translocations) are identified in 50–55% of all older patients with primary AML (3–8). In contrast, about 45–50% of all AML cases are cytogenetically normal (CN-AML) when assessed using conventional banding analysis (3–8). Recent work has identified novel genetic alterations, including gene mutations and changes in gene expression in CN-AML that have improved the classification and risk stratification of this large subgroup of patients (9, 10). Whereas most of these studies were performed in younger patients with CN-AML, some studies have investigated the prognostic significance of genetic alterations in older patients (11–19). Our group reported that nucleophosmin (nucleolar phosphoprotein B23, numatin) (NPM1) mutations are associated with a more favorable outcome in older patients with CN-AML (11), whereas fms-related tyrosine kinase 3 (FLT3) internal tandem duplications (FLT3-ITDs) and mutations in the additional sex combs like transcriptional regulator 1 (ASXL1), runt-related transcription factor 1 (RUNX1) and DNA (cytosine-5’)-methyltransferase 3 alpha (DNMT3A), which affect arginine codon 882 (R882-DNMT3A), are independently associated with worse disease-free survival (DFS) and OS (12–15). We further showed that high expression levels of the brain and leukemia, cytoplasmic (BALC), v-ets avian erythroblastosis virus E26 oncogene homolog (ERG), meningeoma (disrupted in balanced translocation) 1 (MNL1), and DNA (cytosine-5’)-methyltransferase 3 beta (DNMT3B) genes are associated with unfavorable outcome in older patients with CN-AML (16–18).

Significance

Long noncoding RNAs (IncRNAs) are involved in numerous biological roles including epigenetic regulation, apoptosis, and cell cycle. Whereas IncRNAs contribute to epigenetic gene regulation, metastasis, and prognosis in solid tumors, their role in acute myeloid leukemia (AML) has not been hitherto reported. Here, we show that IncRNA expression profiles are associated with recurrent mutations, clinical features, and outcome in AML. A fraction of these IncRNAs may have a functional role in leukemogenesis. Furthermore, IncRNAs could be used as biomarkers for outcome in AML. The identification of patients likely to achieve complete remission with standard therapy alone, based on IncRNA expression, is a significant advance potentially sparing such patients from other toxicities and focusing investigational approaches on postremission studies.


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In addition to genetic alterations in protein coding genes, aberrant expression of noncoding RNAs plays a critical role in leukemia initiation and outcome prediction (19). MicroRNAs (miRNAs) constitute the first class of noncoding RNAs, whose expression was found widely dysregulated in AML and associated with clinical features including outcome (20, 21). We reported unique miRNA expression signatures associated with NPM1, RUNX1, and tet methylcytosine dioxygenase 2 (TET2) mutations in older patients with CN-AML (11, 15, 22). High levels of miR-155 and miR-3151 were also found to be associated with worse outcome in older patients with CN-AML (23, 24).

More recently, another class of noncoding RNAs named long noncoding RNAs (lncRNAs) was discovered (25). LncRNAs are transcripts longer than 200 nucleotides, located within the intergenic stretches or overlapping antisense transcripts of protein coding genes (25, 26). LncRNA genes are typically shorter than protein-coding genes and are predominantly transcribed by RNA polymerase II (26). LncRNAs have emerged as important regulators of gene expression, showing cell-specific expression patterns and subcellular localization and are involved in many biological roles including imprinting, epigenetic regulation, apoptosis, and cell cycle (26, 27). The well-characterized lncRNA HOX transcript antisense RNA (HOTAIR) has been found to be up-regulated in multiple cancers including breast, colorectal, hepatic, gastric, and pancreatic cancers (28–33). HOTAIR modulates chromatin structures by serving as a scaffold and recruiting histone modifiers (28). In breast cancer, HOTAIIR promotes cancer cell invasion and metastasis in vivo through epigenetic silencing of metastasis suppressor genes (29–33). In patients with nonsmall cell lung cancer, high expression of the lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was associated with metastases and poor survival, indicating its importance as a prognostic biomarker (34).

Whereas lncRNAs contribute to epigenetic gene regulation, metastasis, and prognosis in solid tumors, their role in AML has not been reported to date. In this study, we analyzed a large set of older patients with CN-AML using custom lncRNA microarrays and RNA sequencing (RNA-seq) to investigate whether lncRNA expression is associated with clinical features, molecular abnormalities, and outcome.

**Results**

**LncRNA Signatures Associated with Recurrent Mutations in CN-AML.**

To identify lncRNAs associated with recurrent mutations in CN-AML, we compared mutated and wild-type (WT) samples in the training set of 148 older patients with de novo CN-AML. For all of the signatures, we used \( P < 0.001 \) and fold change (FC) \( \geq 1.5 \) (Table S1 and SI Methods and Data show patient clinical and molecular features).

![Heat map of the lncRNA-expression signature associated with NPM1, FLT3-ITD, and CEBPA mutations in older patients with primary CN-AML.](https://www.pnas.org/cgi/doi/10.1073/pnas.1422050112)

(A) The heat map shows expression levels of 205 probe sets found differentially expressed between NPM1-mutated (mut) \( (n = 84) \) vs. NPM1 wild-type (WT) \( (n = 64) \) cases. (B) The heat map shows expression levels of 140 probe sets found differentially expressed between FLT3-ITD \( (n = 48) \) vs. FLT3-WT \( (n = 100) \) cases. (C) The heat map shows expression levels of 57 probe sets found differentially expressed between CEBPA-mut \( (n = 18) \) vs. CEBPA-WT \( (n = 130) \) cases. Rows represent probe sets and columns, individual patients. Patients are grouped according to NPM1, FLT3-ITD, and CEBPA mutation status, and genes are ordered by hierarchical cluster analysis. Expression values of the probe sets are represented by color: green represents expression less than the median value for the given probe set, and red, expression greater than the median value for the given probe set.
**NPM1 LncRNA Signature.** Patients with NPM1-mutated CN-AML (n = 84) have a strong and distinctive lncRNA signature composed of 205 probes corresponding to 180 lncRNAs (Fig. 1A and Table S2). Among the up-regulated lncRNAs, several were antisense transcripts of HOX genes (e.g., HOX-BAS3, MEIS1-AS2) that have been reported to be up-regulated in NPM1-mutated AML (35). The plasmacytoma variant translocation 1 (PVT1) lncRNA, which is expressed at higher levels in NPM1-mutated patients, is associated with poor prognosis in colorectal cancer via apoptosis inhibition (36). Another study reported that PVT1 silencing in breast and ovarian cancer cell lines resulted in a strong proliferation and apoptotic response (37). The coiled-coil domain may inhibit apoptosis (36). Another study reported that PVT1 siRNA knockdown of LOC100505854 is overexpressed in the FLT3-ITD signature, there were two probes corresponding to Wilms tumor 1 antisense RNA (WT1-AS) lncRNA. WT1-AS has been reported to interact with WT1 sense RNA resulting in WT1 protein up-regulation (40). Interestingly, WT1 RNA expression correlates with FLT3-ITD expression in patients with AML (41). Alternative splicing of WT1-AS has been found in AML. Whereas the functional significance of this finding is unknown, aberrations in alternative splicing have been suggested as contributing factors in the development of various diseases including cancer (42).

**FLT3-ITD LncRNA Signature.** We identified 119 lncRNAs (from 140 probes) associated with the presence of FLT3-ITD mutations (n = 48) compared with FLT3-WT (n = 100) (Fig. 1B and Table S3). Because NPM1 and FLT3-ITD mutations frequently coexist, there was considerable overlap in their lncRNA signatures (68 probes) (9). Among the 75 probes that were present only in the FLT3-ITD signature, there were two probes corresponding to Wilms tumor 1 antisense RNA (WT1-AS) lncRNA. WT1-AS has been reported to interact with WT1 sense RNA resulting in WT1 protein up-regulation (40). Interestingly, WT1 RNA expression correlates with FLT3-ITD expression in patients with AML (41). Alternative splicing of WT1-AS has been found in AML. Whereas the functional significance of this finding is unknown, aberrations in alternative splicing have been suggested as contributing factors in the development of various diseases including cancer (42).

**CEBPA LncRNA Signature.** We identified a signature of 51 lncRNAs (from 57 probes) in patients with CCAAT/enhancer binding protein (C/EBP), alpha (CEBPA)-mutated CN-AML (n = 18) (Fig. 1C and Table S4). The most down-regulated lncRNAs in CEBPA-mutated leukemia samples corresponded to HOXB-AS3, already identified as strongly associated with NPM1 mutation. This finding was expected, because CEBPA mutations occur preferentially (>90%) in patients with wild-type NPM1 (9, 10). Among the up-regulated lncRNAs (n = 50) in patients with CEBPA-mutated CN-AML, 47 lncRNAs were differentially expressed in patients with CEBPA-mutated CN-AML and 10 lncRNAs were differentially expressed in patients with CEBPA-mutated CN-AML and WT cases (n = 108) (Table S5). Among these lncRNAs, 19 were differentially expressed in IDH2-wt and 8 in IDH2-mut cases (n = 140). Altogether, the data indicate that there are lncRNAs associated with both types of IDH2 mutations, whereas some lncRNAs are characteristic of only one type of mutation. For example, LOC110058584 is overexpressed in the IDH2-wt subset, but not in the IDH2-mut samples. This lncRNA associates with and represses the tumor suppressor CDKN1A/p21 promoter by recruiting polycomb complex proteins (45).

**RUNX1 LncRNA Signature.** In our patient cohort, RUNX1 mutations were mutually exclusive with NPM1 mutations (Fig. S1), which themselves have strong, characteristic lncRNA gene-expression signatures. To avoid confounding effects due to the NPM1 mutations, we performed a two-class analysis (RUNXI-mutated vs. WT) blocking for RUNX1 mutation cases and using a randomized block design, as described in SI Methods and Data. Using this approach, we identified a signature comprising 76 lncRNAs from 83 probes (Table S6). Among the lncRNAs up-regulated in RUNX1-mutated AMLs, several were located in the proximity of genes characteristic of lymphoid cells such as the B-cell linker (BLNK) and the immunoglobulin heavy locus (IGH) complex. We previously reported that patients with RUNX1-mutated CN-AML exhibit up-regulation of lymphoid genes (15). Another up-regulated lncRNA in mutated RUNX1, vault RNA 1-1 (VTNR1A-1) has been associated with multidrug resistance (46). This could be relevant because patients with RUNX1-mutated CN-AML have a particularly poor outcome (15).
of patients with favorable lncRNA. The patients with an unfavorable lncRNA score also had shorter OS ($P < 0.001, HR = 2.95$; Fig. 2B and Table 1). The OS rate at 3 y was only 10% for patients with unfavorable lncRNA compared with 43% for patients with favorable lncRNA. In multivariable analyses, after adjusting for $BAALC$ and miR-155 expression status, patients with an unfavorable lncRNA score had lower CR rates ($P = 0.007, OR = 0.19$) (Table 2). They also had shorter DFS ($P < 0.001, HR = 3.23$), after adjusting for the European LeukemiaNet (ELN) genetic group status $(8)$, and shorter OS ($P < 0.001, HR = 3.62$), after adjusting for miR-155 expression and the ELN genetic group status (Table 2). All statistical analyses were also performed using the continuous lncRNA score, with similar results (SI Methods and Data shows the methods and results of the analyses of continuous lncRNA score).

Validation of the lncRNA Score by RNA-Seq. To validate the lncRNA score, we performed lncRNA profiling using RNA-seq in pre-treatment BM and peripheral blood (PB) samples from 71 older patients with de novo CN-AML. All patients were treated with intensive cytarabine/daunorubicin-based frontline therapy on CALGB protocols and did not receive HSCT in first CR. There were no significant baseline clinical and molecular differences between the training $(n = 148)$ and validation datasets $(n = 71)$ (Table S8). To validate the impact of lncRNA scores on CR, DFS, and OS, we considered 46 of the 48 survival-associated lncRNAs that were used in the training set, because two lncRNAs were not detected by RNA-seq. The RNA-seq score was derived as a linear combination of the expression of the 46 lncRNAs as described in Patients and Methods. Because we used a different platform (RNA-seq), we explored different approaches to select the best cut point (quartiles vs. median). After creating the Kaplan–Meier plots for quartiles, it was evident that the first and second quartiles grouped together and separated well from the third and fourth quartiles and had a significantly better EFS. We therefore classified patients with scores above the median as favorable and patients below the median as unfavorable. Patients with an unfavorable lncRNA score had a lower CR rate $(P = 0.03, 63\% vs. 86\%)$ compared with patients with favorable lncRNA score and had shorter DFS $(P = 0.009)$ and OS $(P = 0.009)$ (Fig. 2C and D).

### Table 1. Treatment outcomes according to the lncRNA score status in 148 older (aged ≥60 y) patients with de novo CN-AML

<table>
<thead>
<tr>
<th>End point</th>
<th>Favorable lncRNA score $(n = 37)$</th>
<th>Unfavorable lncRNA score $(n = 111)$</th>
<th>$P^*$</th>
<th>OR/HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete response, no. (%)</td>
<td>33 (89)</td>
<td>60 (54)</td>
<td>$&lt;0.001$</td>
<td>0.14 (0.05–0.43)</td>
</tr>
<tr>
<td>Disease-free survival</td>
<td>Median, y</td>
<td>1.5</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>% disease-free at 3 y (95% CI)</td>
<td>39 (23–55)</td>
<td>7 (2–15)</td>
<td>$&lt;0.001$</td>
<td>2.88 (1.74–4.76)</td>
</tr>
<tr>
<td>% disease-free at 5 y (95% CI)</td>
<td>36 (32–52)</td>
<td>2 (0–8)</td>
<td>$&lt;0.001$</td>
<td>2.95 (1.89–4.61)</td>
</tr>
<tr>
<td>Overall survival</td>
<td>Median, y</td>
<td>2.2</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>% alive at 3 y (95% CI)</td>
<td>43 (27–58)</td>
<td>10 (6–17)</td>
<td>$&lt;0.001$</td>
<td>2.95 (1.89–4.61)</td>
</tr>
<tr>
<td>% alive at 5 y (95% CI)</td>
<td>41 (25–56)</td>
<td>5 (2–10)</td>
<td>$&lt;0.001$</td>
<td>2.95 (1.89–4.61)</td>
</tr>
</tbody>
</table>

CI, confidence interval; HR, hazard ratio; OR, odds ratio.

* $P$ values for categorical variables are from Fisher’s exact test, $P$ values for the time to event variables are from the log-rank test.

### Discussion

Despite the advances in understanding both the biology and risk stratification of adult AML, outcome is still poor, particularly in older patients aged 60 y or older (2–6). Whereas recent studies have reported that clinical outcome of older patients with CN-AML is influenced by several recurrent mutations, patients with apparently identical cytogenetic and molecular “makeup” do not have uniform outcomes (11–19). This may result from additional undiscovered genetic alterations, which may identify smaller, clinically meaningful patient groups for which it is possible to identify specific therapeutic targets.

In this work, we investigated the associations of lncRNA expression with clinical characteristics, recurrent mutations, and outcome in older patients with CN-AML. We identified distinctive lncRNA expression signatures associated with the most common recurrent mutations in CN-AML. Patients with $NPM1$ mutations exhibit one of the strongest lncRNA signatures, as evidenced by higher lncRNA fold change and lower false discovery rates. It has been reported that $NPM1$-mutated CN-AML exhibits unique and strong mRNA and miRNA expression profiles characterized by $HOX$ gene and miR-10 family overexpression and CD34 negativity (11, 47). Because ~40% of lncRNAs intersect protein-coding loci and exhibit a strong pattern of coexpression (25), we found many up-regulated lncRNAs overlapping antisense transcripts of $HOX$ genes (e.g., $HOXB-AS3$) in the $NPM1$-mutated lncRNA signature. A previous study has shown that the $HOX$ antisense transcript, known as HOTAIR, is transcribed antisense to the $HOX$ locus and despite its genomic location, it has little effect on the regulation of the sense transcript (28). Instead, HOTAIR was shown to function in trans to negatively regulate $HOXD$ via increased polycomb repressive complex 2 (PRC2) occupancy at the $HOXD$ locus (28). A second group reported that $HOX$ antisense intergenic RNA myeloid-specific 1 (HOTAIRM1) is transcribed antisense to the $HOX$ genes (between $HOXA1$ and $HOXA2$), shows myeloid-specific expression and is up-regulated during granulocytic differentiation (48). Knockdown of HOTAIRM1...
positive and lncRNA profiles. On the other hand, expression levels of the how the different prognostic mutations associate with distinct score as an independent marker for outcome. It is remarkable lncRNA score in both the training and validation cohorts. Mul-

Table 2. Multivariable analysis of outcome according to the lncRNA score in older patients with CN-AML

<table>
<thead>
<tr>
<th>Variable</th>
<th>Categories</th>
<th>Complete response</th>
<th>Disease-free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OR 95% CI P</td>
<td>HR 95% CI P</td>
<td>HR 95% CI P</td>
</tr>
<tr>
<td>LncRNA score</td>
<td>Unfavorable vs. favorable</td>
<td>0.19 (0.06–0.63)</td>
<td>3.23 (1.93–5.43)</td>
<td>3.62 (2.26–5.78)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.007</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BAAALC expression group</td>
<td>High vs. low</td>
<td>0.17 (0.07–0.40)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>miR-155 expression group</td>
<td>High vs. low</td>
<td>0.34 (0.14–0.81)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ELN</td>
<td>Favorable vs. intermediate+*</td>
<td>—</td>
<td>0.5 (0.32–0.79)</td>
<td>0.42 (0.28–0.63)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.003</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Favorable Genetic Group is defined by CEBPA mutated or FLT3-ITD negative and NPM1 mutated status; Intermediate+ Genetic Group is defined by patients that are not in the favorable category: CEBPA wild-type and either FLT3-ITD positive and NPM1 mutated, FLT3-ITD negative and NPM1 wild-type, or FLT3-ITD positive and NPM1 wild-type status (8).

In summary, here we describe lncRNA profiles in older patients with CN-AML and their associations with clinical features, mutations, and outcome. Distinct lncRNA signatures were found associated with recurrent mutations in CN-AML. Future work is needed to investigate whether these lncRNAs are functional and whether they are regulated directly or indirectly by biologic changes promoted by these specific mutations. Additionally, we identified a lncRNA score that is strongly associated with outcome, including treatment response to standard intensive che-

Patients and Methods

Patient Set. Pretreatment frozen BM samples from 148 older (aged ≥60 y) patients with de novo CN-AML (training set) and 71 older frozen samples (52 BM and 19 PB) from older patients with de novo CN-AML (validation set) were obtained from the CALGB/Alliance leukemia tissue bank. Informed consent was obtained from the patients in accordance with the Declaration of Helsinki to procure and bank the cells for future research according to the institutional review boards of each of the participating institutions. Patient characteristics are shown in Tables S1 and S8. Cytoge-

Statistical Analyses. Definitions of clinical end points are provided in SI Methods and Data. The associations of the lncRNA score 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. Estimated probabilities of DFS and OS were calcu-

Multivariable Analyses. Multivariable logistic regression models were generated for attainment of CR, and multivariable proportional hazards models were constructed for DFS and OS, using a limited forward selection pro-

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the list of variables considered for model inclusion). For the time-to-event end points, the proportional hazards assumption was checked for each variable individually.

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12. TET2
