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A Leukemic Stem Cell Gene Expression Signature is Associated with Clinical Outcomes in Acute Myeloid Leukemia

Andrew J. Gentles, PhD, **Sylvia K. Plevritis, PhD**, **Ravindra Majeti, MD, PhD**, and **Ash A. Alizadeh, MD, PhD**
Department of Radiology, Lucas Center for MR Spectroscopy and Imaging (AJG and SKP); Department of Internal Medicine, Divisions of Oncology (AAA) and of Hematology (AAA and RM), Cancer Center, and Institute for Stem Cell Biology and Regenerative Medicine (AAA and RM), Stanford University School of Medicine, Palo Alto, CA 94305, USA

Abstract

Context—In many cancers, specific subpopulations of cells appear to be uniquely capable of initiating and maintaining tumors. The strongest support for this cancer stem cell model comes from transplantation assays in immune-deficient mice, which indicate that human acute myeloid leukemia (AML) is driven by self-renewing leukemia stem cells (LSC). This model has significant implications for the development of novel therapies, but its clinical relevance has yet to be determined.

Objective—To identify a leukemic stem cell gene expression signature and test its association with clinical outcomes in AML.

Design, Setting, and Patients—Global gene expression (microarray) profiles of LSC-enriched subpopulations from primary AML and normal patient samples were analyzed. Patient samples were obtained at Stanford University Medical Center between April 2005 and July 2007. Validation datasets of global transcriptional profiles of AML tumors from four independent cohorts totaling 1047 patients were analyzed retrospectively.

Main Outcome Measures—Identification of genes discriminating LSC-enriched from other subpopulations in AML tumors; association of the LSC-specific genes with overall, event-free, and relapse-free survival, and with therapeutic response.

Results—Expression levels of 52 genes distinguished LSC-enriched from other subpopulations in cell-sorted AML samples. An *LSC score* summarizing expression of these genes in bulk primary AML tumor samples was defined and found to be associated with clinical outcomes in four independent patient cohorts. High LSC scores were associated with worse overall (OS),

Correspondence should be addressed to: Ash A. Alizadeh MD, PhD, Stanford University School of Medicine, Palo Alto, CA 94305, USA. arasha@stanford.edu phone: 650-725-0120; or Ravindra Majeti, MD, PhD, Stanford University School of Medicine, Palo Alto, CA 94305, USA. rmajeti@stanford.edu phone: 650-721-6376.

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event-free (EFS), and relapse-free (RFS) survival, among patients with either a normal karyotype (NKAML), or with chromosomal abnormalities. For the largest cohort of patients with NKAML (n=163), the LSC score was significantly associated with OS as a continuous variable (hazard ratios [HR] 1.15, 95% Confidence Interval [CI] 1.08-1.22, log-likelihood p<0.001). When patients were split into high and low LSC score groups, the absolute risk of death by 3 years was 57% (95% CI 43-67%) for the low LSC score group, versus 78% (95% CI 66-86%) in the high LSC score group (HR 1.9, 95% CI 1.3-2.7, log-rank p=0.002). In another cohort with available data on EFS for 70 patients with NKAML, the risk of an event by 3 years was 48% (95% CI 27-63%) in the low LSC score group vs. 81% (95% CI 60-91%) in the high LSC score group (HR 2.4, 95% CI 1.3-4.5, log-rank p=0.006). The LSC score was associated with poorer outcomes, independently of known prognostic factors including age, *FLT3* or *NPM1* mutations, and cytogenetic risk group, and added to their prognostic value. For OS in three cohorts that included patients with cytogenetic abnormalities, the HRs of the continuous LSC score in multivariate Cox regression with FLT3/NPM1 status, age, and cytogenetic risk group were respectively HR 1.07 (95%CI 1.01-1.13), p=0.02; HR 1.10 (95% CI 1.03-1.17), p=0.005; and HR 1.17 (95% CI 1.05-1.30), p=0.005.

Conclusions—High expression of a leukemic stem cell gene expression signature is independently associated with adverse outcomes in AML.

INTRODUCTION

Acute Myeloid Leukemia (AML) is an aggressive malignancy of the bone marrow characterized by accumulation of early myeloid blood cells that fail to mature and differentiate. The course of the disease is marked by poor prognosis, frequent relapse, and high disease-related mortality[1-2]. Recent clinical investigation has focused on the identification of prognostic subgroups in adult AML with the goal of guiding patients into risk-adapted therapies. Such investigation determined that cytogenetic abnormalities are prognostic, some favorable and others unfavorable[3-4], yet up to 50% of patients have normal karyotype AML with a wide range of clinical outcomes. In these patients, the presence of specific molecular mutations can provide prognostic information, including internal tandem duplications within the *FLT3* gene (*FLT3*-ITD), partial tandem duplication of the *MLL* gene (*MLL*-PTD), mis-localizing mutations of the *NPM1* gene (NPM1c), mutations in the *CEBPA* and *RAS* genes, and increased expression of the *BAALC* and *ERG* genes[5-6]. However, these parameters, and others such as patient age, are only partially successful at capturing risk of relapse and patient outcomes following treatment.

A growing body of evidence suggests that specific cancer cell subpopulations possess the ability to initiate and maintain tumors [7-8]. AML is the paradigm for which this cancer stem cell hypothesis has been advanced, and this model has major implications for the development of novel therapeutic agents [9]. There is significant experimental evidence indicating that AML is organized as a hierarchy of malignant cells initiated and maintained by self-renewing leukemia stem cells (LSC) that comprise a subset of the total leukemic burden (eFigure 1)[7, 10]. These LSC are enriched in the CD34+CD38− fraction (hereon referred to as the LSC-enriched subpopulation), and in turn give rise to CD34+CD38+ leukemia progenitor cells (LPC), which further differentiate into the CD34− leukemic blast

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population[10-11]. A major implication of this cancer stem cell model is that in order to eradicate the cancer and cure the patient, the LSC must be eliminated[7-8]. While AML was the first human malignancy for which this model gained experimental support, its clinical significance has yet to be fully established.

We hypothesized that if the cancer stem cell model accurately reflects the biology of human AML, then patients with LSC enrichment should have worse clinical outcomes, even when accounting for known prognostic parameters, and that this association could be quantified by global gene expression profiling of bulk AML samples.

METHODS

Purification and Genomic Expression Profiling of Normal and Leukemic Cell Subpopulations

Seven human AML tumor samples were obtained at the Stanford University Medical Center between April 2005 and July 2007, according to an approved protocol of the Institutional Review Board after informed consent. Normal human bone marrow mononuclear cells were purchased from AllCells Inc. (Emeryville, CA), and human cord blood was obtained from Stanford University Medical Center. Normal and leukemic subpopulations were purified from peripheral blood and/or bone marrow by fluorescence-activated cell sorting using the antibodies shown in eFigure 1, as follows: AML LSC (n=7), AML leukemic progenitor cells (n=7), AML Blasts (n=7), normal hematopoietic stem cells (HSC; bone marrow and cord blood, n=7), normal multipotent progenitors (bone marrow and cord blood, n=7), normal common myeloid progenitors (bone marrow, n=4), normal granulocyte-monocyte progenitors (bone marrow, n=4), and megakaryocyte-erthythrocyte progenitors (bone marrow, n=4). Global transcriptional profiles were generated for each sample using Affymetrix U133 Plus 2.0 gene expression microarrays. Raw data were deposited at the NCBI Gene Expression Omnibus (GEO, accession GSE24006). Detailed experimental procedures for purification of cell subpopulations have been reported previously[12].

Definition of LSC Signature Based on Gene Expression Microarray Analysis

Global gene expression profiles of 14 paired LSC-enriched and LPC-enriched subpopulations purified from the 7 AML patient samples described above were combined with 16 paired profiles (8 LSC-enriched and 8 LPC-enriched subpopulations) from an independent study [13] to produce one dataset of 30 samples for this analysis. All genes profiled on microarrays were ranked by the mean ratio of their expression between paired LSC-enriched and LPC-enriched subpopulations, and evaluated using Gene Set Enrichment Analysis[14]. This approach assessed whether any pre-defined *groups* of biologically-related genes were concordantly more highly expressed in LSC-enriched or LPC-enriched subpopulations. *Individual* genes expressed more highly in LSC-enriched compared to LPC-enriched subpopulations (or vice versa) were identified by using Significance Analysis of Microarrays[15] (false discovery rate<10%). Ingenuity Pathways Analysis was used to identify interaction networks involving these genes.

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The genes that were more highly expressed in LSC-enriched relative to LPC-enriched subpopulations were summarized by an *LSC signature.* The LSC signature was defined as a single number representing the relative expression of the LSC-enriched genes in a given sample compared to the other samples in the same dataset. The signature was computed as the first principal component of the gene expression data matrix whose rows were the LSC-enriched genes. Each column of the matrix represented one sample, and matrix entries were the gene expression values corresponding to each gene in each sample. By definition, the first principal component of such a data matrix is the weighted sum of the genes' expression levels that explains the maximum possible amount of their total variation across all samples. Thus, for a group of n genes, each sample S is associated with one number: $(\text{LSC signature})_S = a_1 g_{1S} + a_2 g_{2S} + \ldots + a_n g_{nS}$, where g_{iS} is the expression level of gene i in sample S, and is weighted by a_i in the summation. The LSC signature was evaluated in the purified normal and leukemic subpopulations described above to investigate the expression of the LSC-enriched genes beyond the LSC-enriched and LPC-enriched subpopulations used to identify them.

Definition of LSC Score for Testing Association with AML Survival Outcomes

To test associations between the LSC-enriched genes and clinical outcomes, a retrospective training-validation scheme was adopted. Raw microarray data were obtained for four publicly available bulk AML gene expression studies with available clinical annotations[16-20] from NCBI GEO (GSE12417, n=163 normal-karyotype AML only, with OS outcomes; GSE10358, n=184, OS and EFS; GSE14468, n=527, OS, EFS and RFS) and the National Cancer Institute caArray database (accession willm-00119, n=170, OS only). The largest NKAML dataset[16] (n=163) was used as the training set, and the other three datasets were used for validation. The LSC signature was calculated in the training cohort and the *same weights* were then applied to the test cohorts. Because the genes weights were not recomputed in each test cohort to ensure unbiased validation, the resulting number for each sample was referred to as the *LSC score*. In the training set, the LSC signature and LSC score were identical by definition. The median LSC score in the training set was used to partition patients in all cohorts into high versus low LSC score groups.

Statistical Analysis of LSC Score on AML Survival Outcomes

The LSC score was tested for associations with survival outcomes as a continuous variable using Cox proportional hazards regression (log-likelihood test), and as a dichotomous stratification (high vs. low LSC score group) by Kaplan-Meier analysis (log-rank test), using the *survival* package (version 2.35) in *R* version 2.11. Patients with missing data were excluded from analyses. Absolute risk (AR) of events occurring by 3 years was determined from Kaplan-Meier analysis. Given that LSC have been experimentally demonstrated to be chemotherapy resistant[13, 21], the LSC score was tested for associations with primary refractoriness to therapy and disease relapse (event-free and relapse-free survival). For relapse-free survival, only patients who had first achieved clinical remission from disease were included[22].

The robustness of the association between the LSC score and outcomes was evaluated as follows. The training dataset was split in half. Gene weightings defining the LSC score were

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derived in one half of the data, and then applied to the other half to test associations with survival. Results from 1000 random splits of the training set were compared. Furthermore, the uniqueness of the prognostic value of the LSC-enriched genes was tested by comparing it to results obtained by repeating the analysis on 10000 sets of the same number of randomly selected genes.

Independent Prognostic Value of the LSC Score

Prior clinical investigation in adult AML has defined several important prognostic factors including age, karyotype (chromosomal rearrangements) and molecular mutations, particularly internal tandem duplications in the gene *FLT3* (*FLT3*-ITD) and mis-localizing mutations in *NPM1* (NPM1c)[5, 23]. In the current analysis, multivariate Cox regression was used to test whether the LSC score conferred prognostic value independent from these established clinical predictors. For further investigation of how the LSC score added to known prognostic factors, Area Under the Received Operating Characteristic curve (AUC-ROC) was conducted using the *survivalROC* package (version 1.0) in *R* [24]. Because assignments to cytogenetic risk groups were inconsistent between different clinical groups, risk was compared in uniform fashion across datasets, by applying the refined Medical Research Council risk scheme (favorable, intermediate, adverse) based on metaphase karyotypes [23].

Since acute promyelocytic leukemia (APL) is a distinct disease entity, it was excluded from all survival analyses. Association of the LSC score with AML clinical subtypes was also assessed by ANOVA followed by Games-Howell post-hoc test (SPSS12, IBM Inc) for groups with unequal sizes and variances. All statistical tests were two-sided. P-values less than 0.05 were considered significant.

RESULTS

An LSC-Enriched Gene Expression Signature is Shared with Normal HSC

Global gene expression profiles of 15 AML LSC-enriched subpopulations were compared to 15 paired LPC-enriched subpopulations, collected from the same 15 AML samples. The samples were derived from patients representing a diversity of AML subtypes and clinical outcomes (Table 1). Significance Analysis of Microarrays [15] identified 31 genes as more highly expressed in the LSC-enriched than LPC-enriched subpopulations, and 21 genes as more highly expressed in LPC-enriched than LSC-enriched subpopulations (false discovery rate <10%; Figure 1A and eTable 1). Many of these genes were significantly associated with each other in a network-based analysis (eFigure 2). In addition to the CD34 and CD38 cell surface markers used to purify the samples, the group of genes included factors known to be differentially expressed in early hematopoiesis such as *VNN1, RBPMS, SETBP1, GUCY1A3*, and *MEF2C* (all gene names reported are the gene symbol assigned by the Human Genome Organization Gene Nomenclature Committee). Interestingly one other gene more highly expressed in the LSC-enriched subpopulation, the homeobox protein *HOPX,* has known interactions with the induced pluripotency factors *SOX2, POU5F1*, and *NANOG*, as well as the histone deacetylase *HDAC2* (eFigure 3).

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Gene Set Enrichment Analysis[14] showed that genes more highly expressed in LSC were enriched for those expressed in normal CD34+CD38− cells, which include normal hematopoietic stem cells (HSC), compared to normal CD34+CD38+ progenitors (Figure 1B and eTable 2). Notably, genes more highly expressed in LSC were also enriched for genes whose expression in AML has been correlated with high expression of the gene *BAALC,* an adverse prognostic factor[25]. Conversely, proliferation, cell cycle, and differentiation genes were systematically repressed in the LSC-enriched subpopulations when compared to more differentiated LPC-enriched subpopulations, consistent with a tendency for replicative quiescence[10].

The 31 genes more highly expressed in LSC were combined to generate an *LSC signature* as described in the Methods. The LSC signature was computed in the purified subpopulations from primary AML patient samples and in subpopulations from the normal hierarchy of differentiating myeloid blood cells. By definition, the LSC signature was high in LSC compared to LPC, but also relative to downstream, more differentiated CD34− blasts (Figure 1C). Among normal hematopoietic cells from healthy individuals, the LSC signature was high in HSC and multipotent progenitors, compared to more mature myeloid cell populations (Figure 1C). These observations suggest that the LSC signature is shared with normal HSC, implying that it may reflect self-renewal ability and relative proliferative quiescence.

High LSC Score is Associated with Inferior Overall Survival

We next evaluated whether expression of LSC-enriched genes was associated with clinical outcomes using four public datasets of bulk AML expression profiles with available clinical annotations. Details of patient characteristics, primary therapies, clinical responses, remission rates, and outcomes have been reported previously[16-19, 26], and are summarized in Table 2.

The LSC signature was calculated for a training set of 163 AML patients with no chromosomal abnormalities (NKAML)[16], defining the weights for combining expression levels of the 31 LSC genes into a single measure for each sample (eTable 3). Because the same gene weights were applied to the independent test cohorts, we refer to this as the *LSC score*. In the training set, the LSC score ranged from 17.4 to 33.1 (median 24.9), and was associated with overall survival as a continuous variable ($p<0.0001$ log-likelihood, hazard ratio [HR] 1.15, 95% Confidence Interval [CI] 1.08-1.22), with higher LSC score associating with inferior outcome (Table 3). Stratification of patients into high versus low LSC score groups robustly separated survival curves ($p=0.002$ log-rank, HR 1.85, 95% CI 1.25-2.74; Table 3 and Figure 2A). The absolute risk of an event by 3 years (AR[1]) was 57% (95% CI 43-67%) in the low LSC score group versus 78% (95% CI 66-86%) in the high LSC score group. Association of the LSC-enriched genes with OS was supported by internal cross-validation in the training cohort (eFigure 4).

The LSC score was calculated for each sample in the three independent test cohorts. For the NKAML cases in these cohorts, high LSC score was associated with inferior OS as a

[1]Throughout the text, absolute risk is reported at 3 years.

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continuous variable (p<0.012 in all cases; with HR 1.17, 95% CI 1.07-1.28; HR 1.13, 95% CI 1.04-1.22; and HR 1.18, 95% CI 1.04-1.34 in the three cohorts; Table 3). Using the median LSC score from the training set as a pre-specified threshold, stratification of patients into high versus low LSC score groups significantly separated survival curves in each dataset (Table 3, Figure 2B and eFigure 5). For example, in NKAML patients from one well-characterized cohort of adult AML patients with diverse karyotypes primarily treated with induction regimens including cytarabine and an anthracycline (Tomasson et al.)[17, 20], the LSC score ranged from 16.6 to 31.0 and was significantly associated with OS (Table 3 and Figure 2B; see Table 4 for medians and interquartile ranges of the LSC score in all cohorts). This association was significant whether the LSC score was evaluated as a continuous predictor (p=0.003; HR 1.13, 95% CI 1.04-1.22), or a high versus low LSC score split (p=0.002; HR 2.7, 95% CI 1.4-5.1), with those in the low LSC group having a median OS of 56.3 months (AR 0.39, 95% CI 0.20-0.54) compared to 16.3 months (AR 0.81, 95% CI 0.61-0.90) for those in the high LSC group (Table 4). The set of genes comprising the LSC score was significant in its prognostic utility when compared to 10000 randomly selected gene sets of the same size (eFigure 6), supporting the conclusion that the association with clinical outcomes was not a false positive result.

The LSC score ranged from 16.6 to 35.5 among all non-APL AML patients, including those with cytogenetic abnormalities, in the Tomasson et al. cohort (Table 4), and was again associated with OS (p=0.001, HR 1.10, 95% CI 1.04-1.17) as a continuous variable. Patients in the low LSC score group had a median OS of 56.3 months (AR 0.45, 95% CI 0.30-0.57) compared to 16.5 months (AR 0.75, 95% CI 0.64-0.83) in the high LSC score group (p=0.003, HR 2.0, 95% CI 1.3-3.2). Investigation of the LSC score in patients from two additional cohorts including patients with chromosomal abnormalities confirmed its association with adverse OS in both (eFigure 5 and Table 3).

Higher LSC Score is Associated with Inferior EFS, Refractoriness to Treatment, and Disease Relapse

Higher LSC scores were consistently associated with inferior EFS in patients with NKAML from two cohorts with available data (p=0.001, HR 1.15, 95% CI 1.06-1.26; and p=0.007, HR 1.11, 95% CI 1.03-1.21 as a continuous variable). As with OS, the LSC score-high group had inferior EFS, with a median of 10 months (AR 0.81, 95% CI 0.60-0.91) compared to 48 months (AR 0.48, 95% CI 0.27-0.63) in the low LSC score group of the Tomasson et al. cohort[17] (p=0.006, HR 2.4, 95% CI 1.3-4.5; Figure 2C, and Tables 3 and 4). In the second cohort with available EFS data[19], high versus low LSC score groupings separated survival curves (eFigure 5; p=0.02; HR 1.7, 95% CI 1.1-2.7; AR 61%, 95% CI 46-72% in the low LSC score group vs. AR 80%, 95% CI 64-89% in the high group). For the latter dataset[19], LSC score was also associated with RFS in NKAML patients who had achieved an initial clinical remission (p=0.03, HR 1.13, 95% CI 1.01-1.27 as a continuous variable), with median RFS of 66 months (AR 43%, 95% CI 26-56%) in the low LSC score cases, compared to only 10 months (AR 68%, 95% CI 46-81%) in the high group (p<0.06, HR 1.8, 95% CI 1.0-3.3; eFigure 5). This finding is consistent with the demonstrated chemo-resistance of LSC[13].

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Concordantly, the rate of clinical remission (CR) was superior among AML patients with low LSC score compared to those with high LSC score, both in an older cohort (median age 65y, 56% CR for low LSC score vs. 29% for high LSC score, p<0.001 by Fisher exact test)[18], and a younger one (median age 43y, 88% vs. 76%, p=0.02)[19, 26]. Furthermore, LSC scores were significantly higher in patients failing to achieve clinical remission compared to those who reached CR (p<0.001, Figure 2D), a distinction most evident for those patients in whom such remissions were durable.

The LSC Score is Independently Associated with Clinical Outcomes

The LSC score carried prognostic value independent of other known clinical factors including age, FLT3/NPM1 mutations, and cytogenetic risk group. It was significant independent of these factors in multivariate Cox regression, with high LSC score again being associated with adverse OS, EFS, and RFS, in all but one instance (Table 5). Comparisons of Area Under the Receiver Operator Characteristic curves (AUC-ROC, a measure of the accuracy of a prognostic model) showed that the LSC score added to the prognostic value of age, *FLT3*-ITD, NPM1c, and cytogenetic risk in predicting OS at 2 years in all cohorts for both NKAML and non-APL AML (eTable 4). Models that incorporated the LSC score in addition to other known prognostic factors had consistently higher AUC-ROC, when compared to models that did not include the LSC score (see eSupplement for further details).

In NKAML, higher LSC score associated with inferior OS in *NPM1*-wild-type and *NPM1*-mutant cases, despite the fact that the latter are frequently CD34-negative, and in patients with both wild-type *FLT3* and wild-type *NPM1* (eTable 5). Furthermore, when all analyses (including derivation of LSC score gene weightings) were performed excluding CD34-negative cases (defined either as *NPM1* mutant, or as the 40% of samples with lowest CD34 expression), similar results were obtained. Exclusion of CD34 from the model-building and validation resulted in an LSC score with similar gene weightings and prognostic value (eTable 3). Therefore, the LSC score was not simply a proxy for CD34 status.

Taken together, these data indicate that higher LSC score is associated with inferior survival outcomes independent of age, *FLT3*-ITD, *NPM1* mutations, CD34 expression, and cytogenetic risk group, and adds to their prognostic utility.

Lower LSC Score is Associated with Prognostically Favorable AML Subtypes

Though similar across most age groups and morphological subtypes, LSC scores were higher in cases with minimally differentiated myeloblasts (French-American British M0), which typically have poor prognosis[27], consistent with previous reports of high LSC prevalence in this subtype (eFigure 7)[28]. In general, the LSC score was similar in favorable, intermediate, and adverse cytogenetic risk groups, and was not a direct proxy for this factor (eFigure 7C). This is consistent with our findings from survival analysis that the LSC score confers independent prognostic value. When considering specific cytogenetic subgroups, the LSC score had higher than average values in patients with unfavorable −5 or 7(q) abnormalities, and lower than average values among AML harboring anomalies involving

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11q23/*MLL*. Recent studies have reported that self-renewing cells from AML mouse models carrying MLL anomalies reside in more mature cells[29].

We also investigated the relationship of the LSC score to molecular mutations in the largest single cytogenetic subgroup of AML, NKAML. LSC scores were significantly lower in those harboring NPM1c mutations (eFigures 7D,8), in agreement with recent observations that leukemia initiating cells in *NPM1* mutant AML are frequently CD34 negative[30]. Furthermore, LSC scores were significantly lower within the subgroup of patients with wild type *FLT3* but mutant NPM1c, a combination conferring a distinctly favorable prognosis in NKAML (eFigure 7D)[6]. LSC scores were also lower in NKAML with double *CEBPA* mutations, again associated with favorable outcomes [19], relative to cases with single mutants, but not relative to wild-type *CEBPA*. Similar findings were observed in all four independent datasets totaling 1047 patients (eFigure 8 and 9). Of note, no significant differences in LSC scores were observed when patients with AML were stratified according to less common recurrent somatic mutations, including those in the tyrosine kinase domain of *FLT3* (*FLT3*-TKD), or activating mutations in *NRAS, KRAS*, or *IDH1*.

COMMENT

Clinical evidence supporting the significance of the cancer stem cell model for human AML has been lacking despite ample experimental evidence from transplantation assays in immune-compromised mice. In this study, we show that a gene expression score associated with the LSC-enriched subpopulation is an independent prognostic factor in AML, with high score associated with adverse outcomes in multiple independent cohorts. Specifically, high LSC score is associated with poor OS, EFS, and RFS in NKAML, and inferior OS in patients with chromosomal abnormalities. Additionally, the LSC score was associated with primary response to induction chemotherapy, as high scores strongly correlated with lower remission rates. Multivariate analysis demonstrated that the score associated with poor outcomes independently of age, *FLT3* or *NPM1* mutations, and cytogenetic risk group. These findings support the clinical relevance of the cancer stem cell model for AML.

AML stem cells were originally identified by prospectively separating primary leukemic specimens into subpopulations based on expression of CD34 and CD38, surface markers that are differentially expressed in normal hematopoiesis (eFigure 1)[10]. When the function of these tumor subpopulations was assessed by transplantation into immune-deficient mice, leukemia-initiating activity was demonstrated exclusively in the CD34+CD38− fraction[11]. The majority of recent studies indicate that AML LSC activity is enriched in the CD34+CD38− subpopulation, although recent reports have challenged whether this is exclusive [30-31]. The clinical significance of the leukemia stem cell model is suggested by two prior studies, the first of which identified an inverse correlation between the frequency of CD34+CD38− cells at diagnosis and the duration of relapse-free survival[32]. The second study reported that the relative ability of AML cells to successfully engraft in immune-deficient mice (a property associated with LSC) correlated with adverse clinical features[33]. While suggestive, neither of these studies investigated large cohorts of patients with long-term follow-up and diverse clinical features.

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Notably, the LSC signature was highly expressed in purified HSC, and much lower in more differentiated myeloid progenitor cells, suggesting that it may be reflective of self-renewal ability. Despite the observed similarities between the LSC signature and HSC gene expression programs, therapeutic targeting of leukemic stem cells is still possible without toxicity toward normal HSC. Indeed, markers distinguishing LSC from HSC exist and are amenable to targeted therapies, including antibodies to CD47, CLL-1, and CD123[34-36]. Future work is needed to prospectively validate the prognostic ability of the LSC score by evaluating its component genes using RT-PCR in an independent patient cohort. It will also be pertinent to examine the relationship of the LSC score to other gene expression signatures that have been proposed for predicting survival in AML[16, 26, 37].

In addition to the markers CD34 and CD38 which were employed for their purification, LSC were distinguished from LPC by the expression of several other genes known to be differentially expressed during early myelopoiesis. These included three members (*GIMAP2, GIMAP6,* and *GIMAP7*) of a small family of immune-associated nucleotide-binding proteins implicated in survival of hematopoietic cells and leukemia[38]; however, no prior associations with AML have been described. Two genes (*HOPX, GUCY1A3*) in this signature, which have previously been incorporated into AML prognostic models[16, 37], are notable for their distinctive pattern of expression and histone modification in self-renewing cells[39]. *HOPX* is an unusual homeodomain protein known to directly recruit histone deacetylase activity without directly binding DNA[40], and to be directly repressed *in vivo* in malignant cells in response to administration of the histone deacetylase inhibitor panobinostat[41]. The latter is currently being studied in clinical trials for patients with AML. *GUCY1A3,* which encodes a component of the soluble guanylate cyclase enzyme catalyzing the conversion of GTP to cGMP, is repressed during replicative senescence[42], and cGMP has been reported to stimulate HSC proliferation[43].

The cancer stem cell model has been studied in solid tumors in addition to leukemia[7]. Investigation of gene expression in human breast cancer stem cells identified a signature prognostic of metastasis-free and overall survival in multiple carcinomas, suggesting the clinical significance of the cancer stem cell model in these solid tumors[44]. Among other human malignancies, we and others have described prognostic significance of distinctive signatures of self-renewing populations including embryonic stem cells[45-46] and HSC and progenitor cells[47-48]. However, the current work represents the first to directly define a signature of enriched AML-initiating cells, and to relate this signature to expression profiles of diagnostic specimens, allowing a link to corresponding clinical and pathological features of patients. Ultimately, this model has major implications for cancer therapy, most notably that in order to achieve cure, the cancer stem cells must be eliminated[7]. To accomplish this in AML, novel therapies targeting LSC must be developed. Several such therapies are being investigated including small molecules[21, 49-51] and monoclonal antibodies[34-35, 52], which hold promise for improving therapeutic efficacy beyond current conventional treatments.

Our LSC prognostic model requires validation in a prospective study of AML patients treated with a standardized protocol incorporating strict eligibility criteria, a uniform treatment plan, uniform sample collection and handling, and well-defined primary endpoints. Moreover, microarrays are not currently broadly employed in clinical decision

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making [53]. While surrogate methods such as real-time PCR have demonstrated clinical utility[54], their application requires performance assessment in independent laboratories. Flow cytometric analysis of the predictive power of the proteins comprising the LSC score, and comparison to RNA-based models, may help to determine the best platform for future clinical application. However, monoclonal antibodies useful for flow cytometry are not presently available for the full set of encoded proteins.

CONCLUSION

High expression of a leukemic stem cell gene expression signature is independently associated with adverse outcomes in AML. If prospectively validated, the described LSC score may be incorporated into routine clinical practice for predicting prognosis in patients with AML, and used in clinical trials incorporating risk-based stratification or randomization strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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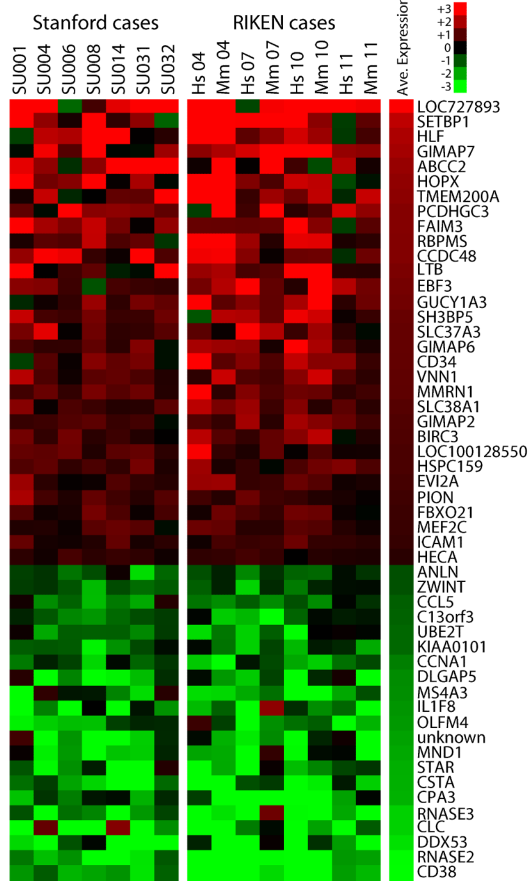

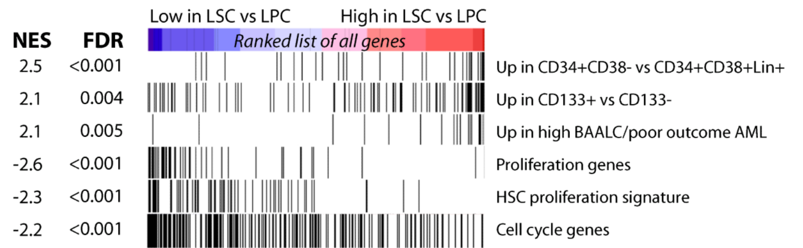

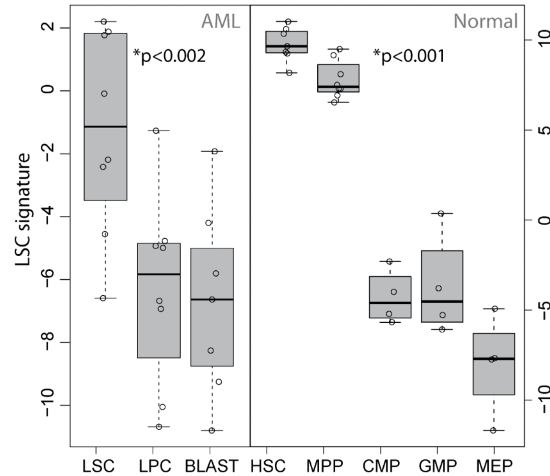

Figure 1. LSC-Enriched Subpopulations Have a Distinct Gene Expression Signature That is Shared with Normal HSC

Genes distinguishing leukemic stem cells (LSC) from leukemic progenitor cells (LPC). **(A)** Gene expression heatmap, with each column representing the difference in expression between LSC/LPC-enriched subpopulations isolated from the same AML patient[12-13]; 'Hs' denotes LSC/LPC profile purified from primary human patient specimen, and 'Mm' represents corresponding samples from mouse xenografts. 52 unique genes were identified as differentially expressed between LSC and LPC at 10% false discovery rate (eTable 1), with red indicating higher expression in LSC. **(B)** Enrichment analysis of relative expression between LSC and LPC of 17119 genes for the samples depicted in panel A (see eTable 2 for gene set definitions). Vertical bars in each of the six rows represent genes from each of the indicated gene sets. All nominal p-values were <0.001. NES: normalized enrichment score[14]; FDR: false discovery rate. **(C)** Expression of the *LSC signature* across AML subpopulations (left) and normal hematopoietic stem and progenitor cell (HSPC) populations involved in myeloid differentiation (right), including AML leukemic stem cell (LSC), leukemic progenitor cell (LPC), and leukemic blast (BLAST) populations, as well as normal hematopoietic stem cell (HSC), multipotent progenitor (MPP), common myeloid progenitor (CMP), granulocyte-monocyte progenitor (GMP), and megakaryocyte-erythrocyte progenitor (MEP). LSC and LPC samples are the same as those whose paired

differences are depicted in panel A (Stanford cases). Boxes span the interquartile range, with median depicted by the thick horizontal bar. Each circle marks one sample. P-values were derived from Wilcoxon test comparing LSC to LPC/Blast, and for HSC/MPP compared to CMP/GMP/MEP.

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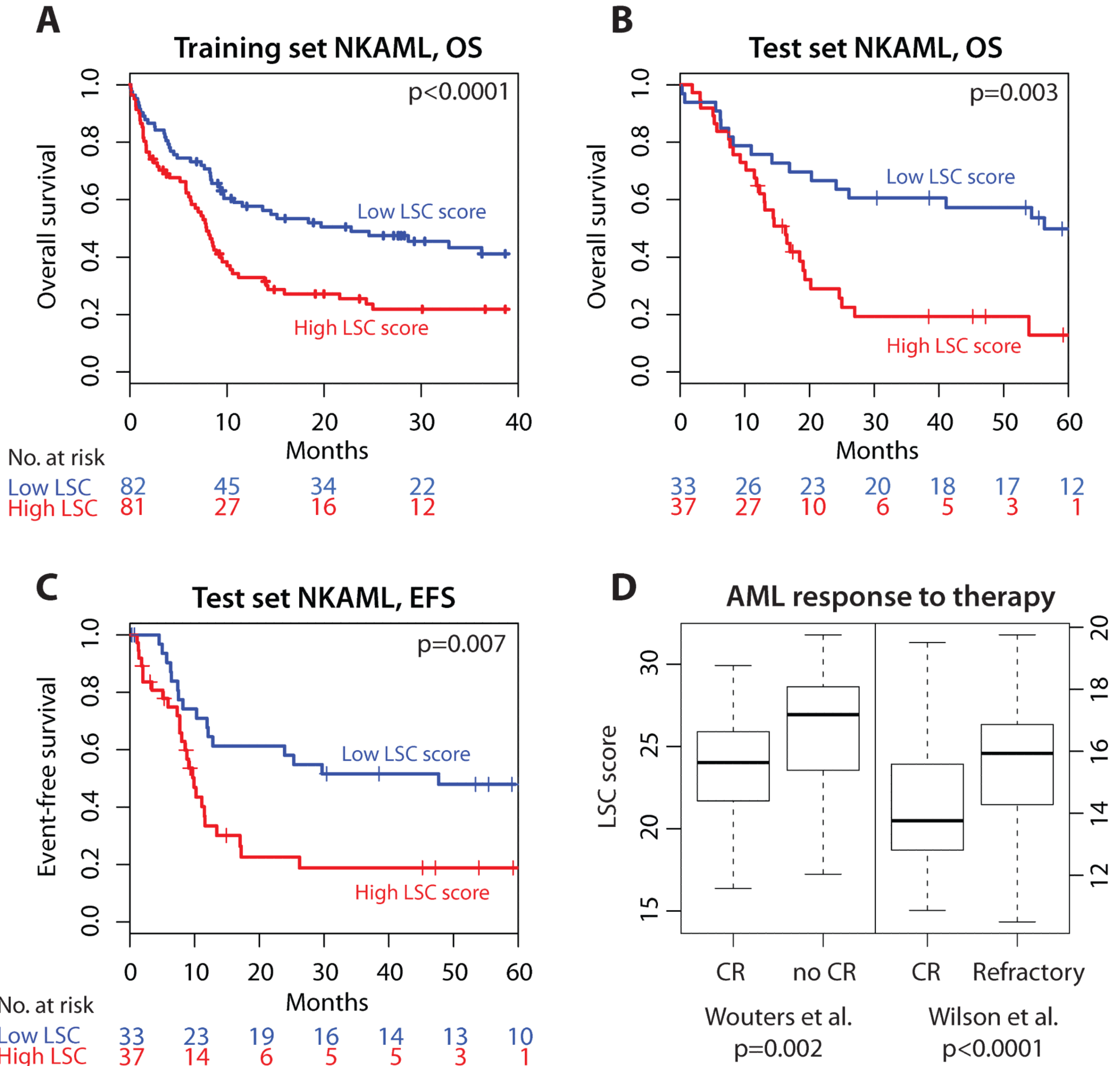

Figure 2. Higher LSC Score is Associated with Worse Outcomes

Kaplan-Meier analysis of the association between the LSC score and survival outcomes in normal karyotype AML (NKAML). Excluding those with acute promyelocytic leukemia (APL), patients were split into high versus low LSC score groups according to the median value of the LSC score in the training cohort. Stratification of outcomes using this approach is depicted for OS of NKAML patients in the training set[16] **(A)**, in NKAML from one of the validation sets[17] for OS **(B)**, and for EFS **(C)**. Vertical ticks on curves indicate censored events, and p-values shown are for the LSC score as a continuous predictor of survival (log-likelihood test; log-rank estimates provided in Table 3). Similar results were obtained in additional independent datasets (eFigure 5 and Table 3). **(D)** The LSC score was

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significantly associated with initial therapeutic response as determined by the ability to achieve clinical remission in two datasets for which this information was available[18-19] (p-values derived from t-test). Boxes indicate the interquartile range, with median shown as the thick horizontal bar. Numbers within boxes indicate the sample sizes. OS=overall survival; EFS=event-free survival; CR=clinical remission.

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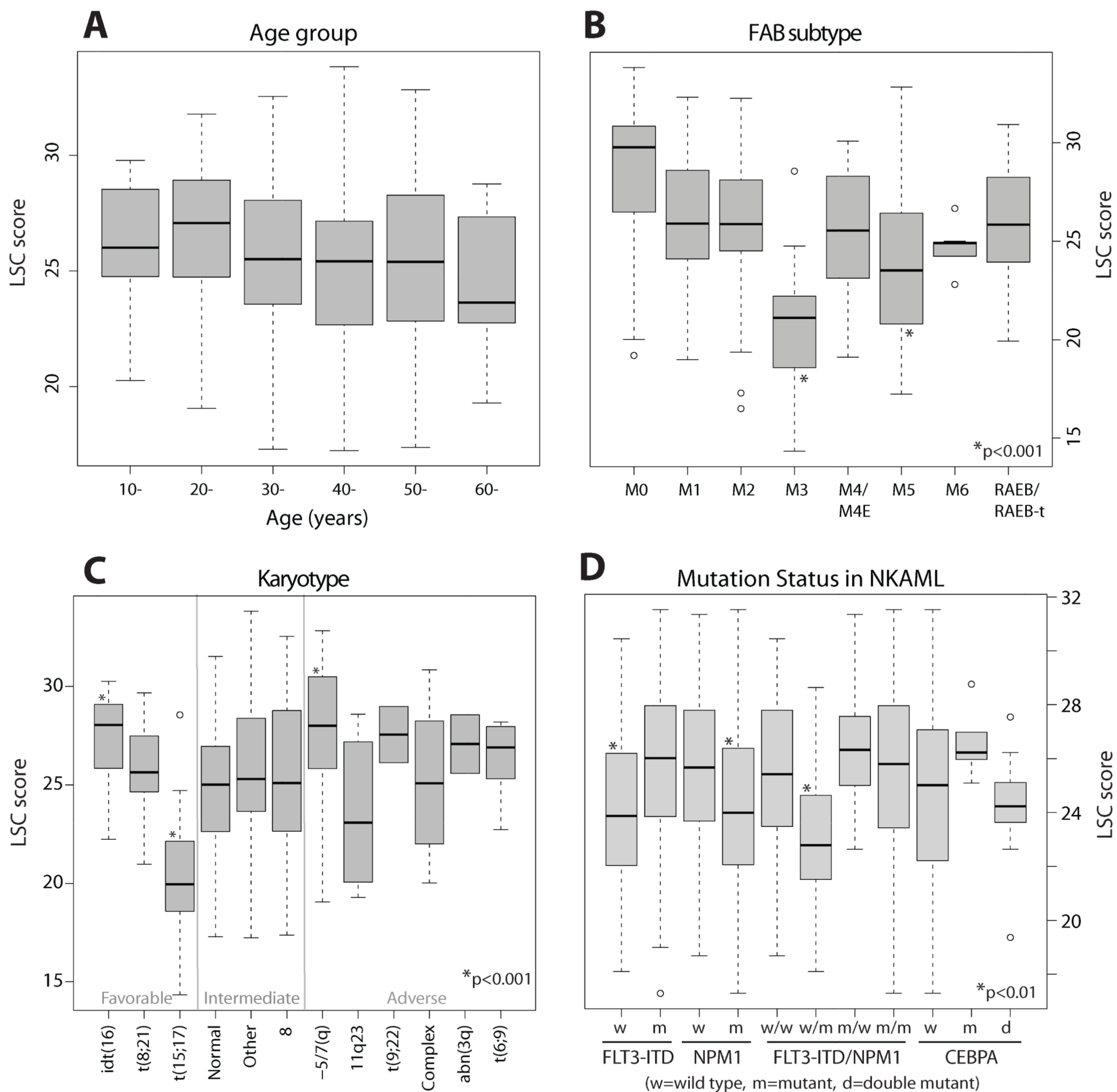

Figure 3.

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Table 1

Characteristics of Patient Samples Used to Identify LSC Signature Genes

For Stanford patients [12], age (at initial diagnosis), gender, cytogenetic abnormalities, FAB subtype, FLT3-ITD status, time from diagnosis to last follow-up, and status at last follow-up are reported. For the RIKEN dataset[13], only FAB subtype and gender was available. Unknown entries are dashed.

Institution	Sample ID	Age	Gender	De Novo/ Relapsed	Cytogenetics	FAB	FLT3-ITD	Time to last follow-up (days)	Status at last follow-up
Stanford[12]	SU001	59	Female	Relapsed	Normal	M2	Negative	32	DEAD
	SU004	47	Female	Relapsed	Normal	M5	Positive	74	DEAD
	SU006	51	Female	De Novo	-	M1	Negative	1196	ALIVE
	SU008	64	Male	De Novo	Normal	M1	Positive	1102	ALIVE
	SU014	59	Male	De Novo	Normal	n/a	Positive	23	ALIVE
	SU031	31	Female	De Novo	Complex	M4	Negative	708	ALIVE
	SU032	47	Male	De Novo	Normal	M5	Negative	226	ALIVE
RIKEN[13]	Hs04	-	Male	De Novo	-	M2	-	-	-
	Hs07	-	Female	De Novo	-	M4	-	-	-
	Hs10	-	Male	De Novo	-	M2	-	-	-
	Hs11	-	Male	De Novo	-	M1	-		

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Table 2

Summary of Four Independent AML Cohorts with Public Gene Expression Data on Bulk Samples

Summary indicates size of study, type of AML samples, age of patients (median and range) and follow-up periods. Microarray platform and database accession (GEO or caArray) are indicated. Dates of study reflect earliest patient enrollment to most recent follow-up as captured by clinical trial registration, enrollment, and publication dates. Primary therapyprotocol and survival data available for each study (response to therapy, OS, EFS, RFS) are summarized. *7 days of infusional cytarabine and 3 days of anthracycline.

	AML Cohort			
	Metzeler[16, 55]	**Wouters[19,26]**	**Tomasson[17, 20]**	**Wilson[18]**
	Adult AML, Normal karyotype	**Adult AML, Mixed karyotypes**	**Adult AML, Mixed karyotypes**	**Adult AML, Mixed karyotypes**
Median age, yrs (range)	60 (17-85)	46 (15-77)	47 (16-81)	65 (20-84)
Patients (n)	163	526	188	170
Median follow-up, months (inter quartile range)	9 (3-27)	17 (7-73)	25 (10-55)	10 (2-22)
Cooperative Group	German AML Cooperative Group (AMLCG)	Dutch-Belgian Hematology-Oncology Cooperative (HOVON)	Washington University (WU) and CALGB	Southwest Oncology Group (SWOG)
Primary Therapy Protocol(s)	AMLCG 1999	Multiple HOVON trials:PMIDs 9396403, 12930926, 15070662	WashU: Primarily 7+3*; CALGB 9621/9222/9191/9710	S9031/ S9333/ S9034/ S9500/ S9126
Dates of study	1999-2007	1992-2008	1993-2007	1993-2004
Patient Outcome Data	OS	Response to Primary Therapy; OS; EFS; RFS	OS; EFS	Response to Primary Therapy; OS
Microarray Platform(s)	Affymetrix HG-U133A&B	Affymetrix HG-U133 Plus 2.0	Affymetrix HG-U133 Plus 2.0	Affymetrix HG-U95Av2
Dataset Accession	GSE12417	GSE14468	GSE10358	NCI-caArray-willm-00119

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Table 3

The LSC Score As a Univariate Predictor of Survival in Four Independent AML Cohorts, Stratified by AML Subtype and Outcome Variable

Prognostic value of the LSC score, FLT3-ITD mutation status, NPM1 mutation status, age, and cytogenetic risk are shown for OS, EFS, and RFS for the datasets described. Shown are the hazard ratios (HR) with 95% confidence intervals, and p-value (p), computed using log-likelihood test for continuous predictors, and log-rank test for discrete predictors. Units for variables are as follows: Age in years; continuous LSC score as log2 of gene expression intensity; dichotomous LSC score as high (1) vs low (0); FLT3 and NPM1 as mutated (1) vs wild-type (0); cytogenetic risk as favorable (1), intermediate (2), adverse (3) per the modified Medical Research Council scheme23. Patients with APL were excluded.

	Cohort	Wouters[19, 26] (test)		Tomasson[17, 20] (test)		Wilson[18] (test)		Metzeler[16, 55] (training)	
	Predictor	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
OS, NKAML	**Number of patients**	**99**		**70**		**65**		**163**	
	LSC score (continuous)	1.17 (1.07-1.28)	0.0007	1.13 (1.04-1.22)	0.003	1.18 (1.04-1.34)	0.011	1.15 (1.08-1.22)	<0.001
	LSC score (dichotomous)	1.86 (1.15-3.02)	0.01	2.70 (1.43-5.10)	0.002	2.55 (1.44-4.51)	<0.001	1.85 (1.25-2.74)	0.002
	FLT3-ITD	1.84 (1.14-2.98)	0.012	2.68 (1.42-5.07)	0.002	1.28 (0.73-2.23)	0.39	2.22 (1.49-3.31)	<0.001
	NPM1c	0.76 (0.47-1.23)	0.26	1.55 (0.86-2.79)	0.14	0.67 (0.38-1.17)	0.16	0.79 (0.54-1.17)	0.24
	Age	1.01 (0.98-1.03)	0.6	1.02 (1.00-1.04)	0.06	1.03 (1.00-1.05)	0.029	1.03 (1.01-1.04)	<0.001
OS, all non-APL	*Number of patients*	**219**		**137**		**170**			
	LSC score (continuous)	1.07 (1.02-1.13)	0.009	1.10 (1.04-1.17)	0.001	1.15 (1.07-1.25)	<0.001		
	LSC score (dichotomous)	1.36 (0.98-1.88)	0.07	2.01 (1.27-3.18)	0.003	1.99 (1.43-2.79)	<0.001		
	FLT3-ITD	1.82 (1.28-2.59)	<0.001	1.82 (1.09-3.02)	0.019	1.12 (0.78-1.62)	0.53		
	NPM1c	0.86 (0.60-1.22)	0.39	1.49 (0.95-2.32)	0.079	0.79 (0.55-1.14)	0.2		
	Age	1.01 (1.00-1.03)	0.082	1.03 (1.01-1.04)	<0.001	1.03 (1.02-1.05)	<0.001		
	Cytogenetic Risk Group	2.04 (1.54-2.69)	<0.001	1.97 (1.34-2.90)	<0.001	2.16 (1.52-3.06)	<0.001		
EFS, NKAML	**Number of patients**	**99**		**70**					
	LSC score (continuous)	1.15 (1.06-1.26)	0.001	1.11 (1.03-1.21)	0.007				
	LSC score (dichotomous)	1.69 (1.07-2.69)	0.02	2.39 (1.26-4.52)	0.006				
	FLT3-ITD	2.12 (1.33-3.38)	0.001	2.44 (1.23-4.82)	0.008				
	NPM1c	0.92 (0.58-1.47)	0.74	1.29 (0.70-2.37)	0.41				
	Age	1.00 (0.98-1.02)	0.96	1.01 (0.99-1.03)	0.25				
RFS, NKAML	**Number of patients**	**85**							

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	Cohort	Wouters[19, 26] (test)		Tomasson[17, 20] (test)		Wilson[18] (test)		Metzeler[16, 55] (training)	
	Predictor	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
	LSC score (continuous)	1.13 (1.01-1.27)	0.03						
	LSC score (dichotomous)	1.78 (0.99-3.25)	0.055						
	FLT3-ITD	2.80 (1.53-5.14)	0.0005						
	NPM1c	1.09 (0.59-2.02)	0.79						
	Age	0.99 (0.97-1.02)	0.63						

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Table 4

Range of LSC Scores Across AML Cohorts Subjected to Survival Analyses

The median and interquartile range (IQR) of the LSC score is reported for each of the four AML cohorts, separately for NKAML and non-APL subtypes. The dataset of Wilson et al. does not have probes for some of the LSC genes, hence the range is different from the other three cohorts. Also shown is median survival for specified endpoints in low and high LSC score groups, together with the comparative risk (percentage of patients having an event) by 36 months in the low and high LSC score groups. Corresponding hazard ratios and p-values are presented in Table 3.

	AML Cohort	n	LSC score, median (IQR)	End-point	Median survival, months		Comparative absolute risk of event by 3 years, %	
					Low LSC score group	High LSC score group	Low LSC score group (95% CI)	High LSC score group (95% CI)
NKAML	**Metzeler** [16, 55]	163	24.9 (22.6-27.0)	OS	22.8	7.9	57 (43-67)	78 (66-86)
	Tomasson [17, 20]	74	25.2 (22.3-27.6)	OS	56.3	16.3	39 (20-54)	81 (61-90)
				EFS	47.7	9.9	48 (27-63)	81 (60-91)
	Wouters [19, 26]	181	25.0 (22.6-27.0)	OS	31.3	8.4	52 (36-63)	73 (57-84)
				EFS	14.0	7.4	61 (46-72)	80 (64-89)
				RFS	65.6	10.4	43 (26-56)	68 (46-81)
	Wilson [18]	65	14.0 (12.8-15.6)	OS	23.7	7.3	58 (39-72)	93 (72-98)
non-APL AML	**Tomasson** [17, 20]	143	25.7 (23.1-28.4)	OS	56.3	16.5	45 (30-57)	75 (64-83)
	Wouters [19, 26]	392	25.6 (23.4-28.2)	OS	25.0	14.5	55 (44-64)	69 (59-76)
	Wilson[18]	170	14.7 (13.4-16.3)	OS	15.9	6.6	67 (56-76)	93 (84-97)

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Table 5

Multivariate Survival Analysis Including the LSC Score in Four Independent AML Cohorts, Stratified by AML Subtype and Outcome Variable

The LSC score was tested as a multivariate predictor in combination with age, FLT3-ITD status, *NPM1* status, age, and cytogenetic risk group using Cox regression. Hazard ratios (HR) and p-values (p), using log-likelihood test, are reported for each variable within the multivariate model. The overall log-likelihood p-value for the model is also indicated. The number of patients (n) differs from those in Table 3, depending on whether information on all covariates was available in each case. Units are as in Table 3. Patients with APL were excluded

		Wouters[19, 26] (test)		**Tomasson[17, 20] (test)**		**Wilson[18] (test)**		**Metzeler[16, 55] (training)**	
	Predictor	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
OS, NKAML	**Patients (n)**	**99**		**70**		**63**		**162**	
	LSC score	1.16 (1.05-1.27)	0.003	1.15 (1.06-1.26)	0.002	1.14 (0.97-1.34)	0.1	1.10 (1.03-1.17)	0.006
	FLT3-ITD	1.94 (1.15-3.27)	0.013	3.00 (1.50-6.00)	0.002	2.05 (1.10-3.85)	0.025	2.19 (1.42-3.37)	<0.001
	NPM1c	0.73 (0.42-1.27)	0.27	1.58 (0.83-3.01)	0.17	0.82 (0.42-1.61)	0.57	0.87 (0.58-1.30)	0.49
	Age	1.02 (1.00-1.04)	0.087	1.02 (1.00-1.04)	0.14	1.03 (1.00-1.06)	0.026	1.03 (1.01-1.04)	<0.001
	Overall		<0.001		<0.001		0.01		<0.001
OS, all non-APL	**Patients (n)**	**219**		**137**		**136**			
	LSC score	1.07 (1.01-1.13)	0.02	1.10 (1.03-1.17)	0.005	1.17 (1.05-1.30)	0.005		
	FLT3-ITD	1.98 (1.35-2.91)	<0.001	2.00 (1.18-3.37)	0.01	1.45 (0.91-2.30)	0.12		
	NPM1c	0.70 (0.46-1.06)	0.094	1.64 (1.01-2.65)	0.045	0.93 (0.55-1.60)	0.8		
	Age	1.02 (1.00-1.03)	0.023	1.02 (1.01-1.04)	0.007	1.03 (1.01-1.04)	0.002		
	Cytogenetic Risk Group	2.02 (1.53-2.67)	<0.001	1.86 (1.26-2.76)	0.002	1.99 (1.37-2.89)	<0.001		
	Overall		<0.001		<0.001		<0.001		
EFS, NKAML	**Patients (n)**	**99**		**70**					
	LSC score	1.14 (1.04-1.24)	0.004	1.13 (1.04-1.23)	0.005				
	FLT3-ITD	2.15 (1.30-3.55)	0.003	2.86 (1.37-5.94)	0.005				
	NPM1c	0.88 (0.52-1.48)	0.62	1.27 (0.67-2.42)	0.46				
	Age	1.01 (0.99-1.03)	0.35	1.01 (0.99-1.03)	0.38				
	Overall		<0.001		0.003				
RFS, NKAML	**Patients (n)**	**85**							

		Wouters[19, 26] (test)		Tomasson[17, 20] (test)		Wilson[18] (test)		Metzeler[16, 55] (training)	
	Predictor	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
	LSC score	1.12 (1.00-1.25)	0.05						
	FLT3-ITD	2.83 (1.47-5.45)	0.002						
	NPM1c	0.95 (0.47-1.91)	0.89						
	Age	1.00 (0.98-1.03)	0.78						
	Overall		0.004						

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