Despite major therapeutic advancements, worldwide death rates of acute myeloid leukemia (AML) remain high, with approximately 20,830 people diagnosed in the USA in 2015, 10,460 (50.21%) of whom were estimated to die from the disease. Several studies have shown that leukemia stem cells (LSCs), the founder cells from which AML arise, are characterized by specific transcriptional and epigenetic profiles which can be applied to predict patient survival and prognosis. The actual model for AML development postulates that LSC arise within the same niches as the normal hematopoietic stem cells (HSCs), taking them over in time as the hematopoietic niche turns into a leukemic niche. While the altered expression of different extracellular matrix (ECM) elements within the leukemic niche has already been investigated, the direct contribution of LSCs to the modification of the niche ECM has not been assessed systematically, and the prognostic relevance of alterations to the ECM homeostasis directly operated by LSCs, and AML cells remains untested. To this aim, we studied the transcriptional profile of ECM-related genes in LSCs, and applied the results to two AML cohorts to verify their prognostic potential.

The raw microarray profiles of normal HSCs, multipotent progenitors (MPPs), committed progenitors (megakaryocyte-erythrocyte progenitors, MEPs, common myeloid progenitors, CMPs, and granulocyte/monocyte progenitors, GMPs), LSCs, leukemia progenitor cells

**Figure 1. The prognostic value of the ECM signature.** (a-b) ECM signature expression clusters leukemic and normal precursors into three groups, according to a) hierarchical clustering with Ward’s method and b) PCA followed by LDA. (c, d) Analysis of c) the overall survival (OS) of the GSE10358 and TCGA LAML cohorts and d) mean event-free survival (EFS) for GSE10358 and mean disease-free survival (DFS) for TCGA LAML, using the early and definitive leukemic types identified by the ECM signature. P-values are from Log-Rank test in (c) and from Mann-Whitney U test in (d). In (d), data are reported as 10-90 percentile with outliers, median (thin internal line), mean (thin internal cross) and standard deviation. (e-f) A risk classification scheme adding the ECM signature to the NCCN classifier outperforms the classifier alone. (e) AUC-ROC analysis of Cox proportional hazard (Cox-PH) and generalized linear models (GLM). (f) Predictive curve, NRI and IDI from GLM.
(LPCs), and blasts were retrieved from the NCBI Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/) through the GEO Series accession number GSE24006. AML patient microarray and clinical data were retrieved from GEO for the accession number GSE10358 or from the GDC Legacy archive (http://gdc-portal.nci.nih.gov/legacy-archive) for The Cancer Genome Atlas AML cohort (TCGA LAML). Raw data from healthy donors (GSE11504 and GSE13159) were used as controls for AML. The raw intensity expression values were processed by Robust Multi-array Average procedure in Chipster software (http://chipster.csc.fi/) and significantly under- and over-expressed genes in leukemic cells (precursors and cohorts) tested with Empirical Bayes test with Benjamini-Hochberg correction. Only the genes which significantly varied in AML precursors vs. normal precursors and in AML cohorts vs. healthy donors were further studied. The list of ECM genes on which we focused was compiled by merging the genes in the Gene Ontology (GO) categories detailed in the Online Supplementary Information. Data standardization, hierarchical clustering (Ward’s method), principal component analysis (PCA), Linear Discriminant Analysis (LDA), Fisher’s Exact test (2-sided), Kaplan-Meier (Log-Rank), Cox multivariate models for survival analysis (Cox-PH) and generalized linear models (GLM) were performed in IBM SPSS Statistics 21. Net reclassification improvement (NRI), integrated discrimination improvement (IDI) and the area under the receiver operating characteristic curve (AUC-ROC) were calculated in R. A value of $P<0.05$ was considered significant. The support Vector Machine (SVM) algorithm used to select the 15 most important genes among the previously-identified ones was trained and tested as reported in the Online Supplementary Information. The retrospective Oulu AML cohort used to assess gene expression in patients was assembled with

![Figure 2. Components of the ECM signature and the CD44-ECM subnetwork.](image)

(a-b) Enrichment of the up- and downregulated genes of the ECM signature according to a) gene ontology (GO) categories and conceptual meta-categories, and b) categories from the Matrisome database. In a) data are presented as the natural antilogarithm of the false discovery rate (-ln FDR). (c) Normalized expression of CD44 in early and definitive leukemic cells. (d-e) Differentially expressed genes in early and definitive leukemic cells (d) and network analysis of genes upregulated in definitive leukemic cells (e). (f) Normalized expression of COL18A1 in leukemic cells vs. normal cells (upper panel) and in early vs. definitive leukemic cells (lower panel). (c, f) Data are reported as 10-90 percentile with outliers, median (thin internal line), mean (thin internal cross) and standard deviation. $P$-values are from Mann-Whitney U test.
In total, 80 ECM genes were found differentially expressed in leukemic vs. normal precursor cells as well as in the two AML cohorts vs. healthy donors (Online Supplementary Table S4). Of them, the differential expression of the 15 most important genes, as resulted from the SVM algorithm, was validated via qPCR in the 65 AML founding patients for the Oulu retrospective procedures. Next, we standardized the leukemic precursors and the patient data together and observed that the samples from the early and definitive groups continued to cluster independently (albeit fragmenting in smaller subgroups), never mixing and partitioning patient data into the same groups (Online Supplemental Figure S3). Patient grouping into “early-type” and “definitive-type” resulted in significant differences in overall survival (Figure 1C), even when data were adjusted for karyotypical or molecular drivers of AML. Notably, other absolute levels of COL18A1 are lower in leukemic cells than in their normal counterparts (Figure 2B), but they are at their local highest in definitive leukemic cells, again remarking the peculiarities of this stage of leukemic development.

In this study, we show for the first time the existence of an “ECM signature” which is shared by leukemia precursor cells and circulating AML cells from patients. The most striking feature of the ECM signature was the upregulation leukemic precursors into two groups, which differed for a restricted set of ECM genes and for the expression of the CD44 receptor. CD44 belongs to a family of transmembrane glycoproteins whose primary function is to bind hyaluronic acid (HA), laminins, collagens, matrix metalloproteinases (MMPs), osteopontin, etc., and we found this receptor has previously been implicated in cell migration, proliferation, differentiation, survival, and bone marrow homing of hematopoietic stem/progenitor cells as well as in the homing of LSCs to extra-medullary niches and in resistance to chemotherapy. We observed that the leukemic precursors with a higher expression of CD44 (the group of cells we called “definitive leukemic cells”) also exhibit a parallel upregulation of genes whose products interact directly (COL18A1, LAMB2, MMP2, COL4A5) or indirectly (MMP1, CTSG, CD44) with CD44 and downregulation of MMP9 (which directly interacts with CD44 but whose levels correlate inversely with patient prognosis), suggesting that the establishment of a “CD44-ECM network”, rather than the expression of CD44 alone, is a crucial step in the progression of leukemic cells towards an aggressive phenotype. This also seems to be supported by the observation that, in two independent cohorts, patients with an ECM profile similar to that of the definitive leukemic cells showed significantly shorter survival in general, and independently from well-known karyotypical or molecular drivers of AML. Notably, other genes upregulated in definitive leukemic cells include the matrix metalloproteinase 2 (MMP2), a disintegrin and metalloprotease domain 17 (ADAM17) and cathepsin G (CTS), suggesting that the proteolytic subnetwork that sits well with the overall upregulation of proteases which we observe in the ECM signature and that others have already reported in AML. Also, MMP2 has been implicated in AML invasiveness, and ADAM17 seems to play a central role in the survival of leukemic cells via the activation of the Lyn/Akt survival pathway. In conclusion, the correlation of the ECM signature with AML outcome and leukemic precursor subtypes suggests a central role for ECM alteration in AML biology and encourages further studies to understand the regulatory mechanisms controlling it.
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