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High expression of neutrophil elastase predicts improved survival in pediatric acute myeloid leukemia: a report from the Children's Oncology Group

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Neutrophil elastase, a serine protease of the chymotrypsin family encoded by *ELANE* (*ELA2*), is primarily found in azurophilic (primary) granules of neutrophils [1,2]. Physiologically, neutrophil elastase plays a role as an intracellular microbicidal agent that, after release, remains exposed at the cell surface and contributes to the extracellular killing of microorganisms; the protease also regulates immune responses by activating specific cell surface receptors and modulating the cytokine network [1,2].

Given its association with developing neutrophils, neutrophil elastase has historically served as a parameter in the differential diagnosis of myeloid and lymphoid leukemias [3–7]. It has long been recognized that high serum levels of neutrophil elastase are found in acute promyelocytic leukemia (APL), where it may play an important pathogenetic role by cleaving the PML–RAR α (promyelocytic leukemia–retinoic acid receptor α) fusion protein and facilitating the leukemogenic potential of this onco-protein [7–10]. More recent studies have pointed to a prognostic role of neutrophil elastase. Specifically, in chronic myeloid leukemia (CML), high expression of *ELANE* in CD34+ cells is a predictor of an indolent course and longer survival in patients with chronic-phase disease, as well as improved survival after allogeneic hematopoietic cell transplantation (HCT) of patients with advanced-phase disease [11,12]. However, the prognostic role of neutrophil elastase in acute

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myeloid leukemia (AML) is unknown. In this study, we therefore evaluated the expression of neutrophil elastase in diagnostic specimens from 113 patients enrolled on the recent Children's Oncology Group (COG) AML pilot protocol AAML03P1 [13], and correlated expression levels with patient demographics, laboratory characteristics and clinical outcome.

AAML03P1 was a pilot study conducted to determine the safety and feasibility of adding gemtuzumab ozogamicin (GO) to intensive chemotherapy [13]. From December 2003 to November 2005, AAML03P1 enrolled 340 eligible children (aged 1 month to 21 years) with newly diagnosed *de novo* AML, excluding those with APL, bone marrow failure syndromes, juvenile myelomonocytic leukemia or Down syndrome. Treatment consisted of remission induction followed by intensification I, II and III, or intensification I and matched related donor HCT with busulfan/cyclophosphamide as conditioning regimen. Cycle regimens were cytarabine/daunorubicin/etoposide (ADE) plus GO (induction I), ADE (induction II), high-dose cytarabine and etoposide (intensification I), mitoxantrone/cytarabine plus GO (intensification II) and sequential high-dose cytarabine and asparaginase (intensification III). Pretreatment ("diagnostic") specimens ($n = 113$) from patients who consented to the biology studies and for whom marrow specimens were available were used for this study. Informed consent was obtained in accordance with the Declaration of Helsinki. The institutional review boards of all participating institutions approved the clinical protocol, and the COG Myeloid Disease Biology Committee approved this study. AAML03P1 was registered at ClinicalTrials.gov as NCT00070174.

A combination of cytogenetic and molecular abnormalities was used to stratify participants enrolled on AAML03P1 into risk groups. Patients were considered low-risk if a chromosomal abnormality/mutation was present in core-binding factors [CBF; t(8;21) or inv(16)/t(16;16)], *NPM1* or *CEBPA* ($n = 43$). Patients were classified as high-risk if they had -5/5q-, monosomy 7 or *FLT3*/internal tandem duplication (ITD) with high allelic ratio ($n = 16$). All other patients having data sufficient for classification were considered standard-risk ($n = 54$).

Total RNA was extracted with the AllPrep DNA/RNA Mini Kit using the QIAcube automated system (Qiagen, Valencia, CA), and quantified with a microvolume spectrophotometer (NanoDrop™; Thermo Scientific, Wilmington, DE). To allow multianalyte profiling directly from purified RNA preparations, *ELANE* mRNA expression was quantified from 125 ng of RNA using a multianalyte sandwich nucleic acid hybridization method employing branched DNA molecules to amplify the signal from captured target RNA [14,15] (QuantiGene™ Plex 2.0; Panomics/Affymetrix, Santa Clara, CA) in combination with xMAP system fluorescent-dyed microspheres (xMAP™ beads; Luminex, Austin, TX), according to the manufacturer's instructions; *ELANE* expression levels were normalized using β -glucuronidase (*GUSB*). Two technical replicates obtained from each specimen were analyzed with xPonent software (Life Technologies, Grand Island, NY).

Clinical outcome data for COG AAML03P1 were analyzed through 31 December 2011. The Kaplan–Meier method was used to estimate overall survival (OS; time from study entry to death) and disease-free survival (DFS; time from end of induction I for patients in complete remission [CR] until death or relapse). Estimates of the cumulative incidence of relapse were calculated from the end of induction I for patients in CR to relapse or death due to progressive disease, where deaths from non-progressive disease were considered competing events. The significance of predictor variables was tested with the log-rank statistic for OS and DFS and with Gray's statistic for risk of relapse (RR). All estimates are reported with two times the Greenwood standard errors. Children lost to follow-up were censored at the date of last known contact. Cox proportional hazards models were used to estimate the

hazard ratio (HR) for specific univariate and multivariate analyses. The χ^2 test was used to test the significance of observed differences in proportions, and Fisher's exact test was used when data were sparse. Differences in medians were compared using the Mann–Whitney test.

Diagnostic marrow specimens from 113 randomly selected participants enrolled on AAML03P1 were used for ELANE mRNA quantification. ELANE mRNA expression levels were undetectable in three samples and varied more than 70 000-fold relative to GUSB in 110 samples [from 0.0005 to 36.32 (median, 0.89); Figure 1(A)]. Of note, most AML specimens expressed ELANE mRNA to a lower degree than normal bone marrow, possibly reflecting the higher proportion of mature myeloid cells in normal marrow. Table I summarizes the characteristics of patients with low (< median expression) and high (> median expression) ELANE expression. Patients with high ELANE expression had lower platelet counts at diagnosis than those with low ELANE expression ($p = 0.048$). They were also more likely to have myelomonocytic cell characteristics (French–American–British [FAB] M4, 50% vs. 15%, $p < 0.001$) and core-binding factor translocations, i.e. t(8;21) ($p = 0.05$) or inv(16) ($p < 0.003$). They had a lower prevalence of megakaryocytic leukemia (FAB M7; 2% vs. 17%, $p = 0.014$). There was no difference in the prevalence of *FLT3*/ITD or *NPM1* mutations. As a result of these characteristics, patients with high ELANE expression were more likely to have low-risk disease ($p < 0.001$) and less likely to have standard-risk disease than those with low ELANE expression, whereas the prevalence of high-risk disease was similar in both groups. Only 19 of the 113 patients underwent allogeneic HCT as consolidative therapy, and there was no difference in the proportion of transplanted patients among those with low (9/56 [16%]) and high (10/57 [18%]) ELANE expression ($p = 0.834$).

Next, the clinical outcome per median ELANE expression was determined for the entire cohort as well as the specific risk groups. For the entire cohort, CR rates were not significantly different for patients with high and low ELANE expression after the first (87% vs. 76%, $p = 0.216$) or second (89% vs. 90%, $p = 0.787$) induction course. Similarly, the likelihood of minimal residual disease (MRD), as prospectively assessed by multiparameter flow cytometry in a centralized laboratory in marrow specimens obtained after induction I, was comparable between both groups (33% for high ELANE vs. 34% for low ELANE expression, $p = 0.882$). However, consistent with the recent notion that high ELANE expression is associated with improved outcome in CML [11,12], patients with high ELANE expression had a significantly higher 5-year OS [$77 \pm 11\%$ vs. $57 \pm 14\%$, $p = 0.045$; Figure 1(B)] and a better DFS ($64 \pm 14\%$ vs. $46 \pm 16\%$) than those with low ELANE expression, although this difference did not reach statistical difference ($p = 0.151$); the cumulative risk of relapse at 5 years was not significantly different for both groups ($27 \pm 13\%$ for high ELANE vs. $40 \pm 15\%$ for low ELANE expression, $p = 0.311$).

Finally, uni- and multivariate Cox models were used to evaluate the role of ELANE expression as predictor of OS. In the univariate model, high ELANE expression was associated with a statistically significantly reduced hazard of death (HR = 0.51 [95% confidence interval, 0.26–1.00], $p = 0.049$). Given the association between cytogenetic risk and ELANE expression, the better outcome for patients with high ELANE expression could at first glance be attributable to the higher prevalence of low-risk disease in this subgroup. Consistent with this possibility, after adjusting for cytogenetic/molecular disease risk, the association between high ELANE expression and lower hazard of death was less pronounced and did not reach statistical significance (HR = 0.66 [0.32–1.36], $p = 0.258$). On the other hand, the subgroup analyses, which were limited by the small sample size, suggested that the association between ELANE expression and outcome differed in dependence on the specific risk subgroup, raising the possibility of effect modification, with

an association primarily limited to high-risk patients. Specifically, in high-risk disease, patients with high ELANE expression tended to have a better 5-year OS than patients with low ELANE expression ($67 \pm 38\%$ vs. $30 \pm 29\%$, $p = 0.308$). By comparison, OS in low- and standard-risk patients appeared similar for patients with high and low ELANE expression ($87 \pm 12\%$ vs. $75 \pm 25\%$, $p = 0.361$ for low-risk; $64 \pm 22\%$ vs. $59 \pm 18\%$, $p = 0.849$ for standard-risk).

In conclusion, this study revealed significant heterogeneity of ELANE expression in pediatric AML and its association with improved OS. This association is at least in part due to the high proportion of AMLs with core-binding factor aberrations in patients with high ELANE expression. Nevertheless, high ELANE expression may predict improved OS even within specific risk groups, in particular high-risk disease, although further studies with a larger patient cohort will be necessary to confirm this finding.

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Figure 1.

(A) Quantitative expression of ELANE relative to β -glucuronidase (GUSB) in the 113 diagnostic bone marrow specimens from patients enrolled in AAML03P1. (B) Kaplan–Meier estimates of overall survival for patients with high (>median) and low (<median) ELANE expression.

Table I

Comparison of baseline characteristics of patients with low vs. high ELANE expression.

Patient characteristic	Low ELANE (n = 56)	High ELANE (n = 57)	p-Value *
Age [median (range)], years	9.1 (0.1–20.8)	10.7 (0.4–18.3)	0.381
Gender, n (%)			
Male	28 (50%)	37 (65%)	0.158
Female	28 (50%)	20 (35%)	
WBC [median (range)], $\times 10^3/\mu\text{L}$	27.6 (2.1–404.8)	25.6 (1.6–296.4)	0.466
Hgb [median (range)], g/dL	8.8 (3.3–14.2)	8.3 (3.7–13.7)	0.242
Platelet count [median (range)], $\times 10^3/\mu\text{L}$	55 (7–520)	36 (4–307)	0.048 *
Bone marrow blasts [median (range)], %	70 (2–95.3)	54.5 (5–100)	0.076
Cytogenetics, n (%)			
Normal	15 (27%)	12 (21%)	0.621
t(8;21)(q22;q22)	3 (5%)	11 (19%)	0.050 *
inv(16)/t(16;16) (p13.1;q22)	3 (5%)	16 (28%)	0.003 *
t(9;11)/11q23	8 (14%)	6 (11%)	0.748
t(6;9)(p23;q34)	1 (2%)	3 (5%)	0.618
– 7/7q –	3 (5%)	1 (2%)	0.364
– 5/5q –	2 (4%)	0 (0%)	0.243
Trisomy 8	7 (13%)	4 (7%)	0.506
Other	14 (25%)	4 (7%)	0.019 *
Risk group, n (%)			
Standard	34 (61%)	20 (35%)	0.011 *
Low	12 (21%)	31 (54%)	< 0.001 *
High	10 (18%)	6 (11%)	0.397
Molecular alterations, n (%)			
FLT3ITD	9 (18%)	6 (11%)	0.430
NPM1 mutation	2 (5%)	2 (4%)	1.000
CEBPA mutation	4 (9%)	3 (5%)	0.696

WBC, white blood cells; Hgb, hemoglobin.

* Significant p-values.