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High Expression of Suppressor of Cytokine Signaling-2 Predicts Poor Outcome in Pediatric Acute Myeloid Leukemia: A Report from the Children's Oncology Group

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Abstract

Deregulated cytokine signaling is a characteristic feature of acute myeloid leukemia (AML), and expression signatures of cytokines and chemokines have been identified as significant prognostic factor in this disease. Given this aberrant signaling, we hypothesized that expression of Suppressor of Cytokine Signaling-2 (SOCS2), a negative regulator of cytokine signaling, might be altered in AML and could provide predictive information. Among 188 participants of the Children's Oncology Group AAML03P1 trial, SOCS2 mRNA levels varied >6,000-fold. Higher (>median) SOCS2 expression was associated with inferior overall ($60 \pm 10\%$ vs. $75 \pm 9\%$, $p=0.026$) and event-free ($44 \pm 10\%$ vs. $59 \pm 10\%$, $p=0.031$) survival. However, these differences were accounted for by higher prevalence of *high-risk* and lower prevalence of *low-risk* disease among patients with higher SOCS2 expression, limiting the clinical utility of SOCS2 as predictive marker. It remains untested whether high SOCS2 expression identifies a subset of leukemias with deregulated cytokine signaling that could be amenable to therapeutic intervention.

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Keywords

SOCS2; cytokine; acute myeloid leukemia; prognostic factor; childhood cancer

INTRODUCTION

It is well recognized that deregulation of cytokine and growth factor signaling pathways is a characteristic feature of human myeloid stem cell disorders and implicated in their pathogenesis [1,2]. In acute myeloid leukemia (AML), elevated cytokine expression has repeatedly been observed, and expression signatures of cytokines and chemokines have been identified as significant prognostic factor [3]. Physiologically, the magnitude and duration of cytokine signaling is closely controlled by the Suppressor of Cytokine Signaling (SOCS) family of proteins via targeting of substrates for ubiquitination and subsequent degradation by the proteasome; for example, SOCS proteins are established negative regulators of the JAK/STAT pathways and are implicated in IL-3, IL-6, EPO, G-CSF, and GM-CSF signaling [4,5].

Given the dysregulation of cytokine signaling in AML, we hypothesized that expression of SOCS family proteins might similarly be altered in this disease and could serve as a prognostic or predictive marker. In fact, for SOCS2, there is evidence of overexpression in advanced stage chronic myeloid leukemia (CML) [6]. On the other hand, high expression of SOCS2 has been associated with favorable disease features and better outcome in patients with breast cancer [7]. However, the prognostic role of SOCS2 in AML is unknown. In this study, we therefore quantified SOCS2 expression in pretreatment (“diagnostic”) bone marrow samples from pediatric patients who underwent intensive chemotherapy for newly diagnosed AML on a recent Children's Oncology Group (COG) trial, and correlated SOCS2 expression levels with patient demographics, laboratory characteristics, and clinical outcome.

MATERIALS AND METHODS

Study Cohort

COG-AAML03P1 (registered at ClinicalTrials.gov as NCT00070174) was a multicenter pilot study determining the safety and feasibility of adding gemtuzumab ozogamicin (GO) to intensive chemotherapy [8]. AAML03P1 enrolled 339 eligible children (aged 1 month to 21 years) between 2003 and 2005 with untreated *de novo* AML, excluding those with acute promyelocytic leukemia, juvenile myelomonocytic leukemia, documented bone marrow failure syndromes, Down syndrome, or secondary/treatment-related leukemia. Treatment consisted of remission induction and, if complete remission (CR) was achieved, either intensifications I-III or intensification I and busulfan/cyclophosphamide-based matched related-donor hematopoietic cell transplantation as follows: 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) [8]. Pretreatment/diagnostic specimens from all 188 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 both the COG Myeloid Disease Biology Committee and the National Cancer Institute's Cancer Therapy Evaluation Program approved this study.

Risk Stratification

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* ($n=68$). Patients were classified as *high-risk* if they had -5/5q-, monosomy 7, or *FLT3* /internal tandem duplication (ITD) with high allelic ratio ($n=24$); as done previously [9], the latter was defined as a *FLT3*/ITD to wild-type *FLT3* ratio of >0.4 as quantified via PCR-based assay on DNA purified from diagnostic bone marrow specimens. All other patients having data sufficient for classification were considered *standard-risk* ($n=82$). Only 14 patients had cytogenetic/molecular data that were insufficient for risk classification.

Quantification of SOCS2 Expression

Total RNA was extracted with the AllPrep DNA/RNA Mini Kit using the QIAcube automated system (Qiagen, Valencia, CA). After quantification with a microvolume spectrophotometer (NanoDrop; Thermo Scientific, Wilmington, DE), 10 ng of total RNA was subjected to quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) using ABI taqman primers per manufacturer's instructions to determine expression of *SOCS2* and, for normalization, β -glucuronidase (*GUSB*). Patient samples were run in duplicate. The $\Delta\Delta C_T$ method was used to determine the relative expression levels of individual genes.

Statistical Analyses

Clinical outcome data for COG AAML03P1 were analyzed through June 30, 2013. The Kaplan–Meier method was used to estimate overall survival (OS; time from study entry to death) and event-free survival (EFS; time from study entry until failure to achieve CR during induction, relapse, or death). 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 EFS 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. All reported p-values were 2-sided, with <0.05 being considered statistically significant.

RESULTS

Diagnostic marrow specimens from 188 participants enrolled on AAML03P1 were available for this study and used for SOCS2 expression quantification. SOCS2 mRNA was detected in all but one sample. When SOCS2 was expressed, it varied >6,000-fold relative to GUSB (0.0017-8.602 [median: 0.188]; Figure 1A). Most AML specimens expressed SOCS2 at lower levels than those found in a small set of normal bone marrows (median relative expression: 0.6687 [range: 0.4128-0.8616], $n=4$). Table 1 summarizes the characteristics of patients with low (<median) and high (>median) SOCS2 expression; this cut-off was chosen *a priori* for our analyses but was supported by exploratory analyses of OS and EFS (see Discussion). Patients with higher SOCS2 expression had lower pre-treatment white blood cell counts ($p=0.028$) and a trend towards higher platelet counts ($p=0.051$). More importantly, they were less likely to have core-binding factor leukemias (inv(16): 10% vs. 22%, $p=0.036$; $t(8;21)$: 8% vs. 18%, $p=0.063$). Conversely, higher SOCS2 expression was associated with higher prevalence of FLT3/ITD mutations (21% vs. 3%, $p<0.001$); in fact, of the 17 patient samples carrying a FLT3/ITD abnormality at a high allelic ratio in this our study cohort, 15 also had high SOCS2 expression. In addition, there was a trend towards higher frequency of leukemias with monosomy 7 (6% vs. 0%, $p=0.059$) in patients with high SOCS2 expression. Consistent with these cytogenetic/molecular differences, patients with high SOCS2 expression less likely had *low-risk* disease (22% vs. 56%, $p<0.001$) and more likely had *high-risk* disease (24% vs. 3%, $p<0.001$).

Having established these associations between patient/disease characteristics and SOCS2 expression, we then determined the relationship between clinical outcome and SOCS2 expression. For these studies, we dichotomized our study cohort into high vs. low SOCS2 expression based on the median SOCS2 expression. Among the entire cohort, CR rates were not significantly different for patients with high or low SOCS2 expression after first (77% vs. 82%, $p=0.397$) or second induction (84% vs. 88%, $p=0.431$); similarly, there was no difference in the proportion of patients having flow cytometric evidence of minimal residual disease (MRD) after induction I (32% vs. 27%, $p=0.466$). However, patients with higher SOCS2 expression had a significantly worse 5-year OS ($60\pm10\%$ vs. $75\pm9\%$, $p=0.026$; Figure 1B) and 5-year EFS ($44\pm10\%$ vs. $59\pm10\%$, $p=0.031$; Figure 1C) than those with lower SOCS2 expression. While both the cumulative risk of relapse at 5 years and the non-relapse mortality from end of induction I were not significantly different for both groups, the risk of relapse and non-relapse mortality appeared to be slightly increased in patients with higher SOCS2 expression (for high vs. low SOCS2 expression $35\pm12\%$ vs. $26\pm11\%$, $p=0.223$ for relapse risk; $12\pm8\%$ vs. $7\pm6\%$, $P=0.311$, for non-relapse mortality). Of note, in these survival analyses, we did not censor patients who underwent allogeneic hematopoietic cell transplantation on study. To determine whether transplantation impacted our findings, we repeated our analyses with censoring of patients at the time of transplantation and found similar differences for patients with higher SOCS2 expression vs. those with lower expression as those in the above analyses (5-year OS: $56\pm11\%$ vs. $73\pm10\%$, $p=0.017$; 5-year EFS: $38\pm11\%$ vs. $56\pm11\%$, $p=0.019$; 5-year relapse risk: $43\pm13\%$ vs. $32\pm13\%$, $p=0.160$; and 5-year non-relapse mortality: $12\pm9\%$ vs. $6\pm6\%$, $P=0.347$).

Finally, we built univariate and multivariate Cox models to further evaluate the role of SOCS2 expression as a predictor of survival in pediatric AML (Table 2). In the univariate model, high SOCS2 expression was significantly associated with OS ($p=0.029$) and EFS ($p=0.033$). Similarly, as expected, *high-risk* disease was associated with shorter OS ($p=0.040$), while *low-risk* disease was associated with longer OS ($p=0.004$) and EFS ($p=0.002$). Given the association between cytogenetic risk and SOCS2 expression, we used a multivariate model to test whether the poor outcome of patients with high SOCS2 expression could be attributable to the lower prevalence of *low-risk* disease and higher prevalence of *high-risk* disease in this subgroup. Indeed, after adjustment for disease risk and age, high SOCS2 expression was no longer associated with decreased OS (HR: 1.68 [0.92-3.07], $p=0.094$) or EFS (HR: 1.40 [0.87-2.24], $p=0.168$), suggesting that SOCS2 outcome associations are largely explained by differences in disease risk group.

DISCUSSION

Dysregulations of major signal transduction pathways in response to cytokines or growth factors have been well characterized in AML [10-16]. In contrast, little attention has so far been paid to SOCS proteins, the pivotal family of negative feedback regulators of cytokine signaling. In this study, we have focused on SOCS2 and, in particular, its possible role as marker for outcome prediction because it has previously been associated with disease characteristics and outcome in other human malignancies [7]. Perhaps not surprisingly, our data obtained in a relatively large patient population demonstrate significant heterogeneity of SOCS2 expression in pediatric AML. More importantly, our findings indicate that SOCS2 expression level could indeed serve as a predictive marker in this disease when used alone, although additional independent studies, ideally in larger patient populations, will be required to confirm this finding. However, because of the tight association between SOCS2 expression and cytogenetic risk, SOCS2 does not offer any predictive information that is independent and goes beyond that provided by widely used cytogenetic and molecular abnormalities, limiting the clinical utility of SOCS2 as biomarker for outcome in AML. In our studies, we compared patients with high ($>$ median) vs. those with low ($<$ median) SOCS2 expression based on our *a priori* intent to dichotomize our study cohort by median SOCS2 expression value. However, for exploratory purposes, we analyzed a Cox model for EFS and OS from study entry for every distinct cutpoint of SOCS2 expression between the 10th and 90th quantile of expression. There were 139 such distinct values in this dataset. We found that many significant values for OS and EFS were just above the median (data not shown), suggesting that using the median value was a reasonable cut off to consider for studies aimed at exploring SOCS2 as biomarker for outcome in AML. However since this is only an exploratory analysis in one data set, additional studies should be performed to validate that the median is an acceptable cutoff value for SOCS2 for this purpose.

Although we found no role of SOCS2 as a predictor of early response to AML therapy, i.e. achievement of morphologic remission or persistence of MRD, high levels of SOCS2 mRNA expression were associated with shorter survival in our study cohort. At first glance, this association may seem counterintuitive given SOCS2's role in dampening cytokine signaling. However, through indirect effects, SOCS2 can lead to stimulation of cytokine and growth factor signaling. Specifically, SOCS2 can target other SOCS family proteins for

degradation via proteasome [17], with the ensuing cross-modulation resulting in increases in signal strength; for example SOCS2 can enhance IL-2 and IL-3 signaling through accelerated degradation of SOCS3 [18]. As another example, SOCS2 has a dual effect on growth hormone (GH) signaling, with both high levels of SOCS2 or SOCS2 deletion leading to increased signaling whereas lower levels of SOCS2 attenuate GH signaling [19-21]. While likely indicative of dysregulated growth factor and/or cytokine signaling, additional studies will be needed to determine the relationships between SOCS2 expression levels and activity through individual cytokine signaling pathways.

As a salient finding of our study, we observed that high SOCS2 expression was highly associated with distinct cytogenetic and molecular abnormalities and, consequently, disease risk. Most notably, in the AAML03P1 study cohort, patients with high SOCS2 expression were much more likely to have *high-risk* disease than those with low SOCS2 expression, while the opposite was true for patients with *low-risk* disease. Particularly striking was the association between high SOCS2 expression and FLT3/ITD with high allelic ratio in our study cohort. The functional relationship between FLT3/ITD and SOCS2 has been well studied in AML cell lines [22,23]. Of note, expression of FLT3/ITD but not wildtype FLT3 has found to constitutively activate STAT5 and increase SOCS2 expression in isogenic BaF3 cells [22]. Thus, our observation of high SOCS2 expression among patients with FLT3/ITD is consistent with these mechanistic studies and suggests that the high SOCS2 levels could be an immediate consequence of dysregulated FLT3 signaling in these leukemias.

In conclusion, our study indicates that high SOCS2 expression is associated with poor survival in pediatric AML. However, high SOCS2 expression is associated with poor-risk disease features, which largely account for the observed poor outcome. Given the involvement of SOCS2 as a negative feedback regulator in cytokine signaling pathways, it is interesting to speculate that leukemias with high SOCS2 may be characterized by particular deregulations of such pathways, offering the possibility of a therapeutic intervention, but further mechanistic studies will be necessary to test this hypothesis.

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A **B** **C**

Figure 1.

(A) Box-and-whisker plot showing quartile distribution of quantitative expression of SOCS2 mRNA, normalized to β -glucuronidase (GUSB) mRNA, across 188 diagnostic bone marrow specimens from patients enrolled in AAML03P1. (B, C) Kaplan-Meier estimates of overall survival (B) and event-free survival (C) of patients with high (>median) and low (<median) SOCS2 mRNA expression.

TABLE 1

Baseline Characteristics of Patients with Low vs. High SOCS2 Expression.

Patient Characteristics	SOCS2 Expression (Quartile)		P-value
	Q1-Q2 n = 94	Q3-Q4 n = 94	
Median Age, years (range)	11.3 (0.1-19.7)	8.8 (0.2-20.8)	0.459
Male Sex, n (%)	56 (60%)	55 (59%)	0.882
WBC $\times 10^3/\mu\text{L}$ - median (range)	38.6 (1.8 - 302)	24 (2.1 - 495)	0.028
Median bone marrow blasts, %	68.3 (10-100)	71.6 (2-100)	0.428
Platelet count $\times 10^3/\mu\text{L}$ - median (range)	44 (7 - 578)	56 (4 - 520)	0.051
Hemoglobin - median (range)	8.6 (3.3 - 12)	8.3 (4.3 - 15.6)	0.713
Cytogenetics, n (%)			
Normal	19 (22%)	18 (21%)	0.821
t(8;21)(q22;q22)	15 (18%)	7 (8%)	0.063
inv(16)/t(16;16)(p13.1;q22)	19 (22%)	9 (10%)	0.036
t(9;11)(p22;q23) or other abn 11q23	13 (15%)	20 (23%)	0.187
t(6;9)(p23;q34)	2 (2%)	4 (5%)	0.682
Monosomy 7	0 (0%)	5 (6%)	0.059
Del7q	2 (2%)	2 (2%)	1.000
-5/5q-	1 (1%)	1 (1%)	1.000
Trisomy 8	4 (5%)	9 (10%)	0.155
Other	10 (12%)	11 (13%)	0.838
Unknown	9	8	
Risk Group, n (%)			
Standard	35 (40%)	47 (54%)	0.068
Low	49 (56%)	19 (22%)	<0.001
High	3 (3%)	21 (24%)	<0.001
Unknown	7	7	
Molecular alterations, n (%)			
FLT3/ITD	3 (3%)	20 (20%)	<0.001
NPM1 mutation	8 (9%)	2 (30%)	0.187
CEBPA mutation	7 (8%)	3 (3%)	0.206
WT1 mutation	7 (8%)	12 (14%)	0.206
Protocol Stem Cell Transplant received?			
Yes	16 (17%)	13 (14%)	0.686
No	78 (83%)	81 (86%)	

TABLE 2

Univariate and Multivariate Cox Regression Models of OS and EFS for Entire Study Cohort

		OS			EFS		
	N	HR	95% CI	P-value	HR	95% CI	P-value
Univariate Model							
SOCS2 Expression							
Q1-2	94	1			1		
Q3-4	94	1.80	1.06 - 3.03	0.029	1.58	1.04 - 2.41	0.033
Age (years)							
3-10	59	1			1		
0-2	42	1.24	0.55 - 2.76	0.603	1.34	0.73 - 2.48	0.347
11	87	2.08	1.10 - 3.94	0.024	1.67	1.01 - 2.77	0.047
Disease Risk[*]							
Standard-risk	82	1			1		
Low-risk	68	0.35	0.17 - 0.72	0.004	0.44	0.27 - 0.74	0.002
High-Risk	24	1.95	1.03 - 3.68	0.040	1.46	0.82 - 2.58	0.196
Multivariate Model							
SOCS2 Expression							
Q1-2	87	1			1		
Q3-4	87	1.68	0.92 - 3.07	0.094	1.40	0.87 - 2.24	0.168
Age (years)							
3-10	59	1			1		
0-2	35	1.09	0.4 - 2.59	0.843	1.31	0.69 - 2.49	0.416
11	80	2.41	1.23 - 4.72	0.010	1.82	1.07 - 3.11	0.028
Disease Risk[*]							
Standard-risk	82	1			1		
Low-risk	68	0.30	0.14 - 0.	0.010	0.43	0.25 - 0.74	0.003
High-Risk	24	1.19	0.89 - 3.34	0.106	1.09	0.57 - 2.07	0.798

* A combination of cytogenetic and molecular abnormalities was used to stratify participants enrolled on AAML03P1 into risk groups (see Methods section for details).