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LETTER TO THE EDITOR

Signaling changes in the stem cell factor–AKT-S6 pathway in diagnostic AML samples are associated with disease relapse

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Although current chemotherapy regimens are effective at eliminating the bulk of acute myeloid leukemia (AML) blasts, the disease relapses in the majority of patients.[1] Whether relapse arises through the selection of a relatively rare subpopulation of cells present at diagnosis having specific characteristics that facilitate chemoresistance or through the induction of changes required to render them resistant to the chemotherapy is uncertain. Identification of the characteristics that confer chemoresistance, whether selected or induced, would allow for the implementation of additional therapies that target the lynchpin of resistance, thus improving disease outcome.

This study evaluated whether single-cell network profiling (SCNP), a process that simultaneously measures post-translational protein modifications (for example, phosphorylation, acetylation and so on) in multiple signaling pathways within a single cell, could be used to functionally characterize intracellular pathways associated with *in vivo* AML chemotherapy resistance.[2,3] Specifically, we hypothesized that leukemic blasts at relapse have different signaling characteristics from those at diagnosis and that assessing intracellular signaling networks

Table 1 Clinical characteristics of patient donors for longitudinally paired diagnosis and relapse samples

Donor	*Age (years)*	*Gender*	*Sample source*	*Secondary AML*	*FAB*	*Cytogenetics*	*Cytogenetic group*	*FLT3-ITD*	*Induction chemotherapy*	*Induction response*	*Relapse*	*CR duration (weeks)*
1	77.8	M	BM	No	M0	46,XY,t(3;21)(q26;q22)	Unfavorable	Neg	IDA+HDAC	CR	Yes	46.143
2	34.8	F	BM	No	M2	t(6;9)	Unfavorable	Pos	IA+ZARNESTRA[a]	CR	Yes	11.143

Abbreviations: BM, bone marrow; CR, complete response; F, female; FAB, French-American-British classification system; FLT3-ITD, FMS-like tyrosine kinase 3 receptor (FLT3) internal tandem duplications (ITD); HDAC, high-dose Ara-C; IDA, idarubicin; M, male; Neg, negative; Pos, positive.
[a]IDA+Ara-C+Zarnestra.

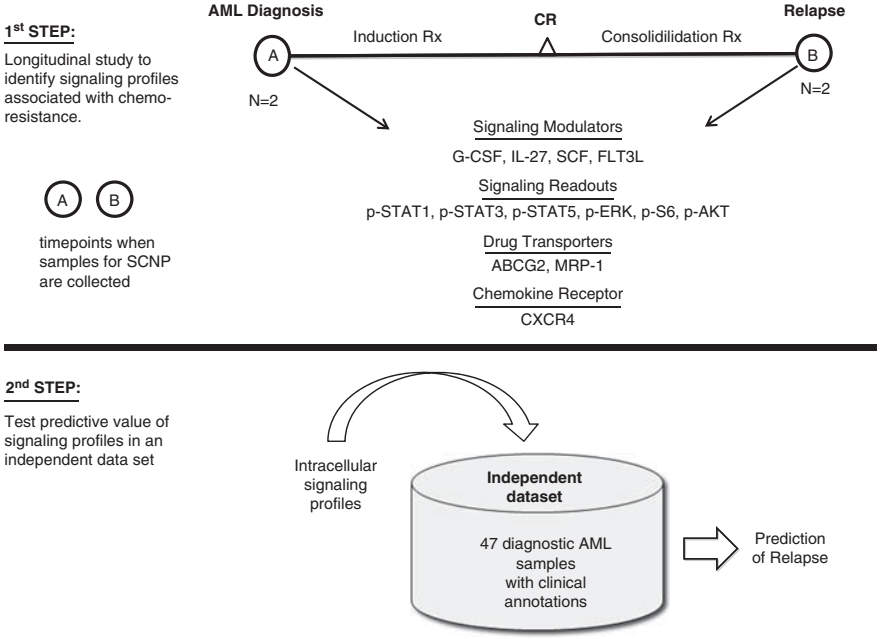


Figure 1 Study schema. The two-step process involves the identification of resistance-associated signaling node subpopulations followed by testing the predictive value of these profiles for disease response in an independent data set. The top portion of the diagram shows a time continuum of AML therapy, with A and B representing the time points (diagnosis and first relapse after complete response (CR) when samples were collected on the two patients for SCNP analysis). The signaling modulators, readouts, drug transporters and chemokine receptor tested are listed. The lower portion of the schema depicts the application of the chemoresistant profiles to an independent data set of 47 patients. CR, complete response; G-CSF, granulocyte colony-stimulating factor; IL-27, interleukin-27; Rx, therapy.

using the SCNP approach in longitudinally paired (that is, from the same patient) AML bone marrow (BM) samples at diagnosis and relapse time points may enable functional characterization of the mechanisms of chemoresistance in cell subpopulations that are dominant at relapse. The above hypothesis was tested by searching for resistance-associated signaling nodes in paired diagnosis/relapse samples from two AML patients and then examining the predictive value of the identified resistance-associated signaling nodes in an independent set of diagnostic AML samples collected from patients that had a complete disease response[4] to standard induction chemotherapy and for which clinical annotation about disease relapse was available.

Clinical characteristics of the two AML patients are shown in Table 1. Both patients received high-dose cytarabine-based induction chemotherapy with complete disease response followed by relapse within 1 year. Cytogenetic analysis revealed prognostically unfavorable translocations of AML1-EVI1 and DEK-NUP214 [t(6;9)] in patients 1 and 2, respectively. In addition, patient 2 had FMS-like tyrosine kinase 3 receptor internal tandem duplications (FLT3-ITD)-positive leukemia, a known poor prognostic marker for relapse risk and overall survival and associated with the DEK-NUP214 translocation in the majority of cases.[5] Healthy control BM mononuclear cells ($N=2$) were derived from young healthy male volunteers (age = 18 and 20 years, respectively).

The cryopreserved BM samples from the above two AML patients were processed (thawed, modulated, fixed, permeabilized and incubated with antibodies to both surface and intracellular proteins)[2,3,6] to assess whether specific cell subpopulations identified using cell surface phenotypes and/or signaling profiles were present in the relapsed sample in a greater percentage than observed in the corresponding diagnostic sample (Figure 1). Next, in an independent and larger group of diagnostic patient samples, blasts with the previously identified cell profiles were examined for their association with disease relapse (Figure 1).

The two longitudinally paired samples were first compared for expression of conventional surface markers used to define myeloblast maturity as shown in Figure 2a.[7] Cell subpopulations

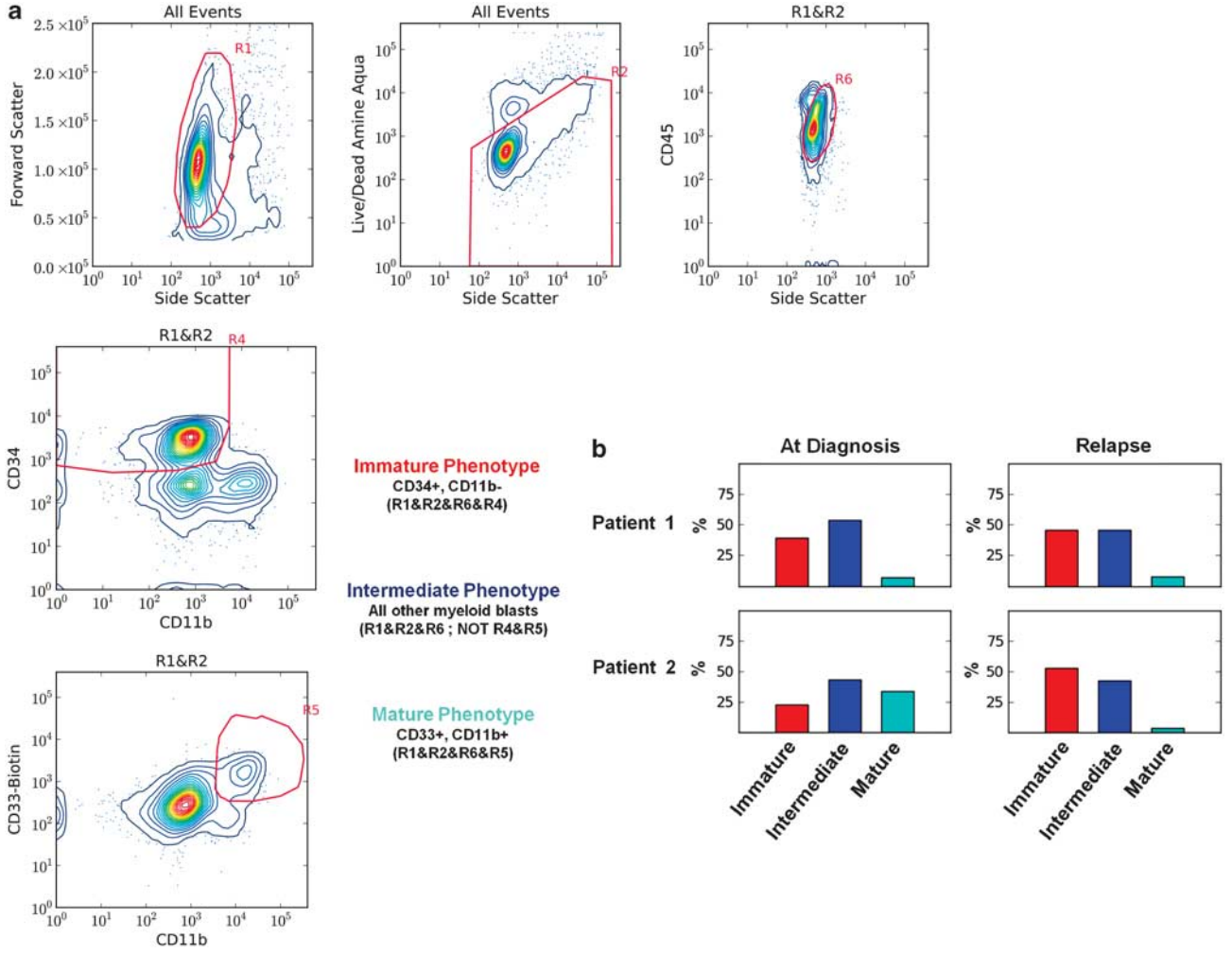
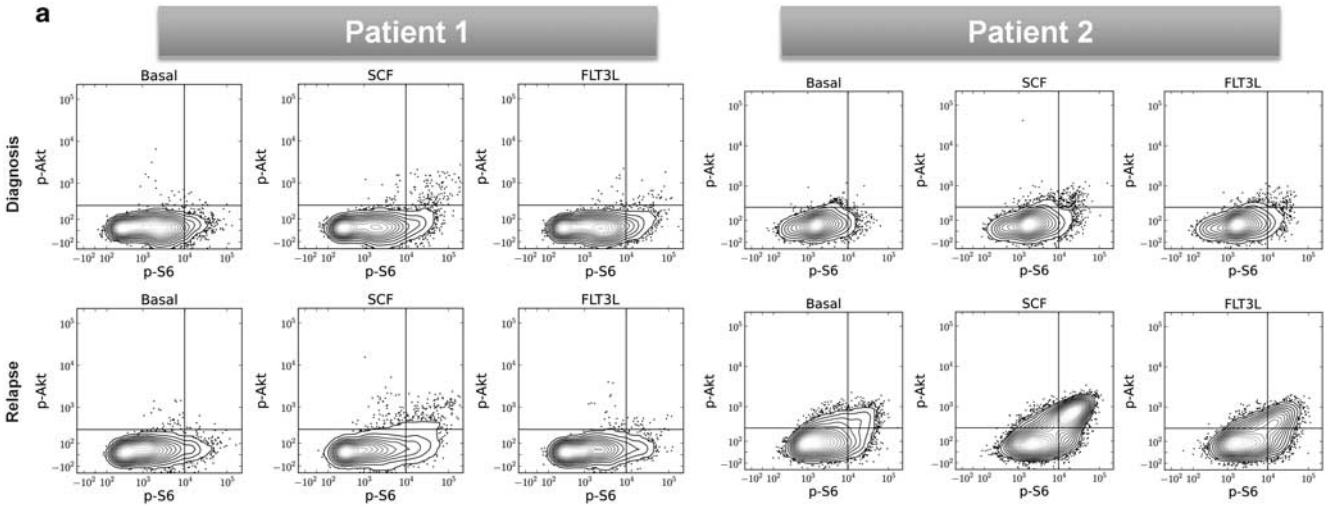


Figure 2 (**a**) Gating analysis to define leukemic blast population. Flow cytometry plots and sequential gating scheme. The first panel is a flow cytometry dot plot indicating how non-cellular debris was excluded using an FSC and SSC gate (R1). The second panel is a flow cytometry dot plot indicating how non-viable cells were excluded and viable cells included with an SSC and Aqua viability dye gate (R2). This gating scheme ensured that only live cells were analyzed for signaling responses. The third panel is a flow cytometry dot plot indicating CD45+ cells (R6) that were then subdivided into groups of immature CD34+ CD11b− (R1&R2&R6&R4), mature CD33+ CD11b+ (R1&R2&R6&R5) and intermediate (R1&R2&R6 NOT R4 &R5) myeloid leukemic blasts. (**b**) Cell surface markers did not identify resistance-associated myeloblast subpopulations. Shown are bar graphs that depict the percentage (of total blasts) of immature, intermediate and mature blasts (as defined above in **a**) found in the diagnosis and relapse samples from patient 1 (top panels) and patient 2 (bottom panels).



b

	% Total Blasts Positive for Both p-Akt and p-S6					
Modulator:	None (Basal)		SCF		FLT3L	
	Diagnosis	Relapse	Diagnosis	Relapse	Diagnosis	Relapse
Patient 1	0.15%	0.21%	1.00%	3.20%	0.40%	0.45%
Patient 2	0.16%	7.91%	1.24%	31.70%	1.07%	18.10%

Figure 3 Examination of signaling profiles revealed differences in relapse and diagnosis samples for SCF and FLT3L. (**a**) Shown are flow cytometry dot plots measuring phospho-S6 (*X*-axis) versus phospho-Akt (*Y*-axis) in response to no modulator/basal, modulation with SCF or modulation with FLT3L for patient 1 (left side) and patient 2 (right side) leukemia samples at diagnosis (top panels) and at first relapse after complete remission (lower panels). (**b**) Figure showing the percentage of cells positive for both p-Akt and p-S6 in diagnostic and relapse samples.

based on these characteristics of myeloblast maturity were not informative of relapse risk for either patient sample (Figure 2b).

Examination of intracellular signaling profiles revealed functionally distinct cell subsets in the otherwise phenotypically similar relapse and diagnosis samples (Figure 3 and Supplementary Figure 1). Specifically, when the relapse samples from patient 1 and patient 2 were modulated using stem cell factor (SCF), both p-Akt and p-S6 were induced in 3.2 and 31.7% of cells, respectively (Figure 3). A similar finding of an increased percentage of myeloblast subpopulations defined by intracellular signaling profiles in relapse versus diagnosis samples was observed when FLT3L (inducing p-S6 and p-Akt, Figure 3) and interleukin-27 or granulocyte colony-stimulating factor (inducing p-signal transducer and activator of transcription (STAT)3 and p-STAT5) were used as modulators (Supplementary Figure 1).

To investigate whether similar cells were present at the time of diagnosis, which would support the concept of selection, or absent, supporting the idea of an induced change, we looked for the presence of cells with similar functional responses to SCF, FLT3L, interleukin-27 and granulocyte colony-stimulating factor in the corresponding diagnosis samples. While no interleukin-27-responsive subpopulation was identified, SCF-, FLT3L- and granulocyte colony-stimulating factor-responsive cells were observed in the diagnostic AML BM samples (Figure 3 and Supplementary Figure 1), although in a lower percentage ($\sim$1%). SCF-responsive cells, defined by the SCF:p-Akt/p-S6 signaling profile, were phenotypically diverse in the relapse AML samples compared with SCF-responsive cells in healthy donor BM samples (Figure 4). In healthy BM samples, the SCF:p-Akt/p-S6 profile existed specifically in a subset of progenitor cells with the CD34+, CD33−, CD11b− phenotype (Figure 4).

After identifying the resistance-associated signaling nodes in the relapsed samples, we hypothesized that detection of the same nodes in the diagnostic samples might be predictive of poor outcome (early relapse). We first applied the SCF:p-Akt/p-S6 gating scheme (as defined in Supplementary Figure 2) to an independent set of diagnostic AML samples. The clinical characteristics of these 47 patients are shown in Table 2. All patients received high-dose cytarabine-based induction chemotherapy with disease response of complete remission. Of these, 27 experienced disease relapse (complete response followed by relapse, CR Rel), while 20 remained in complete continuous remission for two or more years. Patients from whom this independent sample set was obtained were young (41/47; <60) and a high proportion (20/47) had French-American-British classification system M2 AML.

In seven diagnostic AML samples, a subset of leukemic blast cells that responded to SCF modulation by phosphorylation of p-Akt and p-S6 were observed (Table 2, Supplementary Table S1). Of those seven, six patients experienced disease relapse within 2 years ($P=0.21$, Fisher's exact test) from remission, while the seventh patient had a complete remission lasting more than 2 years; interestingly, this AML sample had favorable cytogenetics, t(8;21) (Figure 5 and Supplementary Table S1). Of note, all of the patients with this SCF-responsive profile were less than 60 years old and, with the exception mentioned above, they all had intermediate- or high-risk cytogenetics; six out of seven also had an early myeloid FAB classification of M1 or M2. Also of note, the occurrence of the SCF:pAkt/pS6 subpopulation was independent of the presence of FLT3-ITD: only one of the six samples was positive for FLT3-ITD mutation. Importantly, the predictive value of the combination of FLT3-ITD and SCF:p-Akt/p-S6 for disease relapse was greater than that of either biomarker individually ($P=0.03$, Fisher's exact test, Table 3).

We next examined whether expression of c-Kit, the receptor for SCF, could function as a surrogate marker for the SCF:p-Akt/p-S6 phenotype. Although only samples that expressed c-Kit

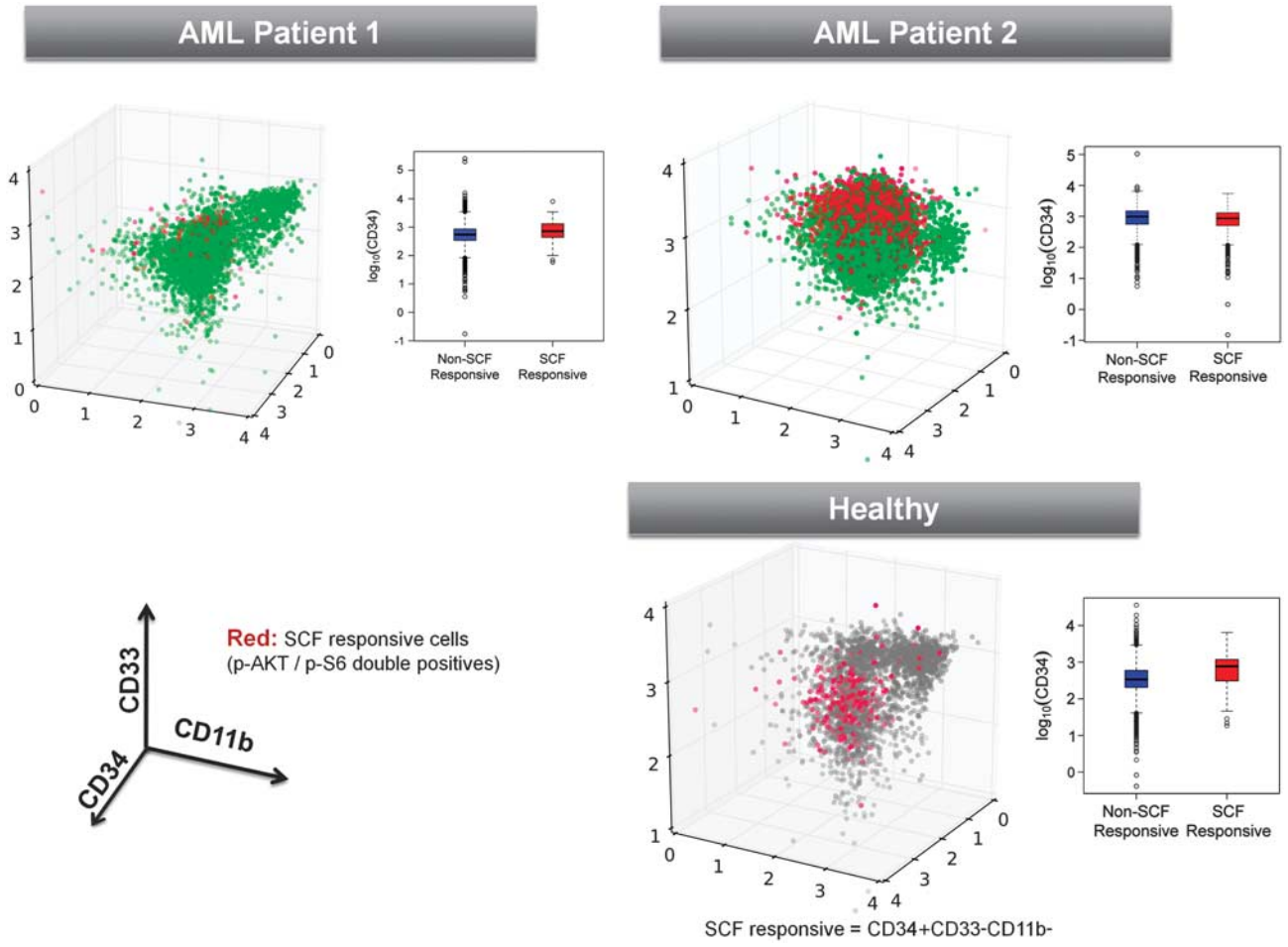


Figure 4 In the relapse samples, the SCF:p-Akt/p-S6 signaling profile is present in a small subset of AML cells that are phenotypically diverse. Myeloid cells (R1&R2&R6 gated—see Figure 2a) are displayed on a three-parameter plot with three fluorescent cell surface markers: CD11b (*X*-axis), CD33 (*Y*-axis) and CD34 (*Z*-axis). Red spheres are cells that stain positive for the SCF:p-Akt/p-S6 signaling profile. Bone marrow cells from a representative healthy individual (bottom right) show that normally the SCF:p-Akt/p-S6 profile exists mostly in a subset of progenitor cells with the CD34+, CD33−, CD11b− phenotype. To the right of each plot are box and whisker plots that show the relative expression ($\log_{10}$ fluorescence) of CD34 in the SCF:p-Akt/p-S6-responsive and non-responsive populations for two relapse AML samples and one healthy sample. In the box and whisker plots, boxes contain 50% of the sample data with the median values indicated by a horizontal bar. Whiskers contain 1.5× the interquartile range and outliers past this range are shown as individual points.

were able to respond to SCF, no association between c-Kit expression levels and likelihood of leukemia relapse (Figure 6a) was observed, suggesting that c-Kit expression is a necessary but not sufficient condition for SCF-induced intracellular signaling. In line with this observation, the removal of non-c-Kit-expressing samples improved relapse prediction (Figure 6b). Furthermore, when blast cells from an AML sample were simultaneously examined for c-Kit and the downstream signaling marker p-Akt, intra-patient heterogeneity in c-Kit expression and response to SCF within c-Kit-expressing cells were observed (Figure 6c).

We also examined whether FLT3L:p-Akt/p-S6, granulocyte colony-stimulating factor:p-STAT3/5 or interleukin-27:p-STAT3/5 signaling nodes predicted poor outcome in the same independent set of diagnostic AML samples. Unlike SCF:p-Akt/p-S6 gate, no association was found with disease relapse (Supplementary Table S2).

Relapse due to chemoresistant residual disease is a major cause of death in both adult and pediatric patients with AML, and aberrant signal transduction within pathways that control cell proliferation and survival is thought to have an important role in secondary chemoresistance. In this study, we used SCNP as a strategy to identify specific signaling pathway profiles associated with *in vivo* chemoresistance using paired diagnosis and relapse samples. Although it was performed on a limited number of paired AML samples, our study provides unique insights into the nature of AML secondary chemoresistance in rare cell populations, identifying a functionally characterized cell subset associated with likelihood of early relapse when the assay was applied in a separate patient cohort.

A subset of leukemia cells with enhanced activity within the phosphoinositide 3-kinase/Akt cascade (SCF:p-Akt/p-S6) was found to be commonly expanded in the two leukemia samples collected at relapse. Importantly, the presence of cell subpopulations expressing this same signaling profile at diagnosis was associated with disease relapse after complete response to induction chemotherapy in an independent sample set of AML diagnostic samples. Although the SCF:p-Akt/p-S6 profile was not present in all patients with relapsed disease, all but one patient with a diagnostic sample containing a subpopulation of ⩾3%

Table 2 Clinical characteristics of independent test set of 47 patient samples

Characteristic	*Total*	*40 non-SCF responsive*		*7 SCF responsive*	
Total samples = 47		*CCR*	*CR Rel*	*CCR*	*CR Rel*
CCR	20 (74%)	19		1	
CR Rel	27 (26%)	21		6	
Age					
<60 years	41 (87%)	17	17	1	6
>60 years	6 (13%)	2	4	0	0
Cytogenetics					
Favorable	6 (12.7%)	4	1	1	0
Intermediate	24 (51%)	11	9	0	4
Unfavorable	17 (29.7%)	4	11	0	2
FAB					
M0	1	0	1	0	0
M1	7	1	2	0	4
M2	20	10	8	1	1
M4	10	5	5	0	0
M4EOS	1	1	0	0	0
M5 (A,B,C)	5	2	3	0	0
M6	1	0	0	0	1
Unknown	2	0	2	0	0
FLT3 mutation					
ITD	10 (21.3%)	2	7	0	1
WT	35 (74.5%)	16	13	1	5
Unknown	2 (4.2%)	1	1	0	0

Abbreviations: CCR, complete continuous remission; CR, complete response; CR Rel, complete response followed by relapse; SCF, stem cell factor; WT, wild type.

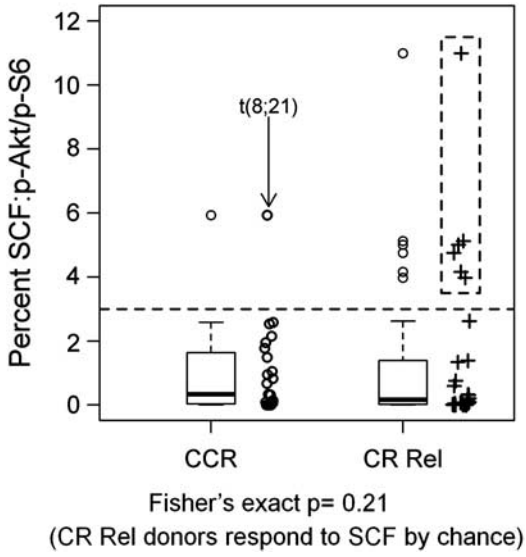


Figure 5 Application of SCF:p-Akt/p-S6 gate identifies a subset of patients with relapsed disease. Shown are box and whisker plots of patients with leukemia that relapsed within 2 years (complete response (CR) Rel, right side) versus those in complete continuous remission for 2 or more years (CCR, left side) and the percentage of SCF-responsive (inducing p-S6 and p-Akt) blasts. To the right of the plots each symbol represents an individual sample. A circle is drawn around the six patient samples whose diagnostic samples showed $\geqslant 3\%$ of SCF:p-Akt/p-S6 cells and who experienced disease relapse. The cytogenetics of the CCR sample with the SCF-responsive subpopulation is depicted. Note: In box and whisker plots, boxes contain 50% of the sample data, with the median values indicated by a horizontal bar. Whiskers contain 1.5 × the interquartile range and outliers past this range are shown as individual points.

Table 3 The SCF: pAkt/pS6 profile is independent of FLT3-ITD status

	SCF non-responsive and FLT3-WT	*SCF responsive or FLT3-ITD*
CCR	17	3
CR Rel	14	13
Fisher's test	$P = 0.03$	

Abbreviations: CCR, complete continuous remission; CR, complete response; SCF, stem cell factor; WT, wild type.

SCF:p-Akt/p-S6 cells relapsed within 2 years of remission. These data support the marked biological heterogeneity at the basis of AML secondary chemoresistance and lend merit to the approach of studying signaling profiles in functionally distinct subpopulations in longitudinally collected AML samples before and after therapy to identify poor-prognostic cell populations.

Currently there are no measures to indicate why patients with similar clinically appearing disease have different responses to therapy, with some remaining disease free while others undergo disease relapse and ultimately succumb. SCNP permits an accurate characterization of each individual's leukemia signaling pathway phenotype and biological heterogeneity, allowing for a more efficient delineation of the normality or pathology of leukemic subpopulations. This study shows that leukemic cell populations differ quantitatively and qualitatively before and after *in-vivo* therapeutic pressure in AML, and that SCNP offers a novel approach to identify chemotherapy-resistant subpopulations that may predispose patients to disease relapse.

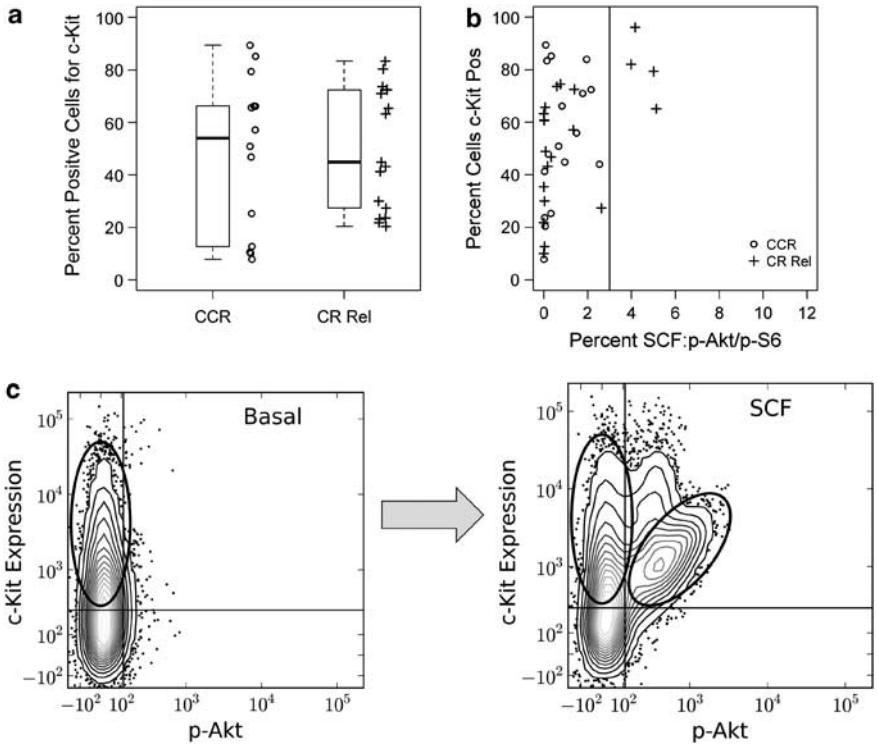


Figure 6 (**a**) c-Kit (SCF receptor) expression is not predictive of SCF responsiveness. Shown are box and whisker plots of patients with leukemia that relapsed within 2 years (complete response (CR) Rel, right side) versus those in complete continuous remission for 2 or more years (CCR, left side) and the percentage of cells with c-Kit surface expression. To the right of the plots each symbol represents an individual sample. Note: In box and whisker plots, boxes contain 50% of the sample data, with the median values indicated by a horizontal bar. Whiskers contain 1.5 × the interquartile range and outliers past this range are shown as individual points. (**b**) Only samples that expressed c-Kit were able to respond to SCF. Scatter plot of SCF-responsive SCF→p-Akt/p-S6 (*X*-axis) and percent-positive cells for c-Kit (*Y*-axis) for relapsed samples (circles) and CCR samples (pluses). (**c**) Simultaneous staining of c-Kit and p-Akt shows multiple c-Kit+ cell subsets that vary in p-Akt signaling in response to SCF. Phospho-Akt versus c-Kit 2-D contour plots with and without SCF for 15-min incubation. Quadrant gate was applied based on the isotype controls.

Conflict of interest

Dr Cohen, Dr Woronicz and Dr Fantl were employees of Nodality during the time the research was conducted. Todd Covey, Dr Putta, Dr Gayko and Dr Cesano were employees of Nodality during the time the research was conducted and are presently employed by Nodality. Dr Kornblau declares no conflict of interest.

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Supplementary Information accompanies the paper on Blood Cancer Journal website (http://www.nature.com/bcj)