ORIGINAL RESEARCH

Patient-derived acute myeloid leukemia (AML) bone marrow cells display distinct intracellular kinase phosphorylation patterns

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Abstract: Multiparametric analyses of phospho-protein activation in patients with acute myeloid leukemia (AML) offers a quantitative measure to monitor the activity of novel intracellular kinase (IK) inhibitors. As recent clinical investigation with FMS-like tyrosine-3 inhibitors demonstrated, targeting IK with selective inhibitors can have a modest clinical benefit. Because multiple IKs are active in patients with AML, multikinase inhibitors may provide the necessary inhibition profile to achieve a more sustained clinical benefit. We here describe a method of assessing the activation of several IKs by flow cytometry. In 40 different samples of patients with AML we observed hyper-activated phospho-proteins at baseline, which is modestly increased by adding stem cell factor to AML cells. Finally, AML cells had a significantly different phospho-protein profile compared with cells of the lymphocyte gate. In conclusion, our method offers a way to determine the activation status of multiple kinases in AML and hence is a reliable assay to evaluate the pharmacodynamic activity of novel multikinase inhibitors.

Keywords: leukemia, AML, bone marrow, flow cytometry

Introduction

Relapsed acute myeloid leukemia (AML) remains a disease with poor prognosis in which new therapies are needed.[1] Targeting intracellular kinases (IK) responsible for critical signal transduction pathways in AML represents a novel approach of developing agents to treat AML.[2] Clinically, activated or phosphorylated IKs are associated with prognosis in patients with AML.[3] One important IK in AML is the FMS-like tyrosine kinase 3 (FLT 3). Various FLT3 inhibitors have been developed to inhibit wildtype or mutated FLT3.[4–11] However, the clinical benefit of selective FLT3 inhibitors has been modest, perhaps because the single pathway inhibition may not be sufficient to affect the AML progression. Based on the observation that selective inhibition of FLT3 indirectly also modulates other IKs such as ERK,[8,12] novel IK inhibitors, such as KW2449,[13] are being developed to target multiple IKs. For the clinical development of such multikinase inhibitors, it is essential to monitor sequentially the activity of IKs simultaneously.

One way to determine the status of IK activation and subsequent alteration by IK inhibitors, is based on the use of multiparametric flow cytometric analysis.[14,15] This approach of analyzing multiple phosphorylated proteins in AML requires a sophisticated isolation, fixation and permeabilitization procedure followed by a rigorous flow cytometric analysis. Analyses protocols have been presented for the IK inhibitor sorafenib in AML patients,[16] but such assessments are limited to specialized laboratories.[17]

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Previous studies have suggested that assessing extracellular signaling-regulated kinase (pERK 1/2) may reflect anti-leukemia activity of sorafenib.[16] Given the advances in flow cytometry, the role of IKs in AML pathology and the rapid development of several IK inhibitors, we here present a multi-parametric flow cytometry assay, which can be conducted at routine clinical pathology laboratories. Our interest was focused on the assessment of pERK, pcKIT (stem cell factor receptor or CD117), pFLT3, pY100 (pan receptor tyrosine kinase marker), ribosomal protein pS6 (ribosomal protein involved in downstream signaling events of several receptor tyrosine kinases) and pAKT, because these IKs are generally activated in some or most AML cells.[3,16] Thus, our assay may be used to monitor sequentially the activity of multi-targeted IK inhibitors in AML patients.

Materials and methods
AML sample preparation (materials)
In compliance with United States (US) Health Insurance Portability and Accountability Act (HIPAA) guidelines, we obtained bone marrow samples from 40 patients with relapsed AML from different hematology practices in the US. All samples were obtained as discarded material (during the course of 2006) along with patient specific pheno-typing results, from the Esoterix Oncology diagnostic lab (Austin, TX) after all diagnostic testing had been performed and patient information had been de-identified. Demographic information noted on requisition forms was used to charac-terize the origin of the bone marrow samples. In each case, automated cell counts were obtained and samples were placed in a 37 °C/5% CO_2 incubator for one hour prior to processing to allow enzyme activity and cellular processes to stabilize. The testing for each sample was obtained for basal levels and an additional aliquot was stimulated with 10 ng/ml of stem cell factor (R&D Systems, Minneapolis, MN) for exactly 15 minutes and immediately fixed/permeabilized per US Patent #7326577. All samples were then placed at 2–8 °C overnight to be stained for phosphorylated proteins the following day as indicated below.

Antibodies (materials)
All antibodies to phosphorylated proteins were obtained from Cell Signaling Technology (CST) (Danvers, MA). Antibody specificity titrations were performed prior to use of the antibodies based on rested and stimulated conditions to obtain the saturating concentrations for each antibody. Thus, the antibodies were used per $1–2 \times 10^6$ cells. The specific antibodies for this study were: phospho-FLT3 (CD135) (Y591)-Alexa Fluor 488 (CST #3459); phospho-c-KIT (CD117) (Y719) (CST#3391L); phospho-p44/42 extracel-lular signaling-regulated kinase 1/2 (ERK1/2) (T202/Y204) Alexa Fluor 647 (CST#4375); phospho-AKT (S473)-Alexa Fluor 647 (CST2337); phospho-Tyrosine-Y100-Alexa Fluor 488 (CST 9414); phospho-S6 Ribosomal Protein (S235/236)-Alexa Fluor 647 (CST, Esoterix custom conjugate). The surface antibodies used for the study were CD11b-Alexa Fluor 700 (Esoterix custom conjugate; Beckman Coulter, Miami, FL); CD34-Biotin (Invitrogen, Carlsbad, CA) and CD45-PE (Immunotech; Beckman Coulter). The isotype controls used for the phospho-proteins were matched to the primary antibodies at the identical concentrations used in the assay. The CD34-Biotin was developed post fixation using streptavidin QDOT 605 (Invitrogen) and the phospho c-KIT was developed using a goat anti rabbit Alexa Fluor 488 (both used at 1 µg per staining reaction). In all cases, the staining reactions were carried out in 50 µl total volumes in phosphate-buffered saline (PBS) with 3.4% bovine serum albumin (BSA).

Staining of AML samples for surface and phosphorylated proteins
The samples were surface stained with the CD34 and CD11b antibodies for 20 minutes. If cells were chosen for *ex vivo* stimulation, they were immediately stimulated with SCF and fixed/permeabilized as indicated above. The cells were spun out of fixative and washed twice in PBS + 2% NCS. Respec-tive phospho antibodies as listed below were added to the cell pellet and allowed to incubate for one hour at ambient temperature in the dark. The excess antibody was removed by washing the cells twice in 2 ml of PBS-2% NCS with 10 minute soaks between each centrifugation. The following combinations were used for each donor: Tube (1) p-cKIT/p44/42/ERK1/2-AF647; Tube (2) pFLT3-AF488/pS6-Ribosomal Protein-AF647; Tube (3) pY100-AF488/pAKT-AF647; Tube (4) isotype Alexa 488/isotype Alexa 647. The secondary antibodies were added to the appropriate tubes in conjunction with CD45-PE in all tubes and incubated for an additional 20 minutes. The excess antibody was removed using same wash sequence as listed above. Finally, the cells were re-suspended in 400 µl of PBS-2% NCS final volume and 100,000 intact events were collected per tube during flow cytometry assessments.

Cytometer information
Flow cytometry was performed on a Beckman Coulter FC500 with custom filter sets designed to optimize the collection of

Alexa 647 and Alexa 700 signals. The instrument was set up using QC3 beads (Bangs Laboratories, Fishers, IN) for the daily monitoring of instrument performance within an accepted "window of analysis". Full spectrum fluorescent calibration particles (Spherotech, Lake Forest, IL) were also run with each assay to determine the molecules of equivalent fluorescence (MEFL) of the phospho-proteins in a methodology that has been described in detail.[18]

Sample analysis

Each sample was analyzed using a direct data exchange (DDE) link between WinList 6.0 (Verity Software House, Topsham, MA) and Mircosoft Excel (Microsoft, Redmond, WA). All data gating, displaying, and processing were conducted in WinList. The pattern recognition of the marrow elements was by eye and experience based on known CD45/SSC patterns. The MEFL of the phospho-proteins was measured in the "blast" population as determined by CD45/SSC and/or CD34$^+$, CD11b$^-$ staining. In samples that contained more than one phenotypic clone of "blasts", each clone was analyzed individually for phospho-protein expression. The SCF stimulated levels were compared to the unstimulated levels in each population.

Statistical analysis

AML phospho-protein expression was analyzed using a mixed effect ANOVA model using the REML method to estimate covariance parameters. A variance components structure was used for the covariance matrix. For normalized untreated AML, the SCF-treated AML, and the ratio of untreated AML to lymphocyte responses the model used the following terms: AML FAB Classification, Patient nested within AML FAB Classification (random factor), IK, phenotype, IK*phenotype, AML FAB Classification*IK, and AML FAB Classification*phenotype. Pairwise comparisons were made using the LSMEANS and pdiff options in SAS Proc Mixed (SAS Institute Inc. Cary, NC). A Fisher's LSD approach was used to approximately control the experiment-wise type I error rate. Also, not all pairwise comparisons were of interest. For significant interaction model terms, the slice option was used in SAS to further protect the experimentwise error rate. Only statistically significant slices were followed up with further pairwise comparisons. The data were analyzed using JMP ver. 6.0.2 and SAS ver. 9.1 (SAS Institute, Inc.).

Results

We obtained bone marrow samples from 40 patients with relapsed AML to test a novel technique of determining phospho-protein expression in AML blasts and to profile AML blast cells based on their ability to express specific phospho-proteins. All samples were received from different hematology practices in the United States, and were evaluated as part of routine hematological evaluation (Table 1). We used samples from relapsed AML patients based on the assumption that AML blasts in advanced disease will show increased phosphorylation levels.[16] From the 40 samples, 19 (47%) had not been classified based on the FAB AML classification, of which eight patients had recurrent AML (8/19; 42%). In addition to AML samples with no clear FAB classification, there were samples from six M1 and M4 AML patients, respectively (each 6/40; 15%). For M2, M3, and M5 AML there were each three samples (3/40; 7%). In 5/40 (12%) patients the bone marrow showed less than 20% blast cells.

Based on this randomly collected material from patients with AML, we evaluated the phospho-protein levels without and with SCF *ex vivo* stimulation. After gating cells on forward scatter (FS) vs. side scatter (SS), cells were gated based on CD45, CD11b and CD34 expression prior to analyzing the phospho-protein expression of pERK, pc-KIT, pAKT, pY100, pFLT3, and pS6 (Figures 1 and 2). Based on the surface marker expression, we categorized the cell population in the following groups: (a) AML phenotype 1, (b) phenotype 2 and (c) lymphocytes. AML phenotype 1 was characterized by CD34^{hi}/CD45^{low}/CD11b$^-$ surface expression (red gate in Figures 2A,B) consistent with an "immature-like" cell population. AML phenotype 2 was characterized by CD34^{low}/CD45^{hi}/CD11b$^+$ surface expression (green gate in Figures 2A, B) which is characteristic for "mature-like" cells. Most samples in this study were AML Phenotype 1 cells (Table 2). Compared to the lymphocytes, both phenotype 1 and 2 AML cells showed high phospho-protein expression (Figures 2–4). Ex vivo stimulation with SCF increased levels of phosphorylation in both phenotypes of AML, but not in lymphocytes (Figures 2C–F). Hence, lymphocytes can serve as negative control for assessing the effect of SCF-induced phosphorylation of IKs in AML cells.

We then investigated whether phenotype 1/2 or cells based on the FAB AML classification were associated with a particular phospho-protein expression pattern (Figures 3, 4). AML cells were immediately assessed after receipt in the clinical pathology lab and without any additional in vitro stimulation (basal condition) (Figure 3). pFLT3, pS6, pY100 and pcKIT were increased compared with p-AKT and p-ERK (Figure 3, Panel A and B). Phospho-protein expression between phenotype 1 and 2 was similar with statistical

Table 1 Demographics of analyzed AML bone marrow cells

FAB classification	Patient ID	History of AML	Cytogenetics	Blasts (%)	HLA-DR	CD34	CD117	CD11b	CD13	CD33	CD14	CD64
NC	408931	Recurrent AML	48,XX,+9 +11 +13	54	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Neg	Neg.
NC	409039	Recurrent AML	Not performed	83	Pos.	Pos.	Pos.	Neg.	Pos.	Part.	Neg	Neg.
NC	410224	Recurrent AML, poor differentiation	46,XY,−11, der (12) t(11;12)(q13;p13), dup(14)(q24;q32),+mar	90	Neg.	Part.	Part.	Pos.	Pos.	Pos.	Neg	Part.
NC	411848	MDS/evolving AML	46,XX,t(1;2)(p10;q10)	19	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Neg	Neg.
NC	412003	Not Available	Not performed	75	Pos.	Neg	ND	Pos.	Pos.	Pos.	Pos	ND
NC	412861	AML with poorly differentiated myeloid blasts	Not performed	68	Pos.	Pos.	Pos.	Part.	Pos.	Pos.	Neg	Neg.
NC	419226	High grade MDS/ evolving AML	Normal	19	Pos.	Neg.	Pos.	Neg.	Pos.	Pos.	Neg.	Neg.
NC	419970	AML	46,XX,add(2)(q11;:2), add(4)(p14), t(14;15) (p11.2; q22), add(18) (q21), der(21)	63	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.	Neg.	Neg.
NC	420052	Recurrent AML	46,XX, del(5)(q13q33) [3]/46−49, sl, +add(1) (q32)−7, add (11)(q23), −14	25	Pos.	Pos.	Pos.	Part.	Pos.	Pos.	Neg.	Neg.
NC	421759	Recurrent AML	Not performed	45	Pos.	Neg.	Neg.	Part.	Neg	Pos.	Pos.	Pos.
NC	424061	AML	45,XY,I(1)(p10), der(5) t(1;5)(q23;q13), −7, +8	75	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Neg.	Neg.
NC	428392	High grade MDS/ evolving AML	44,XX,−3(5)(q22),−14, del(15)(q22), trisomy 8	24	Pos.	Pos.	Pos.	Neg.	Pos.	Part.	Neg.	Neg.
NC	428407	Recurrent AML	Not performed	45	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.	Neg.	Neg.
NC	429214	Recurrent AML	46,XY,inv(16)(p13q22)	79	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Neg.	Neg.
NC	429247	MDS/MPD	46,XX, inv(3)(q21q26), t(9;22)(q34;q11.2)	11	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Neg.	Neg.
NC	430008	Recurrent AML	Normal	12	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Neg.
NC	430764	Myeloproliferative AML/accelerated phase	46,XY,t(9; 22)(q34;q11.2)	15	Pos.	Pos.	Part.	Part.	Pos.	Pos.	Neg.	Neg.
NC	430975	AML with multi lineage dysplasia	46,XY,del(5)(q13q33)	53	Part.	Pos.	Part.	Part.	Pos.	Pos.	Neg.	Neg.
NC	431093	CMML/AML	45,XY,−7	36	Pos.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.
1	408965	AML-M1/M2 or multilineage AML	47,X,idic(X)(q13),+(X) (q13)[20]	75	Pos.	Pos.	Pos.	Part.	Pos.	Pos.	Neg.	Neg.

Group	ID	Classification	Cytogenetics	Blasts (%)								
1	408982	AML-M1/M2	Normal	85	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Neg	Neg.
1	413234	AML-M1	47, XY,+8	65	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.	Neg.	Neg.
1	425931	AML-M1/M2	Not performed	65	Pos.	Part.	Pos.	Pos.	Pos.	Pos.	Neg.	Neg.
1	431237	AML	Chromosome 6 inversion	68	Neg.	Pos.	Pos.	Neg.	Part.	Part.	Neg.	Neg.
1	432689	AML-M1	Normal	65	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.	Neg.	Neg.
2	419252	AML-M2 or multilineage	46, XY, t(8; 21)(q22; q22)	60	Pos	Part.	Pos.	Neg.	Pos.	Pos.	Neg.	Neg.
2	424042	AML-M2 or AML-t (8; 21)	Not performed	49	Pos.	Neg.	Pos.	Neg.	Pos.	Pos.	Neg.	Neg.
2	430797	AML-M2	46, XX, t(8; 21)(q22;q22)	25	Pos.	Pos.	Pos.	Part.	Part.	Pos.	Neg.	Part.
3	412097	AML-M3	46, XX, t(15; 17), (q22; q21)	85	Neg	Neg	Neg	Neg.	Pos.	Pos.	Neg	Neg.
3	425945	AML-m3v	46, XX, add(7)(q32), t(15; 17)(q22; q21)	94	Neg	Part.	Part.	Neg.	Pos.	Pos.	Neg.	Pos.
3	425959	AML-m3v	Not performed	93	Neg	Part.	Part.	Neg.	Pos.	Pos.	Neg.	Pos.
4	409110	AML-M4 or multilineage	Normal	48	Pos.	Neg	Neg	Pos.	Neg	Pos.	Part.	Pos.
4	415524	AML-M4 or multilineage	Normal	60	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Neg.	Neg.
4	415446	AML-M4	Not performed	49	Pos	Neg	Neg	Pos.	Pos.	Pos.	Pos.	Pos
4	417941	AML-M4	47, XX, −8, der(10) t(10; 17)(q26; q21), der(17) t(8; 17) (q13; q21), +21	62	Pos.	Neg	Pos.	Pos.	Neg	Pos.	Neg.	Pos.
4	421245	AML-M4 (possibly arising form MDS)	Normal	39	Pos.	Neg.	Neg.	Pos.	Neg.	Pos.	Part.	Pos.
4	428198	AML-M4	Normal	50	Pos.	Neg	Neg.	Pos.	Neg.	Pos.	Part.	Pos.
5	412058	AML-M5	Not performed	97	Pos.	Neg	Part.	Pos.	Pos.	Pos.	Part.	Pos.
5	420038	AML-M5	Normal	83	Pos.	Neg	Neg.	Pos.	Neg.	Pos.	Part.	Pos.
5	428196	Recurrent AML	Normal	95	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.	Part.

Abbreviations: AML, acute myeloid leukemia; MDS, myelodisplastic syndrome; MS, multiple sclerosis.
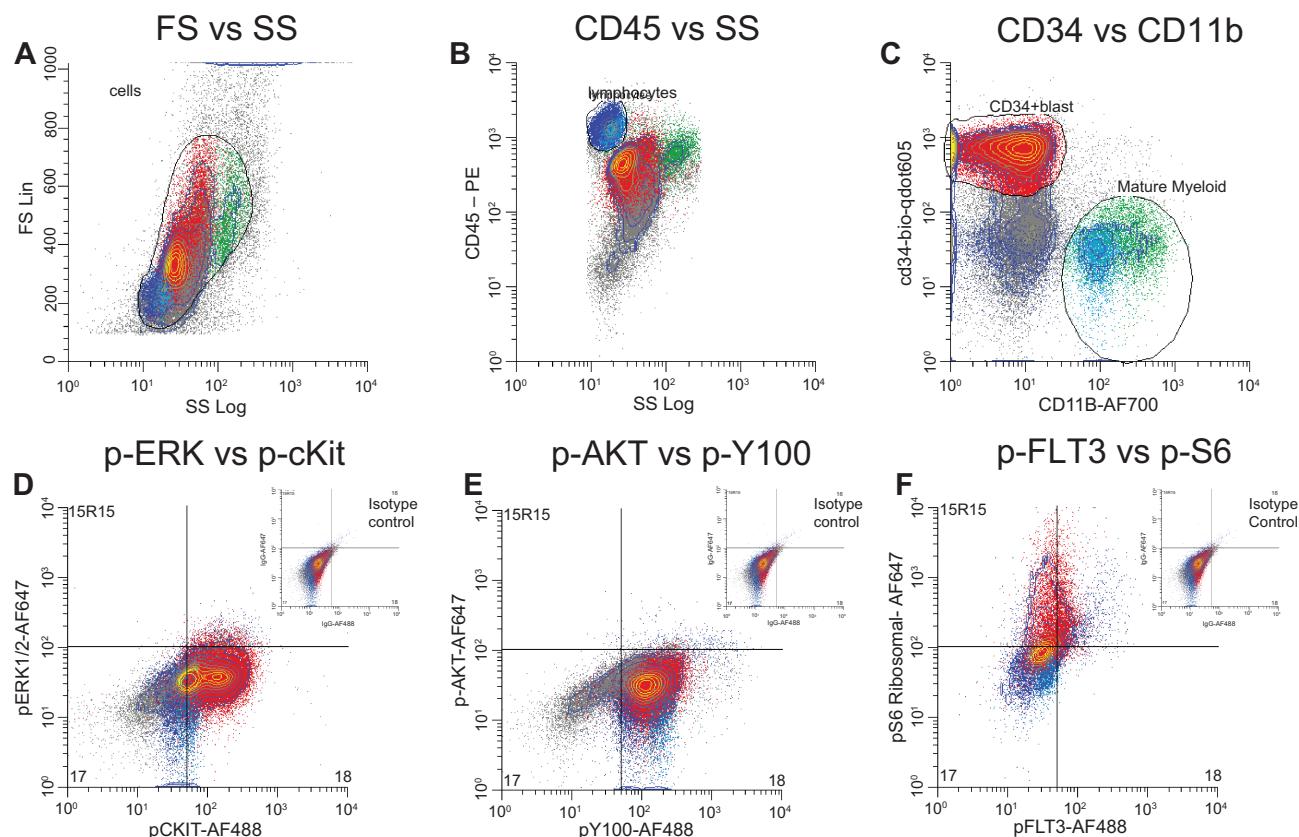
Figure 1 Detection of endogenous phospho-epitope levels. The combination of SSC/CD45/CD34/CD11b allows the delineation of the marrow elements (panels A–C). Cells were first assessed based on forward scatter (FS) versus side scatter (SS) (panel A), then grouped based on the CD45 and SS staining (panel B) and finally subdivided based on CD34 and CD11b staining (panel C). The lymphocytes (blue) lack CD34 and CD11b; the "blasts" (red) exhibit CD34 and lack CD11b whereas the maturing myeloid cells (green) exhibit CD11b and lack CD34. After identifying the different subpopulation based on morphology (FS and SS) and surface marker staining (CD45/CD11b/CD34), "blasts" were assessed for their expression of elevated levels of pc-KIT and pERK (panel D), pAKT and pY100 (panel E) and pFLT3 and pS6. Isotype controls are inserted into each of the panels (cytograms D–F).

differences observed between phenotype 1 and 2 for p-FLT3 ($p = 0.0034$) and p-Y100 ($p = 0.0041$) (data not shown). There were also statistical differences observed in phospho-protein expression for p-FLT3 and p-Y100 among the AML FAB classification (Figure 3C). For these two IKs the M3 AML classification showed lower phospho-protein expression compared to the other subtypes (Figure 3, Panel C).

When the same AML cells were stimulated with SCF (Figure 4), the overall phospho-protein expression was modestly increased compared to un-stimulated cells (Table 3). In particular, pAKT, pS6 and pERK were significantly increased after stimulation with SCF compared to unstimulated cells (Table 3). Phospho-protein expression between phenotype 1 and 2 were similar to the overall pattern, but after stimulation there were statistical differences between phenotype for p-ERK ($p = 0.0345$), p-FLT3 ($p = 0.0047$), p-Y100 ($p = 0.0095$), and Survivin ($p = 0.0186$) (data not shown). Further, there were statistical differences observed in the phospho-protein expression for p-FLT3 among the AML FAB classification. For p-FLT3, the M3

AML classification was statistically lower than all other FAB classifications ($p < 0.05$) (Figure 4C).

In contrast to phospho-protein expression of IKs, survivin belongs to a different group of intracellular signaling proteins and its overexpression has been associated with poor differentiation and prognosis in AML.[19–21] Hence, the survivin expression served as an additional control in this study to differentiate between activation pattern of phospho-proteins and constitutive intracellular activation. Consistent with the previous studies, survivin expression was present in all AML cell and its expression did not increase after SCF stimulation (Figures 3 and 4).

Finally, we also compared the phospho-protein expression to the cells in the lymphocyte gate (Figure 5). We found that all phospho-protein levels are elevated and statistically different when compared to the cells in the lymphocyte gate. All IK signals increased by greater than 2-fold compared to lymphocytes with the greatest differences observed for pAKT (8-fold) and pERK (6-fold) (Figure 5A). This difference is remarkable because both phospho-proteins were low at the
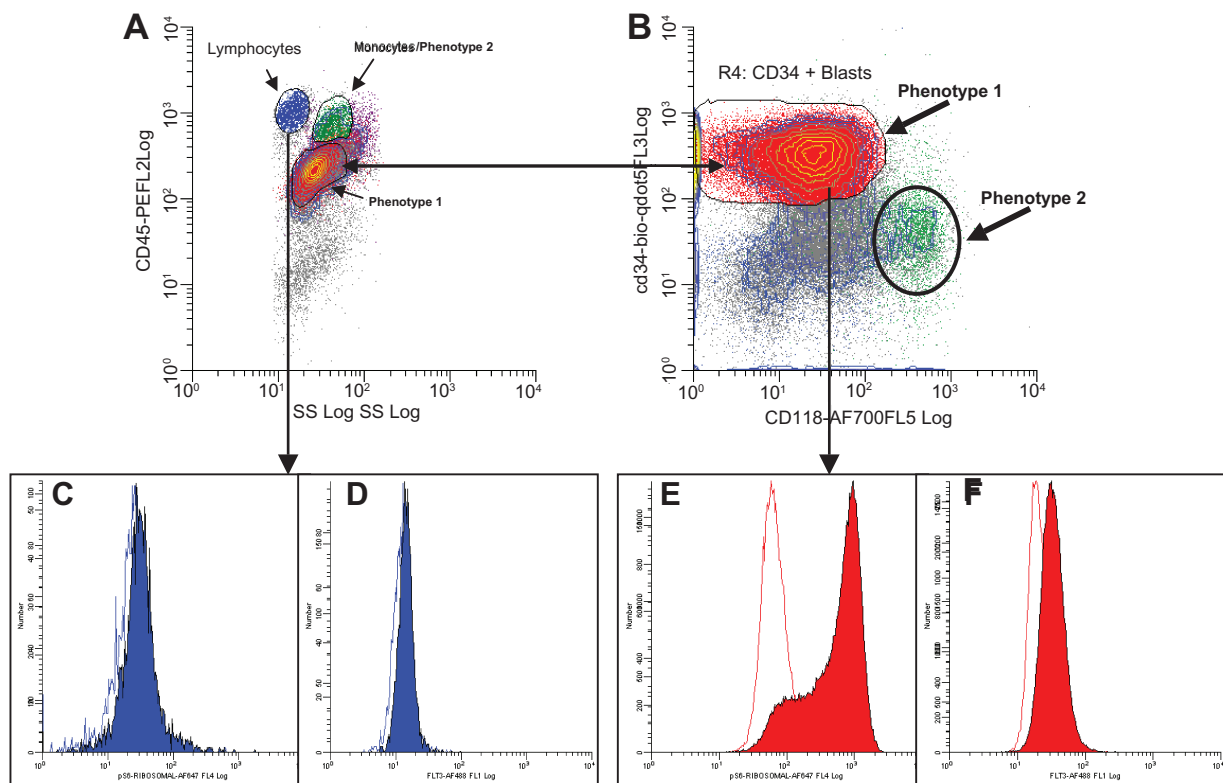
Figure 2 Phospho protein assessment in lymphocytes and AML (phenotype 1) Cells. All cells above a light scatter trigger are displayed in panel A showing the cell population based on CD45 and Side Scatter (SS). Cells denoted by the color blue have dense CD45, low SS and lack both CD34 and CD11b, representing lymphocytes. In contrast, the cells denoted by the color red have moderate SS, low CD45 and express CD34, representing the AML blast phenotype 1. Green colored cells in panels A and B represent CD34 low AML blasts, which are denoted as phenotype 2. Panels C–F represent the phosphorylation status of pS6-ribosomal protein (panels C and E) and pFLT3 (panels D and F) for lymphocytes (blue color) and AML blasts of phenotype 1 (red color) in unstimulated (unfilled peaks) and after stimulated conditions with Stem cell factor (SCF) (10 ng). Because of the marginal increase of phosphorylation in pS6 and pFLT3 after SCF stimulation, lymphocytes can serve as a negative control compared to AML blasts.

basal level for all the AML cells. Comparison to cells in the lymphocyte gate was even more useful to differentiate the phospho-protein expression pattern between phenotype 1 and 2 (Figure 5 B). Compared to the cells in the lymphocyte gate, phenotype 2 AML cells had higher phospho-protein levels than phenotype 1 with statistical differences observed for p-FLT3 ($p = 0.0005$) and p-Y100 ($p = 0.0005$) (Figure 5B). For survivin, phenotype 1 was statistically lower than phenotype 2 ($p = 0.0301$). With regards to the FAB classification, M4 (7-fold, $p = 0.0001$) and M5 (12-fold, $p = 0.0001$)

subtypes showed the greatest activation compared to the cells in the lymphocyte gate (Table 4). Thus, the comparison to cells in the lymphocyte gate appears to increase the resolution to differentiate phospho-protein expression in AML cells from other cell types present in the bone marrow.
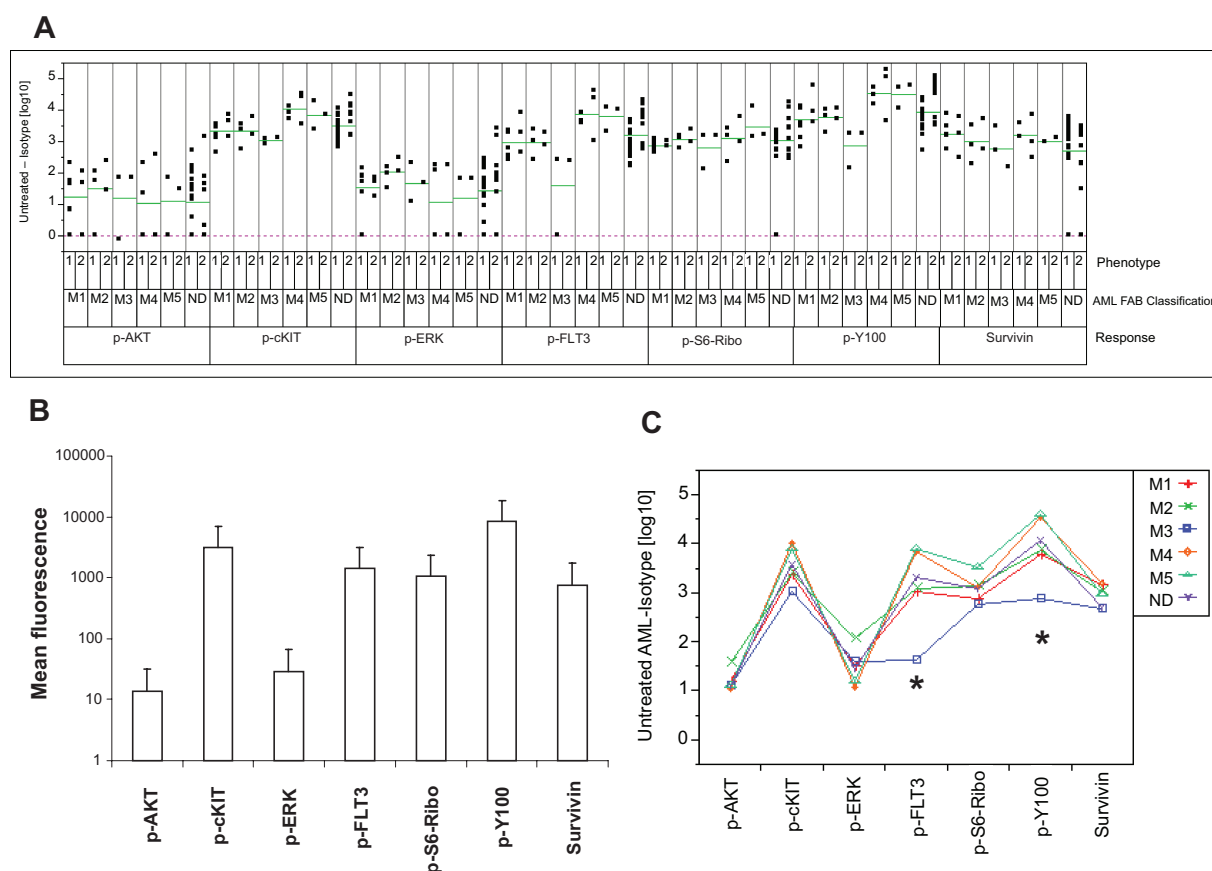
Discussion

In AML, Western Blotting has been used to assess the inhibitory activity of IK inhibitors, such as sunitinib,[8] and recently multiparametric analysis using flow cytometry has been applied to monitor phospho-protein changes after administration of IK inhibitors in patients with AML.[16,22] The advantages of using flow cytometric analysis lie in its ability to provide quantitative assessment of cellular events and to determine pharmacodynamic effects in nearly real-time. In the present study, we evaluated the application of an assay to monitor phospho-protein changes in the setting of a clinical diagnostic pathology laboratory. The present assay is part of developing tools to monitor the pharmacodynamic changes induced by IK inhibitors in patients with AML. Thus, the validation comprised three important

Table 2 Surface marker expression of AML cells allows definition of two categories (Phenotype 1 and 2)

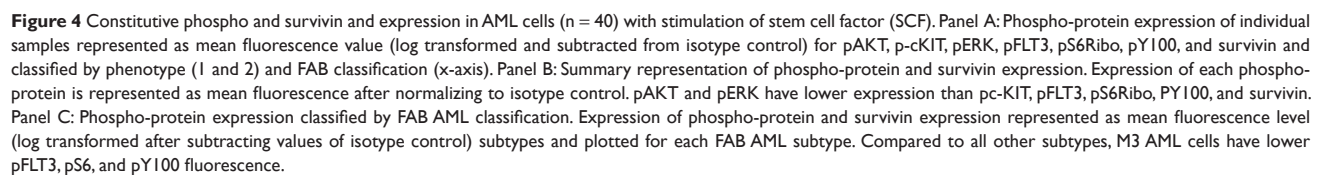| Phenotype of AML cells	Characteristics defined by CD	N (samples)
Phenotype 1	CD34⁺/CD45^low^/CD11b⁻	40
Phenotype 2	CD34⁻/CD45^hi/mid^/CD11b⁺	24

Notes: For Phenotype 1, 29 out of 40 samples were CD34⁺. For remaining 11 samples, CD34 was negative with concomitant CD45 staining being low. For Phenotype 2, 22 out of 24 samples were CD34⁻. All 24 samples from Phenotype 2 showed mid to high levels of CD45 staining.

Figure 3 Constitutive phospho and survivin expression in AML cells (n = 40) without stimulation of stem cell factor (SCF). Panel A: Phospho-protein expression of individual samples represented as mean fluorescence value (log transformed and subtracted from isotype control) for pAKT, p-cKIT, pERK, pFLT3, pS6Ribo, pY100, and survivin and classified by phenotype (1 and 2) and FAB classification (x-axis). Panel B: Summary representation of phospho-protein and survivin expression. Expression of each phospho-protein is represented as mean fluorescence after normalizing to isotype control. pAKT and pERK have lower expression than pc-KIT, pFLT3, pS6Ribo, PY100 and survivin. Panel C: Phospho-protein expression classified by FAB AML classification. Expression of phospho-protein and survivin expression represented as mean fluorescence level (log transformed after subtracting values of isotype control) subtypes and plotted for each FAB AML subtype. Compared to all other subtypes, M3 AML cells have lower pFLT3 and pY100 fluorescence.

development steps: first, the development of appropriate gating protocols to allow for simultaneous evaluation of various phospho-protein changes in patient-derived AML cells; second, a profile of phospho-protein expression across various types of AML cells (including those defined by the AML FAB classification); and thirdly, the differentiation of phospho-protein events from other cell population present in the bone marrow.
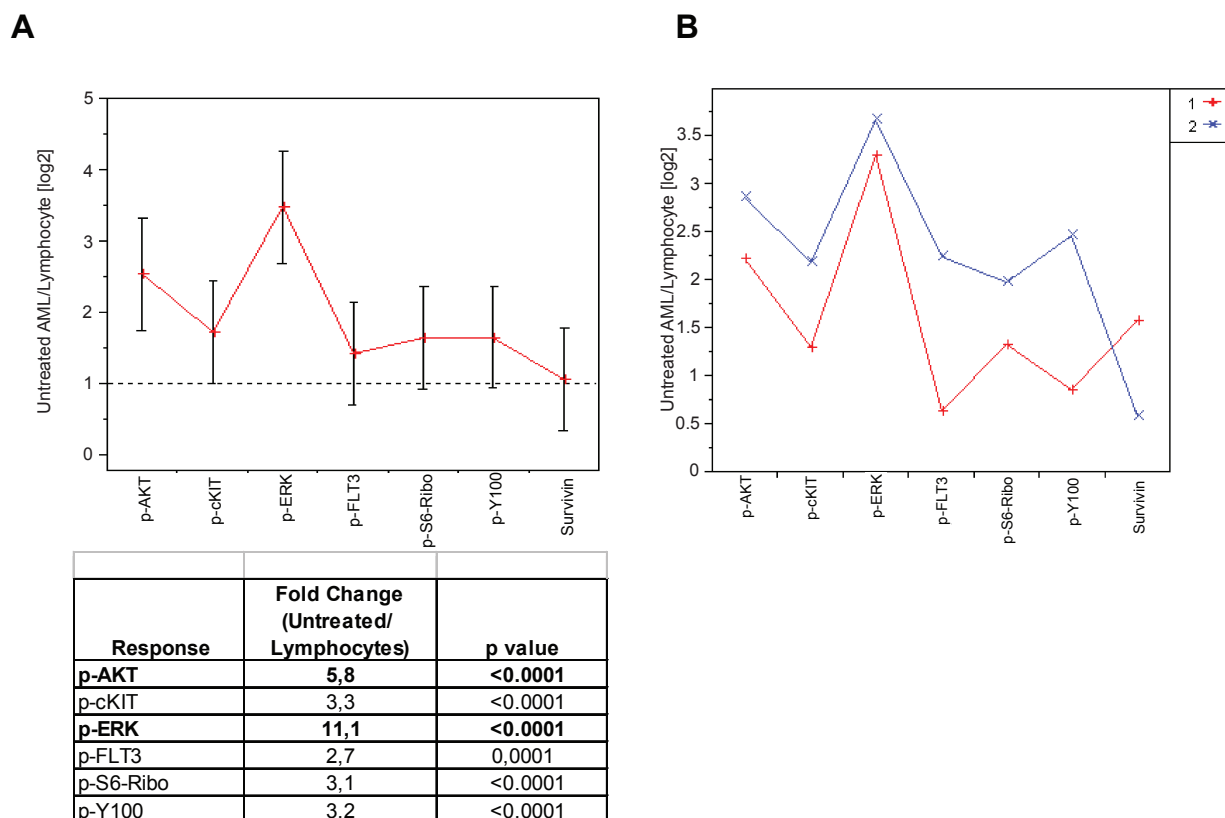
The present protocol allows the assessment of 6 different phospho-proteins in AML cells, including those that have been recently targeted by IK inhibitors (Figures 1 and 2). To our knowledge this is the first time that multiparametric assessment of both basal and stimulated levels of 6 different phospho-proteins has been reported. Setting up the appropriate gates involved the standard approach of defining the cell population based on size (FS vs SS), followed by the subsequent gating on surface marker expression defined by CD45, CD34 and CD11b. This generally is sufficient to gate on the

most relevant AML population with myeloid characteristics, including "immature"- and "mature"-like phenotypes. A third population, which serves as a control, is the cell population in the lymphocyte gate, which is characterized by CD45 and SS^{low} expression. Our categories of phenotype 1 ("immature") and 2 ("mature") were made to reduce the complexity of the sample and to investigate whether AML cells with immature phenotype had a higher phospho-protein expression pattern than those with a more mature phenotype. Although, the "immature" AML cells had a higher phospho-protein expression than those with a more "mature-like" pattern, the numbers were too skewed towards the phenotype 1 to estimate a possible correlation of phenotype 1/2 and a particular phospho-protein profile. The third gated population had the characteristics of lymphocytes. Although we have not formally demonstrated that this gate contains exclusively lymphocytes and given the recent observations of aberrant cell population in relapsed AML patients requiring careful

Figure 4 Constitutive phospho and survivin and expression in AML cells (n = 40) with stimulation of stem cell factor (SCF). Panel A: Phospho-protein expression of individual samples represented as mean fluorescence value (log transformed and subtracted from isotype control) for pAKT, p-cKIT, pERK, pFLT3, pS6Ribo, pY100, and survivin and classified by phenotype (1 and 2) and FAB classification (x-axis). Panel B: Summary representation of phospho-protein and survivin expression. Expression of each phospho-protein is represented as mean fluorescence after normalizing to isotype control. pAKT and pERK have lower expression than pc-KIT, pFLT3, pS6Ribo, PY100, and survivin. Panel C: Phospho-protein expression classified by FAB AML classification. Expression of phospho-protein and survivin expression represented as mean fluorescence level (log transformed after subtracting values of isotype control) subtypes and plotted for each FAB AML subtype. Compared to all other subtypes, M3 AML cells have lower pFLT3, pS6, and pY100 fluorescence.

flow cytometry evaluation,[23] prior studies have consistently demonstrated that this gate contains lymphocytes in patients with AML.[24] This careful differentiation is important in order to attribute the phospho-protein profile to the AML cells. In the future, lymphocyte staining may be necessary if phospho

Table 3 Comparison of phospho-protein and survivin responses between unstimulated and SCF-stimulated AML cells

Phospho-protein and survivin response	Fold change (SCF-stimulated/ unstimulated)	P-value (bold are significant changes)
P-AKT	8.2	<0.0001
P-ckit	1.0	0.9733
P-ERK	2.2	0.02
P-FLT3	1.0	0.9481
P-S6 Ribo	6.3	<0.0001
P-Y100	1.0	0.9129
Survivin	0.9	0.8019

protein staining should also include the evaluation of changes in the lymphocyte population, such as lymphocytic leukemia. By using a semi-physiological stimulation for bone marrow-derived cells with SCF, this selective stimulation of IKs in AML versus lymphocytes can be used to further support the identification of a unique phospho-protein expression in AML cells. The increased phospho-protein levels of pS6 and pAKT after stimulation with SCF is consistent with a differential activation of AML cells (Table 3). Lymphocytes do not typically have receptors associated with phosphorylation of S6 and AKT, which in turn explains that pS6 and pAKT were significantly increased after SCF stimulation (Table 3). Such increases have been also observed in patients with the ITD-FLT3 mutation (Shults, personal communication). By contrast, pERK activation has been observed in lymphocytic lineages or in activated lymphocytes.[25,26] Despite the possibility that pERK activation could also be found in lymphocytes, in this study the 11-fold increase of pERK

A

B

Response	Fold Change (Untreated/ Lymphocytes)	p value
p-AKT	**5,8**	**<0.0001**
p-cKIT	3,3	<0.0001
p-ERK	**11,1**	**<0.0001**
p-FLT3	2,7	0,0001
p-S6-Ribo	3,1	<0.0001
p-Y100	3,2	<0.0001

Figure 5 Panel A: Comparison of phospho-protein expression in AML blasts compared to lymphocyte population. Compared to lymphocytes, the expression of phospho-proteins is on average at least 2-fold higher in AML blasts with pERK, pAKT, and pcKIT showing the highest difference (all responses are statistically different from 0. Since the data are on the log2 scale and all the means are greater than 1 indicate that that all responses are greater than 2-fold higher than the lymphocytes). Panel B: Between the phenotype 1 and 2, phospho-protein expression ratio compared to lymphocytes is generally higher in AML phenotype 2 cells compared with phenotype 1 cells.

over lymphocytes and its increase after SCF stimulation, suggest that pERK was primarily activated in AML cells. Finally, vitality of AML blasts may influence the assessment of phospho protein expression in AML blasts. This may be of particular importance for shipping AML samples from the hematology practices to the test laboratory. Prior to conducting this study we performed a validation study of cell lines and 10 primary AML samples to investigated this

Table 4 Comparison of overall phospho-protein and survivin responses between unstimulated and lymphocytes

AML subtype (FAB classification)	Fold change (Untreated/ Lymphocytes)	P-value
M5	12.4	0.0001
M4	7.1	0.0001
ND	3.5	<0.0001
M2	3.4	0.0086
M1	2.0	0.0411
M3	1.5	0.4908

Abbreviation: ND, not defined.

potential risk. We found that AML blasts had to be assessed within 24 to 48 hours after sampling in order to measure consistent and reproducible phospho protein levels (Shults, personal communication).

While recent guidelines for classifying AML recommend the use of the new World Health Organization (WHO) classification,[27] the comparison of the phospho-protein expression to FAB AML subtypes may contribute to further re-fine outcome in AML patients (Table 1). Although the sample size in this study may be too limited to reach a final conclusion, in this set of data it appears that M4 and M5 subtypes exhibit a higher phospho-protein expression than patients with a M3 subtype. Whether this observation is consistent with previous reports suggesting that FLT3 expression is highest in M4 and M5 subtypes and thus is associated with a poor survival remains to be investigated.[28]

In conclusion, the present assay holds promise to detect pharmacodynamic changes in AML cells after treatment with a broadly active IK inhibitor and should be evaluated for its use in future clinical trials. However, the relevance of determining phospho protein levels as a prognostic marker

was not the objective of this study and future prospective studies have to be conducted to determine its usefulness.

Acknowledgments

Disclosures

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