Phospho-Proﬁling Linking Biology and Clinics in Pediatric Acute Myeloid Leukemia

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Abstract
Aberrant activation of key signaling-molecules is a hallmark of acute myeloid leukemia (AML) and may have prognostic and therapeutic implications. AML summarizes several disease entities with a variety of genetic subtypes. A comprehensive model spanning from signal activation patterns in major genetic subtypes of pediatric AML (pedAML) to outcome prediction and pre-clinical response to signaling inhibitors has not yet been provided. We established a high-throughput ﬂow-cytometry based method to assess activation of hallmark phospho-proteins (phospho-ﬂow) in 166 bone-marrow derived pedAML samples under basal and cytokine stimulated conditions. We correlated levels of activated phospho-proteins at diagnosis with relapse incidence in intermediate (IR) and high risk (HR) subtypes. In parallel, we screened a set of signaling inhibitors for their efﬁcacy against primary AML blasts in a flow-cytometry based ex vivo cytotoxicity assay and validated the results in a murine xenograft model.

Certain phospho-signal patterns differ between genetic subtypes of pedAML. Some are consistently seen through all AML subtypes such as pSTAT5. In IR/HR subtypes high levels of GM-CSF stimulated pSTAT5 and low levels of unstimulated pJNK correlated with increased relapse risk overall. Combination of GM-CSF/pSTAT5high and basa/pJNKlow separated three risk groups among IR/HR subtypes. Out of 10 tested signaling inhibitors, midostaurin most effectively affected AML blasts and simultaneously blocked phosphorylation of multiple proteins, including STAT5. In a mouse xenograft model of KMT2A-rearranged pedAML, midostaurin signiﬁcantly prolonged disease latency. Our study demonstrates the applicability of phospho-ﬂow for relapse-risk assessment in pedAML, whereas functional phenotype-driven ex vivo testing of signaling inhibitors may allow individualized therapy.

Introduction
Acute myeloid leukemia (AML) is a highly heterogeneous disease, the second most frequent leukemia entity in children and adolescents, and deﬁnitely the most aggressive variant. Despite continued progress and reﬁnement of therapeutic approaches, about 35% of pediatric patients with the disease still suffer from relapse.1,2 Constitutive activation of one or more signaling pathways in AML cells is associated with poor prognosis.3 Hence, signal pathway interception in addition to current chemotherapy regimen has emerged as a novel approach for the treatment of adult as well as of pediatric AML.4–6 Recently, midostaurin, a multitargeted kinase- and ﬁrst generation FLT3-inhibitor, has shown to significantly prolong overall and event-free survival among AML patients with a FLT3 mutation in

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combination with standard chemotherapy. The application of signaling inhibitors in AML based solely on the presence of specific underlying genetic aberrations, such as FLT3-ITD or mutations in isocitrate dehydrogenase (iDH) may, however, represent a major limitation for their use since most signaling inhibitors- especially of first- and second generation- are by no means absolutely specific for a single target, but rather inhibit multiple kinases. These ‘off-target’ effects could be therapeutically exploited in patients lacking the primary genetic target aberration. Accordingly, midostaurin shows activity not only in FLT3-mutated but also in FLT3 non-mutated AML patients. In a recent phase II open-label study in patients with relapsed AML the FLT3-ITD mutation status, albeit being predictive for response to AC220 (quizartinib), showed a high error rate as tool for stratification of patients into responders and non-responders.

Hence, there is a need for functional precision medicine moving beyond pure genotypic target selection to explain the heterogeneity of treatment response more accurately and to provide precise prognostic information for risk identification and to guide the use of targeted therapy. Phospho-signal profiling (phospho-flow) has the potential to be a powerful ally to current genomic approaches for prediction of relapse risk as well as of drug responses. Phospho-flow identified G-CSF induced pSTAT3 as predictive for survival in pedAML and fedatinib (FED)- mediated reduction of pSTAT5 levels was identified as a predictive biomarker for in vivo drug-responses.

In a phosphoproteome analysis of primary AML blasts a signature consisting of 5 phosphorylation sites predicted the response of a small cohort of adult AML patients to AC220. A comprehensive model spanning from signal activation patterns in the major genetic subtypes of pedAML to outcome prediction and to testing of signaling inhibitor effects in primary pedAML blasts has, however, not been provided so far. We therefore conducted a retrospective pilot study using phospho-flow based signal pattern profiling of primary bone marrow (BM) derived AML blasts from pedAML patients to determine whether phospho-flow can be used to (i) link distinct phospho-profiles to genetic subtypes of pedAML (ii) refine risk stratification in pedAML and (iii) predict or monitor response to signaling inhibitor treatment ex vivo.

Results

Flow-cytometry screen of phospho-profiles in AML under ex-vivo cytokine stimulation

We exposed 166 primary patient samples to G-CSF, GM-CSF, Flt-3 ligand (FL), SCF, SDF, IL-3, TPO, IFNγ or a combination thereof (“cocktail”) to measure ligand-induced phosphorylation ex vivo. Cytokine concentrations used corresponded to those found in serum of AML patients. Basal signal activation levels (no cytokine stimulation, Fig. 1C-D) served as reference. In the “cocktail” setting, we detected prominent stimulation of pSTAT5, pAKT, pS6 and to a lesser extent of pERK1/2, p4E-BP1, and pSTAT3 (Fig. 2A). In the “cocktail plus” setting (including IFNγ) we additionally observed increased levels of pSTAT1 (Fig. 2A). Distinct cytokines elicited specific phospho-signal(s) (Fig. 2B), but p38, NF-KB and JNK were not further stimulated by any cytokine.

As expected, G-CSF strongly engaged pSTAT3 and pSTAT5, IFNγ mostly pSTAT1 and pSTAT5, GM-CSF, IL-3 and TPO provoked predominantly pSTAT5, SCF, SDF, and FL primarily stimulated AKT, S6 and 4E-BP1 phosphorylation. Phospho-ERK1/2 rose mainly under FL stimulation and to a lesser extent with SCF and G-CSF. Altogether, the most pronounced changes were observed in levels of pSTAT5 which was activated by many cytokines (G-CSF, GM-CSF, IL-3, TPO and IFNγ).

Differences of cytokine-induced signal activation between different genetic subtypes of pedAML were also investigated (Fig. 2C). We excluded GATA-1 patients due to restricted numbers. We found consistent increases in pSTAT5 upon G-CSF, GM-CSF, and IL-3 irrespective of the subtype. NK and RUNXI-RUNXIT1 cohorts reacted with pSTAT5 also upon TPO stimulation. Elevated levels of pSTAT3 were seen in response to G-CSF throughout all subtypes, but with strongest stimulation among RUNXI-RUNXIT1 and PML-RARA samples. More specifically, G-CSF showed its broadest effects in the RUNXI-RUNXIT1 subset, where it stimulated also pAKT, pS6, p4E-BP1, and pERK1/2. These four signaling molecules were broadly stimulated through FL and SCF and among RUNXI-RUNXIT1 and CBFB-MYH11 cases, whereas PML-RARA remained non-responsive, SDF activated pS6 and p4E-BP1 in CBFB-MYH11 and KMT2Ar cases only. Single patient specific profiles are provided in Suppl. Fig. 1B (Supplemental Digital Content, http://links.lww.com/HS/A52).

Phospho-signal levels at diagnosis and risk of relapse

We retrospectively analyzed basal and cytokine induced phospho-signal levels in AML blasts from diagnosis and correlated them with the incidence of relapse.

Due to low numbers of relapses among LR cases (4 among 42) we focused on 90 intermediate / high risk (IR/HR) patients (41 relapses) (Suppl. Fig. 2A and B, Supplemental Digital Content, http://links.lww.com/HS/A52). We found that patients with and without a relapse showed differential activation of 13 signal nodes at diagnosis (Suppl. Fig. 2C, Supplemental Digital Content, http://links.lww.com/HS/A52). More specifically, patients who
relapsed showed higher signal activation in 11 conditions (independently in the three gross genotypic cohorts: KMT2A rearranged (KMT2Ar), normal karyotype (NK); with other genetic abnormalities (other), and lower levels in only two signaling nodes (basal pJNK, in all 3 genotypes; basal pSTAT3, only visible in NK and KMT2Ar). Phospho-STAT5 was involved in four of the 13 nodes. In univariate analysis with correction for multiple testing (Suppl. Fig. 2D, Supplemental Digital Content, 2020) 4:1 www.hemaspherejournal.com
as well as in a multivariate model including signal activation nodes and gross genotypic subsets, 2 signal nodes remained of statistical significance (p < 0.01 each; Fig. 3A and Suppl. Fig. 2E, Supplemental Digital Content, http://links.lww.com/HS/A52): GM-CSF/pSTAT5 (HR 3.0; CI 1.5–6.1) and basal pJNK levels (HR 0.3; CI 0.2–0.6).

This prompted us to estimate the cumulative incidences of relapse (CIR; at 5 years) in the total IR/HR cohort of patients according to these two most relevant signal nodes. Patient cohorts were split into 2 groups with low or high signal activation according to thresholds set to median levels of each node. We validated the use of the median as threshold by Cox-regression with restricted cubic splines assessing the potential non-linearity of the relationship between 5-year CIR and signaling node expression level (Suppl. Fig. 3A, Supplemental Digital Content, http://links.lww.com/HS/A52). Basal pJNKlow and GM-CSF-stimulated pSTAT5high expressers showed a significantly higher 5-years CIR than reciprocal expressers (p < 0.001 each) (Fig. 3B).

This separation of risk groups held true when the three main genetic subgroups were analyzed separately (Suppl. Fig. 3B, Supplemental Digital Content, http://links.lww.com/HS/A52).

Next, we assessed a possible rational association of signal levels with known risk factors including FLT3-ITD status as well as white blood cell (WBC) counts at diagnosis tested as...
continuous variables. As expected, we found that FLT3-ITD status (positive in 12/80, 15% of IR/HR patients) significantly correlated in our cohort with the number of relapses, both in the “other” AML subtype (positive in 4/14 relapses; 29%; \( p < 0.05 \)) as well as in all subtypes together (KMT2Ar+NK-other; positive 9/41 relapses; 22%; \( p < 0.05 \)). However, there was no correlation of the FLT3-ITD status with basal pJNK or GM-CSF/pSTAT5 levels (Suppl. Fig. 4A and B, Supplemental Digital Content, http://links.lww.com/HS/A52). Data on NPM1 mutations were not available. Higher WBC counts, another known risk factor, did not correlate with adverse signal activation of the driver signal represented by high GM-CSF/pSTAT5 levels (Suppl. Fig. 4C, Supplemental Digital Content, http://links.lww.com/HS/A52).

Figure 3. Levels of pAKT, pSTAT5, pS6 and pJNK predict risk of relapse. (A) Univariate Cox regression analysis was performed to obtain hazard ratios (HR) and 95% confidence interval (CI) for 13 signal nodes with differential phospho-signal activation between samples from relapsed/non-relapsed intermediate and high risk patients. Permutation test was used to adjust for multiple testing. Only the correlations of pJNK\_UNSTIM and pSTAT5\_GM-CSF with relapse risk remained significant (orange bars), as opposed to the other 11 signal nodes (blue). (B) We merged data from patients of the KMT2Ar, NK and “other” groups divided patients into “high” and “low” expressers upon individual magnitude of pSTAT5 (upon GM-CSF) and pJNK (unstimulated) levels. Cut-offs were set using median levels of expressions adjusted according to univariate Cox regression analysis using RCS. 5-years CIR are depicted for pJNK\_low and pJNK\_high expressers. (C) Combined assessment of pJNK\_low and pSTAT5\_high in a score-system: 1 point for pJNK\_low, 1 additional point for high pSTAT5 levels resulted in three groups with a score of either 0 (none of these factors is present), 1 (either of these factors is present) or 2 (both factors are present). Patients of the “score 2” – group had the highest risk of relapse and the worst 5-years CIR that significantly differed from those of “score 1” or “score 0” cohorts.
To analyze whether the combined assessment of the two most relevant signaling nodes is of additive prognostic value we established a score-system according to the result of the Cox-regression: 1 point each for pJNKlow or GM-CSF/pSTAT5high levels. This resulted in 3 groups with significantly different relapse rates (5y CIR score 0: 0.16 ± 0.07; score 1: 0.49 ± 0.08; score 2: 0.73 ± 0.09) (Fig. 3C).

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### Phospho-flow and functional testing of inhibitor sensitivity

We further applied phospho-flow to primary AML blasts treated with a set of FDA approved signal inhibitors to test whether individual drug-sensitivity can be predicted by measuring phospho-levels at the time of diagnosis, and/or by changes of phospho-levels upon in vitro drug treatment. We focused on ruxolitinib, dasatinib, midostaurin, sorafenib, sunitinib, rapamycin, everolimus, bortezomib, and erlotinib which all are either used or suggested for treatment of AML. Later in the study we expanded the inhibitor panel and added the in vitro-only inhibitor AG490 along with ruxolitinib because their specificity for JAK-signaling is potentially relevant for AML. Inhibitor sensitivity in terms of in vitro reduction of viable AML blasts (Suppl. Fig. 5A, Supplemental Digital Content, http://links.lww.com/HS/A52) was assessed along with signal expressions in 44 primary AML samples (Suppl. Fig. 5B, Supplemental Digital Content, http://links.lww.com/HS/A52) out of the total cohort.

Midostaurin was most effective in reducing viable AML blasts (Fig. 4A and Suppl. Fig. 5C, Supplemental Digital Content, http://links.lww.com/HS/A52) in 25 of 44 cases irrespective of the AML subtype (genetic or FAB type, Fig. 4B). Similar effects were only seen upon AG490 treatment which also evoked responses (in 8/29 tested samples) throughout all FAB subtypes (mainly in KMT2Ar and NK subgroups).

Midostaurin was also most active in reducing several signals like pS6, pSTAT5 and pAKT (Fig. 4C). However, phospho-signal levels at diagnosis did not indicate inhibitor sensitivity (Suppl. Figs. 6A and 6B, Supplemental Digital Content, http://links.lww.com/HS/A52), and the extent of modulation of phospho-levels by inhibitors did not necessarily predict the degree of reduction of viable AML blasts (Fig. 4C and Suppl. Figs. 7 and 8, Supplemental Digital Content, http://links.lww.com/HS/A52).

### Xenotransplant validation of signaling inhibitor sensitivities

To validate the efficacy of signaling inhibitor treatment in vivo, we performed xenotransplantation experiments in mice (scheme...
Figure 4. Midostaurin treatment kills pedAML blasts and lowers pS6, pSTAT5 and pAKT levels. (A) Histogram showing the sensitivity of AML blasts towards individual signaling inhibitors (ruxolitinib n = 28, AG490 n = 28, dasatinib n = 43, midostaurin n = 44, erlotinib n = 40, rapamycin n = 44, everolimus n = 40, bortezomib n = 39, sorafenib n = 43, sunitinib n = 44). Number of alive blasts following signaling inhibitor treatment was assessed using YO-PRO<sup>TM</sup> apoptotic cell staining and subsequent flow cytometric analysis. Ratio of alive blasts following signaling inhibitor treatment versus vehicle (DMSO) control is shown. (B) Responses of AML blasts to signaling inhibitors according to genetic categories and FAB subtypes. A ratio < 0.5 (viable blasts<sup>inhibitor</sup>/viable blasts<sup>DMSO</sup>) was considered as response. (C) The magnitude of reduction of pS6, pSTAT5 and pAKT (y-axis) following treatment with midostaurin (color) or other signaling inhibitors (grey) and the association with the number of alive blasts (x-axis) is depicted for the different genetic AML subtypes (depicted in different colors).
Fig. 5A). Expanded blasts from 5 KMT2Ar patients (Suppl. Fig. 9A, Supplemental Digital Content, http://links.lww.com/HS/A52) were divided into 5 experimental groups (sorafenib, midostaurin, rapamycin, ruxolitinib or DMSO [vehicle] control), pretreated (overnight) ex vivo and injected into NSG mice. Major shifts of drug sensitivities due to cell expansion in mice were excluded before (Suppl. Fig. 9B, Supplemental Digital Content, http://links.lww.com/HS/A52). The presence of human CD45+ (hCD45+) cells in the peripheral blood of mice was monitored over time (Suppl. Fig. 9C, Supplemental Digital Content, http://links.lww.com/HS/A52). Complete time courses were obtained from three individual patients. In all settings, treatment of midostaurin significantly delayed the expansion of hCD45+ cells. Assessment of hCD45+ cells in the bone marrow (Suppl. Fig. 9D, Supplemental Digital Content, http://links.lww.com/HS/A52) and spleen (data not shown) at the time of terminal disease showed a pronounced high grade leukemic cell infiltration. Whereas survival of the xenografted mice upon sorafenib-, rapamycin- and ruxolitinib-treatment was unaltered compared to the DMSO control, midostaurin treatment significantly prolonged disease latency (Fig. 5B).

Discussion

Our study demonstrates that phospho-flow of primary AML blasts can complement and expand on known prognostic information of pedAML, and is potentially useful to refine current risk assessment strategies and tailor individual therapy.

We investigated the activation status of representative key molecules (STAT3, STAT5, ERK1/2, JNK, p38, and AKT) of the three major signaling pathways related to leukemogenesis, that is, the JAK/STAT, RAF/MEK/ERK (MAPK) and the PI3K/AKT/mTOR pathway.21–27 Most signals delivered by cytokines, or by mutant receptors (eg, FLT3, c-KIT) converge to these molecules, and many new inhibitory agents (like sunitinib, sorafenib, dasatinib) act up-stream of them. NFκB and S6 ribosomal protein act further downstream and confer anti-apoptotic as well as growth-regulating effects. They are activated in AML by the ERK- and in particular by the PI3K/AKT pathway, and may serve as secondary read-out markers of these pathways. Hence, we considered these molecules to be ideal points of convergence for activated signal pathway profiling. All phosphorylation sites examined in the study are indicative for the activation of the given molecules (Suppl. Table 2, Supplemental Digital Content, http://links.lww.com/HS/A52). Several other studies already reported aberrant constitutive activation of individual signaling molecules in AML.28–34 Our study now gives a comprehensive overview on key signaling pathways in pedAMLs with respect to genotype-related heterogeneities. Basal activation of pSTAT5 was found throughout all pedAML subtypes, whereas the basal activation of other signaling molecules differed according to genotypes. The KMT2Ar group was characterized by broadest basal signal activation – similarly as described in,35 followed by the “other” and CBFB-MYH11 groups. In contrast, RUNX1-RUNX1T1 and PML-RARA subsets were comparably silent except for pJNK activation. Elevated pSTAT5 signaling elicited by several
cytokines was found to be a general hallmark of pedAML with strongest stimulation through GM-CSF, which mirrors observations in chronic myeloid malignancies.\textsuperscript{36,37} STAT3 activation by G-CSF was also a consistent finding throughout all subtypes as described before,\textsuperscript{11} whereas cytokine-stimulated patterns of pAKT, pS6, p4E-BP1, pERK1/2 rendered genotype associated signatures.

Both, constitutive or triggered activation of certain signaling molecules like pAKT, pERK, and pSTAT5 has been assigned prognostic relevance in adult myeloid malignancies.\textsuperscript{33,38} In pedAML, G-CSF/IL6 sensitivity of STAT3 signaling and elevated STAT3 sensitivity were found to correlate with good prognosis and low risk genotypes.\textsuperscript{11} Our data confirm previous observations including the fact that broadly elevated signal activation except for pSTAT3 correlates with relapses. High levels of GM-CSF-stimulated pSTAT5 and low basal levels of pJNK were the most significant parameters correlating with increased relapse incidence. This was particularly true for patients with higher clinical risk (subgroups KMT2Ar, NK and “other”). To date most studies have focused on molecular and genetic lesions\textsuperscript{41} for stratifying patients with AML, but outcome as well as relapse risk vary considerably within IR and HR groups.\textsuperscript{42,43} We propose that the combination of these two outcome-predicting signal activation nodes allows separating three distinct risk groups among IR and HR pedAMLs. Testing these parameters at initial diagnosis fuels the possibility of improving stratification by applying phospho-flow. Our data also corroborate that interfering with pSTAT5 may be a broadly applicable signal-dependent intervention strategy against pedAML regarding its salient activation patterns.

To explore further potential applications of phospho-flow we assessed whether the activation status of signaling molecules at diagnosis or their modulation upon treatment with different FDA approved signaling inhibitors predicts drug-responsiveness of AML blasts. We found that neither was the activation of a specific signaling molecule predictive for the cytotoxic efficacy of any of the inhibitors used in our ex vivo short-term drug sensitivity assay, nor was the cytotoxic efficacy of a certain drug exactly predicted by phospho-signal inhibition of singly investigated signaling molecules. We could, however, demonstrate that midostaurin displayed the broadest and most consistent cytotoxic effects as compared to the other inhibitors tested against various genetic subtypes of pedAML including a larger cohort of KMT2Ar cases. Interestingly, midostaurin in particular showed also simultaneous suppressive activity against several signaling molecules implicated above in prognostic aspects (pSTAT5, pAKT, pS6). Other drugs appeared to exert less or more selective activity.

The relevance of our ex vivo drug sensitivity assay was further validated in a murine xenograft model of KMT2Ar pedAML. Herein, again midostaurin showed efficacy in suppressing leukemic engraftment, thus exerting some activity probably even on the leukemic stem-cell level in KMT2Ar AML. Although further investigations are necessary to draw definite conclusions, one could speculate that the superior activity of midostaurin could be a result of multi-kinase signaling interception. Most cancer cells are not addicted to a single overactive kinase pathway and the vast majority of so called “specific kinase inhibitors” exert considerable off-target effects. This is also consistent with the emerging view that sensitivity to an inhibitor may not only be due to activation of the target kinase but also to activation of multiple parallel pathways.\textsuperscript{44} Accordingly, we demonstrate that a functional phenotype-driven ex vivo drug testing approach such as the one presented here is more likely to lead to identification of effective drugs than a purely hypothesis-driven, genomic target-centric approach.\textsuperscript{45,46}

Our investigation has some limitations, including its basic design as a single-laboratory, retrospective study on frozen samples of patients recruited over two full clinical trial periods, during which some changes of diagnostic approaches, stratification and treatment have been made. A full characterization of genetic and molecular features, as would be nowadays necessary for separating three risk groups,\textsuperscript{14,15} was not available. Hence, we summarized IR and HR patients together. Notwithstanding this, we find it striking that a single test still can separate patients at risk well in this otherwise mixed cohort of cases. A more detailed investigation of the capabilities of the test within well-defined genetic subgroups needs to be performed anyway on a much larger, independent cohort and in a multi-centric fashion to warrant implementation into future up-front stratification schemes of pedAML for further treatment personalization. To this end, preliminary technical data confirm that flow-cytometric assessment of phospho-levels on thawed material (as in this study) or on fresh samples renders largely concordant results (Suppl. Fig. 10, Supplmental Digital Content, http://links.lww.com/HSA/A52). Obviously, also our cytotoxicity-oriented drug sensitivity assay has limitations related to usage of single drug concentrations which were, however, oriented on human pharmacokinetic data, as well as to its short-term design probably unable to detect effects not linked with immediate cytotoxic effects. The amazing similarity with the results of xenograft models, still appear to indicate some biologic substance.

We conclude that phospho-flow and the functional phenotype-driven drug sensitivity assay yielded critical insights into potential novel options for improved relapse-risk assessment, stratification, as well for guiding personalized therapy, and should therefore be further validated in pedAML.

Materials and methods

Patients and cells

Sampling as well as subsequent research was covered by appropriate informed consent policies and clearance of study procedures by the involved ethical committees. We analyzed BM samples (cryopreserved after gradient-centrifugation) from 166 newly diagnosed pedAML patients recruited to the AML-BFM-1998 and 2004 treatment protocols in Austria (n = 132); assessed as per availability of frozen samples out of the total cohort of 191 registered patients in the trial periods) and Germany (n = 34; randomly selected upon sample availability). The median age of patients was 10.06 years (range 0.17–17.88), and the initial leukocyte count was 35 G/L in median (range 0.93–521.1 G/L). BM sampling was done as clinically indicated per protocol. We used only leftover materials stripped from further clinical questions. Sampling as well as subsequent research was covered by appropriate informed consent policies and clearance of study procedures by the involved ethical committees. Clinical data including detailed genetic information, events and outcome in long-term follow-up were also collected and were complete in 132 patients (79.5%). Among those 132 patients there were 42 patients fulfilling LR (low/standard risk) criteria as per protocol [RUNXI-RUNXIT1 (n = 15); CBFB-MYH11 (n = 17); PML-RARA (n = 7); GATA-1 (n = 3, all are children with Down syndrome) gene mutations], as well as 90 patients with...
intermediate or high risk (IR/HR) criteria\(^1,2\) [KMT2A rearrangements (n=33); normal karyotype (NK; n=23)]; as well as a remaining cohort with variable genetics - termed “other” (n=34)]. Treatment details of trials AML-BFM-1998 and 2004 have been delineated previously.\(^1,2\) The entire cohort of non-LR patients was summarized as IR/HR patient cohort in order to represent what would nowadays be stratified separately. All cases of the KMT2A\(^*\) group (Suppl. Fig. 2B, Supplemental Digital Content, http://links.lww.com/HS/A52) were treated in the HR group of the respective protocols. Only later after stop of recruitment to the presented study, such patients were split into an IR and HR group, respectively, dependent on the type of KMT2A-rearrangement which is current BFM strategy. FLT3-ITD data were available in a subset of 97 patients; no other mutational results were available due to the retrospective nature of the study. There was no significant difference between the analysed cohort and all registered patients in Austria regarding risk group allocation (LR: 31.8% vs 38.1%; n.s.; IR/HR: 68.2% vs 61.9%, n.s.) as well as regarding relapse rates (34.1% vs. 33.7%). The median follow-up time in analyzed patients without a relapse was 6.25 years (range 0.0–16.5 years). See Suppl. Table 1 (Supplemental Digital Content, http://links.lww.com/HS/A52) for details on patient characteristics.

**Phospho-Flow for measurement of basal and cytokine stimulated signaling**

Cryopreserved AML samples were thawed, split in 1×10⁷ cells/ml aliquots, incubated for 2 hours in serum-free X-Vivo\(^\text{TM}\) 10 medium (Lonza) and stimulated with 20 ng/ml of either G-CSF, GM-CSF, FL, SCF, SDF1, IL-3, TPO, or IFN-\(\gamma\), or with a mixture of cytokines (all from PeproTech) without (“cocktail”) and with IFN-\(\gamma\) (“cocktail plus”) at a common cytokine concentration of 1 ng/ml (except G-CSF: 5 ng/ml) for 15 minutes at 37°C, or left untreated, respectively. Cells were then fixed with 2% formaldehyde (free of MeOH; Polyscience or Thermoscientific) for 15 min at RT and permeabilized in 100% ice-cold MeOH for 15 minutes at −20°C. Cells were centrifuged, MeOH was completely removed and the pellet was washed once and resuspended in PBS/1% human serum albumin. The cell suspension was split into seven micronic tubes and labelled for flow cytometry with CD45, anti-active caspase-3 and with antibodies against respective phospho-sites (see Suppl. Table 2, Supplemental Digital Content, http://links.lww.com/HS/A52, for details on antibodies). All phosphorylation sites examined in the study are indicative for the activation of the given molecules (see Suppl. Table 2, Supplemental Digital Content, http://links.lww.com/HS/A52, for respective references). Cells were stained for 30 min at RT and analyzed by flow cytometry (FACSCalibur, BD). Only viable (according to scatter properties and negativity by anti-active caspase 3 staining) leukemic blasts (median 71%; range 30%–91%) were analyzed by gating on low/intermediate-side scatter cells with low CD45 expression, thus excluding active caspase 3-positive blasts (median 7%; range 1%–35%) as well as residual normal cells.

**Phospho-Flow for measurement of signaling inhibition and flow-cytometric cell survival assay**

Approximately 1×10⁶ cells/ml were incubated for 2 hours with one of the respective signaling inhibitors (see Suppl. Table 3, Supplemental Digital Content, http://links.lww.com/HS/A52) in serum-free X-Vivo\(^\text{TM}\) 10 medium at 37°C followed by 15 minutes stimulation with added cytokine cocktail (as above). DMSO (vehicle) treatment together with cytokine cocktail was used as control. Fixation, permeabilization, staining of the cells and flow-cytometric measurement of phospho-levels were performed as described above. In parallel to flow cytometric measurement of phospho-levels after 2 hours signaling inhibitor treatment, we performed an in vitro cell survival assay. For this purpose, sample aliquots containing 1×10⁶ cells were incubated for 48 hours with individual signaling inhibitors (selection and concentrations as described above) in serum-free X-Vivo\(^\text{TM}\) 10 medium supplemented with cocktail and kept at 37°C in a humidified atmosphere of 5% CO₂. Cells treated with DMSO instead of inhibitor were used as controls. After 48 hours, cells were incubated for 1 hour by adding 1 μg/ml of Vybrant violet stain (freely cell-permeable DNA-dye for cell cycle profiling; Invitrogen/#V35003) to otherwise untouched incubations. Subsequently, entire cell incubations were transferred into BD Trucount Tubes\(^\text{TM}\) (for bead-based absolute counting) and YO-PRO\(^\text{-1}\) iodide (10 μg/ml; apoptotic cell dye; ThermoFisher Scientific) as well as CD45 PerCP-CY5.5 antibody (as before) were added. Measurement and analysis were performed subsequently without a centrifugation step on a BD LSR2 Flow Cytometer equipped with a violet laser using BD FACSDiva software. Absolute viable blast counts (CD45\(^{\text{pos}}\)/SSC\(^{\text{pos}}\)/YO-PRO\(^{\text{neg}}\) were calculated upon individual tube-specific bead-based correction factors.

**Xenotransplantation and in vivo drug studies**

NOD.SCID-IL2R\(^\gamma\)null (NSG) mice were bred and maintained under specific pathogen-free conditions according to FELASA recommendations. Breeding and experiments were approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna and granted by the national authority (Austrian Federal Ministry of Science and Research) according to Section 8ff of Law for Animal Experiments under licenses BMWFUW-68.205/0112-WFV/3b/2016 and BMWFUW-68.205/0093-WFV/3b/2015 and were performed according to the guidelines of FELASA and ARRIVE. Schematic presentation of the experiment is presented in Figure 5A. Patient-derived AML samples were thawed, washed once with PBS, split into three and injected intravenously into NSG mice for cell amplification. Human cell engraftment was evaluated by the presence of human CD45 positive cells in mouse blood using anti-mouse CD45-PE-Cyanin7 (30-F11; ThermoFisher Scientific, ebioscience\(^\text{TM}\) #25-0451-81) versus anti-human CD45-eFluor 450 (HI30; ThermoFisher Scientific, ebioscience\(^\text{TM}\) #48-0459-42). Flow-cytometric measurement was performed on a BD FACSCanato II (BD Bioscience, Heidelberg, Germany) and data analyzed using BD FACSdiva software V8.0. After successful engraftment mice were sacrificed by cervical dislocation. Single cell suspensions were obtained by pressing the spleen trough 100 μm cell strainers or flushing the BM. Cell suspensions of mice containing engrafted patient leukemic cells were frozen in freezing medium containing 10% DMSO and 90% FCS (Capricorn, #PBS-12A) until further usage or injected for further amplification into NSG mice.

For validation of signaling inhibitor sensitivity of AML blasts in vivo murine splenic cell suspension containing engrafted human leukemic cells were split into five experimental groups and incubated with selected signaling inhibitors (midostaurin, rapamycin, ruxolitinib, sorafenib; at concentrations as described in Material and Methods) or DMSO (vehicle) as negative control in X-Vivo\(^\text{TM}\) 10 overnight at 37°C prior to intravenous injection into NSG mice (approximately 2×10⁶ cells/mouse; 2 mice/
inhibitor; 2 independent experiments). Disease onset was monitored by puncturing vena facialis and blood analysis of human CD45 cells as described above. Leukemic mice were sacrificed by cervical dislocation. Patient leukocytes in blood and single cell suspensions of spleen and bone marrow were analyzed as described above.

Statistical analysis
Basal and induced signal protein phosphorylation was assessed according to a method adapted from Irish and Nolan et al.\(^{47}\) using log2 ratios of geometric mean fluorescence intensities (geoMFI) of target expressions versus mock control. Signal assays were performed blinded regarding clinical outcome data. Differences in the distribution of features between groups of patients were investigated using the Chi-Square test (contingency tables with continuity correction).

The construction of a heatmap was performed in R statistical environment using the “pheatmap” package. For hierarchical clustering “euclidean distance” and the “complete clustering” algorithm was used.

For the evaluation of the prognostic value of the signals, signals were dichotomized using the median as threshold. To screen for relevant differences of signal activation in correlation with relapses we performed univariate analysis in the framework of a Cox-model for cause-specific hazard ratios with backward stepwise selection with a p-value greater than 0.05 for removal of variables allowing evaluation of 88 patients. We used the multivariate analysis was restricted to univariately estimate a potential non-linear relationship to the competing events (non-leukemic death, relapses) and to correct for multiple testing. Multivariate analysis was performed blinded regarding clinical outcome data.

Cumulative incidence of relapses (CIR) was estimated taking into account the competing events calculated from the respective time-point of assessment until the date of event or last observation. The CIR was compared with the Gray test. All CIR estimates were set to 5 years of observation. Secondary end-point was event-free survival (EFS) estimated according the method of Kaplan-Meier. In order to validate using the median as threshold, Cox-regression with restricted cubic splines was used to univariately estimate a potential non-linear relationship between the signal level and the cause-specific hazard ratios and the estimated 5-year CIR.\(^{50}\)

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