ARTICLE

Dasatinib response in acute myeloid leukemia is correlated with *FLT3*/ITD, *PTPN11* mutations and a unique gene expression signature

Sigal Tavor,[1] Tali Shalit,[2] Noa Chapal Ilani,[3] Yoni Moskovitz,[3] Nir Livnat,[3] Yoram Groner,[4] Haim Barr,[2] Mark D. Minden,[5,6,7,8] Alexander Plotnikov,[2] Michael W. Deininger,[9,10] Nathali Kaushansky[3,#] and Liran I. Shlush[3,5,11,#]

[1]Hemato-Oncology Department, Assuta Medical Center, Tel Aviv, Israel; [2]G-INCPM, Weizmann Institute of Science, Rehovot, Israel; [3]Department of Immunology, Weizmann Institute of Science, Rehovot, Israel; [4]Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel; [5]Princess Margaret Cancer Centre, University Health Network Toronto, Ontario, Canada; [6]Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada; [7]Department of Medicine, University of Toronto, Toronto, Ontario, Canada; [8]Division of Medical Oncology and Hematology, University Health Network, Toronto, Ontario, Canada; [9]Division of Hematology and Hematologic Malignancies, University of Utah, Salt Lake City, UT, USA; [10]Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA and [11]Division of Hematology, Rambam Healthcare Campus, Haifa, Israel

[#]NK and LIS contributed equally as co-senior authors.

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ABSTRACT

Novel targeted therapies improve the survival of specific subgroups (defined by genetic variants) of patients with acute myeloid leukemia (AML), validating the paradigm of molecularly targeted therapy. However, identifying correlations between AML molecular attributes and effective therapies is challenging. Recent advances in high-throughput, *in vitro* drug sensitivity screening applied to primary AML blasts were used to uncover such correlations; however, these methods cannot predict the response of leukemic stem cells. Our study aimed to predict *in vitro* response to targeted therapies, based on molecular markers, with subsequent validation in leukemic stem cells. We performed *ex vivo* screening of sensitivity to 46 drugs on 29 primary AML samples at diagnosis or relapse. Using unsupervised hierarchical clustering analysis we identified a group with sensitivity to several tyrosine kinase inhibitors, including the multi-tyrosine kinase inhibitor, dasatinib, and searched for correlations between the response to dasatinib, exome sequencing and gene expression in our dataset and in the Beat AML dataset. Unsupervised hierarchical clustering analysis of gene expression resulted in clustering of dasatinib responders and non-responders. *In vitro* response to dasatinib could be predicted based on gene expression (area under the curve=0.78). Furthermore, mutations in *FLT3*/ITD and *PTPN11* were enriched in the dasatinib-sensitive samples as opposed to mutations in *TP53* which were enriched in resistant samples. Based on these results, we selected *FLT3*/ITD AML samples and injected them into NSG-SGM3 mice. Our results demonstrate that in a subgroup of *FLT3*/ITD AML (4 out of 9) dasatinib significantly inhibited leukemic stem cell engraftment. In summary we show that dasatinib has an anti-leukemic effect both on bulk blasts and, more importantly, on leukemic stem cells from a subset of AML patients that can be identified based on mutational and expression profiles. Our data provide a rational basis for clinical trials of dasatinib in a molecularly selected subset of AML patients.

Correspondence:

LIRAN I. SHLUSH
liran.shlush@weizmann.ac.il

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Introduction

AML is an aggressive myeloid neoplasm with complex and heterogeneous genetics that influence prognosis and treatment response. Furthermore, AML is a multi-stage disease with preleukemic, leukemic and late stages.[1] Identification of AML-inducing mutations is required for accurate diagnosis and to tailor therapy according to the genetic profile of individual patients. Recent advances in AML targeted therapy based on driver mutations have improved overall survival.[2,3] Increased understanding of AML molecular pathophysiology provided new opportunities to target specific mutants, such as *FLT3*/ITD (FMS-like tyrosine kinase-3, internal tandem duplications) and *IDH1/2*.[4-6] Moreover, the BCL2 inhibitor, venetoclax, has increased activity in patients with mutated *NPM1c* and *RAD21*,[7] and in combination with hypomethylating agents targets leukemia stem cells (LSC).[8] While these novel therapies can achieve prolonged remissions, most patients eventually relapse. Our recent studies suggest that the origins of AML relapse are heterogeneous, but relapsing clones invariably exhibit stem cell properties.[9] Relapse usually originates from leukemic subclones that were present before therapy, and were either selected due to resistance mechanisms[9,10] or gained competitiveness through further evolution, possibly due to exposure to chemotherapy.[11] Targeted therapies can fail to prevent relapse by addressing one leukemic cell clone, while allowing other clones to expand and cause relapse.[12] In order to prevent relapse, it is crucial to eradicate LSC in the early stages of treatment (induction/consolidation) before they expand and acquire additional mechanisms of resistance. It has been suggested that combination therapies that simultaneously target multiple leukemic subclones may overcome AML heterogeneity. However, combination therapy approaches are limited by tolerability, particularly in older individuals, and it is important to maximize efficacy and minimize toxicity by selecting the right drug for the right set of patients. Given the multitude of therapeutic options, it is essential to design clinical trials based on molecular markers that select for those patients likely to benefit.

Our latest studies demonstrated that, in certain AML patients in whom relapse originated from primitive cells,[9] the cells from the time of diagnosis capable of leukemic engraftment in immunodeficient mice were identical to those that caused relapse. In these patients the bulk of cells were responsive to chemotherapy, but rare LSC that expanded in xenografts could drive relapse. Accordingly, drugs that inhibit the engrafting clones should be useful in preventing relapse, but screening a large number of drugs in a large number of samples in xenografts is not feasible. An alternative approach is to screen for drugs with known mechanistic effects against large and heterogeneous AML cohorts *in vitro*, which allows for high throughput screening of many drugs to establish correlations between response and molecular attributes of AML. Disadvantages include the lack of a microenvironment and immune system and the short duration of exposure to the drug, which reflects the inability of AML cells to grow *in vitro*. Short-term *in vitro* drug sensitivity assays have become feasible in recent years by mirroring normal hematopoietic stem and progenitor cell culture conditions. So far *in vitro* drug testing of primary AML samples have focused on predicting clinical outcome,[13,14] and, in some cases, guiding individualized therapy.[15] Such personalized therapy is particularly important for patients with relapsed/refractory disease.[16] *In vitro* drug studies identified biomarkers of response to specific drugs[17] and clusters of AML samples with similar response profiles.[18] In the current study, we studied *in vitro* drug sensitivity of primary AML samples from patients who achieved complete remission and compared our results to those of the Beat AML study. Our global aim was to identify therapies and biomarkers that could predict which drugs might be added to induction therapy to prevent relapse in specific subtypes of AML and then validate the results *in vivo*. We discovered that samples with *in vitro* sensitivity to dasatinib (a multi-kinase inhibitor) had a specific *in vitro* drug sensitivity pattern and gene expression signature, and were enriched for *FLT3*/ITD and *PTPN11* mutations. Xenograft studies confirmed that dasatinib targets LSC *in vivo*.

Methods

Primary acute myeloid leukemia cells

Frozen mononuclear cells from bone marrow or peripheral blood of AML patients at diagnosis and relapse, when available, were used. Inclusion criteria for sample selection were achievement of complete remission. Patients with a low peripheral blood blast count and acute promyelocytic leukemia were excluded (*Online Supplementary Table S1*). Ethics approval: UHN 01-0573.

Drug library

We selected Food and Drug Administration-approved cancer drugs with demonstrated clinical and/or preclinical efficacy in leukemia and in other cancers. The 46 drugs and their mechanisms of action are detailed in *Online Supplementary Table S2*.

Drug screening technology

Cells were thawed, washed, counted and suspended in RPMI supplemented with 10% fetal calf serum and human cytokines: stem cell factor (100 ng/mL), interleukin 3 (10 ng/mL), interleukin 6 (20 ng/mL) and thrombopoietin (10 ng/mL). Sensitivity profiling was determined by dose-response over several log ranges (*Online Supplementary Figure S1*). Assay-ready plates in 384-well format were arrayed in 12 concentrations for each compound (2 nM -50 μM), using a Labcyte Echo 555 acoustic dispenser, and frozen until use. Patients' samples were dispensed at approximately 1,000 cells per well and cultured for 2 days. Viable cells were quantified using Cell Titer Glo reagent (Promega Madison, WI, USA). Data were normalized using Genedata Screener software: dimethylsulfoxide-treated cells corresponded to 100% viability and samples without cells corresponded to 0% viability. Normalized data were loaded into CDD Vault software to calculate the half maximal inhibitory concentration (IC_{50}), and minimum and maximum responses for each drug and patient. We compared the *in vitro* response to dasatinib in our study with the results of the Beat AML drug sensitivity and resistance testing (DSRT). In the Beat AML study *in vitro* cultures were without cytokines.[17]

RNA and exome sequencing

The workflow for RNA sequencing was performed as previously described[9] and the methods for RNA sequencing, exome sequencing, and gene expression and mutation analyses are detailed in the *Online Supplementary Methods*. For pathway analyses we used enrichr.[19,20]

Prediction of response to dasatinib

Dasatinib responders were defined in our cohort and in the Beat AML cohort as having an IC_{50} <0.01, and dasatinib non-responders in both cohorts as having an IC_{50} >0.8 (*Online Supplementary Table S3*). To identify predictors of response to dasatinib we initially selected genes that were differentially expressed (DE) between dasatinib responders and non-responders. Furthermore, other gene sets were selected based on pathway analysis of the DE genes and genes known to be targets of dasatinib as reported by the KinMap (*Online Supplementary Table S4*, genes with a Kd(nM) <7 [37 genes]). To refine feature selection, we tested which dasatinib target genes were DE between dasatinib responders and non-responders from the Beat AML study (*Online Supplementary Figure S3*, *Online Supplementary Table S4*). Various machine-learning models were tested, all with a cross-validation of 10-fold, on the Beat AML data only. The best prediction model was the k-nearest neighbor algorithm with cosine similarity. This model was validated on the Israel National Center for Personalized Medicine (INCPM) gene expression data (Matlab statistical tool box).

In vivo dasatinib treatment

SGM3 mice were given an intrafemoral injection of $1x10^6$ CD3-depleted AML human cells. From day 35 after the injection the mice were treated with 50 mg/kg dasatinib orally for 21 days and sacrificed on day 56. Fluorescence-activated cell sorting analysis was performed. Bone marrow cell suspensions were analyzed for expression of human CD45 (BV521, clone HL30), CD34 (APCcy7, clone 581), CD38 (PE cy7, clone 303516), CD15 (BV421, clone W6D3) (products from Biolegend), CD33 (APC), CD3 (FITC) and CD19 (PE) markers (products from Becton Dickinson). Human engraftment was defined as >0.1% of cells positive for human CD45 at the time of sacrifice. The percent engraftment of human cells was compared between drug- *versus* control-treated mice using the Wilcoxon test. The Institutional Animal Care and Use Committee of the Weizmann Institute approved the experiments, which were performed in accordance with its relevant guidelines and regulations (11790319-2).

Statistical analysis

Heatmap and clustering of drug sensitivity analysis was performed with R package ComplexHeatmap. Differences between the defined groups were validated by statistical analysis. Enrichment analyses were performed using the Fisher exact test. Analysis of the DE genes was performed using the DESeq2 package (1.10.1)[21] with the betaPrior, coks cutoff and independent filtering parameters set to "False". Raw *P* values were adjusted for multiple testing using the procedure of Benjamini and Hochberg. DE genes were determined by an adjusted $P<0.05$, absolute fold changes >2 and maximum raw counts >30 (Figure 2, *Online Supplementary Tables S5* and *S6*). Overlap between DE genes in the Beat AML and INCPM was tested by the hypergeometric distribution using the following R function sum (dhyper[*t:b, a, n - a, b*]). List a contains the DE genes in the Beat AML dataset, and *b* the DE genes in INCPM. The total number of expressed genes in both datasets was 20,055. The intersection between *a* and *b* is *t* (Figure 3A).

Results

Classification of acute myeloid leukemia samples based on drug response, and mutation profile

The overall goal of the current study was to identify drugs that can be safely given at diagnosis and target LSC. To achieve this goal, we first tried to identify drugs that were more effective at relapse than at diagnosis. Such drugs would presumably target LSC, which are enriched at relapse. Comparing the DSRT between paired AML diagnostic and relapse samples we found that for the majority of tested compounds the IC_{50} was higher for the relapse samples (Figure 1A), although the difference was statistically insignificant in most cases. Clinically relevant drugs that are being used to treat patients at relapse (etoposide,[22] midostaurin[23] and fludarabine[24]) were significantly less effective at relapse (Figure 1A). Next, we took an unbiased approach to identify subgroups of AML sensitive to specific drugs in order to identify patients likely to benefit from specific targeted therapies. We screened 29 primary AML samples by DSRT using a test set of 46 drugs. Five compounds (BEZ235, lenalidomide, visomodegib, EPZ-5676 and AG-120) were excluded from the analysis because of the low variability in the response among the different samples (almost all samples were resistant to these drugs). Unsupervised hierarchical clustering analysis of IC_{50} values demonstrated two distinct drug response patterns (Figure 1B). One group displayed high sensitivity to TKI (dasatinib, MEK inhibitors, PI3K inhibitor [BKM120], pim inhibitor, ruxolitinib, mTORC1/2 inhibitor [INK128], quizartinib, midostaurin), BET inhibitors (OTX015, JQ1) and several other drugs. This group was termed sensitive. The other group was resistant to most compounds. The "resistant" group displayed heterogeneous sensitivity to BCL2 inhibitors (ABT-737 and venetoclax), purine analogs (clofarabine, fludarabine,) and the hypomethylating agent decitabine. Interestingly, samples sensitive *in vitro* to BCL2 inhibitors (ABT-737 and venetoclax) did not cluster in a specific group, consistent with clinical observations (Figure 1B).[8,25] To define the molecular pathways underlying the drug sensitivity of the clustering groups, we analyzed the somatic mutations (exome sequencing) and gene expression of the AML samples. We focused our genetic analysis on genes that are recurrently mutated in AML.[26] Mutations in *FLT3*/ITD were observed in seven of 17 patients in the "sensitive" group and none of 12 in the "resistant" group (P=0.023). No other significant enrichment was identified in the current dataset.

Gene expression and mutation profile of dasatinib-sensitive acute myeloid leukemia cells

Analysis of the *in vitro* DSRT assay identified a subgroup of AML samples sensitive to a broad range of TKI (Figure 1B). Among the TKI, the sensitivity to dasatinib was different between the two groups. In particular, 52% (9 of 17) of the AML samples in the group of "responders" displayed sensitivity to dasatinib (defined as a 10-fold difference in IC_{50} between sensitive and non-sensitive samples) whereas in the group of "non-responders" none of the samples showed *in vitro* sensitivity to dasatinib (P=0.003) (Figure 1B). Based on these results we focused our analysis on dasatinib, which has been safely used for many years as monotherapy or in combination with chemotherapy for Philadelphia-positive leukemia. Next, we tried to define those molecular features that could predict which AML samples would be sensitive to dasatinib. As our cohort was small, we compared our results to those of the Beat AML study.[17] We classified dasatinib responders (n=61) and non-responders (n=134) based on IC_{50} values (*Online Supplementary Table S3*). Other parameters, including age, gender, leukocyte number, time of sample [diag-

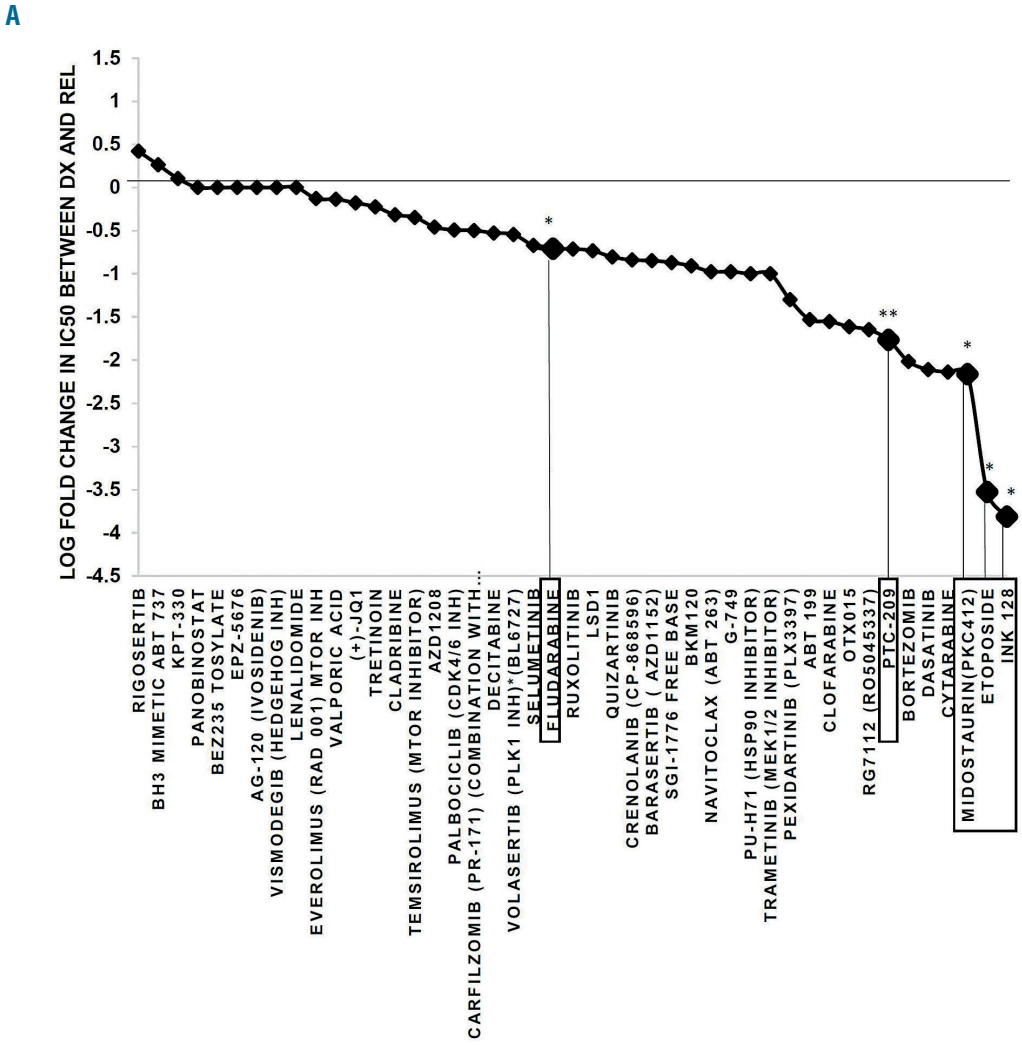

Figure 1. *In vitro* drug resistance. (A) *In vitro* drug resistance to most drugs is acquired after relapse. An average drug response of seven couples of primary acute myeloid leukemia (AML) samples, at diagnosis (DX) and relapse (REL) is shown, comparing the half maximal inhibitory concentration (IC_{50}) of 41 drugs. Each dot represents the response to a specific drug, calculated by the median IC_{50} ratio of diagnosis vs. relapse. (B) Drug sensitivity and resistance testing (DSRT) of primary AML cells. Hierarchical clustering using Pearson dissimilarity and complete linkage was performed. Data are the $\log_2$ IC_{50} +0.001, standardized for each compound by reducing the mean. DSRT for 41 drugs of clinical and preclinical use in AML shows two clustered groups of patients. The age and gender of the patients and the origin of the patients' diagnostic or relapse sample, the name of the drugs, the class of the drug, karyotype and mutation status for *FLT3*/ITD and *NPM1* are shown.

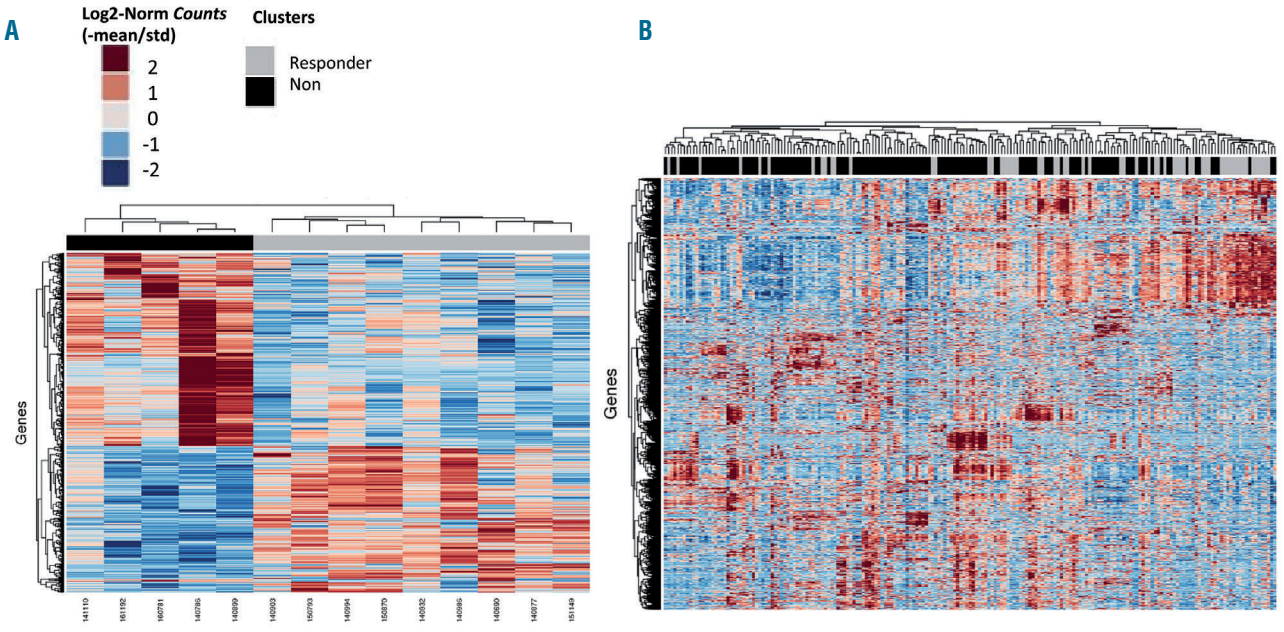

Figure 2. Transcriptome profiling of dasatinib responder samples. Gene expression analysis of whole transcriptome mRNA sequencing comparing dasatinib sensitive to non-sensitive samples of acute myeloid leukemia (AML). (A) Differentially expressed genes between "responders", and "non-responders". (B) In order to extend the tested samples we also applied the same analysis to AML patients' samples from the Beat AML dataset.

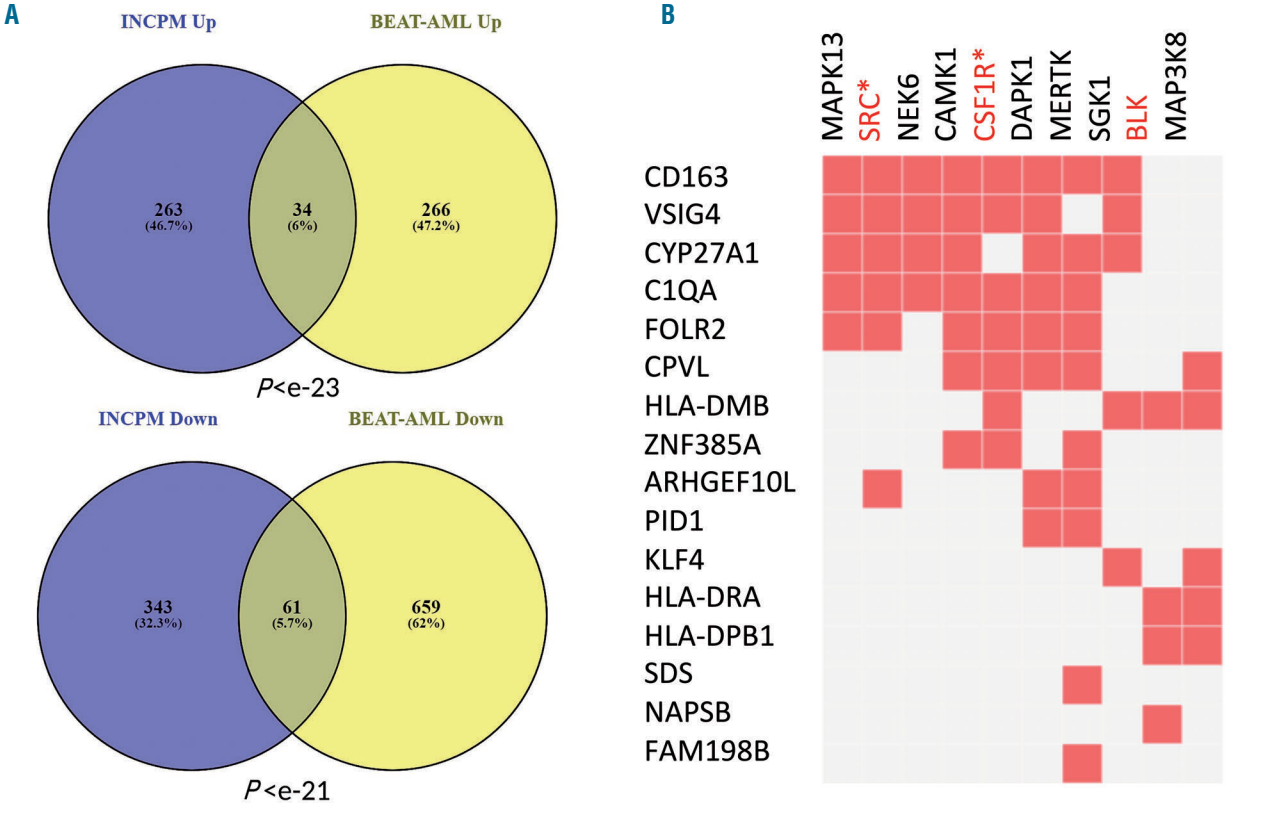

Figure 3. Analysis of differentially expressed genes between dasatinib responders and non-responders from the INCPM and Beat AML datasets. (A) Intersection of upregulated (upper panel) and downregulated (lower panel) differentially expressed genes from the INCPM and Beat AML cohorts. (B) Pathway analysis of intersecting upregulated genes in both cohorts identified significant enrichment of genes that are co-expressed with several dasatinib targets. Dasatinib targets (*CSF1R*, *SRC*, *BLK*) are shown in red and the asterisk marks significant enrichment (false discovery rate<0.05).

nosis/relapse], type of AML [*de novo*/transformed], karyotype and mutation profile were also tested for correlation with response to dasatinib.

Differential gene expression analysis of responders and non-responders to dasatinib in our cohort identified 297 genes significantly overexpressed and 404 genes downregulated in the sensitive group (false discovery rate <0.05) (*Online Supplementary Table S5*). In the Beat AML cohort, we identified 300 significantly upregulated and 720 downregulated genes in the dasatinib-sensitive group as compared to the resistant group (*Online Supplementary Table S6*). Unsupervised hierarchical clustering of all expressed genes enriched dasatinib responders in specific clusters both in our cohort (Figure 2A) and in the Beat AML cohort (Figure 2B). Intersecting the upregulated and downregulated DE genes from the Beat AML and the INCPM cohorts resulted in a significant number of intersecting genes ($P<10^{-23}$ and $P<10^{-21}$, respectively) (Figure 3A). Pathway analysis of intersecting upregulated genes in both cohorts identified significant enrichment of genes that are co-expressed with dasatinib targets (*CSF1R, SRC*) (Figure 3B, *Online Supplementary Table S10*). On the other hand, the same pathway analysis of intersecting downregulated genes did not enrich for dasatinib targets. As we identified upregulation of genes that are co-expressed with dasatinib targets we next performed a focused analysis on the expression of actual dasatinib targets. We identified all known dasatinib targets and performed unsupervised hierarchical clustering on their gene expression in both our cohort and the Beat AML cohort. In both cohorts a cluster enriched with dasatinib responders was identified. In the Beat AML cohort 13 of 21 samples (59%) were responders in this cluster as opposed to 61 out of 195 (31.3%) responders in the entire Beat AML cohort (P=0.016) (*Online*

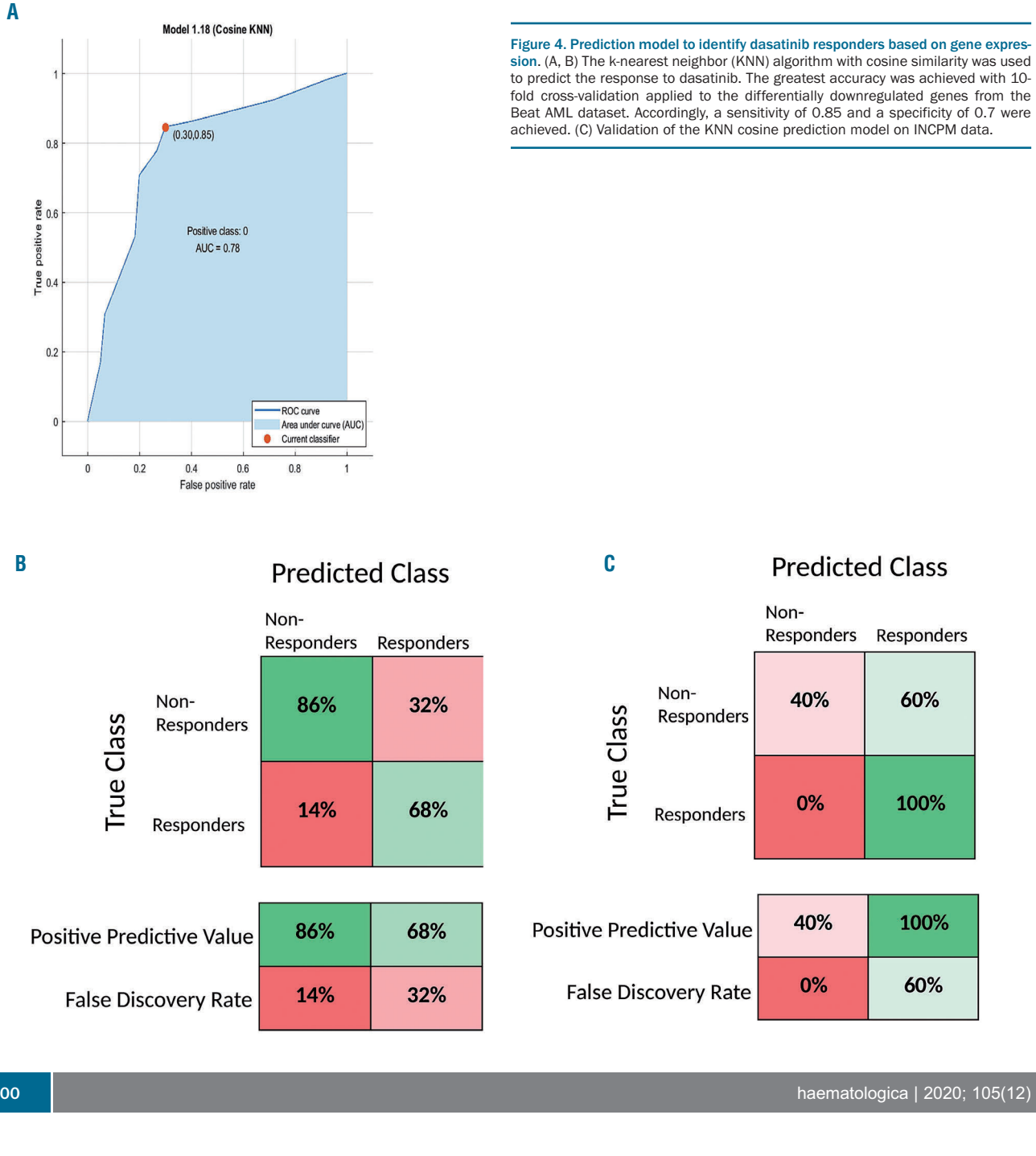

Figure 4. Prediction model to identify dasatinib responders based on gene expression. (A, B) The k-nearest neighbor (KNN) algorithm with cosine similarity was used to predict the response to dasatinib. The greatest accuracy was achieved with 10-fold cross-validation applied to the differentially downregulated genes from the Beat AML dataset. Accordingly, a sensitivity of 0.85 and a specificity of 0.7 were achieved. (C) Validation of the KNN cosine prediction model on INCPM data.

Supplementary Figure S2). In both cohorts the dasatinib target cluster included three SRC family tyrosine kinases *FGR*, *HCK* and *LYN* as well as *PTK6*, *CSK*, *GAK* and *EPHB2*. Several of these genes were significantly overexpressed in dasatinib responders in the Beat AML cohort (*Online Supplementary Figure S3*).

Next, we used the data from the gene expression analysis to establish a prediction model for identifying dasatinib responders *in vitro* based on gene expression. For all experiments we used a cross-validation approach, training the model only on the Beat AML data and validating results on the INCPM data. We tested the following genes sets from the Beat AML data for prediction: all genes, all DE genes, all upregulated genes, all downregulated genes, all dasatinib targets and DE dasatinib targets. The best prediction model, with a sensitivity of 0.85 and specificity of 0.7 (area under the curve=0.78) (Figure 4A,B), was achieved when using the Beat AML downregulated genes. When testing the same model on the INCPM data the positive predictive value was 100% and the false discovery rate was 60% (Figure 4C). These data provide evidence that gene expression can predict *in vitro* response to dasatinib, albeit with limited accuracy.

As gene expression did not provide an ideal prediction for dasatinib response, we tested whether any other parameters from the Beat AML could predict response to dasatinib. We analyzed molecular and clinical data for enrichment in dasatinib responders. We found that samples carrying mutations in *FLT3*/ITD (P=0.011) (*Online Supplementary Table S7*), *IDH2* and *PTPN11* (P<0.05) were enriched in dasatinib responders in the Beat AML cohort. The IC_{50} for dasatinib in carriers of *FLT3*/ITD and *PTPN11* was significantly lower than the IC_{50} of non-carriers (P<0.0001 and P=0.02, respectively) (Figure 5A, B and *Online Supplementary Table S8*), whereas only samples with mutations in *TP53* had significantly higher IC_{50} compared to wild-type samples (P=0.0003) (Figure 5C and *Online Supplementary Table S9*). Adding the mutation status to the gene expression prediction model did not improve accuracy.

Collectively, these data suggest that molecular features (mutations and gene expression profile) identify a group of AML cases responsive to dasatinib *in vitro*. Specifically, the fact the *FLT3*/ITD mutant cases are enriched in dasatinib responders in both the Beat AML and INCPM cohorts prompted us to test the *in vivo* efficacy of dasatinib in this specific group of patients. As our ultimate goal was to test whether *in vitro* response to dasatinib would predict *in vivo* AML response, we chose to focus our *in vivo* dasatinib response studies on samples known to carry *FLT3*/ITD. The focus on *FLT3*/ITD also stemmed from the fact that *FLT3*/ITD AML samples are known to be good AML engrafters.[27]

Dasatinib reduces leukemia stem cells in mice engrafted with *FLT3*/ITD-mutant acute myeloid leukemia

We injected NSG mice (n=5-10/sample) with cells from nine patients with *FLT3*/ITD AML. On day 35 the animals were randomized to dasatinib or a carrier control. Following 3 weeks of treatment we measured human engraftment in the treated and control groups (Figure 6). Fluorescence-activated cell sorting of bone marrow demonstrated leukemic engraftment ($CD33^+$ >90% of human $CD45^+$ cells) in mice from both groups. For four of nine samples, engraftment was significantly reduced in the dasatinib-treated group. This result suggests that dasatinib has anti-LSC activity in a subset of *FLT3*/ITD positive AML cases.

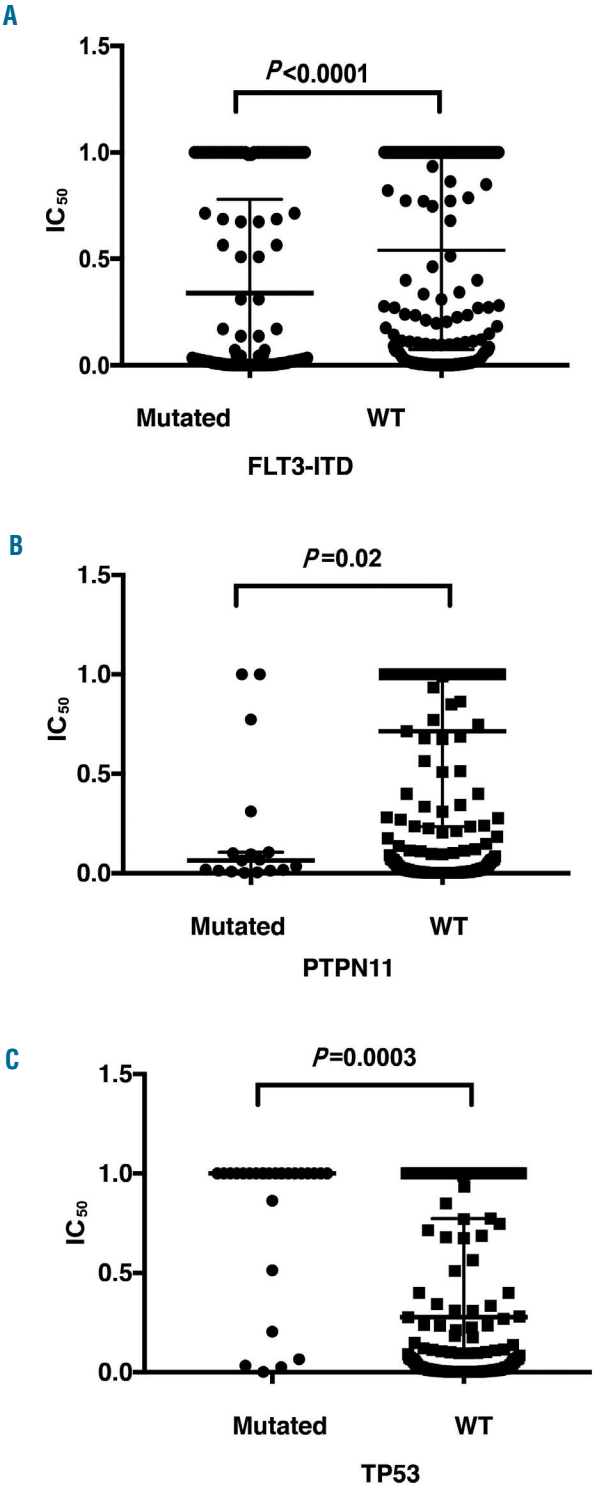

Figure 5. Correlation between *FLT3*/ITD, PTPN11 and *TP53* mutations in acute myeloid leukemia samples and response to dasatinib *in vitro* in the Beat AML study. (A-C) Median dasatinib half maximal inhibitory concentration (IC_{50}) values in cases with mutated *FLT3*/ITD (A), *PTPN11* (B) or *TP53* (C) and wild-type (WT) samples. Differences between groups were validated by the Wilcoxon rank-sum test.

Discussion

We provide evidence that a subset of *FLT3*/ITD$^+$ AML cases and AML subtypes with a unique gene expression pattern are sensitive to dasatinib *in vitro* and that this sensitivity translates into sensitivity *in vivo*, at least in *FLT3*/ITD$^+$ cases. We initially discovered sensitivity to dasatinib using DSRT and validated the DSRT results based on the Beat AML study. DSRT can only measure toxicity to bulk blast cells and cannot detect effects on differentiation and LSC. Accordingly, we tested the response of *FLT3*/ITD$^+$ primary AML samples to dasatinib in a xenograft model and confirmed response *in vivo*.

FLT3/ITD is one of the most common mutations in AML, and is associated with high risks of relapse and mor-

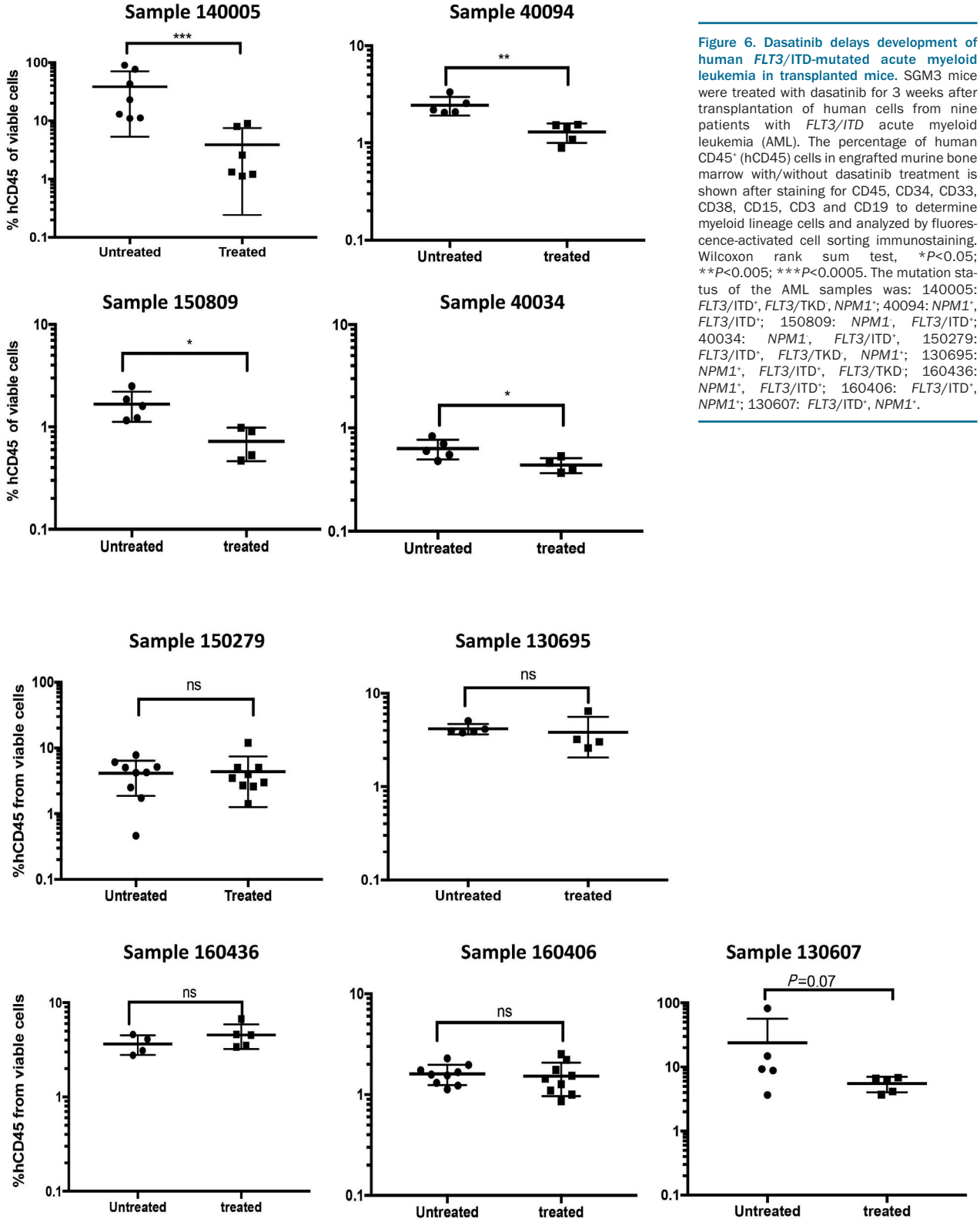

Figure 6. Dasatinib delays development of human *FLT3*/ITD-mutated acute myeloid leukemia in transplanted mice. SGM3 mice were treated with dasatinib for 3 weeks after transplantation of human cells from nine patients with *FLT3/ITD* acute myeloid leukemia (AML). The percentage of human CD45$^+$ (hCD45) cells in engrafted murine bone marrow with/without dasatinib treatment is shown after staining for CD45, CD34, CD33, CD38, CD15, CD3 and CD19 to determine myeloid lineage cells and analyzed by fluorescence-activated cell sorting immunostaining. Wilcoxon rank sum test, **P*<0.05; ***P*<0.005; ****P*<0.0005. The mutation status of the AML samples was: 140005: *FLT3*/ITD$^+$, *FLT3*/TKD$^-$, *NPM1*$^+$; 40094: *NPM1*$^+$, *FLT3*/ITD$^+$; 150809: *NPM1*$^-$, *FLT3*/ITD$^+$; 40034: *NPM1*$^-$, *FLT3*/ITD$^+$, 150279: *FLT3*/ITD$^+$, *FLT3*/TKD$^-$, *NPM1*$^+$; 130695: *NPM1*$^+$, *FLT3*/ITD$^+$, *FLT3*/TKD$^-$; 160436: *NPM1*$^+$, *FLT3*/ITD$^+$; 160406: *FLT3*/ITD$^+$, *NPM1*$^+$; 130607: *FLT3*/ITD$^+$, *NPM1*$^+$.

tality.[28-31] Recently the introduction of *FLT3* TKI has improved overall survival;[32,33] however, not all *FLT3*/ITD$^+$ patients respond to *FLT3* inhibitors, whereas some *FLT3*/ITD$^-$ patients do.[34] We show that *FLT3*/ITD AML cells are sensitive to dasatinib both *in vitro* and in xenograft models. *FLT3*/ITD results in constitutive activation of *FLT3* and activates several signal transduction pathways such as RAS-MAPK, PI3K, JAK-STAT and SRC. This broad signal transduction activation might explain the heterogeneity observed in the response to *FLT3* inhibitors. The current standard of care for patients with *FLT3*/ITD is midostaurin combined with chemotherapy. However, in the RATIFY study only 46.4% of patients achieving complete remission after midostaurin remained disease-free after 4 years with an ongoing risk of relapse, especially during the first year after treatment.[23] The high failure rate even following combination therapy indicates that additional therapy is needed upfront. Previous studies by Weisberg *et al.* demonstrated synergistic effects for the combination of midostaurin and dasatinib in *FLT3*/ITD$^-$ mutated AML cell lines.[35] Our *in vivo* data demonstrate that the anti-leukemic activity of dasatinib extends to *FLT3*/ITD LSC, at least for a subset of patients, and suggest that addition of dasatinib to the current standard of care may be beneficial for *FLT3*/ITD AML patients.

Our results also show that a unique gene expression signature correlates with response to dasatinib. Consistent with mechanistic predictions dasatinib responders overexpressed multiple dasatinib targets. SRC family kinase (SFK) genes, such as *LYN*, *HCK*[36] and *FGR* (which are all dasatinib targets) are overexpressed in dasatinib responders in the Beat AML dataset (Figure 3B, *Online Supplementary Figure S3*), and were shown to be overexpressed in AML-LSC and to contribute to the survival and proliferation of these cells.[37] Exposure to dasatinib treatment inhibited SFK phosphorylation in primitive and committed AML progenitors. In this study the combination of dasatinib and chemotherapy enhanced LSC targeting by p53 signaling.[37] Furthermore recent evidence suggests that *FLT3* activates SFK and these kinases in turn regulate the activity of the RAS/ERK pathways.[38] Altogether, our data suggest that dasatinib may be active not only in *FLT3*/ITD AML but also in other subtypes of AML sharing a similar dasatinib responsive gene expression signature. Similarly, we showed that dasatinib may be effective in treating *PTPN11*-mutated AML, but likely not all *PTPN11*-mutated AML will respond to dasatinib and a larger cohort is needed to identify additional biomarkers. Mutations in *PTPN11* are also found in 35% of patients with juvenile myelomonocytic leukemia and acute lymphocytic leukemia, and in 4-7% of AML patients. *PTPN11* encodes the Shp2 cytoplasmatic protein tyrosine phosphatase, and SHP2 mutations cause increased phosphatase activity that contributes to leukemogenesis by upregulating the RAS, JAK-STAT, and PI3K pathways, leading to aberrant proliferation of myeloid progenitors.[39,40] A recent study demonstrated that *TNK2* activates *PTPN11*; furthermore, mutant myeloid leukemic cells carrying *PTPN11*-activating mutations were sensitive to TNK inhibition by dasatinib *in vitro*.[41] Future studies are needed to better define the subset of *PTPN11*-mutated AML likely to respond to dasatinib and to test the activity of dasatinib activity *in vivo*.

Our results suggest that the addition of dasatinib to the current standard of care for *FLT3*/ITD AML (an *FLT3* inhibitor in combination with cytarabine/anthracycline) may benefit a subset of *FLT3*/ITD$^+$ patients and should be tested in a clinical trial. Similarly, patients ineligible for intensive induction therapy may benefit from combining dasatinib, *FLT3* TKI, hypomethylating agents and venetoclax. Clearly the potential toxicity of such combinations needs to be taken into account. Another possible clinical trial would be to investigate the addition of dasatinib to standard of care in patients with *PTPN11*-mutated AML. A unique gene expression signature, as detected by RNA-sequencing, may predict response to dasatinib, suggesting that selection of patients based on RNA-sequencing may be included in future clinical trials. Given dasatinib's activity against LSC, there is hope that addition of dasatinib during initial therapy will reduce the risk of subsequent relapse.

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References

1. Tuval A, Shlush LI. Evolutionary trajectory of leukemic clones and its clinical implications. Haematologica. 2019;104(5):872-880.
2. McMahon CM, Perl AE. Gilteritinib for the treatment of relapsed and/or refractory FLT3-mutated acute myeloid leukemia. Expert Rev Clin Pharmacol. 2019;12(9):841-849.
3. Perl AE. The role of targeted therapy in the management of patients with AML. Blood Adv. 2017;1(24):2281-2294.
4. Stone RM, Larson RA, Dohner H. Midostaurin in FLT3-mutated acute myeloid leukemia. N Engl J Med. 2017;377(19):1903.
5. DiNardo CD. Ivosidenib in IDH1-mutated acute myeloid leukemia. N Engl J Med. 2018;379(12):1186.
6. Stein EM, DiNardo CD, Pollyea DA, et al. Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia. Blood. 2017;130(6):722-731.
7. Bisaillon R, Moison C, Thiollier C, et al. Genetic characterization of ABT-199 sensitivity in human AML. Leukemia. 2020;34(1):63-74.
8. Pollyea DA, Stevens BM, Jones CL, et al. Venetoclax with azacitidine disrupts energy metabolism and targets leukemia stem cells in patients with acute myeloid leukemia. Nat Med. 2018;24(12):1859-1866.
9. Shlush LI, Mitchell A, Heisler L, et al. Tracing the origins of relapse in acute myeloid leukaemia to stem cells. Nature. 2017;547(7661):104-108.
10. Shlush LI, Chapal-Ilani N, Adar R, et al. Cell lineage analysis of acute leukemia relapse uncovers the role of replication-rate heterogeneity and microsatellite instability. Blood. 2012;120(3):603-612.
11. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012;481(7382):506-510.
12. Estey E, Levine RL, Lowenberg B. Current challenges in clinical development of "targeted therapies": the case of acute myeloid leukemia. Blood. 2015;125(16):2461-2466.
13. Eriksson A, Osterroos A, Hassan S, et al. Drug screen in patient cells suggests quinacrine to be repositioned for treatment of acute myeloid leukemia. Blood Cancer J. 2015;5:e307.
14. Snijder B, Vladimer GI, Krall N, et al. Image-based ex-vivo drug screening for patients with aggressive haematological malignan-

cies: interim results from a single-arm, open-label, pilot study. Lancet Haematol. 2017;4(12):e595-e606.
15. Pemovska T, Kontro M, Yadav B, et al. Individualized systems medicine strategy to tailor treatments for patients with chemorefractory acute myeloid leukemia. Cancer Discov. 2013;3(12):1416-1429.
16. Bose P, Vachhani P, Cortes JE. Treatment of Relapsed/Refractory Acute Myeloid Leukemia. Curr Treat Options Oncol. 2017;18(3):17.
17. Tyner JW, Tognon CE, Bottomly D, et al. Functional genomic landscape of acute myeloid leukaemia. Nature. 2018;562(7728):526-531.
18. Gerstung M, Papaemmanuil E, Martincorena I, et al. Precision oncology for acute myeloid leukemia using a knowledge bank approach. Nat Genet. 2017;49(3):332-340.
19. Chen EY, Tan CM, Kou Y, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics. 2013;14:128.
20. Kuleshov MV, Jones MR, Rouillard AD, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. 2016;44(W1):W90-97.
21. Anders S, McCarthy DJ, Chen Y, et al. Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. Nat Protoc. 2013;8(9):1765-1786.
22. Megias-Vericat JE, Martinez-Cuadron D, Sanz MA, et al. Salvage regimens using conventional chemotherapy agents for relapsed/refractory adult AML patients: a systematic literature review. Ann Hematol. 2018;97(7):1115-1153.
23. Stone RM, Mandrekar SJ, Sanford BL, et al. Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation. N Engl J Med. 2017;377(5):454-464.
24. Fiegl M, Unterhalt M, Kern W, et al. Chemomodulation of sequential high-dose cytarabine by fludarabine in relapsed or refractory acute myeloid leukemia: a randomized trial of the AMLCG. Leukemia. 2014;28(5):1001-1007.
25. DiNardo CD, Pratz K, Pullarkat V, et al. Venetoclax combined with decitabine or azacitidine in treatment-naive, elderly patients with acute myeloid leukemia. Blood. 2019;133(1):7-17.
26. Papaemmanuil E, Gerstung M, Bullinger L, et al. Genomic classification and prognosis in acute myeloid leukemia. N Engl J Med. 2016;374(23):2209-2221.
27. Sanchez PV, Perry RL, Sarry JE, et al. A robust xenotransplantation model for acute myeloid leukemia. Leukemia. 2009;23(11):2109-2117.
28. Kottaridis PD, Gale RE, Frew ME, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001;98(6):1752-1759.
29. Yanada M, Matsuo K, Suzuki T, et al. Prognostic significance of FLT3 internal tandem duplication and tyrosine kinase domain mutations for acute myeloid leukemia: a meta-analysis. Leukemia. 2005;19(8):1345-1349.
30. Thiede C, Steudel C, Mohr B, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002;99(12):4326-4335.
31. Kayser S, Schlenk RF, Londono MC, et al. Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. Blood. 2009;114(12):2386-2392.
32. Sasaki K, Kantarjian HM, Kadia T, et al. Sorafenib plus intensive chemotherapy improves survival in patients with newly diagnosed, FLT3-internal tandem duplication mutation-positive acute myeloid leukemia. Cancer. 2019;125(21):3755-3766.
33. Uy GL, Mandrekar SJ, Laumann K, et al. A phase 2 study incorporating sorafenib into the chemotherapy for older adults with FLT3-mutated acute myeloid leukemia: CALGB 11001. Blood Adv. 2017;1(5):331-340.
34. Rollig C, Serve H, Huttmann A, et al. Addition of sorafenib versus placebo to standard therapy in patients aged 60 years or younger with newly diagnosed acute myeloid leukaemia (SORAML): a multicentre, phase 2, randomised controlled trial. Lancet Oncol. 2015;16(16):1691-1699.
35. Weisberg E, Liu Q, Nelson E, et al. Using combination therapy to override stromal-mediated chemoresistance in mutant FLT3-positive AML: synergism between FLT3 inhibitors, dasatinib/multi-targeted inhibitors and JAK inhibitors. Leukemia. 2012;26(10):2233-2244.
36. Saito Y, Kitamura H, Hijikata A, et al. Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. Sci Transl Med. 2010;2(17):17ra9.
37. Dos Santos C, McDonald T, Ho YW, et al. The Src and c-Kit kinase inhibitor dasatinib enhances p53-mediated targeting of human acute myeloid leukemia stem cells by chemotherapeutic agents. Blood. 2013;122(11):1900-1913.
38. Kazi JU, Ronnstrand L. The role of SRC family kinases in FLT3 signaling. Int J Biochem Cell Biol. 2019;107:32-37.
39. Li L, Modi H, McDonald T, et al. A critical role for SHP2 in STAT5 activation and growth factor-mediated proliferation, survival, and differentiation of human CD34+ cells. Blood. 2011;118(6):1504-1515.
40. Schubbert S, Lieuw K, Rowe SL, et al. Functional analysis of leukemia-associated PTPN11 mutations in primary hematopoietic cells. Blood. 2005;106(1):311-317.
41. Jenkins C, Luty SB, Maxson JE, et al. Synthetic lethality of TNK2 inhibition in PTPN11-mutant leukemia. Sci Signal. 2018;11(539):eaao5617.