Aurora A kinase as a target for therapy in *TCF3-HLF* rearranged acute lymphoblastic leukemia

B-cell acute lymphoblastic leukemia (B-ALL) harboring the t(17;19)(q22;p13) is a rare subtype of leukemia with a dismal prognosis.[1] This translocation produces an aberrant *TCF3-HLF* fusion with distinct gene expression profiles and drug sensitivity. Recent studies have shown that this subtype of B-ALL may be amenable to therapies inhibiting BCL2 and the pre-B cell receptor through inhibition of SRC family kinases.[2-4] Using RNA sequencing (RNA-seq) in combination with *ex vivo* drug sensitivity analyses we identified an overexpression pattern of Aurora A kinase (AURKA) in five of five *TCF3-HLF* ALL samples. This finding further translated to enhanced sensitivity to inhibition by one of the AURKA inhibitors alisertib. Our studies suggest a molecular susceptibility of *TCF3-HLF* ALL to alisertib providing support to pursue further clinical testing within this rare and lethal subtype of ALL.

The t(17;19)(q22;p13) rearrangement encodes for a chimeric transcription factor consisting of type 1 rearrangements with a fusion of exon 16 of *TCF3* with exon 4 of *HLF*; and type 2 rearrangements aligning with a fusion of exon 15 of TCF3 with exon 4 of *HLF*.[5] Because of its rarity and poor prognosis, there are few established recommended therapies. Patients who achieve remission with no evidence of disease historically recur and die of disease even with stem cell transplant as consolidation therapy.[6] Recent advances using immunologic therapies has shown some promise with durable responses targeting expression of CD19 by B-cell leukemia,[7] however not all patients remained in remission and not all may be eligible for immunologic therapies. Therefore, novel therapies remain essential to improve outcomes. We developed an *in vitro* assay using a panel of small-molecule inhibitors to identify patient specific targeted therapies and employed this assay on the diagnostic marrow sample of a patient who presented to our institution with *TCF3-HLF* ALL.[2] We observed *in vitro* sensitivity to the ABL/SRC inhibitor dasatinib with some clinical benefit.[2] Meanwhile, other studies identified targeting the BCL2 family of proteins with venetoclax as a therapeutic potential.[3,4] We thus sought to validate and identify other novel targets across an independent set of samples. We partnered with the Children's Oncology Group (COG) and St. Jude Children's Research Hospital to obtain four separate patient samples combined with our sample which were xenografted and expanded for *in vitro* as well as *in vivo* experiments. All clinical samples were obtained with informed consent with approval by the Institutional Review Board of Oregon Health & Science University, the Children's Oncology Group, and St. Jude Children's Research Hospital. All murine studies were approved by the OHSU Institutional Animal Care and Use Committee (IACUC, protocol #2358).

Standard reverse transcription polymerase chain reaction (RT-PCR), Sanger sequencing and immunoblot analysis of the *TCF3-HLF* fusion identified three samples with a type 1 translocation (10-199, 3.316, and 3.332) with variable insertions corresponding to different protein length, while two others carried a type 2 translocation (3310, 3324) (*Online Supplementary Figure S1A*). We then undertook next-generation sequencing studies with these samples compared to 35 B-ALL samples (*Online Supplementary Figure S1B* and *C*). Using whole-exome sequencing within our ALL cohort, and specifically focusing on variations found in pathways in B-cell ALL,[8] we found that three of the five samples carried an *IKZF1* variant of unknown clinical significance (*IKZF1* I125V; *Online Supplementary Figure S1B*). Further, two of the five samples carried *KRAS* mutations (*KRAS* G12V/S) as previously described.[9] Finally, RNA-seq analysis of the top 1.000 most variably expressed genes across the cohort revealed gene expression signatures that clustered strongly with *TCF3-HLF* ALL (*Online Supplementary Figure S1C*), suggesting relatively uniform gene expression within these five Type 1 and Type 2 *TCF3-HLF* leukemias.

Implementation of our small-molecule kinase inhibitor panel[10] on 69 ALL samples revealed some consistent patterns of responses in *TCF3-HLF* ALL including sensitivity to the class of aurora kinase inhibitors (*Online Supplementary Figures S1D* and *S2*). Harnessing the RNA-seq data for expression identified significantly higher RNA levels for *AURKA*, slightly higher levels of *AURKB* with no difference in AURKC levels in *TCF3-HLF* ALL as compared to the other B-ALL samples (Figure 1A). Increased AURKA expression was further validated by protein expression as compared to three separate B-ALL xenograft samples (Figure 1B). Gene set enrichment analysis (GSEA) of the top 15 up-regulated Reactome pathways also emphasized cell cycle pathways (*Online Supplementary Figures S3A*) as well as pathways involving Aurora kinases in *TCF3-HLF* ALL (*Online Supplementary Figures S3B*). Within the class of Aurora kinase inhibitors, the AURKA selective alisertib appeared to be the most advanced in clinical testing, including pediatric dosing.[11] Utilizing our functional assay, the population of B-ALL appear by and large resistant to alisertib with a median half maximal inhibitory concentration (IC_{50}) >10 μM while the majority of *TCF3-HLF* ALL samples showed some sensitivity (Figure 1D; *Online Supplementary Figures S1D* and *S2*). As AURKA levels can be dependent on the cell cycle we compared the levels of BIRC5 and INCENP, two proteins critical for the chromosome passenger complex during mitosis.[12] Interestingly, both BIRC5 and INCENP levels were increased compared to B-ALL suggesting that these samples may have a higher population of cells progressing through the cell cycle (Figure 1C). These findings correlated with an increase in the number of cells in S phase amongst *TCF3-HLF* ALL and considerable sensitivity to the BIRC5 inhibitor YM155 (Figure 1D).

In order to further interrogate the effects of alisertib on *TCF3-HLF* ALL, HAL01 cells that carry a type 1 fusion was used as a model (Figure 2). We found that HAL01 displayed a low IC_{50} of 1 nM for alisertib when assessing viability, increased apoptosis by annexin V staining and a significant G2/M arrest (Figure 2A to C). Treatment of these cells with alisertib appeared to disrupt the normal assembly of the spindle poles with more cells having only one spindle pole, and some cells with more than two spindle poles (Figure 2D and E). These phenotypes are similar to RNAi depletion of AURKA consistent with alisertib's effects on Aurora A kinase inhibition.[13]

Each *TCF3-HLF* ALL sample was then tested for therapeutic response *in vivo* in cohorts of immunodeficient NSG mice. One week after tail vein injection, cohorts were treated with vehicle, the SRC inhibitor dasatinib (40 mg/kg/day), the BCL2 inhibitor venetoclax (25 mg/kg/day), combination of dasatinib and venetoclax (n=5)[14] or alisertib (30 mg/kg/day)[15] by oral gavage for five days each week (Figure 3). All control animals engrafted rapidly within 2 months after injection. Only sample 3.332 and 3.310 showed significant response to dasatinib, venetoclax, or combination. Interestingly,

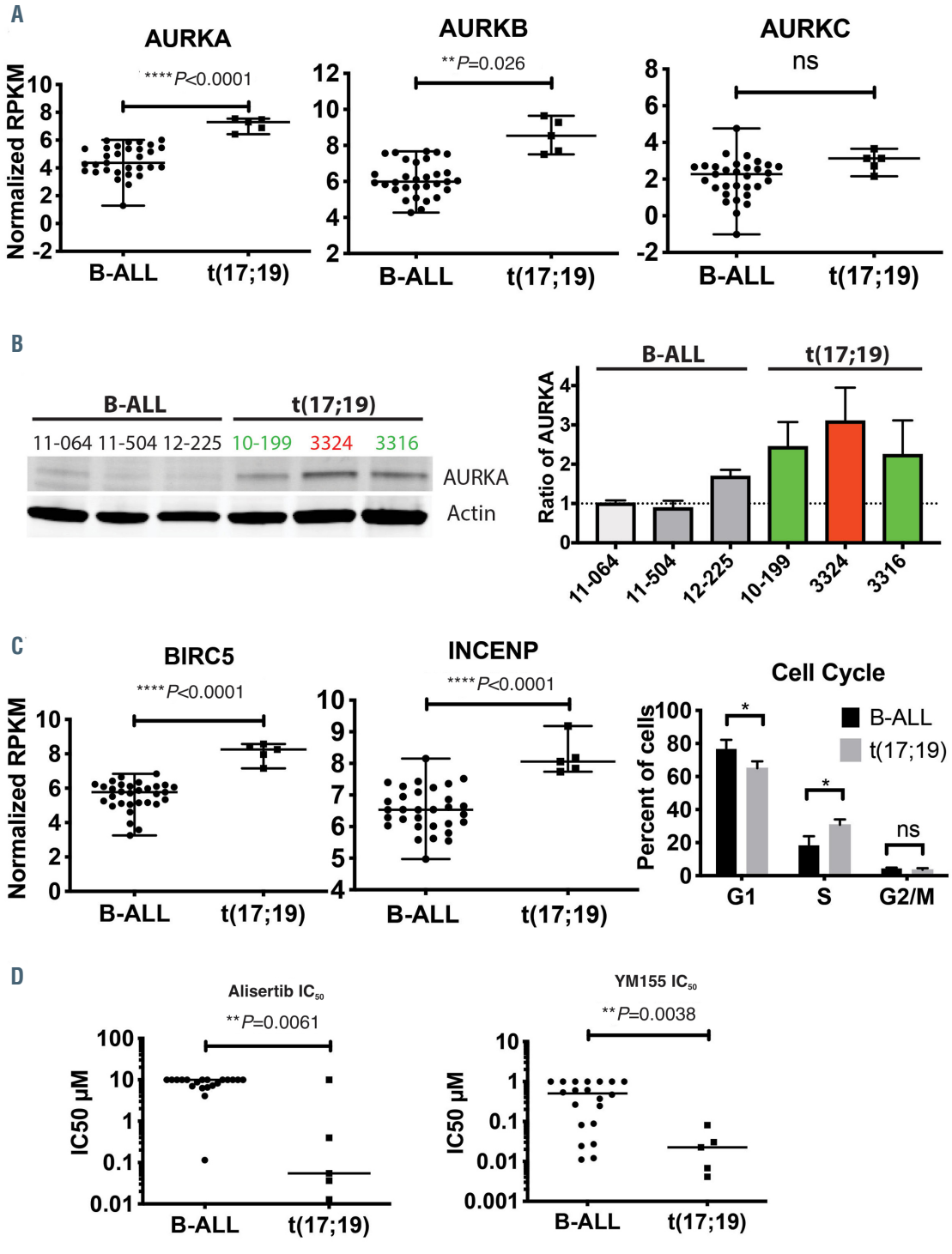

Figure 1. Sensitivity of *TCF3-HLF* ALL to AURKA inhibition. (A) Expression levels of AUK kinases within *TCF3-HLF* acute lymphoblastic leukemia (ALL) samples from RNA sequencing (RNA-seq) analysis. RPKM (reads per kilo base per million mapped reads) levels of *AURKA, AURKB* and *AURKC* were compared between t(17;19) and remaining B-cell acute lymphoblastic leukemia (B-ALL) samples by two-tailed student's *t*-test. (B) Protein expression of *AURKA* in ALL. Protein extract from three primary B-ALL xenograft samples (11-064, *TCF3-PBX1*; 11-504, *KMT2A-rearranged*; 12-225, *BCR-ABL1*) were compared to three t(17;19) *TCF3-HLF* ALL xenograft samples by immunoblot (green denotes type 1 fusions, while red denotes type 2 fusions). Cell lysate was extracted and separated by standard SDS-PAGE. Protein was then transferred to PVDF membranes and immunoblotted using anti-AURKA or anti-actin. Three independent blots for each sample were quantified and AURK level for each sample was normalized to actin. Comparison of the level of AURK to each sample was normalized to the level of AURK in 11-064. Green column indicates type 1 samples, red column indicates type 2. ANOVA ***P=0.0033. (C) Expression from RNA-seq analysis comparing RPKM levels of *BIRC5* and *INCENP* between t(17;19) and remaining ALL samples by two-tailed student's *t*-test. Graphic representation of asynchronous populations within the cell cycle. Cells were fixed in 70% methanol, then stained with propidium iodide (PI), and analyzed for DNA content by flow cytometry. Comparison of the percentage of cells in G1, S and G2/M were performed from three B-ALL xenografted samples compared to three *TCF3-HLF* ALL samples using two-tailed student's *t*-test. (D) Half maximal inhibitory concentration (IC_{50}) comparison of alisertib and YM155 between t(17;19) and remaining ALL samples by two-tailed student's *t*-test.

these two samples do not carry the *IKZF1* variant. In contrast, all animals treated with alisertib did show a significant survival advantage compared to their control counterparts.

Our results continue to confirm that *TCF3-HLF* ALL is a unique subset of ALL with varying degrees of response to prior published targets for therapy. *In vivo* validation in the murine model with our five samples suggests heterogeneity of response to current pursued targets such as BCL2 and SRC. In contrast, we found significant *in vivo* response to the AURKA inhibitor alisertib in all of our samples tested. It is interesting that prior studies using a different set of samples showed significant *in vivo* response to venetoclax[3] compared to our studies. This difference may be due to differences in the methodology used in the *in vivo* studies and/or the biology of the sam-

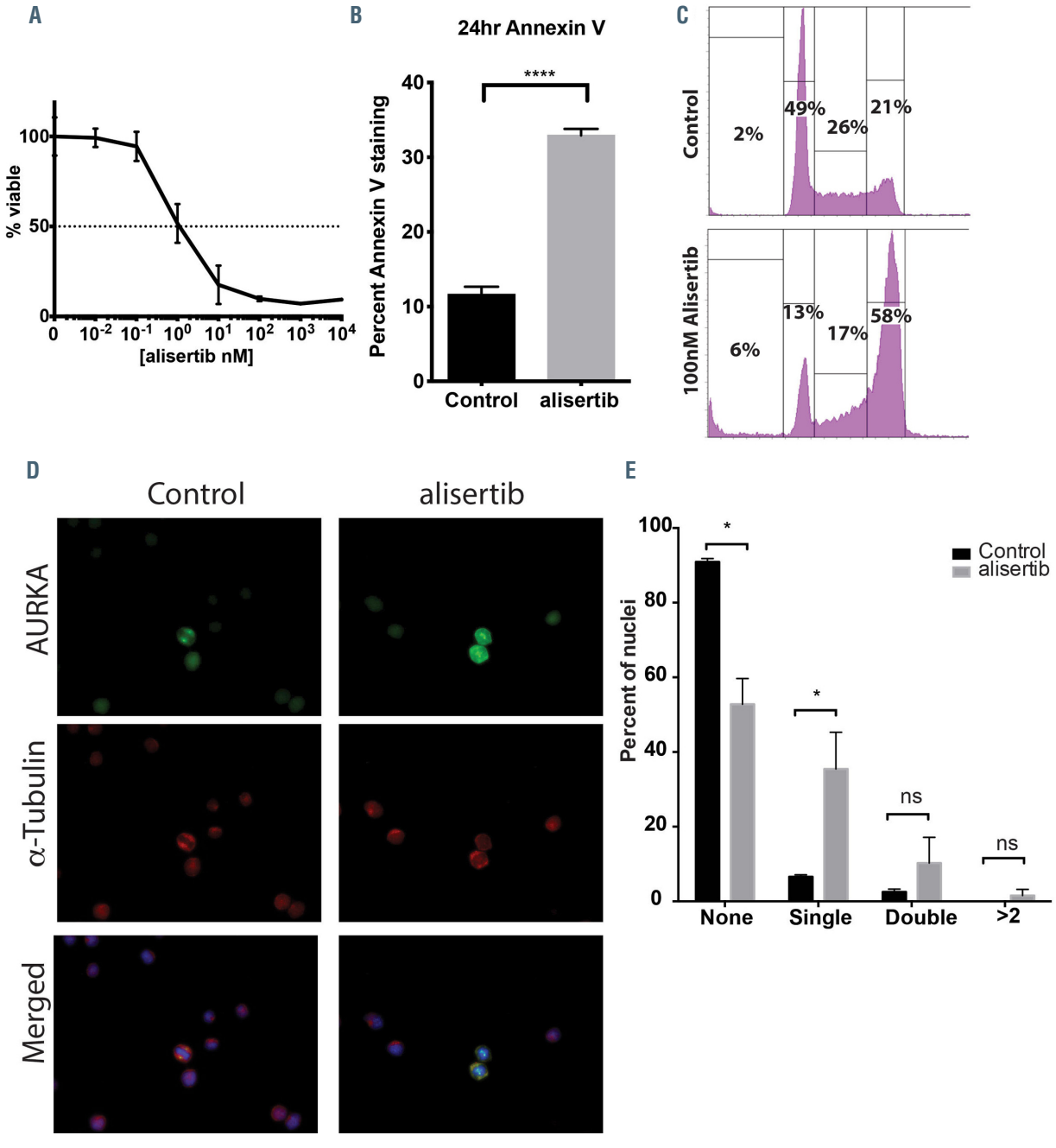

Figure 2. Alisertib effects on *TCF3-HLF* HAL01 acute lymphoblastic leukemia cells. (A) Dose-response to varying concentrations of alisertib. Cells were exposed to alisertib for 3 days then assessed for viability with MTS. Viability was normalized to untreated cells. All data points were performed in triplicate. (B) Assessment of apoptosis with annexin V staining. Cells were exposed to 100 nM alisertib (IC_{90}) for 24 hours, then assessed for annexin V staining using Guava Nexin within the population of cells. All data points were performed in triplicate and compared by unpaired student's *t*-test (*****P*<0.0001). (C) G2/M cell cycle arrest after exposure to alisertib. HAL01 cells were incubated with 100 nM alisertib for 24 hours, fixed with 70% methanol and stained with propidium iodide (PI), then analyzed by flow cytometry for PI intensity. (D) Immunocytochemical fluorescence of AURKA within an asynchronous population of cells. HAL01 cells were exposed to 100 nM alisertib for 24 hours in culture, then fixed with 4% paraformaldehyde, permeabilized with triton X-100 and stained with rabbit anti-AURKA, mouse anti-α-tubulin, secondary anti-rabbit FITC, secondary anti-mouse Texas Red, and DAPI. (E) Quantification of cells with spindle poles. Within an asynchronous population, the number of nuclei with 0 (none), 1 (single), 2 (double), or more (>2) spindle poles were counted. Each population was compared by an unpaired student's *t*-test (**P*<0.05).

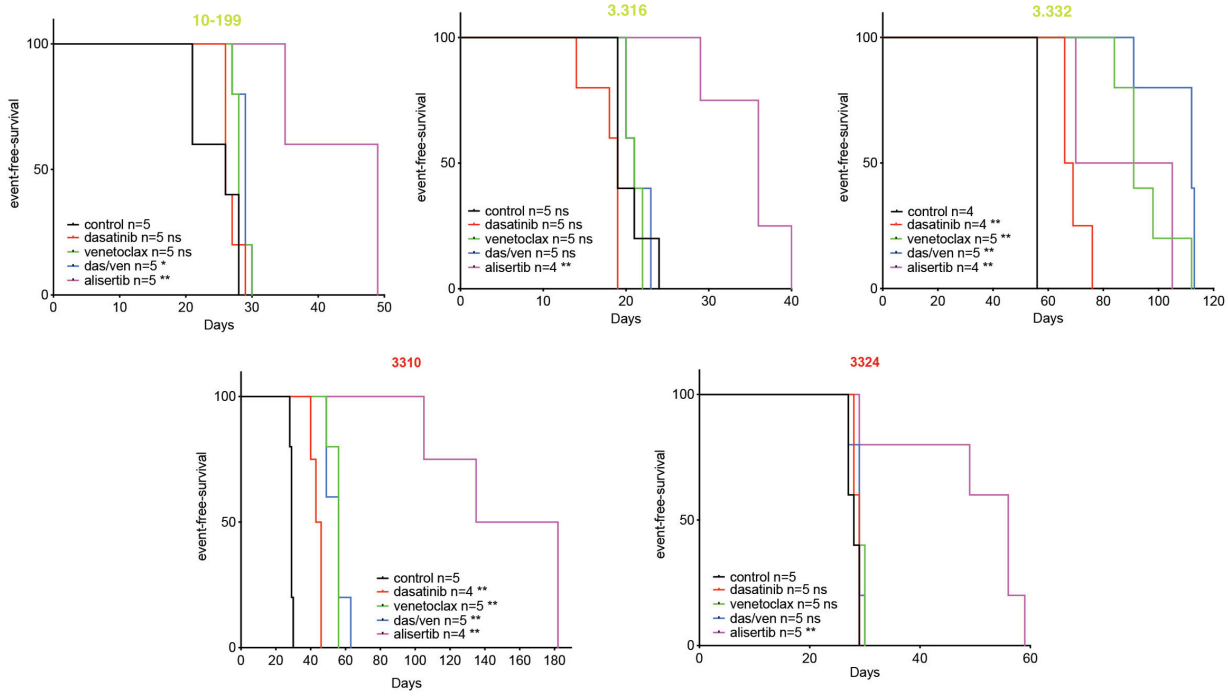

Figure 3. *In vivo* response of *TCF3-HLF* acute lymphoblastic leukemia xenografts. Kaplan-Meier plots for event-free-survival (EFS) comparing control to drug treated cohorts of five separate *TCF3-HLF* acute lymphoblastic leukemia (ALL) samples. Cohorts of five animals were injected with $1x10^6$ leukemia cells per animal by tail vein. Within the first week there was some attrition due to animal loss in samples 3.316, 3.332, and 3.310 (where n= the number of animals per cohort). One week after injection cohorts of four to five individuals began treatment with vehicle (control), dasatinib (40 mg/kg/day), venetoclax (25 mg/kg/day), combination of dasatinib (40 mg/kg/day) and venetoclax (25 mg/kg/day) (das/ven), or alisertib (30 mg/kg/day) by oral gavage for 5 days each week. Animals were monitored weekly for peripheral blood chimerism for human CD45 and murine CD45 by flow cytometry and daily well-being. An event was defined as peripheral blood chimerism ≥10% or if the animal became moribund. The top row represents type 1 *TCF3-HLF* rearrangements, while the bottom row represents type 2 rearrangements. Statistical comparison performed by log-rank test (*P<0.05; **P<0.01).

ples within each study. Further, earlier studies did not identify AURKA as a potential target, although there appear to be similar trends with their studies of other AURK inhibitors.[3,4] One very interesting observation was the sensitivity of *TCF3-HLF* ALL to YM155 both in our studies as well as Fischer *et al.*[3] Unfortunately, this compound is not currently under significant clinical evaluation making the future of this drug unknown. There are also novel immunotherapeutic targets that may be pursued in future therapies including the use of the CD19 target using blinatumomab for therapeutic response followed by stem cell transplantation.[7]

Our studies suggest that *TCF3-HLF* ALL may have clinical benefit from alisertib. This compound has been and is currently under investigation for multiple different disease types. The Children's Oncology Group (COG) phase II study (clinicaltrials gov. Identifier: ADVL0921) achieved their target plasma level, but minimal objective responses were seen with significant toxicities. Their studies concurrently ran xenograft experiments with alisertib using twice daily dosing to show similar therapeutic effects, with their concern that the dose tolerated in the murine model did not reflect what is tolerated in the patient. In contrast we used 30 mg/kg/dose daily in our xenograft studies as described in Manfredi *et al.*[15] this dose was not only well-tolerated for 21 days but had similar to improved response compared to 20 mg/kg given twice daily. These findings would suggest the dosing needed for therapeutic effect for *TCF3-HLF* ALL may be less in patients. Clearly our studies do not describe complete disease control with the single agent alisertib, rather a delay in disease progression as all xenograft animals eventually engrafted and succumbed to their disease. It remains unknown as to whether addition of this drug in combination therapies (e.g., conventional chemotherapy or other targeted agents such as venetoclax) may provide a beneficial response to select tumors that are dependent on AURKA. Because of the rarity of this tumor, it will take collaborative studies to test this compound in patients with *TCF3-HLF* ALL.

Jessica Leonard,[1] Joelle S.J. Wolf,[2] Michelle Degnin,[1] Christopher A. Eide,[1] Dorian LaTocha,[1] Kyle Lenz,[2] Beth Wilmot,[3] Charles G. Mullighan,[4] Mignon Loh,[5] Stephen P. Hunger,[6] Brian J. Druker,[1,7] Marc M. Loriaux,[8] Jeffrey W. Tyner[1,9] and Bill H. Chang[2]

[1]Division of Hematology and Medical Oncology, Department of Medicine and OHSU Knight Cancer Institute, OHSU, Portland, OR; [2]Division of Hematology and Oncology, Department of Pediatrics and OHSU Knight Cancer Institute, OHSU, Portland, OR; [3]Division of Bioinformatics and Computational Biology, Department of Medical Informatics and Clinical Epidemiology, and OHSU Knight Cancer Institute, OHSU, Portland, OR; [4]Department of Pathology, St Jude Children's Research Hospital and University of Tennessee Health Science Center, Memphis, TN; [5]Department of Pediatrics, Benioff Children's Hospital, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, San Francisco, CA; [6]Department of Pediatrics and Center for Childhood Cancer Research, Children's Hospital of Philadelphia and Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; [7]Howard Hughes Medical Institute, Chevy Chase, MD; [8]Department of Pathology and OHSU Knight Cancer Institute, OHSU, Portland, OR and [9]Department of Cell, Molecular, and Cancer Biology, and OHSU Knight Cancer Institute, OHSU, Portland, OR, USA

Correspondence:

BILL H. CHANG - changb@ohsu.edu

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