Focused chemical genomics using zebrafish xenotransplantation as a pre-clinical therapeutic platform for T-cell acute lymphoblastic leukemia

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ABSTRACT

Cancer therapeutics is evolving to precision medicine, with the goal of matching targeted compounds with molecular aberrations underlying a patient's cancer. While murine models offer a pre-clinical tool, associated costs and time are not compatible with actionable patient-directed interventions. Using the paradigm of T-cell acute lymphoblastic leukemia, a high-risk disease with defined molecular underpinnings, we developed a zebrafish human cancer xenotransplantation model to inform therapeutic decisions. Using a focused chemical genomic approach, we demonstrate that xenografted cell lines harboring mutations in the NOTCH1 and PI3K/AKT pathways respond concordantly to their targeted therapies, patient-derived T-cell acute lymphoblastic leukemia can be successfully engrafted in zebrafish and specific drug responses can be quantitatively determined. Using this approach, we identified a mutation sensitive to γ-secretase inhibition in a xenograft from a child with T-cell acute lymphoblastic leukemia, confirmed by Sanger sequencing and validated as a gain-of-function *NOTCH1* mutation. The zebrafish xenotransplantation platform provides a novel cost-effective means of tailoring leukemia therapy in real time.

Introduction

The outcome for high-risk cancers will only be improved by tailoring therapy to individual patients based on the molecular profile of their tumor. Robust pre-clinical and diagnostic platforms are necessary to provide this information in a time-sensitive and cost-effective manner so that meaningful treatment decisions can be determined. T-cell acute lymphoblastic leukemia (T-ALL) serves as an ideal disease paradigm for evaluating such a focused chemical genomic therapeutic approach due to the well-characterized spectrum of drug targetable genetic mutations in this leukemia.

T-ALL is an aggressive hematologic malignancy caused by the arrested development and differentiation of T-cell progenitors. While the overall prognosis for T-ALL patients has improved with the use of intensified chemotherapy regimens, T-ALL is often associated with primary non-responsive, high minimal residual disease (MRD) and relapse.[1-4] For patients with primary relapsed or resistant disease, additional intensification of standard chemotherapy agents leads to unacceptable toxicities, highlighting a desperate need for more molecularly-targeted agents for T-ALL and improved animal models for pre-clinical testing.[3,5]

Many T-ALL patients harbor defined genetic alterations that have emerged as attractive therapeutic targets to improve outcome. Mutations in *PTEN* are found in 20% of adult and up to 60% of pediatric T-ALL cases, and are generally associated with poor outcome.[6,7] Specifically, T-ALL patients with *PTEN* mutations have a 9-fold higher risk of having positive MRD on the ALL-Berlin-Frankfurt-Munster (BFM) 2000 trial with a trend towards inferior event-free survival (P=0.11).[8] Mutations in *NOTCH1* are found in over half of all T-ALL cases[3,9-12] and in contrast are associated with negative MRD and good early response compared to those patients without *NOTCH1* mutations. However, in the same ALL-BFM 2000 trial, amongst the group with T-ALL and *NOTCH1* mutations, 57% had unfavorable MRD early in therapy, although only a small number of these patients subsequently relapsed.[13] Compounds that function to inhibit these pathways are currently available and their efficacy is being evaluated in both pre-clinical models and in clinical trials. However, key to the use of these compounds is the identification of T-ALL patients with targetable mutations who will respond to these therapies, resulting in better outcomes and reduced treatment morbidity.

Personalized cancer therapy approaches have been developed using model organisms that either employ the direct xenotransplantation of patient derived tumors (PDX) in mice to test specific targeted therapies,[14] or utilize transgenic engineering approaches to model putative or validated tumor-associated gene mutations in organisms more amenable to high throughput drug screening, such as the fruit fly, *Drosophila*.[15] Murine models offer the ability to screen and evaluate anti-cancer agents prior to pre-clinical assessment; however, the associated cost is substantial and the time it takes to complete these studies can be extensive and not compatible with patient-directed interventions in an actionable time frame. Primary human T-ALL cells are not sustainable in culture and while T-ALL cell lines have shown utility for drug screening, they are unable to inform individual patient treatment decisions. The zebrafish has emerged as an alternative model for personalized therapy and drug screening that shares a high degree of genetic conservation with humans. Like rodent models but unlike *Drosophila*, zebrafish xeno-

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transplantation (XT) can support the use of patient diagnostic biopsy material, but are better suited to moderate throughput drug-screening than mice.[16,17] Relatively low maintenance costs and short time intervals to complete *in vivo* studies, as well as their size and transparency that facilitate analyses, make the zebrafish an attractive model for the screening of anti-cancer agents.[18] Specifically, zebrafish XT has gained considerable attention as a system to rapidly study and directly observe tumor cell behavior and drug response in a live animal model.[16,17,19-21]

In this study, we demonstrate the feasibility and utility of the zebrafish XT model to elucidate the molecular pathogenesis and drug responses of both established T-ALL cell lines and primary patient samples to drugs targeting NOTCH and the PI3K/AKT/mTOR signaling axis. Importantly, this work sets the stage for the zebrafish XT platform to serve as the first real-time *in vivo* platform for personalizing cancer therapeutics.

Methods

Cell culture and viability assay

T-cell acute lymphoblastic leukemia cell lines were cultured in RPMI 1640 and HeLa cells were cultured in DMEM, both supplemented with 10% fetal bovine serum and 100 U/mL penicillin and streptomycin (Gibco). Mononuclear cells from patient bone marrow samples were obtained by density gradient centrifugation with Lympholyte-H (Cedarlane) and were cultured in MarrowMAX bone marrow medium (Gibco) for 12 h prior to xenotransplantation. Stock solutions of triciribine (Sigma-Aldrich), rapamycin (Sigma-Aldrich) and Compound E (EMD Millipore) were dissolved in DMSO, and maintained at -20°C. Cells were cultured in the presence of vehicle (0.01% DMSO) or drug for 72 h and cell viability was determined using alamarBlue assay (Invitrogen).

Generation of constructs and luciferase assay

pMig-Notch1, pMig-Notch1ΔPEST and JH23a luciferase reporter plasmid were available in the Weng laboratory. Site-directed mutagenesis was performed by overlap extension PCR.[22] Base substitutions within the Notch1 coding sequence were performed in pMig-Notch1 to create pMig-Notch1-A1696D (GCC to GAC). HeLa cells were co-transfected using Neon (Life Technologies) with Notch1 expression constructs, a JH23a firefly luciferase reporter plasmid, and an internal control *Renilla* luciferase plasmid (Promega). Normalized firefly luciferase activities were measured in whole-cell extracts prepared 48 h after transfection with a GloMax 20/20 luminometer (Promega).

Sequencing of patient samples

Selected regions were amplified from genomic DNA by PCR. Amplified fragments were sequenced using Sanger fluorescent sequencing and capillary electrophoresis at the IWK Health Centre in Halifax, Nova Scotia, Canada. Sequence traces were analyzed using MutationSurveyor v.3.97 (Soft Genetics Inc.).

Zebrafish husbandry, embryo collection and embryo staging

casper zebrafish were maintained according to standard protocol,[23-25] collected and grown in E3 embryo media at 28.5°C. Embryos were developmentally staged according to standard protocol.

Xenotransplantation of human leukemia cells into zebrafish embryos, dissociation, and immunofluorescence

Embryos at 48 h post fertilization (hpf) were anesthetized with 0.090 mg/mL Tricaine (Sigma-Aldrich) and used for cell transplantation using a protocol adapted from Haldi *et al.*[26] and Corkery *et al.*[19] Leukemia cells (both cell lines and patient-derived cells) were labeled with CM-DiI (Invitrogen) prior to injection into the yolk sac of zebrafish embryos. Approximately 50-100 cells for cell lines and approximately 500 cells for patient samples were injected per zebrafish embryo. At 48 h post injection (hpi) for cell lines and 96 hpi for primary samples, only embryos with a uniform fluorescent cell mass at the site of injection were used for proliferation studies. A group of 15-20 embryos was sacrificed and the number of leukemia cells was determined to be the base-line number of leukemia cells prior to treatment with vehicle or drug to ensure cells engraft and proliferate in the zebrafish embryos. Groups of 15-20 embryos were treated with vehicle [0.1% (v/v) as a negative control for drug efficacy] or drug for 48 h, and then sacrificed and the number of leukemia cells determined either by enumerating *ex vivo* the number of CM-DiI-labeled leukemia cells, or by counting cells positive for PML bodies (expressed in human cells but not zebrafish cells). For more details, please see the *Online Supplementary Appendix*. Raw data are attached as an *Online Supplementary file.*

Study approval

All studies in zebrafish were approved by the Dalhousie University Committee on Laboratory Animals (Protocol 13-132). All studies using human samples were approved by the Research Ethics Board of the IWK Health Centre (File #1007549).

Statistical analysis

Analyses were performed with GraphPad Prism 5.0. For parameter comparisons between groups, an unpaired 2-tailed Student's

Table 1. Mutation profile of T-ALL cell lines.

	NOTCH1		PTEN	FBXW7	Drug Response		
Cell Line	HD	PEST			GSI	Rapamycin	Triciribine
Jurkat	WT	WT	WT Deficient	Mutated 505 R–>C	Resistant	Sensitive	Resistant
Karpas45	WT	Mutated 7378–>C T	Mutated 1003–>C T	Mutated 505–>R C	Resistant	Resistant	Sensitive
TALL1	WT	WT	WT Deficient	WT	Sensitive	Resistant	Resistant

Numbers correspond to amino acid residues in the genes of interest. WT: wild-type. (Summarized from references 27-31.)

t-test or one-way ANOVA followed by Dunnett's multiple comparison test were used when appropriate (see figure legends). $P<0.05$ was considered significant. Results are reported as mean±SEM.

Results

Three T-ALL cell lines, Jurkat, Karpas45 and TALL1, harboring defined mutations in *NOTCH1* and *PTEN* were

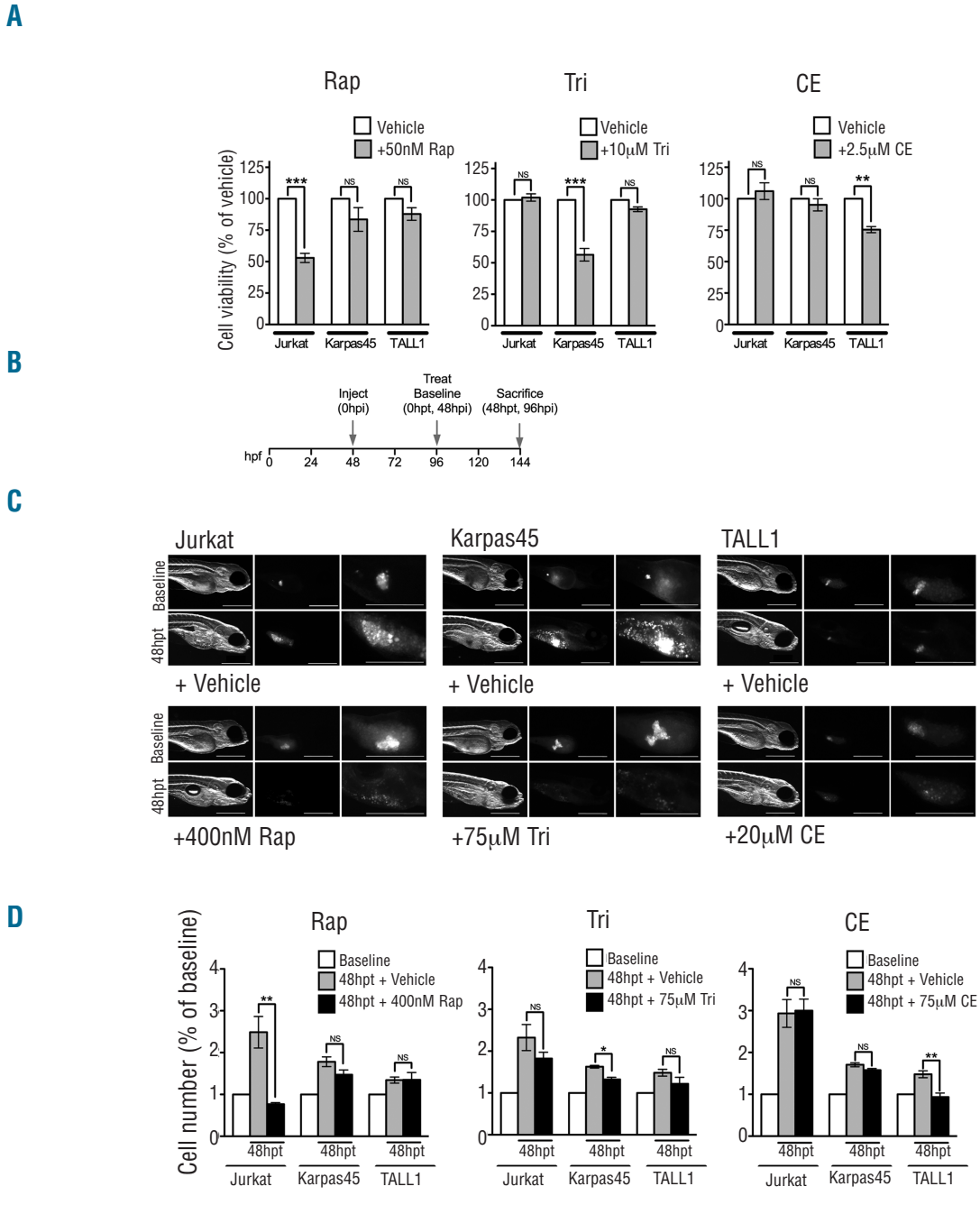

Figure 1. T-cell acute lymphoblastic leukemia cell lines harboring defined mutation in *NOTCH* and *PTEN* have differential responses in vitro and *in vivo* to inhibition of Notch (Compound E), AKT (triciribine), and mTOR (rapamycin). (A) *In vitro* cell viability assay. Cell lines were treated with drug or vehicle (0.01% DMSO) for 72 h, and cell viability determined (via alamarBlue) as a percentage of vehicle control. (B) Schematic of the *in vivo* zebrafish XT cell proliferation assay. (C) Representative brightfield and fluorescent and magnified fluorescent images (from left to right) of zebrafish embryos transplanted with CM-DiI-labeled T-ALL cell lines at 0 hpt (baseline = 48 hpi = 96 hpf) and 48 hpt (96 hpi = 144 hpf) with or without drug. (D) *In vivo* proliferation of T-ALL cell lines in the zebrafish XT model. Groups of 15-20 embryos were used for each time point and treatment, 50-100 cells were injected per fish, and the number of fluorescent cells was enumerated as described in Corkery *et al.*[19] Base-line number of cells was determined at 0 hpt and all drug treatments (at 48 hpt) are shown as a fold change of the baseline. All cell lines engrafted and proliferated in the zebrafish, as represented by the significant increase in number of leukemia cells from baseline to 48hpt with vehicle. Means+SEM; n=3; $P^*<0.05$, $P^{**}<0.01$, $P^{***}<0.001$ for significant decrease in number of cells determined using one-way ANOVA followed by Dunnett's multiple comparison test. N: number of independent experiments, with 15-20 embryos per group per experiment. Hpt: hours post treatment; hpi: hours post injection. Scale bars are 500 μM.

evaluated for their *in vitro* sensitivity to inhibitors targeting the PI3K/AKT/mTOR and Notch signaling pathways (Table 1).[27-31] These cells lines were cultured in the presence of compound E, a γ-secretase inhibitor; triciribine, an AKT inhibitor; and rapamycin, an mTORC complex 1 inhibitor. Cell viability was calculated as a percentage of vehicle control after 72 h of treatment, using an alamarBlue cell viability assay. Of the three cell lines, TALL1 (*NOTCH1* WT) was the only cell line to respond significantly to 2.5 μM of compound E. Karpas45 (*PTEN* mutated) cells responded significantly to treatment with 10 μM triciribine. Jurkat (*PTEN* mutated) cells responded significantly to treatment with 50 nM rapamycin (Figure 1A). While these *in vitro* data reveal specific responses to targeted therapeutic agents, we wanted to validate these responses in an *in vivo* context that more closely recapitulates what might occur in patients using a platform that could be adapted to rapidly evaluate the efficacy of specific compounds to patient-derived samples.

The XT of human cells into zebrafish embryos has emerged as a robust animal model system for directly observing tumor cell behavior and drug responses.[16,17,19-21] We previously demonstrated that a zebrafish human cancer XT platform can robustly detect and quantify the *in vivo* inhibition of leukemia cell proliferation using targeted therapeutics.[19] This approach exploits the conserved genetics and unique imaging opportunities afforded by the zebrafish. Determination of the maximum tolerated dose (MTD) for each of compound E, triciribine, and rapamycin was conducted in non-XT embryos. Embryos were treated with increasing concentrations of each drug, and following 48 h of treatment, were examined for viability (*Online Supplementary Table S1*). No drug treatment exceeded half the MTD (termed MTD50). Double pigment mutant *casper* zebrafish embryos[25] were injected with CM-DiI fluorescently labeled T-ALL cells at 48 h post-fertilization (hpf), and subsequently treated with an individual drug for 48 h starting at 48 h post injection (48 hpi = 96 hpf, referred to as baseline; see Figure 1B for experimental set-up). T-ALL cell lines engrafted and proliferated in zebrafish embryos [indicated by the significant increase in number of leukemia cells from baseline to 48 hpt (= 144 hpf) with vehicle qualitatively shown in Figure 1C and quantitatively in Figure 1D], and had analogous responses to *in vitro* drug treatments. Leukemia cell number was determined by dissociating groups of XT embryos and counting CM-DiI labeled leukemia cells. Previously, we have shown that engrafted CM-DiI labeled leukemia cells are viable and intact using a DRAQ5 viable nuclear stain.[19] Compound E (20 μM) inhibited TALL1 cell proliferation *in vivo* by 37% compared to vehicle, triciribine (75 μM) inhibited Karpas45 cell proliferation *in vivo* by

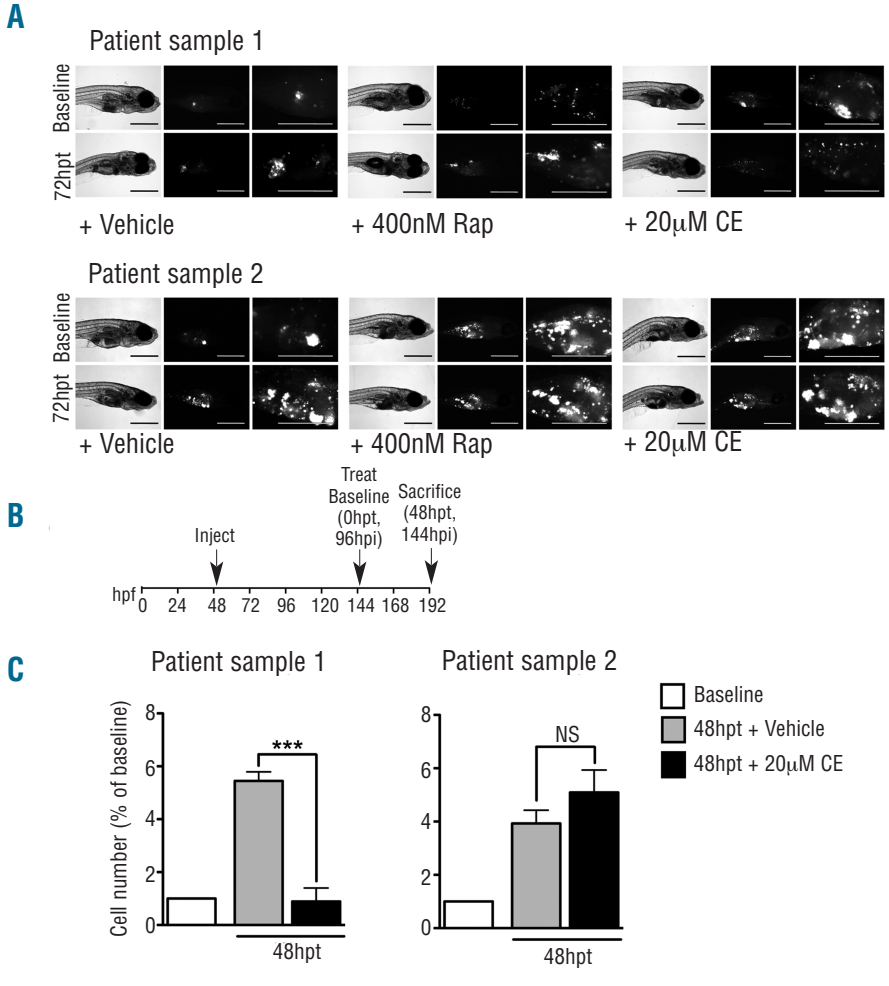

Figure 2. T-cell acute lymphoblastic leukemia patient Sample 1 responds *in vivo* to Notch inhibition with compound E, but not to mTOR inhibition with rapamycin. (A) Representative brightfield and fluorescent and magnified fluorescent images (from left to right) of zebrafish embryos transplanted with patient Samples 1 and 2 at 0 hpt (baseline) and 72 hpt with or without drug. Each embryo was xenotransplanted with approximately 500 cells. (B) Schematic of *in vivo* zebrafish XT cell proliferation assay. (C) Quantification of patient sample engraftment fold change with or without compound E treatment. Patient Sample 1 and 2 engrafted and proliferated in the zebrafish XT model (indicated by the significant increase in number of leukemia cells from baseline to 48 hpt with vehicle). Patient Sample 1 responded significantly to compound E treatment. Engraftment fold change was determined by sacrificing embryos at 0 hpt and 48 hpt and performing cytospins with dissociated embryos and immunohistochemistry for PML bodies (only present in human cells, not in zebrafish cells). Means+SEM; N: 1; $P^{*}<0.05$, $P^{**}<0.01$, $P^{***}<0.001$ for significant decrease in number of cells determined using unpaired 2-tailed Student's t-test. Groups of 15-20 embryos were used for each time point and drug treatment. Scale bars are 500 μM.

19%, rapamycin inhibited Jurkat cell proliferation *in vivo* by 59%, but significant inhibition was not observed with other cell line-drug combinations (Figure 1C and D and *Online Supplementary Figures S1 and S2*). CM-DiI labeling was retained throughout the duration of our experiments. CM-DiI labeled cells respond in an equivalent manner to targeted drug treatments *in vivo* as do XT cells stably expressing GFP (*Online Supplementary Figure S3*).

To evaluate the suitability of employing the zebrafish XT model for the personalization of patient treatment, we injected primary patient-derived T-ALL bone marrow samples obtained routinely at diagnosis. Patient 1 was a three and a half year old male who presented with bruising and hepatosplenomegaly, with an initial white blood cell count of 41.5x10^9/L, hemoglobin of 9.5 g/dL and platelets of 24x10^9/L with 74% circulating blasts. Chest X-ray demonstrated a mediastinal mass. Flow cytometry performed on the bone marrow demonstrated a blast population that was positive for CD2, CD5, CD7, CD8, CD38, CD1a, and negative for myeloperoxidase, TdT and CD79A. Patient 2 was an 8-year old female who presented with progressive respiratory symptoms and was found to have a large mediastinal mass, pleural effusion and lymphadenopathy. At presentation her white blood cell count was 30x10^9/L, hemoglobin 13 g/dL and platelet count 456x10^9/L. Bone marrow flow cytometry demonstrated a blast population that was positive for CD2, CD4, CD5, CD7, CD8, cytoplasmic CD3 and partial TdT, and negative for CD19 and myeloperoxidase. Genotype was not known for either patient.

Cells from the diagnostic bone marrow aspirates of each patient were labeled with CM-DiI prior to injection into zebrafish embryos to facilitate direct screening of targeted drugs. Approximately 500 cells were injected per embryo, and primary bone marrow samples engrafted at 96 hpi (referred to as baseline) and proliferated within the embryo (indicated by the increase in number of leukemia cells from baseline through 48 hpt to 72 hpt with vehicle). Initial qualitative fluorescent imaging of engrafted embryos showed that neither Patient Sample 1 or 2, derived from 2 different children with T-ALL, responded to treatment with rapamycin (Figure 2A) or triciribine (*data not shown*) in zebrafish xenografts, as evidenced by sustained cell proliferation in the presence of drug. However, Patient Sample 1 responded dramatically to treatment with compound E (Figure 2A), which inhibited cell proliferation *in vivo* by 84% (injection and treatment scheme shown in Figure 2B; data in Figure 2C). Patient tumor-drug response was quantified using fluorescent immunocytochemistry to promyelocytic leukemia (PML) nuclear bodies, which exclusively label human leukemia cells (*Online Supplementary Figure S4*). Thus, this *in vivo* chemical genomic approach resulted in the identification of sensitivity to γ-secretase inhibition that strongly suggested a targetable mutation in the NOTCH pathway underlying the leukemia of Patient 1. In agreement with this hypothesis, Sanger sequencing confirmed a heterozygous somatic missense mutation in *NOTCH1* (NM_017617.3) at position c.5087C>A, leading to amino acid change p.A1696D in the C-terminal heterodimerization (HD) domain (Figure 3A). This mutation was previously reported in separate studies in 2 different patient samples; [27,32] however, this is the first time that sensitivity to inhibition of γ-secretase has been directly correlated with this mutation. The biopsy sample from Patient 1 did not have mutations in the *FBXW7* or *PTEN* genes. This mutational status (WT *FBXW7* and WT *PTEN* with p.A1696D *NOTCH1* mutation) was identified in an additional patient sample reported by Kalender et al., suggesting that this mutation alone may be sufficient to drive T-ALL pathogenesis.[27] Mutations in the HD domain of *NOTCH1* are reported in nearly half of all T-ALLs and have been associated with *NOTCH1* activation that remains dependent on γ-secretase complex activity.[33] Ectopic expression of *NOTCH1* carrying the A1696A/D mutation enhanced luciferase expression to a similar extent to a well known activated *NOTCH1* mutant carrying a PEST domain deletion, validating the patient mutation as a gain-of-function mutation in *NOTCH1* (Figure 3B).[34] Consistent with the zebrafish XT results indicating an insensitivity to compound E, sequencing did not reveal mutations in *NOTCH1* in Patient Sample 2.

Discussion

The zebrafish is a powerful and genetically tractable model to study human malignancies, as important signaling pathways regulating cell proliferation, migration, apoptosis and differentiation are well conserved.[35,36] In this study, we have demonstrated the feasibility and utility of the zebrafish as a human cancer xenotransplantation (XT) model and a novel tool for the rapid identification of

A

	Gene sequenced			
	FBXW7	***IL7-R***	***NOTCH1***	***PTEN***
PS 1	**WT**	**WT**	**c.5087c>CA p.A1696A/D**	**WT**
PS 2	**WT**	**WT**	**WT**	**WT**

B

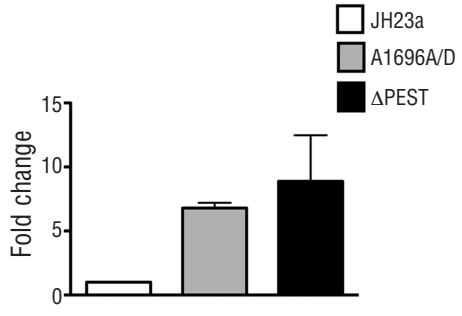

Figure 3. Patient Sample 1 harbors a rare *NOTCH1* mutation. (A) Results from Sanger sequencing of patient samples. Patient Sample 1 (PS 1) had a heterozygous mutation in the heterodimerization (HD) domain of the *NOTCH1* gene (p.A1696D). Patient Sample 2 (PS 2) did not have any mutations in the genes sequenced. (B) p.A1696D mutated *NOTCH1* up-regulates luciferase reporter activity. HeLa cells transfected with JH23a Notch reporter plasmid, Renilla, and+Notch with A1696A/D or the PEST mutation. P.A1696D mutated Notch plasmid up-regulated luciferase reporter activity to a similar degree as constitutively active Notch (with PEST mutation). Means+SEM; N=3; N: biological replicates.

underlying targetable gene mutations, as well as a pre-clinical model well suited to the assessment of known and novel therapeutics for T-ALL.

We demonstrated that human T-ALL cell lines engraft and proliferate in zebrafish embryos, and recapitulate drug responses seen *in vitro*. More importantly, we were able to successfully engraft biopsy material from 2 children with T-ALL in zebrafish embryos without need of immunosuppression, and these human leukemia cells robustly proliferated throughout the course of the experiment. This represents an unprecedented technological advancement over challenging *in vitro* methods for culturing primary patient samples and the formidable task of trying to grow primary patient xenografts in highly immunosuppressed and humanized mice. The ability to engraft sorted human acute myeloid leukemia cells in zebrafish embryos has been previously reported by one study; however, drug-sensitivity was not evaluated nor prolonged engraftment demonstrated.[37] Our efforts go far beyond this previous study by bringing this approach into the clinic and xenografting diagnostic bone marrow samples from children with T-ALL under our care. Moreover, we also demonstrate for the first time that cell proliferation of primary T-ALL can be quantified *ex vivo*, and that the response of a patient's leukemia to targeted therapeutics can be accurately and rapidly determined using this method to define the molecular underpinnings of an individual patient's leukemia using a focused chemical genomics approach. Specifically, as proof-of-principle, we determined a patient's leukemia response to compound E using zebrafish XT, leading to the identification of a targetable *NOTCH1* mutation in Patient Sample 1. While these results did not inform patient treatment in this particular case (both children are on treatment and currently in remission in the maintenance phase of therapy), we note that it was possible to obtain definitive and actionable mutation status to support the potential efficacy of treatment with a γ-secretase inhibitor within just one week following biopsy.

Mouse xenografts currently represent the gold standard pre-clinical assay for evaluating human tumor responses, but are both arduous and costly. Moreover, they require specialized immunosuppressed strains with modifications to variable components of the adaptive and innate immune systems to prevent graft rejection. These highly modified hosts may significantly influence the behavior of cancer cells and affect drug responses leading to results that do not translate to the human patient population. By contrast, the zebrafish XT assay does not require immuno-compromised fish when performed early in development. It is both cost-effective and rapid, providing clinically actionable drug-tumor response data in days rather than the months required for similar murine studies. Zebrafish XT can also be applied early in the drug developmental pipeline to screen and prioritize prospective compounds prior to more definitive pre-clinical testing in rodents and human trials. Thus, while mouse XT responses may influence the drugs to be prioritized in future trials, the zebrafish XT response can uniquely provide data to personalize therapy for a particular patient in real time. Furthermore, the transparency of the zebrafish embryo and the ability to visualize components of the microenvironment through fluorescent labeling, has the opportunity to shed light on how specific therapies impact the tumor niche in addition to the malignant cells.[17,38]

The zebrafish XT approach presents the first *in vivo* method for performing meaningful drug response assays on patient-derived biopsy material in an actionable time frame that can inform clinical decision-making. The opportunities inherent in this platform are provocative and aligned with current efforts to provide more personalized and precise cancer therapies.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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