Relapse-Fated Latent Diagnosis Subclones in Acute B Lineage Leukemia Are Drug Tolerant and Possess Distinct Metabolic Programs

Stephanie M. Dobson1,2, Laura García-Prat2, Robert J. Vanner1,2, Jeffrey Wintersinger3, Esmé Waanders4,5,6, Zhaohui Gu6, Jessica McLeod2, Olga I. Gan2, Illdiko Grandal7, Debbie Payne-Turner6, Michael N. Edmonson8, Xiaotu Ma8, Yiping Fan8, Veronique Voisin1,3, Michelle Chan-Seng-Yue2,10, Stephanie Z. Xie2, Mohsen Hosseini2, Sagi Abelson2, Pankaj Gupta8, Michael Rusch8, Ying Shao11, Scott R. Olsen12, Geoffrey Neale1,2, Steven M. Chan2,10, Mark D. Minden2,15, Quaid Morris1,3,9,16, Charles G. Mullighan6, and John E. Dick1,2

ABSTRACT

Disease recurrence causes significant mortality in B-progenitor acute lymphoblastic leukemia (B-ALL). Genomic analysis of matched diagnosis and relapse samples shows relapse often arising from minor diagnosis subclones. However, why therapy eradicates some subclones while others survive and progress to relapse remains obscure. Elucidation of mechanisms underlying these differing fates requires functional analysis of isolated subclones. Here, large-scale limiting dilution xenografting of diagnosis and relapse samples, combined with targeted sequencing, identified and isolated minor diagnosis subclones that initiate an evolutionary trajectory toward relapse [termed diagnosis Relapse Initiating clones (dRI)]. Compared with other diagnosis subclones, dRIs were drug-tolerant with distinct engraftment and metabolic properties. Transcriptionally, dRIs displayed enrichment for chromatin remodeling, mitochondrial metabolism, proteostasis programs, and an increase in stemness pathways. The isolation and characterization of dRI subclones reveals new avenues for eradicating dRI cells by targeting their distinct metabolic and transcriptional pathways before further evolution renders them fully therapy-resistant.

SIGNIFICANCE: Isolation and characterization of subclones from diagnosis samples of patients with B-ALL who relapsed showed that relapse-fated subclones had increased drug tolerance and distinct metabolic and survival transcriptional programs compared with other diagnosis subclones. This study provides strategies to identify and target clinically relevant subclones before further evolution toward relapse.

See related article by E. Waanders et al.
INTRODUCTION

Despite significant advancements in the treatment of acute lymphoblastic leukemia (ALL), the disease recurs in 15% to 20% of pediatric and 40% to 75% of adult patients, with the prognosis for patients who relapse being dismal (1–3). Analysis of paired diagnosis and relapse ALL samples with increasingly broader and deeper genomic-sequencing methods has shown that classic Darwinian branching evolution of genomically distinct subclones is a hallmark of disease recurrence (4, 5). At both diagnosis and relapse, a single neoplasm may contain multiple genetic subclones related to each other through complex evolutionary trajectories (4–7). Although relapse may evolve from the predominant clone at diagnosis, in the majority of patients relapse arises from preexisting minor subclones within the diagnosis sample or from a rare ancestral clone (4–6). Although the population-level genetic analyses upon which these conclusions are based rely on computational inference of their evolutionary relationships from analysis of bulk leukemic cells, resolution of leukemic subclones at clonal levels, either through isolation of subclones in xenografts or from single-cell analysis, has largely substantiated these predictions (8, 9). Functional studies to explain therapy failure have mainly been undertaken by comparing diagnosis cells with those from relapse and identifying drug-resistance mechanisms present in relapse and absent at diagnosis. However, the properties of the relapse samples have been shaped by exposure to chemotherapy, causing further evolution and mutagenesis. Thus, two critical questions remain: What are the unique properties and mechanisms that contribute to the relapse fate of a particular diagnosis subclone prior to full evolution to drug-resistant relapse disease, and when does drug tolerance arise? Drug tolerance may arise stochastically through genetic or epigenetic mechanisms prior to exposure to therapy, and be selected for by both cell-autonomous and non–cell autonomous processes (10–13). Alternatively, therapy may induce genomic aberrations that are then selected for during disease progression, particularly if such alterations reduce leukemic cell fitness (14) during disease establishment and prior to the administration of therapy (14, 15, 16). Without isolation of the subclones that contribute to disease progression from diagnosis samples, it is not possible to answer these questions and...
uncover the cellular and molecular properties that explain their differing subclonal fates and drug tolerance.

Many therapy resistance mechanisms have been implicated in B-progenitor ALL (B-ALL), including acquisition of stemness programs, dormancy, the protective role of the niche, and the acquisition of resistance driver mutations (14, 17–21). However, the ability to evade drug treatment is only one prerequisite of relapse; surviving cells must also possess significant clonal regenerative capacity to regrow or reproduce disease. For many human cancers, only rare fractions of malignant cells are capable of significant clonal propagation as detected by xenograft-based cancer or leukemia-initiating cell (L-IC) assays (10). Indeed, methods to propagate primary human leukemia samples were first undertaken in B-ALL with patient-derived xenografts (PDX) recapitulating many features of the patient’s disease (22, 23). Subsequent studies used a limiting dilution approach to generate xenografts, thereby tracking the growth properties and genetic identity of single L-ICs (9). Thus, depending on the cell dose transplanted, rare subclones with poorer competitive repopulation properties, compared with more aggressive or numerous L-ICs within the sample, could be identified. By genetic analysis of such xenografts, evidence was found for the existence of ancestral and/or minor subclones, proving that branching evolution and clonal diversity occur at the level of L-ICs; however, these studies did not test for relapse-fated subclones (8, 9). Conceptually, L-ICs with the capacity for clonal propagation should serve as the units of selection during disease progression because only mutations accumulating in clonal propagating cells are relevant for further disease evolution. Thus, the relationship between disease progression and properties of stemness is an active area of investigation (10, 24). The pairing of xenografting assays with genomic studies provides a unique opportunity to enrich for the cellular reservoirs of relapse. However, a direct test of this concept through paired diagnosis and relapse xenografting in B-ALL has been limited (23, 25, 26). Paired studies in acute myeloid leukemia (AML) and T-lineage acute lymphoblastic leukemia (T-ALL) have demonstrated that xenografts can capture distinct subclones present within the diagnosis samples of relapsing patients, some genetically more closely related to the predominant diagnosis subclone and others to a corresponding relapse sample (27–30). These results suggest that latent relapse-initiating subclones possess competitive growth advantages when assessed by xenografting, but remain suppressed by the predominant clone in the patient, making them difficult to study.

Here, we undertook a combined functional and genomic analysis of 14 genetically distinct paired diagnosis and relapse B-ALL patient samples to isolate latent subclones within the diagnosis sample that initiate relapse. Isolation of subclones enabled functional analysis of their growth and drug response properties as well as molecular analysis of their transcriptomic profiles.

RESULTS

Isolation of Relapse-Initiating Subclones in B-ALL

Whole-exome sequencing (WES) and SNP microarray analysis of 6 adult patients and 8 pediatric patients with B-ALL of varying genetic subtypes were undertaken to identify somatic single-nucleotide variants (SNV), insertion–deletion mutations (indels), and DNA copy-number alterations (CNA). The patients encompassed a range of cytogenetic subtypes and varied in the length of their disease remission (range 5.88–94.8 months; Supplementary Table S1). Diagnosis samples had a median of 24 somatic SNV/indels (range 7–100) and 13.5 CNA (range 1–51), whereas relapse samples contained a median of 39.5 SNV/indels (range 22–405) and 16.5 CNA (range 2–58; Supplementary Table S1). Leukemic variants were confirmed by targeted sequencing using a custom capture array of the variants identified by WES (Fig. 1A). Targeted sequencing and WES data were merged, resulting in a coverage of approximately 350×. Computational analysis of the variant allele frequencies (VAF) of leukemic variants comparing diagnosis and relapse samples predicted that the origin of relapse arose from a minor subclone present at diagnosis in 10 patients (patients 1, 3–7, 9, and 12–14; Fig. 1B; Supplementary Table S1) and through further evolution of the major diagnostic subclone in 4 patients (patients 2, 8, 10, and 11; Supplementary Table S1). Broadly, these findings of evolutionary origins are representative of the much larger analysis of 92 paired samples that reports the mutational landscape and patterns of clonal evolution of relapsed childhood ALL as described in Waanders and colleagues (7).

To gain insight into the genetic diversity at the level of L-ICs and uncover rare and/or outcompeted clones, purified leukemic blasts from primary diagnosis and relapse samples were transplanted intrafemorally in a limiting dilution assay into 872 NOD.CB17-Prkdcscid/Il2rgm183Szj (NSG) mice to generate primary PDXs. The frequency of L-ICs varied widely between samples, with L-IC ranging corresponding to those described previously (31, 32); however, paired analysis between diagnosis and relapse samples did not show a consistent trend in L-IC enrichment at either time point (Supplementary Table S2). This collection of PDXs that were engrafted [n = 402, average 28 per patient (range 11–58), 13 per sample (range 0–27)] was used to further study the clonal landscape present in the patient samples. To determine whether PDXs captured the clonal diversity and disease evolution present in the patient samples, human cells were isolated from the bone marrow (BM), spleen, and central nervous system (CNS) of engrafted mice and subjected to targeted sequencing using the custom capture array designed for the patient samples (Fig. 1A). PDXs with sufficient human engraftment for genotyping (>10% human chimerism, 372 PDXs total of 402 engrafted PDXs, average 26 PDXs per patient) were analyzed. Individual PDXs from the same patient sample were often found to vary in their clonal composition in terms of the presence and frequency of variants, suggesting that the L-ICs initiating the grafts derived from genetically diverse subclones often capture the totality of the clonal diversity present in the diagnosis patient sample (Fig. 1C).

Leukemic variants were classified on the basis of the VAF of the variants in the bulk patient diagnosis and relapse samples from which the PDXs were generated: preserved variants (VAF > 30% in both diagnosis and relapse samples, or
Characterization of Relapse-fated Clones in Diagnosis B-ALL

Figure 1. PDXs capture clonal diversity present in paired diagnosis and relapse B-ALL samples. A, Experimental schematic. PDXs were generated for 6 adult and 8 pediatric B-ALL patient samples at diagnosis and relapse by intrafemoral transplantation of sorted leukemic blasts into 30 irradiated NSG mice in a limiting dilution assay. Mice were sacrificed 20 to 30 weeks post-transplant and their engraftment was assessed by flow cytometry. Patient samples were also subjected to genomic analysis including whole-exome sequencing (WES). Variants identified from the WES of patient samples at either time point were used to create custom capture baits for targeted sequencing at a deeper depth in the patient samples and their corresponding PDX. PDXs representing varying clones were identified. B, Schematic representation of the results obtained by mutational clustering of variants based on the variant allele frequencies (VAF) at diagnosis (x-axis) and relapse (y-axis) of patient 1 in 2-D VAF plots showing evolution from a minor subclone as depicted. Each dot represents a variant. Shared variants are shown in gray clusters (clusters a and c). Diagnosis- and relapse-specific variants are shown in the blue cluster (cluster d) and red clusters (clusters d and e), respectively. C, Heat map of the VAFs of leukemic variants at diagnosis, relapse, and in their corresponding PDX for patient 1. Variant classes are labeled with their class and a letter corresponding to the clusters illustrated in B.
Dobson et al.

preserved between samples); diagnosis-specific variants (present at diagnosis and absent at relapse); latent variants (present at diagnosis with VAF < 30% and increasing at relapse); relapse-specific variants (absent at diagnosis and present at relapse; Supplementary Table S3). The limit of detection of our combined sequencing was a VAF of 1%. This analysis revealed three patterns of engraftment in PDXs derived from diagnosis samples (termed dPDXs). In 10 of 13 engrafting diagnosis samples (76.9%, patients 1–7, 9, 12, and 14), latent variants were enriched in dPDXs (>10% increase in VAF in dPDXs) as compared with the diagnosis patient sample demonstrating the regenerative potential of clones marked by these variants (Fig. 2A; Supplementary Fig. S1A and S1B). In 4 of 13 patients (patients 5, 6, 11, and 13), dPDXs were generated where leukemia cells bore relapse-specific variants establishing their existence within the diagnosis sample, despite being at levels below the detectable limit of the sequencing in the patient sample (Fig. 2B). Finally, in one patient (patient 8), whose relapse was predicted to evolve from the major diagnosis clone based on analysis of the bulk patient samples, only diagnosis clones engrafted in dPDXs (Supplementary Fig. S1C). This patient carried an ETV6–RUNX1 translocation and had the longest remission, relapsing 8 years after the initial B-ALL diagnosis. Our approach of generating PDXs with differing cell doses was instrumental in showing the existence of subclones at diagnosis that bear latent (patient 2) or relapse-specific variants indicative of ancestral clones (patient 11) in 2 patients in which genetic analysis of bulk diagnosis and relapse samples had predicted evolution from the major diagnosis clone (Fig. 2B; Supplementary Table S3). Only one diagnosis patient sample (patient 10), a sample predicted to arise from evolution of the major diagnostic clone, did not engraft at the highest transplanted cell dose (250,000 cells). Therefore, xenografting added considerable new insight into the subclonal repertoire of L-ICs in these patients and their evolutionary fates and patterns.

**Genetic Analysis of Xenografts Provides Insight into the Dynamics of Subclone Evolution**

To gain insight into the evolutionary relationships and processes underlying the divergence of subclones, we undertook two approaches: population phylogenetic analysis to examine the genetic similarity between xenografts and patient samples; and generation of mutational trees to reconstruct the clonal hierarchies. We began by using a population genetics approach where phylogenetic analysis was performed for the patient samples and the clones isolated from their PDXs to depict the evolutionary relationships between xenografts and the patient samples (Fig. 3A and B; Supplementary

**Figure 2.** PDXs enrich for latent diagnosis clones. A and B, Heat maps of VAF of the SNV and indel leukemic variants identified by whole-exome and targeted sequencing in diagnosis/relapse patient samples and PDXs, respectively. Variants are clustered as preserved (present in diagnosis and relapse patient samples), diagnosis specific (present in diagnosis patient sample and absent in relapse patient sample), latent (present in diagnosis patient sample with VAF < 0.3 and expanding in relapse sample), and relapse specific (present in relapse patient sample and absent in diagnosis patient sample). PDXs are ordered in decreasing number of transplanted cell doses. A, Representative heat map for the selection of latent variants in diagnosis PDX as observed in patient 9. B, Representative heat map of patient 11 displaying the identification of a relapse-specific variant undetectable in the patient diagnosis sample but present in diagnosis PDX.
Characterization of Relapse-fated Clones in Diagnosis B-ALL

Genetically Diverse Subclones Have Differing Xenograft Repopulation Kinetics

The mutational analysis of the xenografts performed using PairTree helped illuminate the competitive differences in xenograft repopulation kinetics of specific subclones. We compared the predominant diagnosis mutational populations in the patient sample to the mutational populations that engrafted in the xenografts. In all but four patient samples, the dPDX captured the diversity of clones present in the diagnosis samples (Fig. 4; Supplementary Figs. S2A–S2C, S3A–S3C, S4A–S4E, and S5A–S5C). In 2 of these 4 patients (patients 7 and 11), minor diagnosis-specific mutational populations, including a population harboring missense NRAS mutations in patient 7, did not engraft in the dPDX mice (Supplementary Fig. S4A; Supplementary Table S3). In the other two patients (patients 9 and 12), all the dPDXs were initiated from a minor population in the diagnosis sample ancestral to the relapse (Fig. 4E and F; Supplementary Figs. S4C and S5A). These data showed the enhanced competitive repopulation properties of these dRI clones. In patient 9, all dPDXs were initiated from mutational population 5 that corresponded to only 22% of the diagnosis leukemic cells (initially described as population E in the patient sample alone mutational tree) to the patient samples from which they were derived, such as dPDX7 in patient 1 whose clonal composition is more closely related to the relapse than the diagnosis sample (Fig. 4C and D). In addition, the PDXs aided in subdividing single mutational populations into more than a single linearly related population or branch in 11 of 14 patients. For example, population G in patient 9 was expanded into two separate populations (populations 8 and 9) when PDXs were included (Fig. 4E and F; Supplementary Figs. S2A–S2C, S3A–S3C, S4A–S4E, and S5A–S5C).

Figure 3. PDXs identify relapse-fated clones in diagnosis patient samples. Phylogenetic analysis showing relationship of patient 9 (A) and patient 11 (B) patient samples and xenografts, based on VAF of leukemic variants. The distances between symbols on the tree were estimated by a nearest neighbor joining method and represent the degrees of relation between them (Minkowski’s distance). Circles represent patient samples and triangles represent PDX; blue represents diagnosis and red represents relapse. Diagnosis clones on the trajectory to relapse were termed dRI and are indicated by a box with a hatched border.
in comparison with the mutational population 2 lineage that represented 71% of the diagnosis leukemic cells (initially described in population B; Fig. 4E and F). In the dPDXs, population 5 displayed a selective advantage representing at least 93% of the leukemic cells, outcompeting the population 2 lineage (encompassing populations 2–4) and recapitulating the evolutionary dynamics of the patient relapse, where 98% of the leukemic cells came from the population 5 lineage (Fig. 4F). In contrast, in 5 patients (patients 1, 4, 6, 7, and 11), the relapse-fated mutational population represented only a small percentage of the engrafting cells in most dPDXs and only rose to predominance in rare mice. For example, in patient 11, relapse-fated mutational population 3, initially described in population C, corresponded to only 1% of leukemic cells at diagnosis and remained at similarly low levels in most dPDXs. However, in two dPDXs transplanted with a low, near limiting cell dose (10,000 cells), population 3 rose to 92% and 74% of leukemic cells, distinguishing it from population 4 and highlighting the importance of the limiting dilution approach to capture rare subclones by isolating them from other subclones with higher competitive capacity (Fig. 4G and H; Supplementary Fig. S4E). Population 3 was defined by a single variant in the 3′UTR of AHNAK, a gene whose expression has previously been implicated in disease relapse in T-ALL (33). Thus, the xenografting strategy permitted integration of functional and genomic information, providing insight into the distinct competitive growth properties of functionally defined L-IC subclones. Taken together, the phylogenetic analysis and mutational evolutionary trees provide strong evidence that relapse-fated subclones were already present in the diagnosis sample, confirming prior studies of several human acute leukemic diseases and in vitro predictions (4, 5, 28, 29).

Genetically Diverse Subclones Have Differing Immunophenotypic and Migratory Properties

To further examine whether genetically distinct subclones also possessed variation in their immunophenotype or functional properties that might explain why some are fated to contribute to relapse and others are not, we examined their differentiation, growth, and migration properties. We first interrogated the differential properties of the diagnosis clones isolated from patient 9 in which the presence of latent variants, including the known relapse driver CREBBP (21), was segregated between clones. Differences in immunophenotype were observed for dRI-PDXs enriched for these latent variants that were present at low VAFs in the diagnosis patient sample (1%–11%; Fig. 5A and B). The dRI-PDXs exhibited a CD45^−CD34^+ immunophenotype reflective of the relapse sample, whereas others contained both a CD45^−CD34^− and a CD45^−CD34^+ population as observed in the diagnosis patient sample (Fig. 5A). Relapse PDX (rPDX), like the relapse sample itself, were CD45^− (Fig. 5A). dPDXs that were engrafted primarily with CD45^− or both immunophenotypic populations had a genotype that was more closely related to the diagnosis sample (Fig. 5B). The differences noted in immunophenotypic appeared to reflect the rise of the relapse-fated clone and aided in identifying different subclones present within a single xenograft. To validate that the difference in immunophenotype segregated the predominant diagnosis clone from the minor subclone that seeded the relapse, we isolated the two immunophenotypic populations by flow-sorting cells from 6 dPDXs and subjected the populations to targeted sequencing using the custom capture array designed for the patient samples. This analysis confirmed the enrichment of the dRI clone in the CD45^− population, whereas latent variants were absent or very rare (<6%) in the CD45^−CD34^+ population (Fig. 5B). Of note, change in CD45 expression between diagnosis and relapse timepoints was identified in 5 of our patient samples. PDX from patient 9 showed markedly reduced leukemic dissemination from the injected femur to other hematopoietic sites (Supplementary Fig. S6A).

The ability to use differences in immunophenotype to segregate clones enabled us to select evolutionarily related dRI subclones for RNA-sequencing (RNA-seq) analysis from patient 9. We compared the expression profiles of two dRI clones, an ancestral and a daughter clone (clones 1 and 2, respectively), to a representative relapse clone (clone 3). The dRI clone most genetically similar to the relapse, bearing latent leukemic variants including 3 variants in CREBBP (2 missense and 1 silent) as well as variants in TCS2, NRAP, PLXNA4, and PRINS (dRI-PDX clone 2) had an expression profile very closely related to representative relapse clones with only 24 differentially expressed genes (Fig. 5C and D; Supplementary Table S4). In comparison, the ancestral clone not harboring CREBBP (dRI-PDX clone 1^{CREBBP_WT}) showed 479 differentially expressed genes compared with the relapse clones, suggesting that the majority of transcriptional
**A**

Patient 9

Diagnosis | Relapse
---|---

Pt. 9 dPDX

dPDX 1 | dPDX 2 | dPDX 3 | dPDX 4 | dPDX 5 | dPDX 7 | dPDX 12

Pt. 9 rPDX

rPDX 1 | rPDX 2

**B**

Latent Variants

Unsorted | CD45<sub>dim</sub>CD34<sup>+</sup> | CD45<sub>neg</sub>CD34<sup>+</sup>

VAF

0.0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5

**C**

<table>
<thead>
<tr>
<th>Genes</th>
<th>dRI-PDX clone 1&lt;sup&gt;CREBBP_WT&lt;/sup&gt; vs. dRI-PDX clone 2 FDR q-value ≤0.05</th>
<th>dRI-PDX clone 1&lt;sup&gt;CREBBP_WT&lt;/sup&gt; vs. rPDX clone 3 FDR q-value ≤0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene 1</td>
<td>Relative expression</td>
<td>Relative expression</td>
</tr>
</tbody>
</table>

**D**

Edge: Overlapping genes between gene sets

Jaccard overlap combined: 0.375

- Green: Gene sets enriched in clone 1 clones
- Purple: Gene sets enriched in clone 2 and/or clone 3
- Black: AutoAnnotate clusters

**E**

Percentage of human engraftment (%CD19<sup>+</sup> or %CD10<sup>+</sup>) in PB (%)

Weeks

0 | 5 | 10 | 15 | 20 | 25

Published OnlineFirst February 21, 2020; DOI: 10.1158/2159-8290.CD-19-1059
changes that are seen at relapse had already occurred in dRI-PDX clone 2 at diagnosis prior to exposure to chemotherapy (Supplementary Table S4). Broadly, the changes in gene expression of dRI-PDX clone 2 in relation to dRI-PDX clone $^{1\text{CREBBP_WT}}$ centered on deregulation of histone variants and inflammation-related genes as well as downregulation of genes involved in morphogenesis (TGFβ signaling, GATA3), the T-cell receptor and B-cell receptor pathways (CD19, CD79A, CD4, etc.) and antigen processing and presentation pathway, while heatshock response (HSR) and unfolded protein response (UPR) pathways were enriched (Supplementary Table S4). Thus, the early acquisition of additional leukemic variants in this relapse-fated subclone, including the CREBBP mutations, caused significant changes in gene expression. Interestingly, tyrosine kinases such as EPHB2, LTK, and ERBB2 were upregulated after acquisition of CREBBP variants in both dRI-PDX clone 2 and the relapse clone, suggesting possible vulnerabilities to tyrosine kinase inhibitors. Despite this striking change, the dRI subclone remained dormant within the patient for many years, as this patient displayed a long remission (4 years).

PDXs from the majority of samples showed extensive migration of leukemic cells to other hematopoietic sites and other tissues including the spleen, CNS, and peripheral blood (Supplementary Fig. S6B and S6C). Because patients with B-ALL may present with leukemic dissemination to the CNS and testes where they can provide a sanctuary for relapse, we investigated whether there were differences in the dissemination of subclonal populations to each site. Targeted sequencing analysis showed that there was genetic discordance between CNS and bone marrow in 40% (44 of 111) of xenografts and between spleen and BM in 17.8% (28 of 157) of xenografts (Supplementary Table S5). In one patient (patient 7), our analysis of the genetic discordance revealed the presence of a dRI clone engrafting in the CNS (VAF > 39%) of a xenograft transplanted with a high cell dose (250,000 cells), in which the clone was a minor clone barely detectable in the BM (VAF of < 3%) and outcompeted by a more predominant diagnosis clone (Supplementary Fig. S6B). The identification of the dRI subclone in the CNS of dPDX is consistent with the ability of relapse-fated cells to disseminate and cause disease recurrence in the CNS of patients with B-ALL. We also noted the occurrence of a difference in peripheral blood dissemination of dRI in an additional patient (patient 4), with delayed mobilization of the dRI-PDX subclone as compared with the representative diagnosis clone (Fig. 5E). Collectively, the combined functional and genotyping studies show that individual subclones possess distinct immunophenotypic, competitive repopulation, proliferative, and migration properties and that dRI subclones can already possess distinct biological properties at diagnosis, before exposure to chemotherapeutic agents.

dRI Subclones Display Differential Response to Chemotherapeutic Agents

To directly test the functional properties of dRI subclones for their ability to survive and contribute to relapse, we compared the drug sensitivities of individual subclones for 5 of the genetically distinct patients. Multiple secondary PDXs were generated from dPDXs with known dRI clones (dRI-PDX), predominant diagnosis clones (dPDX), or representative relapse clones (rPDX). Treatment of patients with B-ALL includes combination chemotherapy with supportive care; however, it is not possible to replicate human therapy precisely in xenografts. Our interest was to determine whether there was any variation in the responsiveness of different subclones to individual drugs used in these chemotherapeutic protocols. Following engraftment, PDXs received single-agent treatments of dexamethasone, vincristine, L-asparaginase, or saline for 4 weeks (Fig. 6A and B; Supplementary Fig. S7A–S7F). Differences in therapeutic responses to one or more drugs between dRI and representative diagnosis clones were observed for four patient samples. In three patient samples (patient 1, 6, 7), dPDXs harboring a dRI clone (dRI-PDX) were compared with dPDXs repopulated with the predominant diagnosis clone and demonstrated reduced sensitivity to 2 or 3 of the 3 chemotherapy agents tested (Fig. 6A and B; Supplementary Fig. S7A–S7D). Reduced sensitivity to a single chemotherapeutic agent was also observed in one additional patient (patient 4; dexamethasone, significant in injected femur and trend in BM and spleen; Supplementary Fig. S7E). In contrast, there was no difference in the therapeutic sensitivity of two dRI-PDXs from patient 11 defined by the presence of the AHNK 3′UTR variant. This patient has a strong driver KMT2A (MLL) translocation, and the presence of the single relapse-specific variant does not appear to confer sufficient evolution of the leukemia to alter the therapeutic sensitivity in this context (Supplementary Fig. S7F). Purification of human cells from the secondary PDXs post-therapy and targeted sequencing confirmed their genotype and did not reveal the selection of any further relapse-specific variants (Supplementary Fig. S8A–S8C). The observed differences in
Figure 6. dRI subclones display decreased sensitivity to commonly used chemotherapeutic drugs. A, Phylogenetic analysis showing clonal relationships in patient 7 based on VAF of leukemic variants shows clear evidence of the isolation of a relapse-fated, dRI clone in dPDX 20. The distances between symbols on the tree were estimated by a nearest neighbour joining method and represent the degrees of relation between them (Minkowski’s distance). Circles represent patient samples and triangles represent PDXs; blue represents diagnosis and red represents relapse. dRI-PDX 20 is indicated by a hatched border box. Purified human cells from primary dPDX 7, dRI-PDX 20, and rPDX 5 (representative relapse genetics) xenografts were injected into secondary NSG mice and allowed to engraft. Mice were randomized into 4 groups (with 4 to 5 mice per group) and treated with either saline, dexamethasone, l-asparaginase, or vincristine. After 4 weeks of treatment mice were sacrificed and engraftment in the IF, BM, and spleen were analyzed by flow cytometry. Ratio of human chimerism in the BM of drug-treated mice in comparison with saline controls is shown.

B, Ratio of human chimerism in the BM of drug-treated mice in comparison with saline controls of dPDX, dRI-PDX, and rPDX of patient 1. C, Representative flow plots of the CD19 and CD33 immunophenotype of dPDX and dRI-PDX dexamethasone-treated mice from patient 1. Lines represent mean and SD. Only significance between dPDX and dRI-PDX are shown. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; unpaired two-sided t tests.

Drug response could not be accounted for by any consistent changes in L-IC frequency (Supplementary Table S6).

Unexpectedly, in 2 of 5 patient samples used for drug testing, we found that the dRI subclones exhibited immunophenotypic plasticity as compared with the predominant diagnosis clone when exposed to drugs. In the secondary recipients of dRI-PDX of patient 1 and dRI-PDX of patient 7, both patients harboring DUX4 translocations at diagnosis, there was the emergence of a distinct CD33+CD19dim− population upon dexamethasone treatment but not in saline controls or upon treatment with the other two drugs (Fig. 6C; Supplementary Fig. S9A and S9B). This population was very rare or not observed in the primary recipients. The CD33+CD19dim− cells resembled myeloid cells with respect to their cell surface marker expression, size, and granularity (Supplementary Fig. S9C). An immunophenotypic shift toward the myeloid lineage especially with steroid challenge has previously been reported in ERG/DUX4 patients,
but our finding of a subclone-specific switch is of interest (34). The emergence of CD33\(^{+}\)CD19\(^{-}\) cells was not due to the outgrowth of a different subclone, as these cells were genetically identical to their CD19\(^{+}\) counterpart (Supplementary Fig. S9D and S9E). We speculate that this propensity for immunophenotypic plasticity might be linked to chromatin remodeling and the ability to evade therapy, as other studies have shown (35–38). Overall, our data provide strong evidence that prior to exposure to chemotherapy, dRI subclones already possess distinct preexisting functional properties including differential sensitivity to standard chemotherapy agents.

**Transcriptional Analysis of dRIs Reveals Metabolic Rewiring and Enrichment of a Stem-Cell State in Progression to Relapse**

To gain mechanistic insight into the molecular pathways present in dRIs, RNA-seq analysis was performed on cells from dRI-PDX, dPDX, or rPDX of 4 patients (patients 1, 4, 6, 7; \(n = 14\) dPDXs, 15 dRI-PDXs, and 13 rPDXs; Supplementary Table S7). This analysis confirmed the placement of dRIs as intermediates between diagnosis and relapse, sharing transcriptional programs with both timepoints (Supplementary Fig. S10A). Given the distinct B-ALL subtypes/cytogenetics of the patient samples analyzed, as expected only a few (\(n = 23\)) differentially expressed genes reached significance when comparing dPDX versus dRI-PDX across all samples (Supplementary Table S7). Surprisingly, one of the genes upregulated in the dRI-PDX is asparaginase (ASPG), a catalytic enzyme that hydrolyzes asparagine to aspartic acid, albeit nonrobustly in human cells. The human ASPG protein can display cytotoxic activity in human leukemic cell lines (39), suggesting that the dRI-PDX cells may have altered their response to cytotoxic stress or metabolic requirements, thereby explaining why some of the dRI clones are less sensitive to l-asparaginase treatment than dPDX clones.

As pathway enrichment analysis is more sensitive than differential gene expression for finding differences between populations, we undertook this approach to uncover significantly differentially enriched pathways that were shared among all patients. Gene set enrichment analysis (GSEA) comparing the pathways present in dRI-PDX versus dPDX revealed that most pathways significantly enriched in dRI-PDX (FDR \(q\)-value \(\leq 0.05\)) were also present or further enriched in the comparison of dPDX to rPDX; these are termed dRI-PDX/rPDX common pathways (Fig. 7A and B; Supplementary Fig. S10B–S10D; Supplementary Table S7). Network analysis revealed that these pathways were centered on a metabolic signature composed of genes involved in cellular and mitochondrial metabolism including amino acid metabolism, tricarboxylic acid cycle, oxidative phosphorylation, mitochondrial translation and transport, and lipid metabolism (Fig. 7A and B; Supplementary Fig. S10B–S10D). In concordance with a previous study on ALL relapse (26), the central regulator of growth and metabolism, mTOR, was enriched in dRI and relapse clones (Supplementary Fig. S10B; Supplementary Table S7). Pathways identified as uniquely enriched in rPDX versus dPDX (rPDX-unique) included a large network of highly interconnected nodes involved in cell-cycle regulation such as cell-cycle checkpoints, DNA replication, DNA repair, and microtubule organization (Fig. 7A; Supplementary Table S7).

To validate the metabolic signature observed in dRIs that was often further enhanced at relapse, we performed flow cytometry and immunostaining analysis in the PDX from five patient samples (Fig. 7C and D; Supplementary Fig. S10E and S10F). This analysis confirmed the convergence of similarities in metabolic rewiring of dRI-PDX/rPDX in comparison with dPDX in all 5 patients with B-ALL. This functional validation showed that there was an increase in total mitochondrial mass, with individual mitochondria having similar mitochondrial membrane potential in most of the patients analyzed. However, despite this increase in absolute mitochondrial abundance, the levels of reactive oxygen species (ROS) were unexpectedly found to be lower in dRI-PDX and rPDX cells as compared with dPDX cells. The reduced ROS levels are suggestive of the presence of increased antioxidant defense upon progression toward relapse. This interpretation was concordant with our gene-expression data that showed an enrichment of ROS-defense and peroxisomal activity genes (i.e., catalase), which degrade toxic hydrogen peroxide and metabolize drugs, in the dRI-PDX and rPDX samples (Fig. 7A and C; Supplementary Fig. S10B, S10D–S10F; Supplementary Table S7). Significant enrichment for chromatin remodeling and cell stress response (such as the UPR) were also identified as dRI-PDX/rPDX common pathways (Fig. 7A; Supplementary Fig. S10B–S10D). The chromatin remodeling pathways including expression of histone variants and isoforms were likely instrumental in the plasticity observed in the progression to relapse, including the immunophenotypic plasticity we observed in dRI-PDXs of patients 1, 7, and 9 (Figs. 4A and 5C), whereas the expression of the cell stress response pathways identified could contribute to enhanced survival of dRI subclones. To independently validate the results obtained from the PDX-identified pathways, we directly evaluated bulk diagnosis and relapse patient samples. We found enrichment (FDR \(q\)-value \(\leq 0.05\)) of pathways involved in metabolism, mitochondrial regulation, and cell cycle at relapse (Fig. 7E; Supplementary Fig. S10G; Supplementary Table S7). Furthermore, we found significant enrichment of a signature present at minimal residual disease (18) in dRI-PDX and rPDX compared with dPDX, lending further support for the relevance of the dRI-PDX/rPDX pathways for understanding the mechanisms of relapse disease initiation and their presence prior to chemotherapeutic challenge (Supplementary Fig. S10H). Collectively, our transcriptional analysis has for the first time uncovered the utilization of chromatin remodeling, stress responses, and metabolic pathways in dRI subclones whose activity could serve to protect them during chemotherapy treatment and contribute to their ability to further progress to relapse disease. As such, these newly identified pathways represent rich areas to investigate for new therapeutic strategies to target dRI specifically.

To investigate whether the broader cellular state of the dRIs contributes to their functional differences, we performed gene set variation analysis (GSVA) comparing the transcriptomic profiles of representative clones with normal hematopoietic cell populations isolated from human umbilical cord blood using two independent datasets, one from our own newly generated data and the other published previously (ref. 40;
Supplementary Fig. S11A; Supplementary Table S7). dRI-PDX and rPDX exhibited a transcriptome profile significantly enriched for hematopoietic stem cell (HSC) genes and a slight reduction in B-cell genes as compared with dPDX (Fig. 7F; Supplementary Fig. S11B and S11C). Depletion of B-cell genes and enrichment of HSC genes at relapse were also observed in the bulk patient samples from our cohort (Fig. 7F). Furthermore, the leading-edge genes of common pathways enriched in both dRI-PDX and rPDX are upregulated in HSCs as compared with lymphoid cells (Supplementary Fig. S11D). The enrichment of mitochondrial metabolism and stemness at relapse were also validated in the larger Waanders and colleagues cohort of paired diagnosis-relapse patient B-ALL samples (7) encompassing several genetic subtypes (Supplementary Fig. S11E and S11F). These findings are in line with previous studies in the normal and leukemia stem-cell fields (25, 41, 42) where several metabolic and stress response pathways such as those identified here (mitochondria metabolism, UPR, antioxidant defense) have been described to be crucial to the maintenance of stem-cell homeostasis and function. In addition, enrichment of stemness signatures is also a hallmark of high-risk B-ALL (43). Thus, our findings report a link for the acquisition of HSC stemness properties in combination with metabolic rewiring in dRI as part of progression to relapse in lymphoid B-ALL (Fig. 7C).

**DISCUSSION**

Our study provides new insight into the leukemogenic process of human B-ALL through a deep analysis of the functional and molecular properties of genetically diverse diagnosis subclones isolated through xenografting from pediatric and adult patients with B-ALL. By combining xenografting with sequencing, broad clonal structures were unambiguously demonstrated and additional subclones were uncovered. We identify relapse-fated dRI subclones, prior to chemotherapy exposure, with the capacity for clonal propagation and leukemia initiation in B-ALL that are both genetically and transcriptionally related to the relapse. Our data extends prior B-ALL studies that relied on genetic analysis and computational methods of bulk leukemia samples without functional evolution. Pathways regulating mitochondrial dynamics and stress response pathways such as those identified here (mitochondria metabolism, UPR, antioxidant defense) have been described to be crucial to the maintenance of stem-cell homeostasis and function. In addition, enrichment of stemness signatures is also a hallmark of high-risk B-ALL (43). Thus, our findings report a link for the acquisition of HSC stemness properties in combination with metabolic rewiring in dRI as part of progression to relapse in lymphoid B-ALL (Fig. 7C).

**Figure 7.** dRI subclones share a common metabolic and stem cell profile. A, Plot showing the normalized enrichment score (NES) for the top differentially enriched gene sets (FDR q value ≤ 0.05) of dRI-PDX unique, dRI-PDX/pPDX common, and pPDX unique groups from GSEA of the following comparisons: dPDX vs. dRI-PDX and dPDX vs. pPDX. B, Heat maps showing the expression of leading-edge genes (subset of genes found in the ranking at or just before the maximal enrichment score in GSEA) for selected gene sets enriched in dRI-PDX and pPDX from enrichment map in A. Relative expression was generated from variance stabilized normalized counts C, dPDX, dRI-PDX, and pPDX from Patient 1, Patient 4, and Patient 7 were stained with Mitotracker and CellROX dyes and analyzed by flow cytometry. MFI for each sample and dye is represented as ratio to dPDX for each patient (Patient 1: dPDX n = 5, dRI-PDX n = 5, pPDX n = 4; Patient 4: dPDX n = 5, dRI-PDX n = 4, pPDX n = 4; Patient 7: dPDX n = 5, dRI-PDX n = 5, pPDX n = 5). D, Immunostaining analysis for TOMM20, MRPS18B, and SOXI in dPDX, dRI-PDX, and pPDX cells from Patients 1, 3, and 7. The Integrated Density (IntDen = Area × MFI) for 40 to 50 cells from each clone was analyzed using Fiji. The mean for each clone was normalized and calculated as a ratio to the dPDX for each patient separately. Representative images for Patient 7 are shown. (Patient 1: dPDX n = 1, dRI-PDX n = 2, pPDX n = 1; Patient 7: dPDX n = 2, dRI-PDX n = 2, pPDX n = 1). E, GSEA enrichment plots from the following comparisons: dPDX vs. dRI-PDX (n = 4 pts); dPDX vs. pPDX (n = 4 pts); and diagnosis (Dx) versus relapse (Rel) patient samples from our cohort were generated for mitochondrial translation and oxidative phosphorylation gene sets. F, Bar plot of the aggregated GSVA scores for B-cell genes and HSC genes in each sample. GSVA scores for samples in each category were summed and scaled from 0 to 1. The numbers above the bars represent how many times the observed score was higher than random scores obtained in 1,000 permutations using a list of 1,000 random genes. G, Schematic diagram of dRI with altered metabolic and stem-cell programs preexisting in diagnosis patient samples that survive chemotherapy and seed the relapse disease. *P < 0.05; **P < 0.01; ***P < 0.001; unpaired two-sided t test.
we have undertaken from purified AML stem-cell populations (24, 45). The finding of stemness signatures already present in dRI subclones, together with the identified metabolic changes, provides the molecular basis to explain why dRI subclones may both survive therapy and possess the regenerative capacity to initiate disease relapse after a period of dormancy. Future studies that explore the transcriptome of dRI subclones from across an even wider spectrum of B-ALL patient samples would give insight into how the timing of acquisition of these molecular pathways leads to relapse.

The ability to isolate and characterize dRI subclones provides an important first step in understanding the basis for therapy resistance and clonal propagation. Such subclones isolated in this way are not simply an in silico depiction; rather, they can be viably preserved cells or serially propagated xenografts for future studies. Whole-genome sequencing, methylation, and chromatin accessibility studies could be undertaken to build upon our transcriptomic analysis, thereby exploring more deeply the mechanisms that drive their functional properties. The signatures developed from isolated dRI clones could yield biomarkers to improve the classification of patients who are at increased risk of relapse and to better monitor residual disease. The immunophenotypic plasticity we linked to different genetic subclones points to the need to understand the breadth of cell-surface phenotypes present to ensure that all leukemic subclones are properly tracked during flow cytometry-based residual disease monitoring. Finally, further investigation of the dRI transcriptomic profile and metabolic rewiring may be used to uncover the vulnerabilities of dRI subclones, resulting in new therapeutic targets. Improved eradication of dRIs during early treatment phases before the subclones evolve would prevent progression to more aggressive, therapy-resistant disease. Prior studies from us and others have shown that xenografting of a wide spectrum of primary patient samples provides a powerful tool to evaluate novel therapeutics and develop biomarkers (23, 25, 46, 47). Our study suggests that extending the xenograft-based drug development paradigm by including genetic analysis to uncover subclonal responses to drug treatment will open up avenues to evaluate whether relapse-fated clones are effectively targeted.

**METHODS**

**Patient Samples**

Patient samples were obtained at diagnosis and relapse from 6 adult patients and 8 pediatric patients with B-ALL according to preestablished guidelines approved by the Research Ethics Board of the University Health Network and the St. Jude Institutional Review Board, respectively, and were conducted in accordance with recognized ethical guidelines. Adult samples were collected at the Princess Margaret Cancer Centre (Toronto, Ontario, Canada) and pediatric samples were collected at St. Jude Children’s Research Hospital (Memphis, TN). Written informed consent was obtained from all sample donors, and the samples were collected after approval from the institutional review boards at the University Health Network and the St. Jude Institutional Review Board, respectively, and were conducted in accordance with preestablished guidelines approved by the Research Ethics Board of the University Health Network. The ability to isolate and characterize dRI subclones provides an important first step in understanding the basis for therapy resistance and clonal propagation. Such subclones isolated in this way are not simply an in silico depiction; rather, they can be viably preserved cells or serially propagated xenografts for future studies. Whole-genome sequencing, methylation, and chromatin accessibility studies could be undertaken to build upon our transcriptomic analysis, thereby exploring more deeply the mechanisms that drive their functional properties. The signatures developed from isolated dRI clones could yield biomarkers to improve the classification of patients who are at increased risk of relapse and to better monitor residual disease. The immunophenotypic plasticity we linked to different genetic subclones points to the need to understand the breadth of cell-surface phenotypes present to ensure that all leukemic subclones are properly tracked during flow cytometry-based residual disease monitoring. Finally, further investigation of the dRI transcriptomic profile and metabolic rewiring may be used to uncover the vulnerabilities of dRI subclones, resulting in new therapeutic targets. Improved eradication of dRIs during early treatment phases before the subclones evolve would prevent progression to more aggressive, therapy-resistant disease. Prior studies from us and others have shown that xenografting of a wide spectrum of primary patient samples provides a powerful tool to evaluate novel therapeutics and develop biomarkers (23, 25, 46, 47). Our study suggests that extending the xenograft-based drug development paradigm by including genetic analysis to uncover subclonal responses to drug treatment will open up avenues to evaluate whether relapse-fated clones are effectively targeted.

**PDX Generation and Limiting Dilution Analysis**

Twenty-nine clinical samples obtained from the 14 patients (1 patient having two relapse samples) were stained with the following antibodies: anti-CD19 PE (BD Biosciences, clone 4G7), anti-CD2 FITC (BD Biosciences, clone SK7) or anti-CD3 APC (Beckman Coulter clone UCHT11), anti-CD45 APC (BD Biosciences, clone 2D1) or anti-CD45 FITC (BD Biosciences, clone 2D1), and anti-CD34 APC-Cy7 (clone 581). Each sample was sorted on a FACS Aria III (BD Biosciences) for leukemia blasts (CD19+CD45dim+, CD34−), CD34+ (CD19−CD3−CD45hi), T cells (CD3+CD45hi), and B cells (CD19+CD3−CD45hi). Each sample was sort on a FACS Aria III (BD Biosciences) for leukemia blasts (CD19+CD45dim−, purity >90%) and sorted T cells (CD3+CD45hi, purity >90%) using the Gentra Puregene Cell Kit (Qiagen). T cells from patients 2, 3, and 5 were whole-genome amplified (REPLI-g Mini Kit, Qiagen) due to limited material. DNA from the pediatric samples with >90% leukemic blasts was extracted from diagnosis, remission, and relapse samples using phenol–chloroform. Exomes were captured using the TrueSeq Exome Library Prep Kit (67 Mb, 1 μg DNA input) or the Nextera Rapid Capture Expanded Exome (62 Mb, 50 ng DNA input; Illumina). Paired-end sequencing was performed with the HiSeq 2500 genome sequencer (Illumina). The data was mapped to human reference genome hg19 and variant calling was performed using the Bambino variant detector as described previously (50). Bambino variants including SNVs and indels were detected by running the variation detection module of Bambino. The output contained detailed read counts for each variant with columns for tumor/normal status, allele, and strand. Variants were not filtered for coverage prior to combination with targeted-sequencing results.

**Copy-Number Analysis**

Patient copy number aberrations were determined using SNP6.0 microarrays according to manufacturer’s instructions (Affymetrix). Data was analyzed as described previously (51) using optimal reference normalization (52) and circular binary (53, 54) segmentation with Genotyping Console (Affymetrix) and dCHIP (build Apr 2010; ref. 55). Detection of loss of heterozygosity and allelic ratios were performed using Nexus 7.5.2 software (BioDiscovery Inc). All segments were manually curated.

**Recovery of Human Cells and DNA Isolation from Xenografts**

Cells from the injected femur (IF), BM, and spleen were frozen via- bly after sacrifice. The IF and BM of mice engrafted with >10% human cells were combined. These cells as well as cells from diagnosed PDX spleens were depleted of mouse cells using the Miltenyi Mouse Cell
Depletion Kit (Miltenyi Biotec; samples with ≥20% engraftment) or by cell sorting with human CD45 and human CD19 and or CD34 cell-surface antibodies to a purity of >90% as determined by post-processing flow cytometry. CNS cells from mice with greater than 60% engraftment were used directly for DNA isolations. DNA was isolated using the QIAamp DNA Blood Mini or Micro Kit (Qiagen).

**Targeted Sequencing**

All somatic SNVs and indels identified by WES were validated in the patient samples using NimbleGen SeqCap Target Enrichment according to the manufacturer’s instructions (Roche, NimbleGen). Library preparation was completed using 250 to 500 ng of DNA using the NEXTIlex DNA-SEQ Library Prep Kit (BioScientific) with NEXTIlex-96 DNA Barcodes (BioScientific). Sequencing was performed on a HiSeq 2500 genome sequencer to a mean coverage >350× for patient samples and >200× for PDXs.

**Targeted-Sequencing Data Analysis**

Final patient variant calls used the combined results of WES and capture validation. Variants were filtered out if the VAF in the germline was greater than 10% or if there was a dbSNP frequency of greater than 1%. Variants were classified based on the VAFs in the bulk patient diagnosis and relapse samples as: preserved variants (VAF > 30% in both diagnosis and relapse samples, or preserved between samples); diagnosis-specific variants [present at diagnosis (>1%) and absent at relapse (<1%); latent variants (present at diagnosis with VAF < 30% and increasing at relapse); relapse-specific variants [absent at diagnosis (<1%) and present at relapse (>1%)]. For xenograft analysis, variants with less than 5x coverage or uncovered in numerous xenografts were removed. Results were analyzed by visualization in heat maps (i.e., Fig. 1C). Phylogenetic analysis showing genetic relationships of patient samples and xenografts were estimated using Minkowski’s distance calculated from the VAFs and represented visually using a nearest neighbor joining algorithm. Genetic concordance between different tissues of the same xenograft were determined by visual assessment by three independent and blinded individuals. Discordance was called only when all three investigators were in agreement.

**Generation of Mutational Trees from Patient Samples and Xenografts**

Two independent computational analyses were performed—first for patient-only tissue samples, and then for patient samples augmented with xenografts (BM and spleen)—using an early version of the PairTree algorithm (Wintersinger and colleagues, in preparation). PairTree uses variant read counts to estimate the posterior probability distribution over four possible ancestral relationships between every variant pair (A, B). The four ancestral relationships are as follows: variant A and variant B occurred in the same cells, such that no cell possessed one variant but not the other; variant A is ancestral to variant B, such that some cells have A but not B; variant B is ancestral to variant A, such that some cells have B but not A; or neither is the ancestor of the other, such that A and B are on different branches of the evolutionary tree.

To permit temporal ordering of mutations, the infinite sites assumption was made, such that variant A could never be the ancestor of variant B if A’s cancer cell frequency (CCF) was lower than B’s in any sample, after their CCFs were estimated from each mutation’s VAF. To simplify the estimation of CCF from VAF, variants were discarded if they lay in CNA-affected regions determined by SNP6.0 VAF. To simplify the estimation of CCF from VAF, variants were discarded if they lay in CNA-affected regions determined by SNP6.0 VAF. To simplify the estimation of CCF from VAF, variants were discarded if they lay in CNA-affected regions determined by SNP6.0 VAF. To simplify the estimation of CCF from VAF, variants were discarded if they lay in CNA-affected regions determined by SNP6.0 VAF. To simplify the estimation of CCF from VAF, variants were discarded if they lay in CNA-affected regions determined by SNP6.0 VAF.

**RNA-seq**

**For PDX.** Cell pellets for PDX RNA extraction were frozen in 1 mL of TRIzol (Invitrogen) and kept at −80°C. Total RNA was purified by phenol/chloroform and integrity and concentration were verified on a Bioanalyzer Pico Chip (Agilent Technologies). cDNA conversion was performed using SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara; 1 ng total RNA input) and libraries were prepared using Nextera XT DNA Library Preparation Kit (Illumina; 1 ng input of cDNA). Equimolar quantities of libraries were pooled and sequenced 4 cDNA libraries per lane on a High Throughput Run Mode Flowcell with v4 sequencing chemistry on the Illumina HiSeq 2500 following manufacturer’s protocol generating paired-end reads of 126-bp in length to reach depth of 65 million reads per sample.

**For Patient Samples.** RNA was extracted from sorted leukemia blasts (CD19+CD45dim−, purity >90%) using RNeasy Micro Kit (Qiagen) for adult patient samples. From these samples approximately 10 ng of total RNA was processed using the SMART cDNA synthesis protocol including SMARTScribe Reverse Transcriptase (Clontech) as per the manufacturer’s instructions. The amplified cDNA was subject to automated Illumina paired-end library construction using the NEBNext paired-end DNA sample Prep Kit (NEB) and libraries were sequenced on HiSeq2000 (Illumina) to an average of approximately 161 million Chastity-passed paired reads of 75 bp in length per sample. For pediatric samples RNA was extracted from patient samples with >90% leukemic blasts using TRIzol (Life Technologies) and quality and quantity assessed by Qubit (Thermo Fisher Scientific) and RNA6000 Chip (Agilent). One microgram of RNA was used for library preparation with TruSeq RNA Library Prep Kit v2 (Illumina) and 2 × 100 bp paired-end sequencing was performed on a HiSeq 2500 (Illumina). Patient RNA-seq samples were aligned against the human genome (hg19) using STAR v2.3 with default parameters. All PDX samples were aligned with STAR v2.5.2b (56) against the human genome build version GRCh38 and the ensemble v90 gtf file. Default parameters were used except chimeric segments were screened with a minimum size 12, junction overlap 12, and segment reads gap maximum 3; splice junction overlap 10, aligned mates gap maximum 100,000, aligned intron maximum 100,000 and alignSingleMatchNmax 5 and alignMatchNmax 5. For both patient samples and PDX, transcript counts were obtained using HTSeq v0.7.2 (57). Data was library size normalized using RLE, followed by a variance stabilizing transformation using estimatim v2.1.2 (58). Principal component analysis plots were generated on a per sample basis using the top 1,000 variable genes. For downstream visualization, differential expression, and
pathway analysis, the mean expression of each sample clone condition was utilized. On a per patient level, differentially expressed genes were identified. Genes with adjusted P value of < 0.05 and absolute log2 fold change of > 1 were considered significant. All visualizations were generated using R v3.5.1 and the heatmap v1.0.10 and lattice v0.20–38 packages and ggplot2 v3.1.0 packages.

Pathway Enrichment Analysis and Visualization

Pathway enrichment analysis and visualization was performed as described previously (59). Briefly, a score to rank genes from top upregulated to downregulated was calculated using the formula sign(logFC) × log2(P). The rank file from each comparison was used in GSEA (http://software.broadinstitute.org/gsea/index.jsp) using 2,000 permutations and default parameters against a pathway database containing MsigDB c2 and c3, NCIC, IOB, NetPath, HumanCyc, GO BP, Reactome, and Panther (http://baderlab.org/GeneSets, version June 2018). GSEA progressively examines genes from the top to the bottom of the ranked list, increasing the enrichment score (ES) if a gene is part of the pathway and decreasing the score otherwise. These running sum values are weighted, so that enrichment in genes with more moderate ranks is not amplified. The ES is calculated as the maximum value of the running sum and normalized relative to pathway size, resulting in a normalized ES (NES) that reflects the enrichment of the pathway in the list. Positive and negative NES values represent enrichment at the top and bottom of the list, respectively. A permutation-based P value is computed and corrected for multiple testing to produce a permutation-based FDR value.

Cyc, GO BP, Reactome, and Panther (http://baderlab.org/GeneSets, version June 2018). GSEA progressively examines genes from the top to the bottom of the ranked list, increasing the enrichment score (ES) if a gene is part of the pathway and decreasing the score otherwise. These running sum values are weighted, so that enrichment in genes with more moderate ranks is not amplified. The ES is calculated as the maximum value of the running sum and normalized relative to pathway size, resulting in a normalized ES (NES) that reflects the enrichment of the pathway in the list. Positive and negative NES values represent enrichment at the top and bottom of the list, respectively. A permutation-based P value is computed and corrected for multiple testing to produce a permutation-based FDR value.

GSEA score for each patient and each category were plotted on a box plot and a strip chart. GSEA scores were summed for each mixed profile category and standardized from 0 to 1. One thousand permutations with a random gene list of size 1,000 were performed on the mixed profile and percentages calculated to indicate how many times the observed score was higher than the random scores. These results were confirmed using the HSC and B-cell expression profiles from Novershtern and colleagues (ref. 40; Supplementary Table S7).

Transcriptomic Validation Experiments

Staining for mitochondria content, ROS, mitochondrial membrane potential, and mitochondrial superoxide levels was performed by incubating thawed PDX cells at 37°C with 1 mmol/L of MitoTrackerGreen (M7514; dilution 1:10,000); 5 mmol/L CellROX deep red (C10422; dilution 1:500); 1 mmol/L TMRE (T668; dilution 1:1,000), or 5 mmol/L MitoSOX Red (M36008; dilution 1:1,000) following the manufacturer’s instructions (Thermo Fisher Scientific) and directly analyzed on a BD LSRII cytometer. Mean fluorescence intensity (MFI) for each sample and dye is represented as ratio to dPDX for each patient. Immunostaining analysis for TOMM20, MRPS18B, and SOD1 were performed on PDX cells. Briefly, cells were spun onto poly-L-lysine (Sigma)-coated slides (Ibidi, 200 μm diameter), fixed 1/200; SOD1: ab8866, dilution 1/100; and MRPS18B: ab191891, dilution 1/200) in blocking solution overnight at 4°C. Secondary anti-mouse AF568, anti-rabbit AF488, and anti-sheep AF647 (Invitrogen, 1:400) antibodies were added (PBS, 0.025% Tween, Sigma, 1.5 hours, room temperature). After washing, nuclei were stained with 1 μg/mL DAPI (Invitrogen) and slides were mounted (Fluoromount-G, Invitrogen). Images were captured using a Zeiss LSM700 Confocal (oil, 63×/1.4NA, Zen 2012) and analyzed with ImageJ/Fiji. The Integrated Density (IntDen = Area × MFI) for 40 to 50 cells from each clone was analyzed. The mean for each clone was normalized and calculated as a ratio to the dPDX for each patient separately.

Secondary Transplantations for Drug Assays and Limiting Dilution Assays

Human purified cells from the primary recipients were thawed and transplanted on 8–12-week-old female NSG mice subcutaneously as described for the primary recipients. The number of mice used for secondary transplantation experiments/drugs was determined by cell and mouse availability and feasibility. Intrafamilial
Characterization of Relapse-fated Clones in Diagnosis B-ALL

injections of 26,000 to 100,000 leukemic blasts were performed for drug assays and a range of 10,000 to 100,000 leukemic blasts were injected for secondary limiting dilution assays. After 4 weeks, mice were bled from the saphenous vein and human chimerism was evaluated by flow cytometry. Once human engraftment in the peripheral blood reached between 1% and 10%, or after 10 to 14 weeks for those samples in which leukemic blasts did not mobilize to the peripheral blood, mice were randomized and single-agent treatments were started. Dexamethasone (15 mg/kg), i-asparaginase (1,000 KU/kg), and saline were given daily by intraperitoneal injection 5 days a week. Vincristine (0.5 mg/kg) was given once a week by intraperitoneal injection. All four arms of the drug treatment were performed for 4 weeks and mice were sacrificed the following day (vincristine/dexamethasone/saline) or 1 week after the last treatment (vincristine). Analysis of the secondary limiting dilution assay was performed 16 weeks posttransplant.

Human cell engraftment in the injected femur, bone marrow, and spleen were assessed using human-specific antibodies for CD45 (FITC, BD Biosciences,clone 2D1; v500, BD Biosciences,clone H130), CD19 (BD Biosciences, PE, clone 4G7), CD33 (PE-Cy7, BD Biosciences,clone P67-6), CD3 (APC, BD Biosciences,clone UCHT1), CD10 (Qdot605, BD Biosciences,clone H10A), and CD38 (BV421, BioLegend,clone HI2T2). CD34 (APC-Cy7, BD Biosciences,clone S81). Mice were considered to be engrafted when >0.1% of cells in the injected femur were positive for one or more human B-ALL-specific cell-surface markers (CD45, CD44, CD19, CD34). Response to therapy was analyzed as a ratio of human engraftment of drug-treated versus saline-treated mice to eliminate intercolonial differences in engraftment levels. Ratio of human engraftment in each individual drug-treated PDX to the average engraftment level of all saline controls was calculated. Lineage stains were performed on xenografts expressing CD33 (APC, BD Biosciences clone P67-6) and CD19 (PE, BD Biosciences, clone 4G7) includingCD14 (PE-Cy7, Beckman Coulter, clone 52), CD15 (v450, BD Biosciences,clone MMA), CD10 (Qdot605, BioLegend,clone HI2T2), and CD34 (APC-Cy7, BD Biosciences,clone S81). The confidence intervals for the frequency of L-ICs was calculated using ELDA software (49). Statistical analysis was performed using PRISM 6 (GraphPad Software).

**FACS from Xenografts**

Leukemic cells from primary xenografts were sorted for immunophenotypic populations on a FACSAriaIII (BD Biosciences). Cells from the IF and BM were pooled and stained with CD19, CD34, CD45, CD10, and CD33 and collected at a sort purity of >99%.

**Data Availability Statement**

The datasets generated during this study are included in this published article and its supplementary information files.

**Code Availability Statement**

Code used in this study is available at https://www.github.com/morrislab/pairtree

**Disclosure of Potential Conflicts of Interest**

J.S. Danska reports receiving commercial research support from Trillum Therapeutics, Inc. and has ownership interest (including patents) in the same. M.D. Minden reports receiving commercial research support from Kura and has received speakers bureau honoraria from Astellas. C.G. Mullighan is a scientific advisory board member for Illumina, reports receiving commercial research grants from AbbVie, Pfizer, and Loxo Oncology, and has received speakers bureau honoraria from Illumina, Amgen, Pfizer, and Attitude Health. J.E. Dick served on the SAB at Trillum Therapeutics, reports receiving a commercial research grant from Celgene, and has ownership interest (including patents) in Trillum Therapeutics Inc. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

**Conception and design:** S.M. Dobson, C.G. Mullighan, J.E. Dick

**Development of methodology:** S.M. Dobson, J. Wintersinger, I. Grandal, G. Bader, J. Easton, J.S. Danska, Q. Morris, C.G. Mullighan, J.E. Dick

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S.M. Dobson, L. García-Prat, R.J. Vanner, E. Waanders, O.I. Gan, I. Grandal, S.Z. Xie, M. Hosseini, S.R. Olsen, G. Neale, S.M. Chan, J. Easton, C.J. Guidos, J.S. Danska, M.D. Minden, C.G. Mullighan, J.E. Dick


**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** S.M. Dobson, L. García-Prat, J. Wintersinger, E. Waanders, O.I. Gan, S.Z. Xie, S. Abelson, J. Easton, J.S. Danska, M.D. Minden, Q. Morris, C.G. Mullighan, J.E. Dick

**Writing, review, and/or revision of the manuscript:** S.M. Dobson, L. García-Prat, R.J. Vanner, J. Wintersinger, E. Waanders, O.I. Gan, S.Z. Xie, S. Abelson, J. Easton, J.S. Danska, M.D. Minden, Q. Morris, C.G. Mullighan, J.E. Dick

**Acknowledgments**

We thank the patients and their families and physicians who made this study possible. We also thank N. Simard, S. Laronde, L. Jameson, A. Khandani, T. Velanthapillai, and S. Zhao at the UHN/SickKids Flow Cytometry Facility; P. Lo and R. Lopez from the UHN Animal Resources Centre; the Genome Sequencing Facility of the Hartwell Centre for Bioinformatics and Biotechnology, and the Biorepository of St. Jude Children’s Research Hospital. We thank J. Ho, E. Lechman, A. Mitchell, L. Jin, M. Doedens, J. Loo-Yong-Kee, K.L. Woo, M.C. Shoon, and J. Roth for their technical assistance; J.A. Kennedy for clinical annotation; and K. Kaufmann for data analysis script. This work was supported by grants to J.E. Dick from the Princess Margaret Cancer Centre Foundation, Ontario Institute for Cancer Research, with funding from the Province of Ontario, Canadian Institutes for Health Research, Canadian Cancer Society Research Institute, Terry Fox Research Institute Program Project, Genome Canada through the Ontario Genomics Institute, and a Canada Research Chair; and to C.G. Mullighan from the American Lebanese Syrian Associated Charities of St. Jude Children’s Research Hospital, the St. Baldrick’s Foundation Robert J. Arceci Innovation Award, the Henry Schueler 419 Foundation, the NCI grants P30 CA021765 (St. Jude Cancer Center Support Grant) and Outstanding Investigator Award R35 CA197695. Q. Morris was supported by funds from the Natural Sciences and Engineering Research Council, a Compute the Cure award from the NVIDIA charitable foundation, and the Vector Institute. E. Waanders is funded by the Dutch Cancer Society (KUN2012-5366).

Received September 11, 2019; revised December 21, 2019; accepted February 18, 2020; published first February 21, 2020.

**REFERENCES**


Characterization of Relapse-fated Clones in Diagnosis B-ALL

Relapse-Fated Latent Diagnosis Subclones in Acute B Lineage Leukemia Are Drug Tolerant and Possess Distinct Metabolic Programs

Stephanie M. Dobson, Laura García-Prat, Robert J. Vanner, et al.


Updated version
Access the most recent version of this article at:

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2020/02/21/2159-8290.CD-19-1059.DC1

Cited articles
This article cites 59 articles, 19 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/10/4/568.full#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://cancerdiscovery.aacrjournals.org/content/10/4/568.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerdiscovery.aacrjournals.org/content/10/4/568.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.