Published in final edited form as:

Pediatr Hematol Oncol. 2017 ; 34(6-7): 355–364. doi:10.1080/08880018.2017.1395937.

Investigating the biology of relapsed acute leukemia: Proceedings of the Therapeutic Advances for Childhood Leukemia & Lymphoma (TACL) Consortium Biology Working Group.

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Abstract

During the 2016 Therapeutic Advances for Childhood Leukemia & Lymphoma (TACL) Consortium investigators' meeting (Los Angeles, CA), a Biology Working Group was established to support the consortium's mission of developing innovative therapies for currently incurable childhood leukemias and lymphomas. The charge of the Biology Working Group was to address how TACL could advance biological investigations of pediatric relapsed/refractory hematologic malignancies while undertaking forward-looking therapeutic trials. To this end, the TACL Biology Committee was established to provide the scientific platform needed to further develop preclinical and translational studies that will advance the understanding and treatment of relapsed and refractory disease. The Biology Committee will focus on ensuring state-of-the-art studies that

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Declaration of Interest

The authors do not have any relevant conflicts of interest

address biological components of early phase clinical trials, and developing a central biology bank of materials from these early phase trials for interrogations into the mechanisms of disease resistance.

Keywords

leukemia; relapse; biology; genomics; epigenomics

Introduction

Over the past decade, there has been an explosion of genetic information resulting in rational drug design for many malignancies. As modern genetic and computational studies evolved interrogating both diagnostic and relapsed tumor samples, a plethora of data has been elucidated regarding somatically acquired genetic lesions such as copy number alterations, insertions and deletions, and epigenetic and transcriptional changes within the leukemic cell¹⁻⁴. Unfortunately, outside of a handful of situations, most drug discoveries targeting specific genomic lesions have not lived up to their promise. Much of this disappointment can be attributed to complex pathway addictions and heterogeneity of the leukemia cells. This review will highlight specific knowledge of the biology of relapsed acute leukemia as well as discuss the continued need for future studies.

Acute Lymphoblastic Leukemia (ALL)

The interplay of several biologic mechanisms in pediatric ALL leads to disease recurrence and chemoresistance. Insights into the molecular changes acquired by the leukemic genome during therapy have recently shed light on pathways that may be switched “on” or “off” to evade certain chemotherapeutic agents. Recent studies have begun to unravel clonal diversity and we are in the early phases of deciphering the complex relationships between evolving clones. In addition, the epigenetic landscape, and the role it plays in chemoresistance, remains an enigma, but there is promise of benefit to restoring the epigenetic equilibrium by epigenetic modifying therapy. The protection of residual leukemic cells from chemotherapy offered by the bone marrow microenvironment is fundamentally important to the development of drug resistance, and will be the subject of a separate article in this series. Lastly, continued exploration of the role of the host immune system and inherited genetic variants in influencing the response to therapy⁵ is important for a comprehensive understanding of resistance and for developing systematic approaches to counter such mechanisms.

Genomic changes at relapse - Clonal and subclonal relationships

Paired diagnosis-relapse samples are a valuable resource in biologic studies of ALL. With the advent of next-generation sequencing, several studies have demonstrated significant clonal diversity in ALL^{6,7}. Clonal evolution from diagnosis to relapse follows complex branched patterns in individual cases⁸⁻¹¹. Often a minor clone that survives therapy and acquires additional genetic lesions emerges as the predominant clone at relapse⁸. In general, clonal diversity and the number of mutations increase with each consecutive relapse.

The analysis of allelic frequency and clonal diversity at diagnosis and relapse can reflect on the importance of specific aberrations in the generation of relapse. Somatic lesions with stable high allelic frequencies are either the initiating events (e.g. the typical chimeric fusion translocations), “passenger” somatic mutations that were present at the founding pre-leukemic clone, or mutations that not only cause the leukemic evolution of the primary clone but also “drive” relapse. An example of the latter is aberrations in *IKZF1* (*IKAROS*) that are generally stable between diagnosis and relapse and are not only a prognostic biomarker but also can be a molecular driver of relapse.^{8,10}

In fact, there is an especially high diversity of genetic events that activate signaling pathways between diagnosis and relapse. These events are almost ubiquitous, possibly reflecting the requirement for proliferative “drivers” in leukemia. However subclones with differing signaling mutations co-exist and evolve between diagnosis and relapse^{8-10,12}. In general, there is an increase in the prevalence of RAS mutations from diagnosis to relapse (and to subsequent relapses) which supports a role for the RAS pathway in chemoresistance⁹. In some B-ALLs, especially high-hyperdiploid ALL and *CRFL2*-negative ALL of Down syndrome, RAS activating mutations often co-occur with loss-of-function mutations in the histone acetyltransferase *CREBBP*, likely conferring resistance to chemotherapy^{10,12}. In certain cases, clones with mutations that confer sensitivity to chemotherapy seem to disappear at relapse. For example, subclones carrying activating mutations in JAK enzymes, in particular JAK2, which are sensitive to upfront chemotherapy are not identified at relapse^{9,10,13}. Interestingly other components of this pathway, including the upstream receptors *CRLF2* and *IL7RA*, have more variable kinetics^{10,14}. Thus the presence of JAK2 activating mutations at diagnosis does not explain the poor prognosis reported in Philadelphia chromosome-like (Ph-like) ALLs^{15,16}.

The third class of mutations is those that appear to be selected at relapse. These are possibly the most relevant mutations from a therapeutic perspective, as they confer resistance to upfront chemotherapy. Examples include, mutations conferring resistance to corticosteroids (e.g. *RAS*, *CREBBP*, *NR3C1*), mutations in the DNA damage response pathway (e.g. *TP53*, mismatch repair genes) and, specifically to ALL, mutations conferring resistance to 6-mercaptopurine (6MP) and 6-thioguanine (*NT5C2*, *PRPS1*).^{3,7,9,11,12,17-20} Therapy for pediatric ALL uniquely includes a prolonged maintenance phase consisting of metronomic therapy with oral 6MP taken daily for 2-3 years. Acquisition of relapse-specific mutations in *NT5C2* is noted in 5-45% of patients with ALL at early relapse^{8,20,21}, while mutations in *PRPS1* are identified in approximately 7%¹⁷. Both of these gain-of-function mutations confer resistance to thiopurines either by increased clearance of cytotoxic nucleotides by the former or disruption in the normal feedback inhibition of purine synthesis by the latter. These mutations are often, but not always, the dominant clone at relapse. Interestingly, acquired mutations in *PRPS1* were detected by ultra-deep sequencing up to 500 days prior to an overt relapse, suggesting this mutation may be a driver in disease recurrence.¹⁷ However, a complete understanding of the dynamics of leukemia clonal selection during maintenance therapy remains to be determined.

Mutations in the mismatch DNA damage recognition and repair system (MMR) are detected in about 10% of patients with relapsed ALL⁸⁻¹⁰. These abnormalities are expected to confer

resistance to nucleoside analogs while introducing a high number of new mutations at relapse. This subgroup of relapses may be amenable to therapy with immune-checkpoint inhibitors (e.g. anti-PD-1, anti-PD-L1, anti-CTLA-4) that were recently approved for solid cancers with MMR phenotype.

Epigenetic deregulation

By modulating gene expression, epigenetic changes can contribute significantly to leukemic transformation and progression. These changes are reversible and can be targeted with epigenetic modifying agents such as histone deacetylase inhibitors (HDACi) or DNA methyltransferase inhibitors (DNMTi). Interestingly, exposure to a histone deacetylase inhibitor (vorinostat) reversed a global “relapse-specific” gene expression signature.²² Gene silencing by DNA methylation also influences chemoresistance in ALL as evidenced by increased global promoter methylation at relapse compared to diagnosis.³ Therapy with a DNMTi (decitabine) led to re-expression of the hypermethylated genes and restored chemoresistance in an experimental model.²² In addition, microRNAs which are differentially expressed at relapse with a potential role in driving leukemogenesis and resistance are associated with CpG islands and may be targeted by similar therapy.²³

Somatic mutations in key epigenetic regulators (e.g. *CREBBP*, *WHSC1*, *SUZ1*, *SETD2*) are enriched at relapse^{8,10,19,24}. In a study of matched diagnosis-relapse samples from 30 patients, 17 were noted to harbor mutations in epigenetic regulators, predominantly two histone modifiers in *CREBBP* and *SETD2*¹⁹. *CREBBP* encodes a histone acetyltransferase and transcriptional co-activator where deletions or mutations impair both functions; such genetic alterations affect the transcriptional regulation of *CREBBP* target genes such as glucocorticoid receptor responsive genes leading to resistance to glucocorticoids. While resistant to dexamethasone, *CREBBP* mutant T-ALL cell lines were found to be sensitive to vorinostat in clinically achievable concentrations²⁵. *SETD2* is the only human gene responsible for trimethylation of H3K36. Inactivating mutations of *SETD2* cause mislocalization of *MSH6*, disrupt DNA mismatch repair, and cause microsatellite instability leading to chemoresistance¹⁸. Thus the MMR phenotype may be caused by mutations outside the typical mismatch repair machinery.

Protein deregulation

Another area that can assist with interpretation of increased genomic instability is the assessment of protein expression. In contrast to the large heterogeneity of the genetic and epigenetic landscape, proteins have more direct manifestations of the genetic landscape with a myriad of genetic and epigenetic changes expressing themselves through a finite number of changes in proteins with a limited number of post-translational modifications. Recent work has demonstrated that recurrent protein expression patterns correlates with both clinical outcome²⁶ and clinical risk factors in pediatric ALL.²⁷

Implications for therapy

The increased genomic instability in clonal diversity at relapse poses a significant therapeutic challenge. The initial sensitivity of most first B-ALL relapses to the same drugs used in upfront protocols likely reflects the high proliferative potential endowed by signaling

mutations. Furthermore, targeting a specific signaling pathway is unlikely to have a dramatic impact on cure because of the high promiscuity and the subclonal nature of signaling mutations and may have a limited role in treatment of relapse with two notable exceptions: (a) targeting a dominant resistance mutation or (b) targeting a major initiating leukemia aberration that drives relapse. Potential options for the latter may include Casein Kinase II inhibitors²⁸, or FAK inhibitors²⁹ for IKZF1 deleted ALLs, once such drugs become available; or TSLPR antagonists to leukemias dominated by stable CRLF2/IL7R aberrations^{30,31}. Our major therapeutic goal for pediatric ALL should remain the prevention of relapse and also perhaps earlier molecular diagnosis of relapse.

The alternative approach to therapy targeted at specific genetic aberrations, is eradication of the phenotype common to all the leukemic cells. Such therapies may overcome the daunting genomic instability and clonal diversity seen in relapsed ALL. Particular successful approaches that target the leukemia immunophenotype include chimeric antigen receptor (CAR) T-cell therapy and bi-specific T-cell engager antibodies^{32,33}. The long-term success of these approaches remains to be determined; however resistance mechanisms that include clonal selection, alternate splicing and lineage switch are being unraveled.^{34,35} Potential novel “phenotypic” therapies are immune-checkpoint inhibitors for relapse ALL with MMR phenotype (generating many neo-antigens), devising approaches to target the leukemic metabolic phenotype,³⁶ as well as epigenetic therapies (possibly combined with immune checkpoint inhibitors). A recent example of dual epigenetic targeting strategy tested within TACL was the pilot study combining decitabine and vorinostat with chemotherapy in children and young adults with relapsed or refractory ALL.³⁷

Acute Myeloid Leukemia (AML)

One of the intriguing findings in the biology of AML has been the age-dependent differences in the genetic and epigenetic profiles, highlighting the importance of pediatric specific studies within AML for a better understanding of the true drivers of leukemogenesis. As an example, mutations in IDH and DNMT3A are found more often in adult AML, while fusions in methyltransferase genes appear to be more prevalent in pediatric AML suggesting that different mechanisms are utilized to lead to epigenetic changes.³⁸

Genomic changes at relapse

An important study that highlights some of the genetic differences in adult AML and pediatric AML at relapse is the recent NCI/COG cooperative TARGET AML initiative that interrogated 20 matched diagnostic, remission and relapse specimens using whole exome capture³⁹. Prior studies in adult AML had shown that copy number alterations increase from diagnosis to relapse⁴⁰. In contrast, the pediatric TARGET study revealed that copy number alterations were similar between diagnosis and relapse.³⁹ Evaluation of genomic alterations identified that 42% of mutations at relapse were newly evolved variants that could not be detected at diagnosis³⁸. Although recurrent individual mutations were not identified, over 90% of patients had mutations within the RAS/MAPK/MEK pathway suggesting possible targetable pathways. Like ALL, clonal evolution patterns from diagnosis to relapse were

quite variable, and may be reflective of the mutant cell of origin (ancestral versus distant clone) as well as selective pressure of therapy.

Methylation landscape and its relationship to resistance

Methylation changes to the DNA promoter regions of cancer cells, including AML, have long been known to play a role in oncogenesis. These alterations involve biochemical changes to DNA through aberrant hypermethylation of the promoter CpG islands resulting in changes in gene expression. These changes frequently result in increased resistance to cell death through alteration of tumor suppressor (TSP) genes. One TSP gene that can be inactivated through hypermethylation in AML is the Src homology-2 containing protein-tyrosine phosphatase 1 (SHP-1) which is a negative regulator of the JAK/STAT pathway. In patients with FLT3-ITD mutated AML, SHP-1 expression can be altered resulting in chemoresistance to the FLT3 inhibitor lestaurtinib (CEP-701).⁴¹ Chemoresistance can be reversed with the hypomethylating agent 5-azacitidine which has been shown to restore SHP-1 expression and sensitivity toward CEP-701. Another example of aberrant hypermethylation leading to chemoresistance in AML was reported by Yang and colleagues.⁴² Primary AML cells from patients with relapsed or refractory AML were found to be resistant to adriamycin through the down-regulation of the beta subunit of mitochondrial ATP synthase as a result of hypermethylation. This resistance was reversible using 5-azacitidine which restored ATP synthase mRNA and ultimately chemosensitivity to adriamycin. Epigenetic alterations are common in AML and likely responsible, in part, to the chemoresistance that is particularly prevalent at relapse.^{43,44} As new discoveries continue to identify the role of aberrant DNA hypermethylation on chemoresistance in AML, we will need to develop effective strategies to reverse these changes to make an impact on treatment outcomes.

Establishment of the TACL Biology Committee

As we test novel therapeutics for relapsed and refractory disease, how do we better understand the mechanisms of resistance, globally and specifically to targeted therapies? With all that is currently unknown about the mechanisms of leukemia resistance it becomes difficult to prioritize promising therapeutics. Additionally, as novel agents are utilized, these malignancies have the capability to develop further resistance. It is critical to ensure that our therapeutic trials seek to understand these mechanisms. To address these challenges, a Biology Committee was created within the TACL consortium to assist in the development of early phase trials targeting resistance mechanisms and, importantly, to develop a TACL biobank of samples collected from these trials for more in-depth interrogation. In general, TACL novel agent trials include biology aims specific to the study agent. To complement existing efforts, the TACL Biology Committee will assist in the development of biological and translational correlative studies that will be incorporated into all future TACL trials to ensure the incorporation of state-of-the-art investigations to address biological components of these trials. In the future, concerted analysis of serial samples from novel agent trials may lead to important insights into escape mechanisms and pave the way for combinatorial therapies specifically designed to overcome these mechanisms.

TACL Biology Bank

Increased sample availability is critical to achieve the goals of the TACL Biology Committee. The Biology Working Group recognized that many TACL centers have previously established their own leukemia banks with specific strategies for interrogation. A major need was identified by the Biology Working Group to coordinate efforts of these established banks to centralize and increase the total number of samples available for cell expansion and study. To this end, we have proposed to establish a TACL Central Biological Repository (CBR) to receive and process sequential samples from patients enrolled on all future TACL trials. Through this effort, samples would be banked for future studies and submitted for murine xenograft expansion. TACL has the unique opportunity as an international consortium to obtain a large number of samples from patients with relapsed/refractory leukemia that are enrolled on early phase trials using targeted therapies for serial relapses. This would allow for the CBR to obtain, interrogate, and store patient samples including serum/plasma, leukemic blasts and germline tissue for future studies to validate and develop novel trials testing compounds for these patients. The CBR would maintain a database of samples including clinical and biological characteristics. The Biology Committee would support the repository and review studies submitted for use of these banked leukemia samples.

A key mission for a TACL centralized bank is to have sufficient samples to perform preclinical testing. This includes samples for genetic, functional, as well as preclinical therapeutic testing. To this end, there will be a need to obtain and store patient serum/plasma, leukemia cells, micro-environmental immune cells (e.g. T-cells, NK cells), and germline samples. Samples would be frozen viably to enable the extraction of micro-environmental immune cells from leukemic cells. Germline studies can be performed with a variety of cells including mesenchymal stem cells from the bone marrow sample, skin biopsies and blood samples obtained from patients in remission.

A major obstacle to adequate sample collection is the quantity and quality of cells obtained from patients with relapsed or refractory disease. Experience from prior TACL studies has shown that the sample quality can be poor both in cell number and cell viability. At present the majority of investigators who perform leukemia cell expansion, expand their patient samples using the immunodeficient NOD/SCID/Gamma IL2R null (NSG) murine xenograft. In our experience almost 90% of ALL samples and 50% of AML samples with more than one million cells will engraft within 6 months of xenograft injection. Prior studies have shown that even lower doses of ALL cells can engraft in this model.⁴⁵ In contrast, a better murine model for AML may consist of the NSG mice expressing human SCF, GM-CSF, and IL (NSG-S) for improved engraftment.⁴⁶ To this end, the CBR would plan to send all available samples for xenograft expansion in future TACL studies to build up our repository.

To prove feasibility, TACL is developing a biology study to confirm the CBR can centralize the collection of samples to correlate functional and molecular results with potential targetable lesions within our patient population of relapsed or refractory acute leukemia (Figure 1). These studies will assist in developing functional data and correlating it with state-of-the-art sequencing techniques for acute leukemia to verify the validity of targeting pathways for personalized patient treatment.

Conclusions

TACL, as an international consortium pursuing early phase clinical trials in relapsed/refractory acute leukemia and lymphoma has the unique opportunity to develop a CBR for patient samples that can be used to better understand the mechanisms of treatment resistance. One effort from the 2016 TACL Investigators' Meeting was the establishment of a TACL Biology Committee. A challenge for this committee is to add a unique and novel perspective, currently not employed by other pediatric cancer consortium tumor banks. As TACL is currently the only international pediatric consortium solely dedicated to the treatment of relapsed/refractory leukemia and lymphoma, we believe we are poised to do so. It is our goal that rather than competing with other resources, the Biology Committee and CBR will complement current studies and consortia working to improve biologic understanding and advance the care of pediatric patients with relapse/refractory hematologic malignancies.

Acknowledgements

We gratefully acknowledge the TACL Operations staff, clinical trial participants and their families, referring physicians and care teams, and TACL member site clinical research teams, faculty, and staff.

Financial Support

This work was supported in part by Children's Hospital Los Angeles and the Higgins Family Foundation and by NCI award P30CA014089.

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Figure 1:
Platform for TACL Biobank Representation of a biological platform. A patient with leukemia consented for biologic studies will have samples sent for storage within the TACL biobank. Red line represents feasibility of obtaining, storing and expanding for patient-xenografts. Samples can be stored or sent for functional molecular interrogation (black lines represent future studies). Results can be used computational interrogation to identify way

dependence. Samples can then be used for patient derived xenografts for in vivo validation of targeting. Results can also be used for clinical trial design.

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