Advances in the Genetics of High-Risk Childhood B-Progenitor Acute Lymphoblastic Leukemia and Juvenile Myelomonocytic Leukemia: Implications for Therapy

Mignon L. Loh1 and Charles G. Mullighan2

Abstract

Hematologic malignancies of childhood comprise the most common childhood cancers. These neoplasms derive from the pathologic clonal expansion of an abnormal cancer-initiating cell and span a diverse spectrum of phenotypes, including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), myeloproliferative neoplasms (MPN), and myelodysplastic syndromes (MDS). Expansion of immature lymphoid or myeloid blasts with suppression of normal hematopoiesis is the hallmark of ALL and AML, whereas MPN is associated with proliferation of 1 or more lineages that retain the ability to differentiate, and MDS is characterized by abnormal hematopoiesis and cytopenias. The outcomes for children with the most common childhood cancer, B-progenitor ALL (B-ALL), in general, is quite favorable, in contrast to children affected by myeloid malignancies. The advent of highly sensitive genomic technologies reveals the remarkable genetic complexity of multiple subsets of high-risk B-progenitor ALL, in contrast to a somewhat simpler model of myeloid neoplasms, although a number of recently discovered alterations displayed by both types of malignancies may lead to common therapeutic approaches. This review outlines recent advances in our understanding of the genetic underpinnings of high-risk B-ALL and juvenile myelomonocytic leukemia, an overlap MPN/MDS found exclusively in children, and we also discuss novel therapeutic approaches that are currently being tested in clinical trials. Recent insights into the clonal heterogeneity of leukemic samples and the implications for diagnostic and therapeutic approaches are also discussed.

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Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is the commonest childhood malignancy and, despite progressive improvements in the outcome of therapy, remains the leading cause of cancer-related death in children and young adults (1). ALL is characterized by accumulation of immature lymphoid progenitors in the bone marrow, peripheral blood, and central nervous system and results in death from the consequences of bone marrow failure: anemia, neutropenia and infection, and thrombocytopenia and bleeding. Although these features are common to all patients, ALL represents a remarkably diverse range of subtypes characterized by distinct constellations of gross and submicroscopic genetic alterations, including chromosomal translocations, structural variants, and DNA sequence mutations. Many of these lesions are central events in leukemogenesis, and several are also important determinants of the risk of treatment failure and relapse. General overviews of the genetic basis of ALL may be found in several recent excellent reviews (2–6). This review is part of a series of articles in this issue reviewing new genetic insights and therapeutic opportunities in childhood malignancies (7–11). Here, we briefly review key chromosomal rearrangements in ALL and then focus on several high-risk subtypes, which have hitherto been poorly understood and for which recent detailed genomic-profiling approaches are providing important insights into the genetic basis of disease, with potentially important implications for therapy.

Approximately three quarters of childhood ALL cases exhibit aneuploidy (hyperdiploidy or, less commonly, low hypodiploidy) or chromosomal rearrangements, such as t(12;21) ETV6-RUNX1, t(1;19) TCF3-PBX1, t(9;22) BCR-ABL1, rearrangement of MLL to a diverse range of fusion partners, and rearrangement of the enhancer elements of the immunoglobulin heavy chain locus (IGH@) to a range of partner genes, including the cytokine receptor gene CRLF2 (Fig. 1; refs. 2, 12). Several of these alterations are associated with favorable (e.g., hyperdiploidy with greater than 50 chromosomes, ETV6-RUNX1) or poor outcome (BCR-ABL1, MLL rearrangement, hypodiploidy), yet many of these alterations are insufficient to induce leukemia in...
experimental models, suggesting the presence of cooperating genetic lesions. Moreover, a substantial number of childhood ALL samples, including many from those with high-risk features (such as older age, male, and high-pre-sentation blood leukocyte count) and those who experience relapse, lack one of these known chromosomal alterations. Consequently, the past 5 years have witnessed great activity in the use of genomic approaches to identify genetic alterations not evident on cytogenetic analysis (cryptic or sub-microscopic lesions; ref. 13). These approaches include microarray-based profiling of structural genetic alterations, such as array-based comparative genomic hybridization and single-nucleotide polymorphism (SNP) microarrays, candidate gene sequencing, and more recently, next-generation sequencing of all coding exons (exome sequencing), the expressed genome (transcriptome sequencing or RNA-seq), and whole-genome sequencing. Importantly, these studies have often incorporated integration with gene expression profiling data derived from microarrays and clinical data.

ALL genomes typically acquire fewer large structural genetic alterations than many solid tumors, but they harbor more than 50 recurring regions of genetic alteration, most commonly focal deletions that involve few or a limited number of genes (14–16). Many of the genes targeted encode proteins with roles in key cellular pathways, including lymphoid development and differentiation (e.g., PAX5, IKZF1, EBF1, LEF1, and VPREB), cell-cycle regulation and tumor suppression (CDKN2A, CDKN2B [INK4/ARF], TP53, PTEN, RB1), lymphoid signaling (BTLA, CD200, TOX), transcription factors and transcriptional coregulators (ERG, TBL1XR1, CREBBP), regulation of apoptosis (BTG1), and drug receptor genes (NR3C1). Importantly, several of these genes are targeted by multiple mechanisms of mutation, most notably the lymphoid transcription factor PAX5, which is involved by loss-of-function and dominant negative deletions, sequence mutations, and translocations. Furthermore, many lesions are significantly associated with the presence of chromosomal rearrangements and risk of treatment failure in ALL (Table 1), including several sub-types of ALL. These studies have also identified genetic lesions that define novel subtypes of ALL with distinct gene expression profiles, such as subtypes characterized by rearrangement of CRLF2 (17, 18), intrachromosomal amplification of chromosome 21 (19), and focal deletions of ERG (20).

**BCR-ABL1 leukemia**

Expression of the constitutively active tyrosine kinase BCR-ABL1 is a hallmark of chronic myeloid leukemia (CML), an expansion of relatively mature granulocytes, and a subset of ALL, which is typically an aggressive form of
leukemia that responds poorly to therapy. Genomic profiling has shown that BCR-ABL1–positive lymphoid leukemia, either de novo ALL or CML at the progression to lymphoid blast crisis, is commonly accompanied by deletion or, less frequently, sequence mutation of the IKZF1 gene (21, 22), which encodes a lymphoid transcription factor required for normal lymphoid development. These alterations are loss of function or dominant negative, usually mono-allelic, and accelerate the onset of leukemia in murine models (23). In contrast, IKZF1 alterations are rare in chronic phase CML and at myeloid blast crisis, indicating that these alterations are important determinants of Table 1. Recently identified genetic alterations in B-progenitor acute lymphoblastic leukemia

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alteration</th>
<th>Frequency</th>
<th>Pathway and consequences of alteration</th>
<th>Clinical relevance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAX5</td>
<td>Focal deletions, translocations, sequence mutations</td>
<td>31.7% of B-ALL</td>
<td>Transcription factor required for B-lymphoid development. Mutations impair DNA binding and transcriptional activation.</td>
<td>—</td>
<td>(14, 15, 21)</td>
</tr>
<tr>
<td>IKZF1</td>
<td>Focal deletions or sequence mutations</td>
<td>15% of all pediatric B-ALL cases</td>
<td>Transcription factor required for development of HSC to lymphoid precursor. Deletions and mutations result in loss-of-function or dominant negative isoforms.</td>
<td>—</td>
<td>(14)</td>
</tr>
<tr>
<td>JAK1/2</td>
<td>Inherited variants</td>
<td>—</td>
<td>More than 80% BCR-ABL1 ALL and 66% CML in lymphoid blast crisis</td>
<td>Associated with poor outcome</td>
<td>(21, 22, 108)</td>
</tr>
<tr>
<td>CRLF2</td>
<td>Rearrangement as IGH@-CRLF2 or P2RY8-CRLF2 resulting in overexpression</td>
<td>5%–16% pediatric and adult B-ALL, and &gt;50% DS-ALL</td>
<td>Associated with mutant JAK in up to 50% of cases. CRLF2 mutations and JAK mutations cotransforming in Ba/F3 cells results in constitutive STAT activation.</td>
<td>—</td>
<td>(17, 18, 28, 29)</td>
</tr>
<tr>
<td>CREBBP</td>
<td>Focal deletion and sequence mutations</td>
<td>19% of relapsed ALL. Also mutated in non-Hodgkin lymphoma</td>
<td>Mutations result in impaired histone acetylation and transcriptional regulation.</td>
<td>Mutations selected for at relapse and associated with glucocorticoid resistance.</td>
<td>(114, 115)</td>
</tr>
</tbody>
</table>

Abbreviations: CIR, cumulative incidence of relapse; HSC, hematopoietic stem cell.
lymphoid disease lineage and progression. IKZF1 alterations are also associated with poor outcome in BCR-ABL1–negative lymphoid leukemia (24, 25), suggesting an important role in treatment failure, and provide a potential explanation for the inferior outcome of BCR-ABL1 ALL compared with CML. The mechanistic basis for IKZF1 mutation–induced treatment failure is poorly understood, but it may relate to loss of IKZF1 activity, resulting in acquisition of a primitive, stem cell–like phenotype that is more resistant to therapy (24). These findings suggest that modulation of IKZF1 activity, or the downstream pathways regulated by this gene, might further improve the outcome of this subtype of ALL.

"BCR-ABL1–like" acute lymphoblastic leukemia

Two years ago, 2 groups, the Children’s Oncology Group (COG)/National Cancer Institute Therapeutically Applicable Research to Generate Effective Treatments (TARGET) consortium (24) and a Dutch group led by Monique Den Boer and Rob Pieters (26), identified a subgroup of BCR-ABL1–negative childhood ALL cases, characterized by a gene expression profile similar to that of BCR-ABL1 ALL, which has poor outcome. These cases usually have alterations of IKZF1, but until recently, the genetic alterations substituting for BCR-ABL1 were unknown. These "BCR-ABL–like" or "Ph-like" ALL cases have at least 2 broad groups of genetic changes, resulting in constitutive activation of cytokine receptor and/or tyrosine kinase signaling.

Up to 50% of Ph-like cases harbor rearrangements of CRLF2 [encoding cytokine receptor-like factor 2 or the thymic stromal lymphopoietin receptor (TSLPR); Fig. 2; ref. 27]. CRLF2 forms a heterodimer with interleukin receptor α (IL-7R) for the cytokine TSLP. CRLF2 is located at the pseudoautosomal region of Xp/Yp, and the rearrangements are either translocation to IGH@ at 14q33 (18) or a focal deletion upstream of CRLF2 that results in a novel fusion, P2RY8-CRLF2, in which the first noncoding exon of P2RY8 is fused to the entire open reading frame of CRLF2.
Both result in aberrant expression of CRLF2 by leukemic cells that may be detected by routine flow cytometric immunophenotyping at the time of diagnosis (17, 18). Less common is a point mutation, CRLF2 p.Phe232Cys, which also results in receptor overexpression (28, 29). Half of CRLF2-rearranged cases have activating mutations in Janus kinases (17, 18, 29), most commonly at or near R683 in the pseudokinase domain of JAK2, but also in the kinase domain of JAK2, and in both the kinase and pseudokinase domains of JAK1 (30). Coexpression of CRLF2 and JAK mutant alleles transforms model cell lines (17, 29), and human CRLF2-rearranged leukemic cells exhibit JAK-STAT activation (18). This JAK-STAT activation, in either experimental models or primary human leukemic cells, is attenuated by the use of commercially available selective JAK inhibitors (17). Several studies of high-risk ALL cohorts have shown that CRLF2/JAK alterations are associated with IKZF1 deletion and/or mutation and that these cases have poor outcome (27, 31). Thus, the addition of JAK inhibitor therapy to conventional multiagent chemotherapy is attractive and is being explored in a dose-finding phase I trial of the JAK inhibitor ruxolitinib in relapsed and refractory childhood tumors (the COG ADVL1011 trial). These important findings represent one of the first novel targeted therapies to be implemented in ALL since the dramatically important findings represent one of the first novel targeted childhood tumors (the COG ADVL1011 trial). These findings are important, as Ph-like ALL is common (15%, or at least 3 times as common as BCR-ABL1–positive ALL) and is also present at a similar or higher frequency in adolescents and young adults (M.L. Loh and C.G. Mullighan; unpublished observations), in whom the outcome of ALL therapy is inferior to that of younger children. Moreover, although the genetic landscape of Ph-like is complex, the Ph-like phenotype is readily detectable by expression profiling of a limited set of genes or by flow cytometric analysis of pathway activation (e.g., CRKL phosphorylation for JAK2-rearranged cases). Implementation of these approaches and the development of trials to treat Ph-like ALL with tyrosine kinase inhibitor therapy are being vigorously pursued.

Hypodiploid acute lymphoblastic leukemia

Hypodiploid ALL is an uncommon subtype of ALL that is associated with a very high risk of treatment failure (39–43). These cases have multiple whole chromosomal losses and have been subclassified according to the degree of aneuploidy: near-haploid cases with 24 to 31 chromosomes and low-hypodiploid cases with 32 to 44 chromosomes. Apart from the documentation of aneuploidy, the genetic basis of this subtype of ALL has been very poorly understood. To investigate this, in conjunction with the COG, we performed detailed genomic profiling of a large cohort of hypodiploid ALL cases, incorporating microarray analysis of structural genetic variation and gene expression, extensive candidate gene sequencing, and next-generation sequencing (44).

These studies have shown that near-haploid ALL cases have a very high frequency of deletions and sequence variations, known or predicted to activate Ras signaling, and that near-haploid and low-hypodiploid cases have...
distinct and mutually exclusive lesions inactivating lymphoid transcription factors. Several of the lesions, such as alterations of the IKAROS gene family, have only been rarely observed in other leukemias. Conversely, several of the genes involved in regulation of Ras signaling, including NF1, KRAS, NRAS, and PTPN11, are mutated in other disorders, such as juvenile myelomonocytic leukemia (JMML). Moreover, careful analysis of the frequency and nature of these lesions and analysis of leukemic transcriptome have shown that near haploid and low hypodiploid are distinct diseases at the genetic level. As hypodiploidy confers a high risk of treatment failure and satisfactory therapies are lacking, the high frequency of Ras pathway activation suggests that targeted therapies designed to disrupt downstream Ras signaling should be pursued in this disease.

Clonal Heterogeneity in Leukemia

Most genomic-profiling studies in leukemia have examined unfractionated leukemic cell samples, most commonly obtained at the time of diagnosis. These studies have clearly identified multiple novel lesions that are present in the majority of leukemic cells that are important determinants of leukemogenesis and the outcome of treatment. However, as these profiling techniques become increasingly sophisticated, it has become evident that many patients harbor multiple leukemic clones that have distinct constellations of genetic alterations that influence responsiveness to therapy. Many of these insights have been obtained from sequential genomic profiling of leukemic cells obtained at diagnosis and subsequent disease progression.

Cytogenetic analyses performed many years ago showed that many leukemic samples acquire additional chromosomal alterations at the time of relapse, suggesting that the leukemic clone is not static but evolves over time, possibly in response to the selective pressure of treatment (45). Support for this notion has been obtained from multiple studies that did SNP array analysis of DNA copy number alterations of matched samples obtained at diagnosis and relapse (25, 46–49). Genetically identical diagnosis and relapse clones are uncommon (Fig. 3). Similarly, the emergence of a completely distinct leukemia at relapse is infrequent, although it may be more common in very late relapse and in T-lineage ALL (50). In contrast, most samples retain at least some of the genetic lesions from diagnosis to relapse but also exhibit differences, either as the acquisition of new alterations, suggestive of simple clonal evolution, or more commonly, a more complex picture in which some lesions present at diagnosis are lost, and others are gained. This latter scenario suggests that a “prediagnosis” or “ancestral” clone that acquires some, but not all, lesions required for leukemogenesis undergoes divergent evolution, in which one clone acquires additional lesions and expands to form the predominant clone present at diagnosis (Fig. 3). Other clone(s) acquire different lesions, comprise a minority of cells at diagnosis, but may expand during therapy and form the predominant clone at relapse. The genes targeted by novel lesions at relapse include genes previously

Figure 3. Clonal relationship of diagnosis and relapse samples in ALL. Most relapse samples have a clear relationship to the presenting diagnosis leukemic clone, either arising through the acquisition of additional genetic lesions or, more commonly, arising from an ancestral (prediagnosis) clone. In the latter scenario, the relapse clone retains some, but not all, of the lesions found in the diagnostic sample, while acquiring new lesions. Lesion-specific backtracking studies have shown that, in most cases, the relapse clone exists as a minor subclone within the diagnostic sample prior to the initiation of therapy. In only a minority of ALL cases does the relapse clone represent the emergence of a genetically distinct and thus unrelated second leukemia. Figure originally published in Mullighan et al. (46).
implicated in poor outcome and leukemogenesis, including CDKN2A/B, IKZF1, and ETV6. Notably, some copy number alterations, such as those involving PAX5 and EBF1, are lost from diagnosis to relapse, whereas others, such as IKZF1, are consistently retained, suggesting that they are acquired early during leukemogenesis and that they are important determinants of resistance to therapy.

These genetic data are supported by recent studies that have modeled clonal heterogeneity by detailed FISH of leukemic cells, which have shown that individual leukemic cells have distinct genetic alterations and that the patterns of lesions between cells can be used to construct a branching pattern of clonal evolution in individual ALL cases (51). Transplantation of diagnostic BCR-ABL1 ALL samples into immunocompromised mice, followed by comparative genetic profiling of the engrafted tumors with the primary diagnostic samples, has also shown that distinct subclones engraft different mice, and as for the FISH analyses, the patterns of genetic lesions between individual engrafted samples can be used to infer patterns of clonal evolution (52).

These findings have important implications for diagnostic approaches in ALL, as they suggest that a subset of patients have genetic alterations present in minor subclones at diagnosis that will not be detected by profiling of the unfractionated tumor sample by conventional approaches. Strategies to detect lesions present at very low levels will be required, such as quantitative PCR approaches for individual lesions or next-generation sequencing approaches capable of detecting lesions at very low levels.

Sequence analysis of relapsed acute lymphoblastic leukemia

Genome-wide studies of sequence variation have lagged behind those of DNA copy number variation, but they are now feasible with advances in the yield and cost-effectiveness of next-generation sequencing. Although whole-genome sequencing studies of ALL have not yet been reported, candidate gene sequencing efforts using Sanger sequencing have identified a number of novel targets of sequence mutation in high-risk B-ALL (Fig. 4; ref. 53). To extend our understanding of the genetic basis of treatment failure in ALL, we sequenced 300 genes in 23 matched diagnosis-relapse ALL samples. A key finding of this study was the presence of deleterious mutations in CREBBP, encoding CREB-binding protein (CREBBP), in almost 20% of relapsed ALL cases. CREBBP is a multifunctional protein with roles in histone and non-histone acetylation and transcriptional regulation, by acting as a scaffold of a diverse range of transcription factors. Many of the mutations were located in the histone acetyltransferase domain and impaired the normal acetylation activity of CREBBP. We also showed that the CREBBP mutations impaired the normal transcriptional response to glucocorticoids, which are widely used in ALL therapy, suggesting that CREBBP mutations may have an important role in determining resistance to these agents. Moreover, like IKZF1 alterations, CREBBP mutations were preserved from diagnosis to relapse, or they were present in subclones at diagnosis and emerged in the predominant clone at relapse, supporting the notion that these mutations provide a selective advantage during treatment. Histone deacetylase inhibitors are being explored as potential therapeutic agents in a range of tumors, and preliminary studies using leukemic cell lines showed that many, including those with CREBBP mutations, are exquisitely sensitive to these agents. These findings suggest that histone deacetylase inhibitors may represent a novel therapeutic approach in high-risk leukemia.

Juvenile Myelomonocytic Leukemia

JMML is a rare, but highly aggressive, myeloid malignancy of childhood (54–56). JMML can be a difficult diagnosis to
make, as many patients present with nonspecific clinical features that mimic infections or other conditions. However, it is now known that up to 85% of patients harbor a molecular alteration in 1 of 5 genes, making it easier for clinicians to accurately identify patients with JMML. The World Health Organization (WHO) diagnostic criteria still comprise a range of clinical and laboratory findings, but the addition of genetic mutations creates an easier diagnostic algorithm for clinicians to follow (Table 2). These 5 genes (NF1, NRAS, KRAS, PTPN11, and CBL) encode proteins that when mutated are predicted to activate the Ras/mitogen--activated protein kinase (MAPK) pathway (Fig. 5) and, thus, provide additional avenues for therapeutic approaches, although to date, only hematopoietic stem cell transplant (HSCT) is known to be curative.

One of the hallmark laboratory features of JMML, hypersensitivity of myeloid progenitor cells to granulocyte macrophage colony-stimulating factor (GM-CSF) in colony forming assays (57), can also be present in certain viral infections, thus providing a sensitive, but nonspecific and laborious, adjunctive diagnostic tool (58). However, the presence of the colony assay to assess GM-CSF hypersensitivity has proven invaluable as a laboratory tool to validate newly discovered JMML mutations in either mouse or human cells and can also provide a platform upon which to screen new compounds.

**Patients with genetic syndromes provided important clues to unraveling the molecular pathogenesis of juvenile myelomonocytic leukemia**

Children affected by specific congenital syndromes, including neurofibromatosis type 1 (NF1), Noonan syndrome, or Cbl syndrome are at a higher risk of developing JMML or a related transient myeloproliferative disorder in infancy, and the identification of genes associated with these syndromes has provided unique insights into the pathogenesis of this myeloid malignancy.

In 1978, patients with NF1 were noted to be particularly prone to developing a CML or acute myelomonocytic leukemia in the first decade of life (59). Subsequently, NF1 was cloned and found to encode neurofibromin, which hydrolyzes the active, GTP-bound conformation of Ras to the inactive, GDP-bound conformation and, thus, functions as a GTPase-activating protein (60). In 1994, Shannon and colleagues (61) showed loss of the wild-type allele (later shown to identify acquired isodisomy of the mutant allele) in the diseased bone marrows of children with JMML affected by NF1, thus establishing *NF1* as a tumor suppressor gene (61, 62). In subsequent mouse models engineered to conditionally delete the *Nf1* allele, elevated levels of Ras-GTP occur, and a myeloproliferative neoplasm (MPN) develops (63, 64).

Somatic, oncogenic mutations in *NRAS* and *KRAS2* (RAS) were subsequently identified that year in independent cohorts of patients with JMML, thus providing additional genetic evidence to support that hyperactivation of the Ras/MAPK pathway was essential for the pathogenesis of this disease (Fig. 5; refs. 65, 66). Additional animal models have subsequently revealed that *Kras* mutant mice also develop fatal myeloid disorders that resemble JMML in vivo and in vitro (67, 68).

Noonan syndrome is another congenital disorder that is inherited in an autosomal dominant fashion 50% of the time (reviewed in ref. 69). Interestingly, some infants with Noonan syndrome also display a hematologic phenotype, including a self-resolving myeloproliferative disorder that resembles JMML (70, 71). In 2001, Tartaglia and colleagues identified germline mutations in *PTPN11*, a gene encoding the nonreceptor tyrosine phosphatase protein SHP-2, as causative in approximately 50% of children with Noonan syndrome (72). The observations of a JMML-like MPN in

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**Table 2. Current diagnostic criteria for juvenile myelomonocytic leukemia**

<table>
<thead>
<tr>
<th>Category 1 (all of the following)</th>
<th>Category 2 (at least 1 of the following)</th>
<th>Category 3 (2 of the following if no category 2 criteria are met)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of the BCR-ABL fusion gene</td>
<td>Somatic mutation in <em>RAS</em> or <em>PTPN11</em> and</td>
<td>White blood cell count &gt;10,000 (10^9/L) and</td>
</tr>
<tr>
<td>&gt;1,000 (1 x 10^9/L) circulating</td>
<td>Clinical diagnosis of NF1 or NF1 gene mutation</td>
<td>Circulating myeloid precursors</td>
</tr>
<tr>
<td>monocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20% blasts in the bone marrow</td>
<td>Monosomy 7</td>
<td>Increased hemoglobin F for age</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td></td>
<td>Clonal cytogenetic abnormality</td>
</tr>
<tr>
<td></td>
<td></td>
<td>excluding monosomy 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GM-CSF hypersensitivity</td>
</tr>
</tbody>
</table>

*Current WHO criteria.

*Proposed additions to the WHO criteria that were discussed by participants attending the JMML Symposium in Atlanta, Georgia, 2008 (116). Note that CBL mutations have since been discovered and should be screened for in the work-up of a patient with suspected JMML. Patients who are found to have a category 2 lesion need to meet criteria in category 1, but they do not need to meet category 3 criteria. Patients who are not found to have a category 2 lesion must meet the category 1 and 3 criteria. Only 7% of patients with JMML will NOT present with splenomegaly, but virtually all patients develop this within several weeks to months of initial presentation (C.M. Niemeyer; personal communication).
patients with Noonan syndrome (primarily those arising as spontaneous mono-allelic germline events) led to the identification of mono-allelic, activating somatic mutations in \( \text{PTPN11} \), occurring in as many as 35% of nonsyndromic JMML (73, 74). A comparison between the \( \text{PTPN11} \) mutations in \textit{de novo} and syndromic JMML (Noonan syndrome) reveals that many of the same amino acids in exons 3, 4, and 13 are affected (75), but with different codon substitutions, leading investigators to hypothesize that the transforming ability of mutations in Noonan syndrome are ‘weaker’ and, thus, may be tolerated as germline events. For example, the most common \( \text{PTPN11} \) mutation in \textit{de novo} JMML is the c. 226G > A, resulting in p. Glu76Lys, an alteration that has never been documented as a germline lesion in Noonan syndrome.

To identify additional novel mutations in patients with JMML, we did Affymetrix SNP 6.0 array analysis on samples from JMML patients with and without known Ras pathway abnormalities (76). We identified a region of 11q that acquired uniparental isodisomy in 5 of 27 cases. Importantly, recent reports had uncovered similar lesions in adults with MPN and myelodysplastic syndrome, and subsequent molecular analysis identified homozygous \( \text{c-CBL} \) mutations (77–79). All 5 of our patients also displayed homozygous \( \text{CBL} \) lesions. The most common \( \text{CBL} \) mutation in JMML is the c. 226G > A, resulting in p. Glu76Lys, an alteration that has never been documented as a germline lesion in Noonan syndrome.

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A number of children with JMML harboring these homozygous \( \text{CBL} \) mutations shared phenotypic features, suggesting that these lesions might first occur as germline events (82–84). Additional studies indicate that these mutations are autosomally inherited in a dominant fashion approximately 50% of the time (82). Importantly, genotyping of myeloid colonies grown from 1 patient’s cord blood revealed the presence of rare homozygous clones at birth, supporting our previous finding that oncogenic JMML mutations can arise \textit{in utero} (75, 76).

One striking phenomenon about patients with JMML and homozygous \( \text{CBL} \) mutations is their high rate of spontaneous resolution (82–84). However, of 5 patients reported with spontaneously resolving JMML and the germline \( \text{Cbl} \) syndrome, 4 developed signs consistent with serious vasculopathies by the end of their second decade of life, as evidenced by optic atrophy, hypertension, cardiomyopathy, or arteritis (82).

The relationship of \( \text{Cbl} \) to Ras/MAPK signaling is poorly understood, and furthermore, the function of mutant \( \text{Cbl} \) proteins in malignancy is likely complex. There are 3 members of the \( \text{Cbl} \) family of E3 ubiquitin ligases (\( \text{CBL} \), \( \text{CBLB} \), and \( \text{CBLC} \)), all of which are known to mark activated protein tyrosine kinases and other associated proteins for degradation by ubiquitination, but \( \text{Cbl} \) proteins also retain many important adaptor functions (reviewed in ref. 85). Most of the mutant \text{Cbl} proteins reported to occur thus far in myeloid malignancies result from missense mutations or splice site variants in \( \text{CBL} \), in which the majority have been shown to result in an encoded protein that lacks E3 ligase activity only in the absence of the wild-type protein (79, 82). Two recently generated mouse models indicate possible...
mechanisms by which mutant Cbl proteins exert their phenotypic effects (86, 87). The first is a Cbl, Cblb double-deficient mouse that develops a rapidly fatal MPN in comparison with a Cbl null mouse, which does not develop disease, suggesting that mutant Cbl proteins may confer a dominant negative effect on Cbl b (86). The second is a heterozygous Cbl RING finger mutant knock-in mouse that develops a more latent, but equally fatal, MPN on a Cbl-null background (87). This suggests that mutant Cbl proteins may act as gain-of-function oncoproteins that continue association with certain proteins but that cannot function to degrade protein tyrosine kinases because of the loss of the negative regulatory E3 ligase function, thus constitutively activating growth pathways. Further work should fully interrogate the consequences of mutant Cbl proteins in myeloid malignancies is under way.

Other mutations have been recently reported to occur rarely in JMML, such as ASXL1 or FLT3 (88, 89). In contrast to other MPNs of adulthood, neither JAK2 nor TET2 mutations occur in JMML (M. L. Loh; unpublished data; ref. 81). Taken together, these data would suggest that the current spectrum of commonly occurring mutations in NF1, RAS, PTPN11, and CBL converge on the Ras/MAPK pathway in a highly specific way in JMML, which remains to be fully elucidated.

**Approaches to therapy**

Although the current standard of care for JMML is allo-geneic HSCT, approximately 50% of children will still suffer from relapse. No standard chemotherapy regimen used prior to HSCT has been shown to affect the incidence of relapse after HSCT (90). Based on the spectrum of mutations that were first characterized by loss of NF1 and oncogenic lesions in RAS, it would be reasonable to hypothesize that suppression of Ras/MAPK signaling might have therapeutic efficacy in JMML. However, 3 issues should be mentioned. First, direct inhibition of hyperactive Ras has been problematic for several reasons. For example, Ras is activated when localized to the cellular membrane, which requires covalent binding of a farnesyl group to a conserved CAAX motif by farnesyl transferases (FTase). As prenylation of Ras is catalyzed by FTase, FTase inhibitors (FTI) were developed to prevent Ras localization to the plasma membrane and inhibit Ras signaling (91). However, alternative geranylation can substitute for prenylation, and thus the clinical promise of FTI therapy as Ras inhibitors has been disappointing. The COG sponsored a phase II trial, AAML0122, incorporating a window phase of an FTI followed by chemotherapy only and HSCT (92). The event-free survival on AAML0122 was 40%, with an overall survival of 55%, with a median follow-up of 4 years (T. Cooper; personal communication). Second, the relationships of SHP2 and CBL to Ras signaling are less well understood, and it may be that the biochemical consequences of these lesions will render them more sensitive to alternative therapeutic approaches. Finally, the importance of Ras signaling in diverse physiologic processes in normal cells raises the concern of nonspecific systemic toxicities with any therapies in which normal Ras proteins or their effectors are inhibited. Ideally, one hopes to capitalize on the existence of “oncogene addiction” and identify inhibitors that preferentially treat mutant rather than normal cells. However, amplification of signals upstream or parallel to Ras may occur, raising the possibility that combination therapy to overcome resistance will be necessary to achieve therapeutic effects (93).

One example of a Ras effector pathway involved in JMML is the Raf/MAP–extracellular signal–regulated kinase (ERK) kinase (MEK)/ERK pathway. Ras-GTP induces a cascade of activation from the Raf kinases, to MAPK kinase (MEK1 and MEK2), to ERK, which stimulates diverse nuclear and cytosolic effects. Raf mutations are found in melanoma and renal cell carcinoma, and it is reasonable to hypothesize that inhibiting the Raf/MEK/ERK cascade has therapeutic potential in JMML. To date, the largest clinical experience with Raf inhibitors is with sorafenib (Bay 43-9006), which is U.S. Food and Drug Administration (FDA) approved for treatment of advanced renal cell carcinoma and hepatocellular carcinoma (94, 95). Sorafenib is a novel bi-aryl, multikinase inhibitor with activity against VEGF receptor-2 and receptor-3, platelet—derived growth factor receptor b, fibroblast growth factor receptor 1, FLT-3, and c-KIT (96), as well as Raf-1 and B-ras (97), and it is currently being used in conjunction with chemotherapy for acute myeloid leukemia (AML) in the COG. However, the recent data to support the emergence of skin tumors in patients treated with Raf inhibitors highlight the complexity of signaling networks in cancer cells (93).

MEK is also an attractive target, based on the frequency of Ras and Raf mutations in human cancer. Selective inhibition of MEK, resulting in inhibition of its only known substrate, ERK1/2, can be achieved with non-ATP competitive inhibitors. In recent reports, the use of a MEK inhibitor, PD0325901 (PD901; Pfizer), in a model of JMML and chronic myelomonocytic leukemia, characterized by the conditional expression of the KrasG12D mutant allele, had encouraging results. PD901 treatment resulted in a sustained reduction of peripheral white blood count, improved anemia, and enhanced survival in the treated mutants compared with the wild-type controls (98). Importantly, PD901 abrogated the classic GM-CSF hypersensitivity exhibited by myeloid progenitors in in vitro assays. MEK inhibition may also have a role for patients with refractory JMML who transform to AML. This is suggested by the inhibition of NF1 mutant acute myeloid leukemia colony growth at low doses of CI-1040 and prolonged survival of mice transplanted with NF1 mutant AML when treated with CI-1040 (99). Given the recent description of Ras pathway mutations in hypodiploid ALL, it would be reasonable to assess the clinical utility of MEK inhibition for both of these diseases.

Finally, we and others described the phosphorylation of STAT5 to low-dose GM-CSF in subsets of JMML cells (100, 101). The discovery of activating JAK2 mutations in other MPNs, including the majority of adults with polycythemia vera, essential thrombocytethemia, and primary
myelofibrosis (102–104), led to the development of a number of specific and nonspecific agents that inhibit wild-type and mutant JAK2. Ruxolitinib (formally INCB018424; Incyte/Novartis) has recently been FDA approved for primary myelofibrosis patients, showing excellent tolerability and clinical responses (105, 106). In collaboration with Incyte/Novartis, the COG is determining the maximum tolerated dose of ruxolitinib in children (ADVIL1011) on the basis of recent data showing the presence of JAK lesions in approximately 10% of children with high-risk ALL (30), the known JAK lesions in polycythemia vera and essential thrombocythemia, and the documented signaling abnormalities in JMML (100). Correlating the effect on colony formation, cell growth, and signaling networks in the presence of the inhibitor will help evaluate JAK-STAT signaling in disease pathogenesis and may reveal previously unappreciated relationships between the JAK/STAT5 and Ras/MAPK pathways.

Summary and directions for future work

Advances in genomic technologies have identified a number of lesions that have transformed our understanding of the molecular pathogenesis of leukemia, revealing a number of targetable lesions in both lymphoid and myeloid diseases, particularly in subsets of patients for whom cure is currently elusive or for whom cure is associated with substantial morbidity.

Results from next-generation sequencing efforts have indicated that identification of all genetic alterations contributing to leukemogenesis and treatment failure will require comprehensive analysis of sequence and structural variations with these technologies and deep sequencing of samples at multiple disease time points to identify critical lesions in tumor subclones that confer resistance to therapy.

Ongoing investigations of targeted therapies will require continued study of the biochemical and functional consequences of the genetic lesions occurring in these high-risk diseases and will also require rigorous clinical trials to assess both target inhibition and outcome. However, recent efforts will undoubtedly continue to provide fruitful and productive collaborations among basic, translational, and clinical scientists.

Disclosure of Potential Conflicts of Interest

M.L. Loh is a consultant for Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other author.

Authors’ Contributions

Conception and design: C.G. Mullighan, M.L. Loh
Development of methodology: C.G. Mullighan, M.L. Loh
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.G. Mullighan, M.L. Loh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.G. Mullighan, M.L. Loh
Writing, review, and/or revision of the manuscript: C.G. Mullighan, M.L. Loh
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.L. Loh
Study supervision: M.L. Loh

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References


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