and catheter-based convection enhanced delivery, or strategies to increase BBB permeability by using focused ultrasound or hyperosmotic solutions.[2] Ferreri et al use NGR-hTNF, a drug that binds to CD13, a metalloproteinase, almost exclusively expressed by tumor blood vessels, thereby selectively increasing vessel permeability (and "opening" the BBB) at the level of the tumor, and, in theory, increasing intratumoral delivery of R-CHOP.

As noted, the disruption of the BBB is heterogenous within different regions of PCNSL and additionally the BBB is intact in tumor-infiltrated, nonenhancing regions. It is critical to deliver chemotherapy across all these regions. Ferreri et al observed CD13 expression on tumor vessels in all 12 cases and low baseline values of BBB permeability in the peritumoral areas. These same peritumoral regions demonstrated increased permeability after infusion with NGR-hTNF. In theory, this increased permeability may result in increased delivery of the component drugs of R-CHOP in these regions. However, this remains unproven. As expected, drug delivery to the subarachnoid space was unchanged likely the result of lack of impact of NGR-hTNF on the blood–CSF barrier. Thus, the CSF may emerge as a sanctuary site and source of future relapse in these patients. The blood–retina barrier was not assessed in this study. The efficacy assessments are preliminary but encouraging, with complete and partial radiographic responses observed in 8/12 and 1/12 patients, respectively. However, further assessment is necessary to determine the durability of these radiographic responses.

Disruption of the BBB can be associated with increased cerebral edema as well as increased hydrostatic pressure within the tumor, which could cause seizures or other focal neurological symptoms and signs as well as diminished delivery of drugs, respectively.[2,8] However, in this small study, there was no neurotoxicity of any grade reported after 62 infusions.

Nearly all cases of PCNSL most closely resemble the activated B-cell subtype of DLBCL by gene-expression profiling. Patients with the activated B-cell subtype of DLBCL have inferior outcomes after R-CHOP vs the germinal center B-cell subtype of DLBCL.[9] Hence, increasing the delivery of R-CHOP alone across the BBB may ultimately prove less effective vs the development of novel agents and combinations that target the known oncogenic drivers of PCNSL. Moreover, whether this strategy will be effective in the 10% to 20% of PCNSL patients with concurrent brain, CSF, and eye involvement is an open question given the uncertainty around the effect on the blood–CSF and blood–retina barriers.

As noted by Ferreri et al, NGR-hTNF failed to demonstrate an improvement in survival in a randomized phase 3 trial in malignant pleural mesothelioma.[10] However, the rationale and safety data in PCNSL are compelling to continue further development of NGR-hTNF in PCNSL. Moreover, confirmation of these encouraging, but preliminary, results in the ongoing expansion phase of the INGRID trial would set the stage for reassessment of this "augmented" version of R-CHOP for PCNSL.

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MYELOID NEOPLASIA

Comment on Garg et al, page 263

Triple-mutant AML: too clever by HLF?

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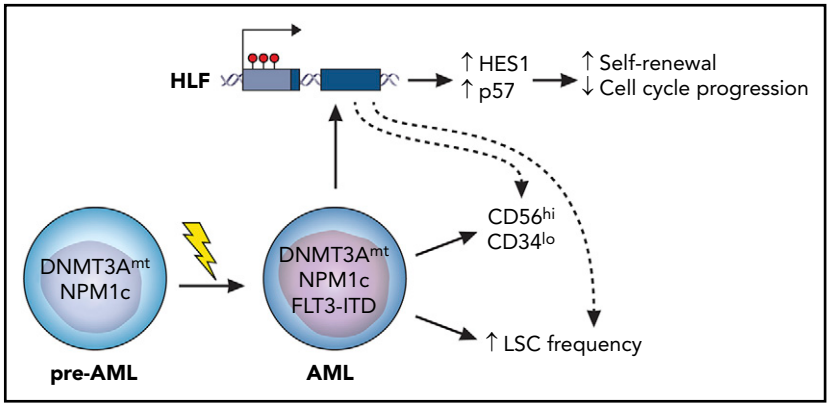
In this issue of *Blood*, Garg et al report on their investigation of the molecular interaction between mutations affecting *DNMT3A* and *NPM1*, and internal tandem duplications (ITDs) of *FLT3* in acute myeloid leukemia (AML). They found that when the 3 mutations cooccur in AML, they synergize to drive increased expression of hepatic leukemia factor (*HLF*), a transcription factor, and are associated with particular characteristics such as increased leukemic stem cell (LSC) frequency and an aberrant immunophenotype (GPR56^{high}CD34^{low}), which seem to be at least in part attributable to overexpression of *HLF*. Their findings establish *HLF* overexpression as an important mediator of the adverse phenotype and propose that HLF or its downstream effectors represent potential therapeutic vulnerabilities of this poor-prognosis AML subtype.[1]



The cooccurrence of oncogenic mutations affecting the *DNMT3A* and *NPM1* genes, together with ITDs in *FLT3*, is one of the most common combinations of genetic events in AML and seems sufficient to generate the disease. The reasons for why there is a relatively high frequency of this cooccurrence and why it is associated with a worse prognosis for patients are poorly understood. Garg et al report that the 3 mutations synergize to upregulate the *HLF* gene in association with hypomethylation of its promoter. In turn, HLF activates a set of genes that includes *HES1* (a known NOTCH target) and the cell cycle inhibitor p57 (*CDKN1C*). These changes are associated with enhanced self-renewal of AML cells, an increased LSC frequency, and an aberrant immunophenotype ($CD56^{high}CD34^{low}$) that is characteristic of the triple-mutant cells. Genetic targeting of *HLF* sensitized AML cells to antileukemic drugs, suggesting that targeting this pathway may represent a novel therapeutic strategy in this and potentially other AML subtypes.

Large-scale sequencing studies have revealed the considerable genetic heterogeneity of AML and have identified recurrent mutations in more than 75 genes and chromosomal regions.[2,3] Within this complex landscape, patterns of significant cooccurrence and mutual exclusivity allude to mechanistic interactions between different mutations and help define genetic subtypes of AML. Perhaps the most striking such pattern is the 3-way cooccurrence of mutations affecting *DNMT3A* (particularly at the R882 codon), *NPM1* (cytoplasmic mutants, NPM1c), and *FLT3* (predominantly *FLT3*-ITD). The pattern is seen in 6% to 8% of all cases of AML,[2,3] a strikingly high percentage for a 3-gene comutation, yet little is known about its precise molecular underpinnings. Furthermore, clinical outcome data show that *DNMT3A/NPM1/FLT3*-ITD triple-mutant AML has a particularly bad prognosis with a long-term survival below 20%,[2] the basis for which is not understood.

To provide insights into these observations, Garg et al initially studied surface expression of the LSC marker GPR56 (which they identified previously[4]) on these AML cells and found that the aberrant $GPR56^{high}CD34^{low}$ immunophenotype was specific to triple-mutant leukemic cells and was not seen in normal hematopoietic stem and progenitor cells (HSPCs) or in AML subclones that did not harbor all 3 mutations. They proceeded to study the transcriptome of triple-mutant AML cells using RNA sequencing and, after focusing specifically on differentially expressed transcription factors, they identified *HLF* overexpression as a characteristic feature of these leukemias. *HLF* was originally identified as a partner in the *E2A-HLF* fusion gene in pediatric acute lymphoblastic leukemia,[5] and it also has important roles in normal hematopoiesis,[6,7] but it has not been reported to have a role in AML. By examining publically available CpG methylation data sets in AML, they found that *HLF* overexpression was in fact associated with hypomethylation of its promoter, a feature encountered more commonly in AMLs with *DNMT3A* R882 mutations than in those with wild-type *DNMT3A*.

Having identified *HLF* overexpression to be characteristic of triple-mutant AML, Garg et al investigated whether this had a pathogenetic role by using xenotransplantation into immunodeficient mice. They report that genetic disruption of *HLF* with CRISPR-single guide RNA (*sgHLF*) in a human triple-mutant AML xenograft was associated with reduction in the LSC-rich $GPR56^{+}CD34^{+}$ and expansion in the LSC-poor $GPR56^{-}CD34^{-}$ cell compartments. This phenotype was associated with reduced ability of engrafted *sgHLF*-targeted AML cells to proliferate when reintroduced into culture ex vivo and a further reduction in the $GPR56^{+}CD34^{+}$ compartment upon secondary transplantation.

To understand the mechanistic basis of these leukemogenic effects of *HLF* overexpression, the authors used label-retention and 5-ethynyl-2-deoxyuridine (EdU) pulse-chase studies to characterize the impact of *HLF* loss on cell cycle and proliferative properties of AML cells. They report that *sgHLF*-targeted cells progressed through the cell cycle faster, divided more often, and were more sensitive to anti-AML drugs, including cytarabine and daunorubicin, than were control cells. To delve into the molecular basis for this phenomenon, they studied gene expression changes upon short hairpin RNA–mediated inhibition of *HLF* in the same triple-mutant AML cells. Among other effects, this resulted in downregulation of the cyclin-dependent kinase inhibitor gene *CDKN1C* and the NOTCH target gene hairy and enhancer of split 1 (*HES1*), which are plausible mediators of the ability of HLF overexpression to slow down cell cycle progression and maintain the LSC-rich $GPR56^{+}CD34^{+}$ compartment, respectively (see figure). Garg et al propose that HLF plays an important leukemogenic role in triple-mutant AML and is likely to be at least in part responsible for the poor prognosis of triple-mutant AML. Thus HLF and its effectors represent possible therapeutic vulnerabilities of this and potentially other AML subtypes.

Beyond providing novel insights into the molecular pathogenesis of AML, the article by Garg et al also represents an important paradigm in the study of carcinogenesis by demonstrating that the combined molecular effects of multiple oncogenic mutations can have unpredictable consequences that can influence cellular phenotype, clinical behavior, and responsiveness to therapy. Researchers and clinicians need to bear this in mind when considering the large number of AML cases driven by other combinations of 3 or more mutations, which are rare or even unique and cannot easily be studied in the same way. Although this is a sobering thought, it may also be safe to assume that the reason some mutations cooccur only rarely is that they are not able to synthesize powerful molecular effects in combination, unlike the 3 mutations studied here.

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TRANSPLANTATION

Comment on Cuvelier et al, page 304

Joint effort to target the orphan of the orphan

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In this issue of *Blood*, Cuvelier and colleagues from the Applied Biomarkers of Late Effects of Childhood Cancer (ABLE) Consortium evaluate, for the first time, the performance of the National Institutes of Health Consensus Criteria (NIH-CC) for diagnosing chronic graft-versus-host disease (cGVHD) in pediatric patients in a prospective multicenter trial. They demonstrate the utility and limitations of the criteria and provide a benchmark for incidence and severity distribution of late-acute GVHD (L-aGVHD) and cGVHD in a large pediatric population.[1]

Clinical research in cGVHD after allogeneic hematopoietic stem cell transplantation (alloHSCT) is increasingly focused on new drug development fostered by the NIH consensus conferences in 2005[2] and 2014.[3] Until now, pediatric cGVHD has been largely ignored for several reasons. First, the average number of pediatric alloHSCTs per center is significantly lower than adult alloHSCTs and represents only 20% of the total transplantations. Second, the indications for pediatric alloHSCT are more heterogeneous with up to 40% to 50% of patients receiving transplants for nonmalignant diseases. Finally, the frequency of cGVHD is lower in children compared with adults for reasons that are not well understood. As a consequence, until recently, pediatric cohorts for evaluating cGVHD were either small or did not use the NIH-CC, which prevented any reliable conclusion on the current incidence of cGVHD and performance of NIH-CC in pediatric cGVHD.[4] To overcome these issues, the ABLE Consortium, consisting of 27 pediatric transplant centers, performed a multicenter trial to validate the performance of NIH-CC for diagnosis of L-aGVHD and cGVHD and to develop biomarkers to diagnose and predict the course of cGVHD. Even with 27 centers, it took almost 4 years to recruit 302 patients, indicating that large international multicenter activities are a crucial prerequisite for success because single centers or even national cohorts would fail to recruit sufficient numbers of pediatric patients with cGVHD.

The ABLE trial provides several valuable results. First, it confirms the low (21%) incidence of cGVHD in pediatric cohorts, which is basically half the incidence in adult patients.[5] Second, in this study population, the significance of aGVHD is noticeable because of a low rate of de novo cGVHD and a higher incidence of L-aGVHD (24%, mostly persistent or recurrent) than of cGVHD, late acute being the major reason for re-classification, which implicates the relevance of aGVHD as the dominant risk factor for pediatric cGVHD. The specific role of aGVHD in the pediatric population is highlighted by the effect of age older than 12 years as a risk factor for cGVHD but not for L-aGVHD. Moreover, the results emphasize the importance of applying NIH criteria to prevent incorrect classification. Even within the trial, a review committee re-classified a significant proportion (25%) of patients. This also indicates that physicians continue to diagnose cGVHD by day posttransplant (ie, after day 100), most likely for its simplicity, even if diagnostic or distinctive symptoms of cGVHD are lacking. This emphasizes the need for rigorously monitoring the classification of all GVHD cases within clinical trials.

Third, the ABLE trial (for the first time) validated the 2014 NIH-CC in bronchiolitis obliterans syndrome (BOS)[3] in pediatric patients; this demonstrates a significant failure rate in diagnosing BOS, mostly because of the age limitations of lung function testing, nonspecific changes in the computed tomography chest scan, and inconclusive histopathology. Because these obstacles are unlikely to be easily resolved, the development of diagnostic biomarkers to identify patients with BOS is an area of great medical need.[6,7]

Fourth, the authors also captured immunologically driven events such as immune thrombocytopenia or nephrotic syndrome that do not meet the NIH-CC but require immunosuppression, which have been recently labeled as "undefined other cGVHD."[8] The latter has typically been ignored in previous prospective trials, although events such as these often require immunosuppressive treatment.

Last but not least, the trial provides important evidence regarding the incidence of cGVHD during the first year after alloHSCT. However, some patients with L-aGVHD may develop the quiescent onset of cGVHD after the 1-year assessment. Finally, the long-term outcome after diagnosis of cGVHD in pediatric patients, including crucial information on