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and submitted to sequencing for barcode identification and quantification. A significant proportion of hematopoiesis (≈9%-50%) was derived from the labeled $CD34^+$ cells in each animal, was stable over time, and was derived from 3000 to 5000 different clones.

The authors document waves of short-lived cell-type–restricted clones immediately after transplantation which are replaced by long-lived ones after 2 to 4 months. They detected thousands of these long-lived clones which variably contributed to hematopoiesis. A fraction of them contributed disproportionally to hematopoiesis. This was true for all cell types. Remarkably, these long-lived clones were quantitatively stable over the observation period. There was no evidence of clonal succession, exhaustion, or expansion. Only the T-cell fraction saw the emergence of several new large clones, probably responding to adaptive immune response to antigenic stimulation. These results are in line with those reported by Biasco et al, who performed in vivo tracking of the clonal dynamics of 4 patients with Wiskott-Aldrich syndrome treated with lentiviral gene therapy.[3] However, they partially contradict the results obtained by Sun et al, who labeled murine native hematopoiesis using Sleeping Beauty transposase.[4] In the granulocytic fraction, Sun et al documented bursts of successive clones with no long-term stability and little correlation with B or T cells. These discordant results may be explained by the size of the model, the difference between native and posttransplantation hematopoiesis, and the different technological approaches used to label HSPCs. These discrepancies will be difficult to resolve, the study of native hematopoiesis in humans is limited to noninvasive clonal markers such as X-chromosome inactivation (XCI) ratio analysis.[5] That being said, the stable marathon-like behavior concept for HSPCs is appealing.

The results presented by Koelle et al raise an interesting paradox between early replicative advantage and long-term maintenance of hematopoiesis. After the initial posttransplant period characterized by clonal diversity, stable clones emerge. The relative clone size is different. Because the barcode is unique and corresponds to a single HSPC, this indicates that some HSPCs replicated more actively than others during this early phase, generating larger clones. The paradox resides in the fact that, right after this replicative period, no change in clone size is observed. There is institution of a status quo. Minimally, this indicates that there is a genetic diversity among HSPCs,[6] and it also raises the question of the equilibrium between expansion and maintenance of the clone. This equilibrium is probably dependent on the complex interactions between genetic, epigenetic, telomere biology, and HSC niche determinants.[7] The identification of factors that promote clonal expansion and break this equilibrium is of high clinical relevance. It may be key to understanding why some aging individuals develop clonal hematopoiesis,[8] which has been associated with risk of hematological cancer progression.[9]

Koelle et al also allowed us to reevaluate the proximity of the end lineage cell type. As expected, granulocytes and monocytes are highly correlated and originate from the same HSPCs. Initially, the correlation between B cells and the myeloid fraction was weaker, but it continuously increased with time. In fact, the correlation between B cells and myeloid cells was greater than the correlation between B cells and T cells. This was due to the emergence of significant T-cell clones potentially associated with immune response. Interestingly, we have obtained identical correlations between cell subtypes studying a large cohort of female subjects using an XCI method.[10] Taken together, this indicates that: steady-state hematopoiesis is derived by multipotent progenitor cells, the human blood hierarchy paradigm may be slightly different than initially thought, and the paradigm may need to be revisited.

The recently developed clonal tracking methodology used by Koelle et al is complex and costly. However, it is robust and highly quantitative. As demonstrated in the article, it has the potential to unravel several mysteries regarding HSC biology in health and in disease.

*Conflict-of-interest disclosure: The author declares no competing financial interests.* ■

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● ● ● LYMPHOID NEOPLASIA

Comment on Ahn et al, page 1469

# Ibrutinib-resistant CLL: unwanted and unwonted!

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In this issue of *Blood*, Ahn et al find intriguing clinical and molecular differences between early and late relapsing patients in a phase 2 trial of chronic lymphocytic leukemia (CLL) treated with ibrutinib.[1]

The introduction of ibrutinib has changed the paradigm of CLL management. Treatment is moving away from cytotoxic chemotherapy and toward targeting biological pathways like B-cell receptor signaling and induction of apoptosis. Ibrutinib, a covalent inhibitor of Bruton tyrosine kinase (BTK), has been approved for treatment of

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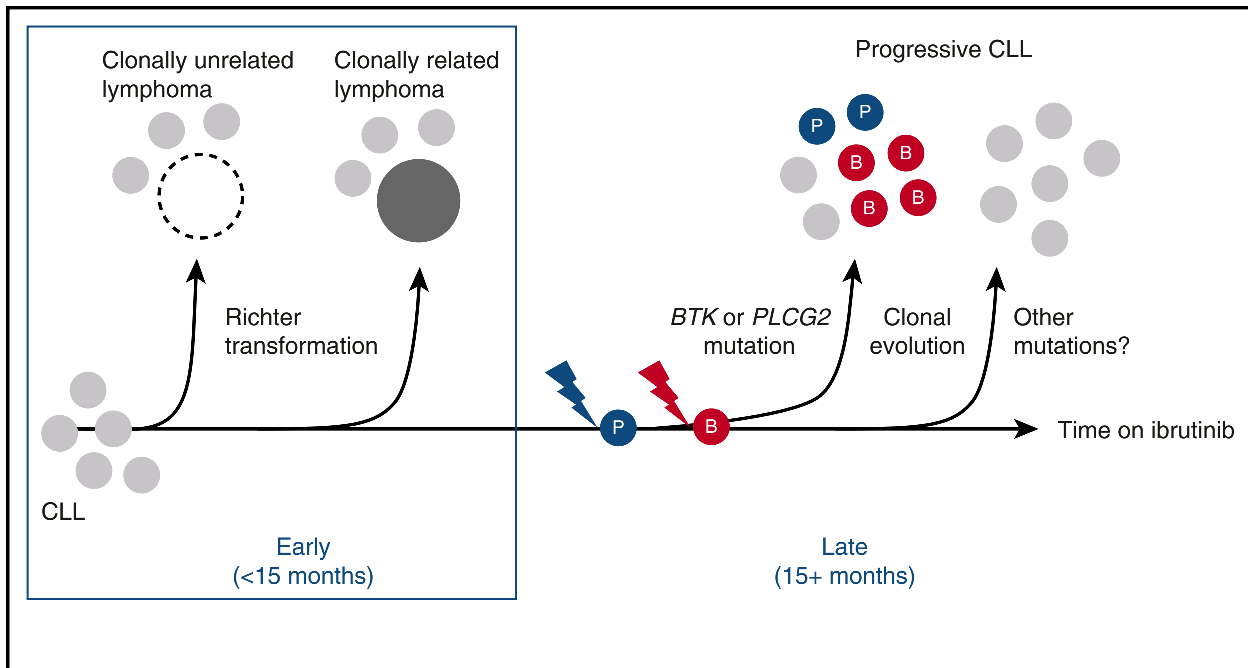


Development of resistance against ibrutinib in CLL. Treatment of CLL patients with ibrutinib has to be stopped because of development of clonally nonrelated aggressive lymphoma (Richter transformation), Richter transformation of a CLL subclone (both mostly occurring within 12 to 18 months of treatment initiation), progressing CLL concurring with acquisition of BTK and/or PLCG2 mutations (mostly occurring after 12 to 18 months of treatment), and possibly late progression in which no mutations of BTK or PLCG2 can be detected. The figure has been adapted from Figure 5 in the article by Ahn et al that begins on page 1469.

CLL that harbors mutations in *TP53* or deletion of 17p for relapsed or refractory disease[2] and also for first-line treatment.[3]

Resistance to ibrutinib, however, occurs in a subset of patients, particularly in heavily pretreated patients and in high-risk CLL.[4,5] Unfortunately, this resistance correlates with aggressive disease, either progressive CLL or Richter transformation, that is difficult to treat. It is therefore of prime importance to not only understand the molecular mechanism of resistance, but also to detect the emergence of resistant disease early or even preemptively, because affected patients will require biologically tailored alternative treatment strategies.

Ahn et al and others have commented on two distinct types of resistant disease that are of special interest (see figure).[6] Although patient numbers are small, in the first group of patients, Richter transformation occurs within 12 to 18 months of treatment initiation and, at transformation, *BTK* and *PLCG2* mutations are not common. In contrast, a second group of patients with later progression of CLL that is frequently associated with mutations of *BTK* and *PLCG2*. There may be a third group of patients with progression of CLL without *BTK* or *PLCG2* mutations. However, the majority of patients who have CLL progression while receiving ibrutinib seem to harbor mutations in their CLL tumor cells in the targeted *BTK*, in phospholipase *PLCG2*, or both.[4]

The article by Ahn et al reports a phase 2 trial that included elderly patients with CLL and patients with CLL who harbored del(17p) and were treated with first-line ibrutinib. In that cohort, 15 (17.9%) of 84 patients progressed with a median follow-up of 34 months. Similar to previous studies, it is always the target cysteine residue C481 that is mutated in *BTK*. The variety of amino acid changes in this position point to the mode of action and specificity of ibrutinib: abrogation of the covalent binding to C481 with retention of the full-length functional BTK points to the importance of full (irreversible) inhibition vs incomplete (competitive) inhibition as a therapeutic mechanism.

In *PLCG2*, it is the autoinhibitory SH2 domain whose function is lost in resistant cells by deletion or mutation, leading to an induction of PLCG2 activity that is independent of BTK. Two of these amino acid changes resulted in a hyperactivated form of PLCG2 via the GTPase RAC.[7] An unresolved issue is the oftentimes low allelic fraction of mutations of *BTK* and *PLCG2* that has been observed in patients with resistant CLL.[1,8] Concurrent mutation of *BTK* and *PLCG2* suggests either that the mutations are present in different subclones or that an additional activation of PLCG2 is advantageous for bypassing BTK inhibition. In addition, the sequence of events is not strictly defined because mutation of *PLCG2* does not occur first in all patients.

However, the fact that *BTK* and *PLCG2* are specifically mutated in ibrutinib-resistant CLL underlines the critical importance of the BCR pathway for survival of CLL cells, that *BTK* within CLL cells is the bona fide target of ibrutinib, that inhibition of the BCR pathway with its critical elements BTK and PLCG2 is the therapeutic principle, and that therapeutic success depends on inhibition of this pathway.

Ahn et al, along with others, noted that development of resistance to ibrutinib seems to be a selection process by cells harboring *BTK* and/or *PLCG2* mutations that are present, at least in some cases, before initiation of ibrutinib treatment.[9] If these unusual (unwonted) cells are only present or subsequently only increase in patients that do develop resistance—which remains to be tested—their detection could be used to instruct treatment decisions. In their analysis of consecutive samples, Ahn et al could show that under selection pressure with ibrutinib, CLL clones with a mutation of *BTK* grow faster than clones with a mutation of

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*PLCG2*, although this could be tested only in a small number of patients. In contrast, growth rates of clones with mutations of *BTK* in the same patient were identical. Thus, from a sensitive detection of the numbers of unwonted CLL cells harboring *BTK* or *PLCG2* mutations before treatment, in theory the development of resistance could be predicted in patients prospectively.

Upon development of resistance against ibrutinib, salvage treatment with PI3K inhibitors (eg, idelalisib) or BCL2 inhibitors (eg, venetoclax[10]) lead to prolonged survival, whereas treatment of patients with Richter transformation remains largely ineffective.[1] Therefore, identification of this latter group of patients at or even before initiation of ibrutinib treatment is an urgent unmet need.

How can these findings inform clinical management? Unfortunately, the quality of response to ibrutinib seems to be no measure of the probability of relapse as a result of the emergence of resistance. Serum levels of CCL3 and CCL4 were tested by Ahn et al as prognostic factors but did not increase in all patients with disease progression. However, mutations of *BTK* and *PLCG2* predated clinical progression. It could therefore be advantageous to monitor mutations of *BTK* and *PLCG2* in CLL patients in a clinical setting to prevent development of resistant CLL.[6] To target these unwonted cells that are pretherapeutically present and that harbor mutations of *BTK* or *PLCG2*, combinatorial treatment is possibly the best choice (eg, CLL2-GIVe trial of the German Chronic Lymphocytic Leukemia Study Group and Jones et al[10]). With the molecular mechanisms of resistance against the novel targeted therapies being described in detail, it becomes more and more clear that these therapies maybe best used when complementing each other. They are the likely route to highly effective treatment, with the ultimate goal of a cure for patients suffering from CLL.

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● ● ● MYELOID NEOPLASIA


Comment on Liu et al, page 1491


# *miR-125b* promotes leukemogenesis via *VEGFA*


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In this issue of *Blood*, Liu et al provide evidence that *miR-125b* may contribute to leukemogenesis by activating an autocrine loop involving *VEGFA*.[1]


The *miR-125* family contains 3 genes (*MIR125A, MIR125B1*, and *MIR125B2*) that are located on different chromosomes but share identical seed sequences. These genes are among the most highly expressed microRNAs in hematopoietic stem cells (HSCs), with expression levels declining during hematopoietic maturation. There is substantial evidence implicating *miR-125* in the regulation of HSC function. Enforced expression of *miR-125* family members enhances HSC engraftment and self-renewal activity and promotes skewing toward the myeloid lineage.[2] Indeed, expression of *miR-125a* confers long-term repopulating activity on human and mouse multipotent progenitors.[3]

There is evidence implicating *miR-125b* in the pathogenesis of hematologic malignancies. *miR-125b* is highly expressed in a subset of acute myeloid leukemia (AML), including those with t(2:11)(p21;q23), AML1/ETO, and PML/RARα translocations. High *miR-125b* expression has also been observed in myelodysplastic syndrome with 5q deletions and acute megakaryoblastic leukemia associated with Down syndrome. Enforced expression of *miR-125b* in mice typically results in a myeloproliferative disorder characterized by leukocytosis, anemia, splenomegaly, and eventual death. Moreover, overexpression of *miR-125b* cooperates with *BCR-ABL* to induce a rapidly fatal myeloproliferative disease in mice.[4] However, the mechanism(s) by which *miR-125b* cooperates with other oncogenes to promote leukemia initiation and maintenance remains poorly understood.

In the current study, Liu and colleagues developed a new transgenic mouse model with doxycycline-inducible *miR-125b* expression to explore the mechanisms through which *miR-125b* cooperates with other oncogenes to induce AML. They focused on the *MLL-AF9* oncogene because *miR-125b* is highly expressed in a subset of AMLs with *MLL* translocations. Liu et al show that coexpression of *miR-125b* modestly shortens the latency of *MLL-AF9*–induced AML and increases the penetrance of AML after secondary transplantation. The latter effect was partially




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