Preleukemic Hematopoietic Stem Cells in Human Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) is an aggressive malignancy of the bone marrow characterized by an uncontrolled proliferation of undifferentiated myeloid lineage cells. Decades of research have demonstrated that AML evolves from the sequential acquisition of genetic alterations within a single lineage of hematopoietic cells. More recently, the advent of high-throughput sequencing has enabled the identification of a premalignant phase of AML termed preleukemia. Multiple studies have demonstrated that AML can arise from the accumulation of mutations within hematopoietic stem cells (HSCs). These HSCs have been termed “preleukemic HSCs” as they represent the evolutionary ancestors of the leukemia. Through examination of the biological and clinical characteristics of these preleukemic HSCs, this review aims to shed light on some of the unexplored questions in the field. We note that some of the material discussed is speculative in nature and is presented in order to motivate future work.

Keywords: leukemia, myeloid, acute, preleukemic hematopoietic stem cell, clonal hematopoiesis, clonal evolution, premalignant lesions

The earliest evidence for a preleukemic phase of acute myeloid leukemia (AML) came from clonality studies in adult and pediatric patients (1–15). Collectively, these experiments demonstrated that leukemogenic mutations arise in multipotent hematopoietic cells and have been thoroughly reviewed previously (16, 17). The current model for preleukemic clonal evolution has resulted from multiple lines of scientific evidence ranging from mouse models to high-throughput sequencing of primary human specimens. This model (18) posits that the first leukemogenic mutation must either occur in a cell that is capable of self-renewal or confer self-renewal upon the cell. If the first mutation fails to meet one of these two criteria, it will be lost over time due to terminal differentiation.

This model has been investigated over the past 5 years, beginning with the first prospective identification of preleukemic HSCs (19). These initial observations were enabled by the identification of cell surface markers, TIM3 and CD99, which allow for prospective separation of normal HSCs from leukemic cells (20, 21). Utilizing these markers, immunophenotypic HSCs isolated from leukemia patients are capable of generating bi-lineage engraftment in immunodeficient mice, demonstrating that they represent bona fide HSCs (19). From targeted deep sequencing, these HSCs were identified to harbor some, but not all, of the leukemia-specific mutations. Moreover, single-cell-derived colonies...
GENETIC, MOLECULAR, AND CELLULAR CHARACTERISTICS OF PRELEUKEMIC HSCs

Follow-up studies provided additional support for these conclusions through investigation of expanded patient cohorts and targeted sequencing experiments (22, 23). In particular, these studies identified patterns of mutation acquisition whereby the earliest mutations in leukemia evolution occur predominantly in genes that regulate the epigenome, while the latest mutations occur predominantly in genes that lead to activated signal transduction and proliferation pathways (22–28). The most common preleukemic mutations occur in the DNA methyltransferase 3A (DNMT3A) and ten-eleven translocated 2 (TET2) genes (22–24). Additional genes mutated during the preleukemic phase include isocitrate dehydrogenase 1 and 2 (IDH1/2) (22, 29) and the members of the cohesion complex (30). The most common late (non-preleukemic) mutations occur in Fms-like tyrosine kinase 3 (FLT3) and Kirsten rat sarcoma viral oncogene homolog (KRAS). Mutations in other common leukemia-related genes such as nucleophosmin 1 (NPM1), CCAAT/enhancer-binding protein alpha (CEBPA), and Wilms tumor 1 (WT1) have been found to occur as both preleukemic and late events (22, 23).

In addition, recent work has demonstrated that the penetrance of preleukemic mutations varies greatly across patients (31). We have previously introduced the concept of “preleukemic burden,” which we define as the percent of HSCs in a leukemia patient that harbor at least the first preleukemic mutation. In this way, patients whose preleukemic HSCs have expanded greatly will have a high preleukemic burden (Figure 1A). It is now clear that the preleukemic burden across AML patients can vary from 100% to below the limit of detection of standard high-throughput sequencing methodologies (~1%) (Figure 1B) (31, 32). To illustrate this point clearly, a preleukemic burden of 100% indicates that a single HSC expanded to outcompete all other HSCs after acquisition of the first preleukemic mutation. This highlights some of the key characteristics of preleukemic HSCs—the ability to survive, outcompete normal HSCs, and undergo clonal evolution through the acquisition of multiple additional mutations, eventually leading to frank leukemia. Mutations in both TET2 and DNMT3A have been shown to be significantly associated with high preleukemic burden in AML (31). Nevertheless, it remains unclear how some patients develop AML with undetectable preleukemic burden while others exhibit full reconstitution of their HSC pool with mutated HSCs. Some of this difference may be mediated by the preleukemic mutations acquired and the time since mutation acquisition, but this is only one piece of a very complicated puzzle (33). Moreover, the same mutations can sometimes lead to highly divergent preleukemic burdens. For example, mutations in DNMT3A have been shown to lead to preleukemic burden ranging from undetectable to 100% (31). One intriguing hypothesis is that this difference is mediated by epigenetic differences in the cell of origin, with certain epigenetic profiles being more primed for clonal competition than others. Future work investigating how and why preleukemic burden is so variable will be crucial to our understanding of this phase of the disease.

The precise mechanisms that mediate this clonal outcompetition remain incompletely understood. From an evolutionary standpoint, an increase in the “fitness” of a stem cell would likely come from an increase in self-renewal. More specifically, a stem cell that produces more daughter cells whose self-renewal potential is at least as great as the parental cell would have an increased fitness. In the context of a preleukemic stem cell, it is not sufficient to merely produce more daughter cells. Rather, those daughter cells must retain the ability to self-renew if they are to persist long enough to acquire additional preleukemic and eventually leukemic mutations. This idea has been functionally tested in preleukemic HSCs isolated from AML patients, demonstrating...
that preleukemic HSCs resist enforced differentiation in vitro in comparison to both cord blood- and adult bone marrow-derived hematopoietic stem and progenitor cells (31). This observation supports the hypothesis that mutations in certain genes (i.e., DNMT3A, TET2, IDH1/2, and the cohesin complex) occur predominantly during the preleukemic phase because they function in part to prevent differentiation. Presumably, these mutations simultaneously enable the HSCs to persist long enough to acquire additional mutations and prevent full differentiation during the leukemic phase of AML. Mechanistically, mutations in these epigenetic regulators could lead to modest but impactful alterations in key lineage defining genes that lead to clonal outcompetition (34, 35). This model of preleukemic evolution is supported by additional studies that demonstrate that certain preleukemic mutations prevent differentiation, both in mouse models and in in vitro culture (30, 36–42).

**CLONAL HEMATOPOIESIS (CH) AND PRELEUKEMIA**

Since the discovery of preleukemic HSCs, multiple groups have identified an age-associated syndrome that has been termed clonal hematopoiesis of indeterminate potential (CHIP) (43–50). CHIP was identified by searching for mutations in genes that occur in hematologic malignancies in blood cells from individuals with no history of hematologic disease that had been sequenced for genomic studies of other conditions. CHIP is characterized by the clonal outgrowth of mutated hematopoietic cells. The most frequently mutated genes in CHIP are DNMT3A and TET2, echoing their role during the preleukemic phase of AML (43, 44, 50). These studies have shown that the incidence of CHIP is associated with age, with very few individuals under the age of 40 showing detectable CH and more than 10% of individuals over the age of 70 showing detectable CH (43, 44). In fact, a small-scale follow-up study using targeted error-corrected sequencing for more sensitive mutation detection (≥0.0003 VAF) identified CHIP in 95% of individuals between the ages of 50 and 60 years old (51). Most individuals only have one detectable mutation in a gene known to be involved in hematologic malignancy. Importantly, the presence of CHIP with a variant allele fraction of at least 0.10 is associated with a 49-fold higher relative risk of developing a hematologic malignancy. However, the absolute risk of hematologic malignancy remains small, with only 4% of persons with CHIP progressing to malignancy (43, 44). These findings raise the possibility of leukemia prevention if therapeutics are developed that can target these pre-malignant cells (discussed below).

In addition to being associated with an increased risk of hematologic cancer, CHIP is also associated with other adverse health outcomes. Of particular note, after controlling for age, sex, and diabetes, the presence of CHIP is associated with an increased all-cause mortality (hazard ratio, 1.4). Contributing to this increase in all-cause mortality, carriers of CHIP have a 1.9-fold higher risk of coronary heart disease, potentially due to an increased secretion of several cytokines and chemokines from mutant hematopoietic cells that contribute to atherosclerosis (52, 53). Intriguingly, a recent study of more than 8,000 individuals has shown an association of CH with solid tumor malignancies (54). Of all cancer patients, 25% carried CH, with 4.5% harboring a presumptive leukemia driver mutation. In this study, CH was associated with increased age, prior radiation therapy, and tobacco use. This indicates that CH may be caused by environmental factors as well as age-dependent stochasticity. The mechanisms accounting for the increased association of CH with solid tumors are unclear, but we propose the intriguing possibility that CH affects the immune system in such a way as to inhibit immune surveillance of cancer. Similar studies have implicated CH as a risk factor for the development of therapy-related myeloid neoplasms (55, 56). Certainly, this will be an important area for further investigation.

**DURATION OF THE PRELEUKEMIC PHASE**

An important topic that remains poorly understood is the duration of the preleukemic phase of AML. To date, no studies have provided concrete evidence to suggest an upper and lower bound for the period of time between the acquisition of the first leukemogenic mutation and the onset of disease. However, multiple lines of anecdotal evidence exist to provide an estimate. Recently, multiple studies have tracked the development of leukemia in allogeneic bone marrow transplant donors and recipients (57, 58). In one study, both the donor and the recipient were diagnosed with AML more than 7 years posttransplant. Both patients harbored mutations in DNMT3A and this mutation was retrospectively identified in the donor prior to transplant (VAF = 41%) (58). In the second study, both donor and recipient developed DNMT3A-mutant AML within 2 years of transplantation and the donor was retrospectively found to have a mutation in DNMT3A (VAF = 46%) at the time of transplant (57). These studies indicate that preleukemic evolution takes at least 7 years and, in reality, probably many more as the DNMT3A HSC clone had already expanded substantially in the donor at the time of transplant. Research from our own group has identified a single patient where we believe the preleukemic phase lasted for at least 15 years (31). This particular patient was diagnosed with AML at age 29 and harbored a preleukemic IDH1 mutation. Intriguingly, this mutation was also present at high penetrance (VAF = 25%) in T cells. As the vast majority of an individual’s T cell repertoire is established prior to puberty and progressive thymic involution (59), this indicates that this preleukemic clone likely arose prior to adolescence. In this case, the preleukemic phase could have lasted more than 15 years.

These temporal dynamics of the preleukemic phase of AML raise multiple interesting, and as of yet unanswered questions. Even if the preleukemic phase lasts 20–30 years, why are the majority of AML patients over the age of 65? If leukemia is capable of developing in just 20 years, why do we not observe more cases of AML in younger adults? Are aged HSCs more susceptible to mutation? Are aged HSCs more capable of accepting the epigenetic consequences of a mutation in DNMT3A or TET2? Perhaps, the progressive myeloid bias observed during aging (60, 61) plays a role in this process as well. Answering these questions will lead...
to clear advances in our understanding of the preleukemic phase and identify opportunities for therapeutic intervention prior to the onset of AML.

**PRELEUKEMIC HSCs IN REMISSION AND RELAPSE**

The identification of preleukemic HSCs as the reservoirs for remission status and to clear advances in our understanding of the preleukemic phase and identify opportunities for therapeutic intervention prior to the onset of AML.

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motivate future work on the impact of persistent preleukemic cells during remission on patient outcome.

**TARGETING PRELEUKEMIC MUTATIONS AND PRELEUKEMIC HSCs**

The identification and characterization of preleukemic HSCs has raised the question of how this knowledge should influence therapy development and treatment decisions. As discussed above, recent work on CHIP has shown that carriers have an increased risk for developing hematologic malignancies. This indicates that, if these cells could be targeted without adverse side effects, it could be possible to prevent the onset of AML. However, it remains unclear how best to approach this problem. First and foremost, successful targeted therapeutic intervention would require identification of a dependency unique to preleukemic HSCs. Recent work has identified minimal consistent transcriptional and epigenetic differences between healthy and preleukemic HSCs (31), making it unlikely that these cells will be universally sensitive to the same intervention. This means that any targeted preleukemic therapy would likely be based on the genetic mutations present in preleukemic cells and therefore target DNMT3A or TET2. Though no approved targeted therapeutics exist for either of these genes, studies have demonstrated proof-of-concept targeting of DNMT3A-mutant cells with an inhibitor of the DOT1-like histone lysine methyltransferase (72). Effective therapies would preferentially tip the scales in favor of differentiation of preleukemic HSCs, leading the mutations to exhaust as the clone undergoes lineage commitment (Figure 2A).

Importantly, no therapies have been designed with preleukemic HSCs as the primary target and it is likely that therapies that are effective against AML cells would be ineffective against preleukemic cells (Figure 2B). Even if a targeted preleukemic therapy existed, there are situations where therapeutic intervention during the preleukemic phase could be highly detrimental. For example, in patients with very high preleukemic burden, up to 100% of HSCs could harbor preleukemic mutations. If these HSCs were induced to differentiate, the patient could suffer widespread bone marrow failure that would only be treatable by bone marrow transplantation (Figure 2C). As most of these individuals would be of advanced age, this type of therapy would likely be poorly tolerated. Some of these problems are exemplified by the treatment

![Figure 2](image-url)
of chronic phase chronic myelogenous leukemia (CML) with the tyrosine kinase inhibitor imatinib. Imatinib targets the BCR-ABL tyrosine kinase fusion protein that is present in every CML cell, including the preleukemic HSCs. However, while progenitor cells are highly sensitive to treatment (73), CML-ABL-positive HSCs remain resistant (Figure 3) (74). CML patients treated with imatinib can achieve complete morphologic remission, durable for many years, but retain HSC clones that harbor the BCR-ABL translocation that is the hallmark of the disease (75). Upon removal of tyrosine kinase inhibitor therapy, these patients can relapse due to the residual HSCs harboring the BCR-ABL fusion (75, 76). Successful eradication of preleukemic HSCs will require consideration of these many caveats to therapy design.

CONCLUSION

Our understanding of preleukemia is still in its infancy. Much of the work that has been performed has aimed at understanding the genetic component of preleukemia, identifying which mutations occur during this protracted evolutionary phase and which mutations occur during the progression to frank leukemia. We have learned that these cells persist during remission, contribute to remission hematopoiesis, and have the potential to generate relapsed disease. We have identified associations between preleukemic burden and patient outcome. However, there is still much to learn about the clinical relevance of these preleukemic HSCs. Future work will serve to demonstrate whether therapeutic intervention during the preleukemic phase is feasible and safe, potentially opening the door to preventative treatments for AML. A more rigorous understanding of these cells could lead to therapeutic interventions that have the potential to stop AML before it starts.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript and contributed to the design and overall outline.

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