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Clonal evolution of pre-leukemic hematopoietic stem cells precedes human acute myeloid leukemia

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Abstract

Massively parallel DNA sequencing has uncovered recurrent mutations in many human cancers. In acute myeloid leukemia (AML), cancer genome/exome resequencing has identified numerous recurrently mutated genes with an average of 5 mutations in each case of de novo AML. In order for these multiple mutations to accumulate in a single lineage of cells, they are serially acquired in clones of self-renewing hematopoietic stem cells (HSC), termed pre-leukemic HSC. Isolation and characterization of pre-leukemic HSC have shown that their mutations are enriched in genes involved in regulating DNA methylation, chromatin modifications, and the cohesin complex. On the other hand, genes involved in regulating activated signaling are generally absent. Pre-leukemic HSC have been found to persist in clinical remission and may ultimately give rise to relapsed disease through the acquisition of novel mutations. Thus, pre-leukemic HSC may constitute a key cellular reservoir that must be eradicated for long-term cures.

Keywords

Acute myeloid leukemia; hematopoietic stem cells (HSC); pre-leukemic HSC; clonal evolution

Introduction

Acute myeloid leukemia (AML) is an aggressive malignancy of bone marrow progenitors that is associated with the clonal accumulation of immature myeloid blasts defective in their maturation and function [1]. Patients generally come to medical attention with symptoms related to bone marrow failure: fatigue resulting from anemia, infection due to leukopenia, or bleeding due to thrombocytopenia. Current standard of care in medically fit patients involves high-dose chemotherapy and in many cases allogeneic hematopoietic cell transplantation. Both of these therapies result in significant morbidity and mortality,

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Conflict of interest statement

The author has no conflict to declare.

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particularly in patients over the age of 60 years who constitute the majority of cases. Even with these aggressive therapies, 5-year overall survival is approximately 30%, and is much lower in elderly patients due to both refractory disease and inability to tolerate therapy. Strikingly, the standard of care in AML has not changed in decades, despite extensive mechanistic and clinical research [2].

Genomics

The advent of massively parallel next-generation DNA sequencing has been rapidly applied to the investigation of cancer genomes resulting in the identification of recurrently mutated genes in human cancer [3–5]. This is particularly true for human AML where several hundred patient samples have been analyzed using these methods. A landmark study from The Cancer Genome Atlas (TCGA) AML group reported the results of whole genome or whole exome sequencing of 200 cases of human AML [6]. These investigators identified the landscape of recurrently mutated, and therefore likely driver, genes including several novel genes that are now being intensely investigated. These genes were classified into 9 distinct groups based on known and/or predicted functions, and mutations within the same group tended to be mutually exclusive. In addition, this work has led to a broader understanding of mutational burden in AML where the TCGA reported that on average, each case of AML harbors 5 mutations in recurrently mutated genes. This observation raises several important questions: (1) How do these multiple mutations accumulate in a single lineage of cells? (2) Which mutations are the founder mutations, initiating the leukemogenic process? and (3) What is the nature and genomics of pre-leukemic cells in de novo AML?

Human hematopoiesis

In order to start addressing these questions, it is first necessary to consider normal human hematopoietic development. Human hematopoiesis is organized as a cellular hierarchy initiated and maintained by long-term self-renewing hematopoietic stem cells (HSC) [7]. HSC in turn give rise to multipotent progenitors, which produce lineage-restricted progenitors, and eventually all the mature, differentiated cells of the blood. In normal myeloid development, mature monocytes and granulocytes have short life spans measured in hours to days, while lineage-restricted and multipotent progenitors survive for only a few weeks [8].

The short life span of these cells links to the first question: How do multiple mutations accumulate in a single lineage of cells? We have hypothesized that since most cells in the myeloid differentiation hierarchy are short-lived, then leukemogenic mutations must serially accumulate in successive clones of self-renewing HSC [9, 10]. According to this model, in AML patients, the rare residual HSC compartment will consist of some normal cells lacking all mutations, but will also contain some pre-leukemic HSC that contain some, but not all, of the mutations identified in the downstream leukemia. Moreover, our model proposes that these mutations will be serially acquired in clones of HSC. Thus, if the leukemia is associated with 5 mutations in genes 1–5, clones of pre-leukemic HSC contain mutation in gene 1 alone, genes 1 and 2, genes 1–3, and genes 1–4 should be present that will indicate the history of mutational acquisition in the individual AML.

We set out to investigate this model through the investigation of de novo AML. This study was facilitated by recent advances in the identification of surface markers that distinguish leukemia cells from normal hematopoietic stem cells [11, 12]. Our overall approach was to: (1) purify residual HSC from AML samples taken at diagnosis using the novel cell surface markers in FACS; (2) genotype the individual leukemia by exome sequencing using purified patient-matched T cells as the reference; (3) design primers to amplify genomic regions harboring mutations and subject each to targeted deep resequencing to identify the presence and frequency of leukemia-specific mutations present in residual HSC – these would be pre-leukemic mutations and indicate the presence of pre-leukemic HSC; and (4) isolate single clones of HSC and genotype using SNP-specific Taqman assays in order to determine the presence of a specific mutation in an individual cell. From this analysis, we speculated that we might identify single cells with one mutation, and other cells with 2 or more mutations. In this way, we could track the order in which the mutations were acquired during leukemogenesis.

Clonal evolution in AML

Using this approach, we investigated 19 cases of de novo AML with a diversity of cytogenetic and molecular abnormalities from which we could isolate residual HSC based on differential expression of TIM3 and CD99 surface markers [13, 14]. Exome sequencing of these 19 cases identified mutations in most of the common recurrently mutated genes in AML including: FLT3-ITD, FLT3-TKD, NPM1, DNMT3A, IDH1, IDH2, TET2, ASXL1, SMC1A, SMC3, STAG2, RAD21, CTCF, NRAS, KRAS, PTPN11, CEBPA, RUNX1, WT1, and others. We then conducted targeted deep re-sequencing of all these leukemia-specific mutations in the patient-matched residual HSC. Consistent with our hypothesis, we found that many of the leukemia-mutations, but not all, could be detected in the residual HSC. In particular, we identified pre-leukemic mutations in TET2, IDH1, IDH2, DNMT3A, RAD21, SMC1A, CTCF, ASXL1, and NPM1.

The frequency in which these mutations were detected varied. TET2 pre-leukemic mutations were found at a very high frequency, being present in nearly 100% of the residual HSC. This is consistent with the competitive advantage observed with TET2-deficient mouse HSC in transplantation assays [15, 16]. Other pre-leukemic mutations were found at much lower frequencies, being present in a minor subset of the residual HSC. Notably, the pre-leukemic frequency of the same mutation was found to vary across different patients. For example, mutations in either IDH1 or IDH2 were found to be pre-leukemic in 12 out of 15 patients investigated; however, the frequency of mutant allele ranged from 1% to >60%. The significance of these differences either to leukemogenesis or clinical responses remains to be determined.

Strikingly, when considering the 19 patients as a whole, patterns in mutation acquisition were detected. All leukemia-specific mutations in recurrently mutated genes were assigned to 1 of the 9 categories defined by the TCGA. We found that pre-leukemic mutations were significantly enriched in genes involved in regulating DNA methylation, chromatin modifications, and the cohesin complex (Figure 1A) [17]. Moreover, genes involved in regulating activated signaling were significantly lacking in the pre-leukemic mutations

(Figure 1B). From this, we propose that leukemia development is initiated in normal HSC that acquire mutations in landscaping genes (DNA methylation, chromatin modifications, or the cohesin complex). These pre-leukemic HSC in turn give rise to hematopoietic clones including progenitors and mature blood cells. It is likely in a progenitor that a pre-leukemic clone acquires progressor mutations in proliferative genes (activated signaling) that result in the establishment of a frankly leukemic clone (Figure 2).

Clinical implications of clonal model

What are the potential clinical implications of this clonal evolution model? If one considers a patient diagnosed with AML who presents for treatment, his or her bone marrow consists of a rare pool of residual HSC, which in turn likely contains some normal HSC and some pre-leukemic HSC clones. At the same time, the leukemia likely consists of a dominant leukemic clone, as well as several subclones. Such a patient is treated with induction chemotherapy and nearly 70%–80% will enter a clinical remission. However, this remission is often short-lived and it is relapsed disease that ultimately leads to death. The question to consider is, what is the clonal basis of relapse? Is it (A) recurrence of the dominant leukemic clone, not completely eradicated by chemotherapy? Is it (B) recurrence from a minor subclone that may exhibit chemotherapy resistance? Is it (C) further clonal evolution with new mutations in an existing leukemic clone? or Is it (D) further clonal evolution with new mutations occurring in an existing pre-leukemic clone that gives rise to a distinct relapse? In addition, it is possible that more than one of these mechanisms can occur in the same patient. Finally, one provocative consideration is whether or not the acquisition of new mutations (options C and D above) could be caused by the genotoxic chemotherapy administered as part of the induction protocol.

Clonal basis of relapsed disease

In order to investigate the clonal basis of relapsed disease, we started by investigating the persistence of pre-leukemic HSC in clinical remission following induction chemotherapy with a standard regimen of an anthracycline in combination with cytarabine. Of the 19 patients we initially investigated at diagnosis, we identified 10 from whom we had obtained bone marrow samples at the time of remission. These remission samples were subject to targeted resequencing of all leukemia-specific mutations identified by exome sequencing of the diagnostic sample within CD34+ progenitor cells, CD19+ B cells, and a mixed population of mature myeloid cells. Of these 10 patients, 6 had evidence of pre-leukemic HSC at diagnosis—and of these 6, pre-leukemic mutations were detected in 4 at remission. In all 4 cases, all pre-leukemic mutations from diagnosis were present at remission. Moreover, pre-leukemic mutations were detected in all 3 cellular compartments, indicating that not only do they persist in the bone marrow, but they contribute to ongoing normal hematopoiesis during remission. Thus, pre-leukemic HSC are not sensitive to standard induction chemotherapy, persist in remission, and may contribute to relapse through the acquisition of new mutations (option D above).

To directly investigate the clonal basis of relapse, we turned again to our cohort of patients and identified 3 for whom we had obtained samples from diagnosis and remission. In these 3

patients, we identified 3 different clonal mechanisms for relapse. In one patient, the same mutations were identified at diagnosis and relapse indicating that this relapse occurred from the dominant clone present at diagnosis (option A described above). In fact, analysis of the remission sample identified the same mutations, suggesting that while this patient achieved a morphologic remission, there were still leukemic cells present. This is consistent with the clinical course, as the patient relapsed only 2 months after entering remission. In the second patient, the pre-leukemic mutations were retained into remission, but a FLT3-TKD leukemic mutation at diagnosis was not present at relapse. Instead, the patient had a mutation in KRAS. Going back to diagnosis, targeted resequencing identified KRAS mutations at a low allele frequency. Thus, this patient relapsed from a minor clone present at diagnosis (option B described above). Finally, in a third patient pre-leukemic mutations were also preserved at relapse. However, a PTPN11 mutation present at diagnosis was not detected at relapse, and a new WT1 mutation was present. Going back to diagnosis, targeted resequencing failed to identify the WT1 mutation, indicating that it was not a relapse from a minor clone. Thus, the WT1 mutation was novel at the time of relapse and could have arisen either from a leukemic (option C described above) or pre-leukemic (option D described above) clone.

Discussion

These results have numerous implications for the development of curative therapies in AML, which rely on the premise that all clones contributing to relapse must be eradicated for cure. First, targeted therapies must be directed against mutations present in all clones, as therapies targeting subclones will be ineffective. Based on the analysis described here and recently published, pre-leukemic mutations are the best potential targets given that they are present in all leukemic cells [13, 18]. Second, if relapsed AML can arise from pre-leukemic clones, as suggested by the case described above, then not only will it be necessary to eliminate leukemic clones, but it will also be necessary to eliminate pre-leukemic clones. Notably, these are cells that possess the same mutation as the downstream leukemic cells, and should therefore, be affected by the same targeted therapies. However, this may not always be the case as cellular context may matter. The same targeted therapy may eliminate leukemic cells, but not affect pre-leukemic cells. Such a scenario has been observed in chronic myelogenous leukemia (CML), where abl-kinase inhibitors are effective at killing progenitor cells leading to long-term remissions, but are ineffective at eradicating the HSC/LSC compartment [19]. If the same holds true for targeted therapies in AML, it shifts focus away from frankly leukemic cells alone, to also include pre-leukemic cells as key targets for curative therapies. The investigation of therapeutic targeting of pre-leukemic HSC is an area of our active research.

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A	TCGA Mutation Category	Mutant Genes Found to be Pre-Leukemic
	TF Fusions	inv(16)
	NPM1	NPM1 (2)
	Tumor Suppressors	
	DNA Methylation	DNMT3A (2), IDH2 (3), TET2 (4)
	Activated Signaling	FLT3
	Myeloid TFs	IKZF1, PHF6
	Chromatin Modifiers	SAP130, MIER3, ASXL1, MTA2, KAISO, CTCF
	Cohesin	RAD21, MAU2, SMC1A (2)
	Spliceosome	
B	TCGA Mutation Category	Mutant Genes Not Found to be Pre-Leukemic
	TF Fusions	
	NPM1	NPM1 (6)
	Tumor Suppressors	
	DNA Methylation	DNMT3A, IDH1
	Activated Signaling	PTPN11, FLT3 (9), CBL, KRAS, AKAP12, NRAS
	Myeloid TFs	CEBPA (4)
	Chromatin Modifiers	MLL-ENL
	Cohesin	
	Spliceosome	

Figure 1. Classification of recurrent mutations as pre-leukemic or not in a cohort of 19 patients with de novo AML

Mutant genes found to be pre-leukemic (A) or not pre-leukemic (B) were assigned to the 9 mutation categories defined by the The Cancer Genome Atlas (TCGA). The numbers in parentheses indicate the number of times a mutation in that gene was identified.

Figure 2. Schematic model for the acquisition of mutations in pre-leukemic HSC leading to AML arising in progenitors

Pre-leukemic mutations in landscaping genes affecting DNA methylation, chromatin modifications, and the cohesin complex are serially acquired in clones of self-renewing HSC (blue and green). Progressor mutations in genes involved in active signaling occur in progenitors leading to the development of AML clones (orange and red).