Mutation Position Within Evolutionary Subclonal Architecture in AML

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Cytogenetic data suggest that acute myeloid leukemia (AML) develops through a process of branching evolution, especially during relapse and progression. Recent genomic data from AML cases using digital sequencing, temporal comparisons, xenograft cloning, and single-cell analysis indicate that most, if not all, AML cases emerge through branching evolution. According to a review of the current literature, the balanced translocations (t[15;17], t[8;21], and inv[16]) and nucleotide variants in DNMT3A and TET2 most commonly occur in the founding clone at diagnosis. These mutations are rarely gained or lost at relapse, and the latter 2 mutations are observed in elderly subjects with mosaic hematopoiesis antedating overt leukemia. In contrast, +8, +13, +22, −X, −Y, and nucleotide variants in FLT3, NRAS/KRAS, WT1, and KIT frequently occur in subclones and are observed either to emerge or to be lost at relapse. Because drugs that target mutations within a subclone are unlikely to eliminate all leukemic cells, it will be essential to understand not only which mutations a patient has but also how they organize within the leukemic subclonal architecture.

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N early 4 decades ago, Nowell suggested that cancer emerged through a process of branching evolution, based on karyotype evaluation of diverse cancers.1 Shortly thereafter, Testa et al2 found that select cases of acute myeloid leukemia (AML) developed complex, branching, subclonal evolution that could be tracked by using metaphase cytogenetics and that the greatest cytogenetic evolution occurred during relapse. However, karyotypic abnormalities are not observed in all cases of AML; nearly one half of patients with AML present with a normal karyotype, and of those patients who relapse, nearly one half again retain a normal karyotype.2–5 Thus, AML is not necessarily a disease of an unstable genome, and metaphase cytogenetic techniques have insufficient genomic resolution to determine subclonal architecture in the majority of cases.

The application of nucleotide-level technologies with increasing resolution has shown that most, if not all, cases of AML emerge through a process of constrained clonal evolution.6,7 Southern blot analysis, spectral karyotyping, fluorescence in situ hybridization (FISH), comparative genomic hybridization arrays, single nucleotide polymorphism arrays, polymerase chain reaction (PCR) assays, and next-generation sequencing have all improved our ability to detect subclones and to integrate mutations into a clonal hierarchy.7–13

To fully integrate patient-specific mutations and targeted agents into clinical care, it will be imperative to understand each mutation on 2 axes: whether it is a “driver” versus “passenger” event and “initiation” versus “progression” event. Driver mutations directly affect the biology of the cell, whereas passenger mutations do not. Initiation mutations exist within the founding clone and are found in all AML cells. Progression mutations emerge later in leukemic evolution, can be found in subclones, and exist in only a fraction of AML cells. Thus, selection pressure, in the form of chemotherapy, can favor the elimination or outgrowth of different branches within the AML evolutionary tree (Figure 1).

The relationship of mutations within a mutation evolutionary hierarchy can be determined either by direct measurement or by inference based on temporal changes. Subclonal mutations can be gained or lost over time, whereas founding mutations exist in all leukemic cells at all time points. Thus, pairwise analysis of cases at diagnosis and at relapse may identify mutations that were present at diagnosis and lost at relapse, and these must have existed in a subclone that was eliminated (Figure 1A). Likewise, mutations that are absent at diagnosis and present at relapse may have existed in a resistant subclone.
that existed below the level of detection at diagnosis, or it may have been acquired by a cell that randomly escaped chemotherapy, thus forming a new subclone (Figure 1B). Therapy that eradicates all leukemic cells must target a population of cells that share a set of susceptible mutations. Therapy that targets a mutation within a subclone is likely to apply selective pressure against that subclone only, leaving the patient at risk for relapse from a new branch in the evolutionary tree.

It will not be sufficient to simply correlate mutation status with clinical outcome; interpreting the outcomes of targeted therapies will require an understanding of mutations within the evolutionary subclonal architecture, evolution dynamics, and selection pressures. The present article reviewed the available data regarding the subclonal organization of AML mutations and their dynamic change at relapse, with a focus on which mutations are most commonly observed in subclones or inferred to be in subclones because they are gained or lost at relapse.

**STRUCTURAL VARIANTS AND SUBCLONAL ARCHITECTURE**

Karyotype analysis provides a single-cell, low-resolution analysis of genome-wide structural variants. This technology can be applied to identify subclonal variants either by observing concurrent structural alterations or through serial sampling (eg, evaluation at diagnosis and relapse).
We recently reviewed the available literature for co-occurrence of structural variants. These data suggest that the balanced translocations are almost universally founding clone variants. Indeed, balanced translocations have been shown to be gained or lost at relapse in very few cases and can recur at relapse despite long remission intervals.

One of the most commonly occurring subclonal structural variants identified through metaphase cytogenetic analysis is +8. Between 25% and 40% of cases with t(15;17), t(8;21), or inv(16) also present with concurrent +8. In nearly one half of these cases, +8 exists in a subclone (leukemic cells are identified with both the balanced translocation and +8, as well as with the balanced translocation but without +8). Less frequently, −7 is observed with t(15;17), −X, or −Y with t(8;21), and +13 or +22 with inv(16). Again, the balanced translocation in these cases is noted in all leukemic cells, whereas the chromosomal gains and losses are frequently noted in subclones. Consistent with this finding, in t(15;17) and t(8;21) leukemias, +8 is the most frequently observed chromosomal gain at relapse (22 of 236 separate cases). Similarly, at relapse, gains of +13, +22, and losses of X or Y are observed in inv(16) and t(8;21) leukemias, respectively.

The additional cytogenetic abnormalities do not seem to alter clinical outcomes compared with patients presenting with isolated t(15;17), t(8;21), or inv(16). This approach remains expensive, and only a limited number of cases have been studied. Subclonal variants were observed only within the founding clone of individual cases. This approach remains technically challenging. To increase DNA yield, colonies can be grown from single AML cells, immunophenotypic subclones flow-sorted, or leukemia fractions engrafted in xenografts, which are subsequently analyzed. However, this approach may be biased toward subclones based on their ability to grow ex vivo or grow in xenografts or on the immunophenotype.

Multiple groups have observed variance in the allelic burden of FLT3 mutations according to PCR, with some patients presenting with low allelic burden and some with high allelic burden. Schnittger et al reported the largest study, analyzing 689 subjects with FLT3 mutations identified within 3365 cases. They observed marked diversity in the FLT3 allelic burden, with many patients presenting with a low allelic burden, suggesting that the mutation existed within a subclone, which may represent only a small fraction of the AML cells. Furthermore, they observed that 139 of 689 AML patients carried 2 to 3 independent FLT3 mutations, again suggesting late, or parallel, evolution of these events.

In contrast, in AML cases with DNMT3A mutations, the DNMT3A allelic burden is almost universally high (usually directly proportional to the blast count). Bisling et al addressed this phenomenon further by applying sensitive PCR-based methods to cases that were DNMT3A R882 wild type by using Sanger sequencing to determine whether DNMT3A mutations might exist in rare subclones below the level of Sanger detection. In 17 unique cases, they were unable to find any with subclonal DNMT3A mutations at low allelic burden.

Our group has used deep-digital read-counts recently to quantify somatic mutations in individual AML patients identified during whole-genome sequencing. We found that one half of the AML cases had ≥1 subclone in addition to a founding clone. We identified cases with NRAS, FLT3, ETV6, and EWSRI1 mutations clustering within distinct subclones, whereas DNMT3A, NPM1, IDH1, and SMC1A variants were observed only within the founding clone of individual cases. This approach remains expensive, and only a limited number of cases have been studied. Subclonal architecture requires multiple variants per subclone to accurately define the subclone. Thus, exome sequencing is typically inadequate in AML cases due to the small number of exome variants per genome (typically 10-20). Furthermore, it remains technically challenging to quantify the subclonal identity of structural variants and indels (small insertions and deletions) by using this approach.

Temporal analysis of nucleotide variants has been performed by multiple groups, most of whom assessed a population of cells); clusters of VAFs can then be used to identify mutations that occur in subclones versus in the founding clone. However, mutations that occur in <5% of sampled cells are likely to be missed, and subclones with overlapping average VAFs are indistinguishable. Single-cell analysis can be performed by using FISH and, recently, by using PCR. However, FISH studies are limited to structural abnormalities, and single-cell multiplexed PCR remains technically challenging. To increase DNA yield, colonies can be grown from single AML cells, immunophenotypic subclones flow-sorted, or leukemia fractions engrafted in xenografts, which are subsequently analyzed. However, this approach may be biased toward subclones based on their ability to grow ex vivo or grow in xenografts or on the immunophenotype.

NUCLEOTIDE VARIANTS AND SUBCLONAL ARCHITECTURE

Diverse technical approaches have been applied to AML cases that can be leveraged to understand the subclonal architecture of nucleotide-level mutations. These approaches have included quantitative PCR analysis, digital sequencing, paired analysis at diagnosis and relapse, analysis of subfractions of bone marrow cells, and single-cell analysis. Digital sequencing quantifies the variant allele frequency (VAF) [how commonly a mutation occurs within a population of cells]); clusters of VAFs can then be used to identify mutations that occur in subclones versus in the founding clone. However, mutations that occur in <5% of sampled cells are likely to be missed, and subclones with overlapping average VAFs are indistinguishable. Single-cell analysis can be performed by using FISH and, recently, by using PCR. However, FISH studies are limited to structural abnormalities, and single-cell multiplexed PCR remains technically challenging. To increase DNA yield, colonies can be grown from single AML cells, immunophenotypic subclones flow-sorted, or leukemia fractions engrafted in xenografts, which are subsequently analyzed. However, this approach may be biased toward subclones based on their ability to grow ex vivo or grow in xenografts or on the immunophenotype.

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single gene for gains and losses in paired samples at diagnosis and at relapse. Meta-analysis of these results is summarized in Figure 2. The mutations most commonly gained at relapse were: FLT3, KIT, NRAS/KRAS, WT1, and IDH1/2. In contrast, several other genes do not appear to gain mutations at relapse: NPM1, DNMT3A, and TET2. Similarly, loss of a mutation at relapse has been observed in FLT3, NRAS/KRAS, CEBPA, KIT, WT1, CEBPA, and IDH1/2. Of note, in several cases of CEBPA4 mutations, the leukemia remained mutated at relapse, although one of the alleles had been lost and another mutation gained.69 These results are consistent with the recent results of 440 paired AML cases presented at a 2012 American Society of Hematology meeting, which identified only 1 of 424 cases that gained TET2 mutations at relapse and 0 of 17 that lost TET2 mutations at relapse.17 Collectively, these data suggest that FLT3, NRAS/KRAS, KIT, WT1, CEBPA, and IDH1/2 mutations frequently occur in subclones (eg, they are cooperating events) that may emerge or disappear at relapse.

Due to sampling limitations, it is technically challenging to know whether a mutation that is gained at relapse existed below the level of detection in a minor subclone at diagnosis or whether the mutation was gained after therapy in a cell that randomly survived chemotherapy (Figure 1B). Higher sensitivity PCR-based platforms have detected KIT mutations that were missed by Sanger sequencing. Wakita et al identified 3 patients with AML clones containing KIT mutations that were not detected at diagnosis by using Sanger sequencing but were detected at relapse. In all 3 cases, the mutation could be detected at diagnosis by using highly sensitive PCR methods, suggesting that these mutations preexisted in rare cells at diagnosis (eg, minor subclones). Likewise, in retrospective analysis using patient-specific, highly sensitive PCR, FLT3 mutations could be identified that preexisted in a small population of cells at diagnosis in patients who relapsed with “new” FLT3 variants.45,70 Intriguingly, patients with new FLT3 variants at relapse tend to relapse more quickly than patients without new FLT3 mutations (6.6 vs 13.5 months).70,73 This short window of time from treatment to relapse again suggests that FLT3 mutations which are “gained” at relapse are likely to have preexisted in an undetected subclone, rather than to have been acquired later in a founding clone cell that stochastically survived.

Paired whole-genome sequencing at diagnosis and relapse followed by deep digital sequencing improves identification of variants within subclones. This approach was applied to 7 cases of AML, and it identified FLT3, IDH1, and ETV6 variants within leukemic subclones, whereas variants in NPM1, DNMT3A, SMC3, WT1, RUNX1, and IDH2 were identified in individual founding clones at diagnosis and relapse.74 Similarly, 2 groups have reported paired analysis of myelodysplastic syndrome (MDS) and subsequent secondary AML, which identified WT1, PTPN11, RUNX1, SMC3, FLT3–tyrosine kinase domain (TKD), RAS, and CEBPA in subclones gained in at least 1 secondary AML case but absent in the corresponding MDS sample.75,76 In contrast, mutations in TET2, IDH1, IDH2, STAG2, TP53, and U2AF1 were observed in the MDS samples, suggesting that these mutations existed in an early MDS clone which gave rise to the subsequent secondary AML.
Jan et al\textsuperscript{13} combined exome sequencing of leukemic cells with flow-sorting of residual “normal” hematopoietic stem cells (HSCs) to identify evolutionary early versus late variants. Five FLT3-positive cases were assessed; in all 5 cases, the FLT3 variant was identified in the leukemic sample but not in the HSC samples. In contrast, variants in NPM1, TET2, and SMC1A were identified in both the leukemic and HSC samples of at least 1 case each, suggesting that these variants occurred early during leukemic evolution and are likely to be founding events. This group recently extended these findings into a larger set of cases and through sequence analysis of single HSC-derived colonies.\textsuperscript{77} They again identified mutations in IDH2 and DNMT3A in preleukemic HSCs. Of particular interest, case SU353 presented with both DNMT3A and ASXL1 mutations at VAF ~50% in both the leukemic and normal HSC samples but <2% in T lymphocytes; the NPM1 and FLT3–internal tandem duplication (ITD) mutations were only detectable in the leukemia sample. These findings suggest that the DNMT3A and ASXL1 mutations were sufficient to lead to clonal dominance within the HSC compartment but were insufficient to cause leukemia without additional cooperating mutations.

Similarly, Shlush et al\textsuperscript{42} performed sequence analysis of flow-sorted hematopoietic progenitor populations at diagnosis, remission, and relapse. Using this approach to analyze 11 patients who had both DNMT3A and NPM1 mutations, the authors observed cell fractions in all cases that contain only the DNMT3A mutation and not the NPM1 mutations, suggesting that the NPM1 mutation was acquired later in clonal evolution. This finding is consistent with data reported by Krönke et al,\textsuperscript{72} who identified 5 of 53 cases with both DNMT3A and NPM1 mutations at diagnosis, which lost the NPM1 mutation at relapse. Interestingly, 5 of these cases profoundly reorganized their mutational complement: 1 case lost a FLT3-ITD mutation, gained a new FLT3-ITD mutation, gained an NRAS mutation, and gained an MLL-PTD mutation; 1 case lost a FLT3-TKD mutation, gained an NRAS mutation, gained an IDH1 mutation, and gained an MLL-PTD mutation; and 1 case lost an NRAS mutation, gained a new NRAS mutation, and gained an MLL-PTD mutation. These 3 cases suggest that the DNMT3A mutation was a very early evolutionary event, and the new branching evolution was responsible for relapse.

Klco et al\textsuperscript{42} integrated xenograft analysis with mutational tracking. This approach revealed that although subclones can be derived from the founding clone, they may become the most abundant leukemic cell population in the bone marrow. Furthermore, although most of the cases they evaluated presented with equivalent distribution of founding clone and subclonal variants in both the blood and bone marrow, they observed a case of acute monocytic leukemia with differential distribution of subclones within the blood versus bone marrow and within separate myeloid compartments. This case had mutations in DNMT3A, NPM1, IDH1, and FLT3 at diagnosis, and it lost the IDH1 and FLT3 mutations at relapse. Intriguingly, this subclonal pattern was recapitulated within immunophenotypic compartments (monocytes carried the clonal mutations that were lost at relapse, whereas immature blast cells carried the relapse mutations). Furthermore, when engrafting these AML cells into either NSG or NSG-SGM3 mice, individual mice tended to engraft only 1 subclone, and some AML samples exhibited subclonal bias in engraftment, with absence of FLT3-TKD, FLT3-ITD, and IDH1 subclones in NSG engraftment but not in NSG-SGM3 engraftment. This suggests that subclones may have selective engraftment potential in different xenograft systems, and again, that in these cases, the FLT3 and IDH1 mutations were late evolutionary events.

Three groups have analyzed genetic changes in the context of mosaic hematopoiesis. X-inactivation ratios in female subjects have been known to develop age-associated skewing, especially in the hematopoietic compartment.\textsuperscript{28,27} Furthermore, this phenomenon tends to be myeloid biased.\textsuperscript{79} Bartram et al\textsuperscript{43} analyzed RAS mutations in AML patients in remission in the context of X-inactivation studies. Of 9 patients, all exhibited monoclonal hematopoiesis at leukemia diagnosis by either hypoxanthine phosphoribosyltransferase or phosphoglycerate kinase restriction fragment length polymorphisms. Most (5 of 7) reacquired polyclonal hematopoiesis during remission. Of the 2 cases that retained monoclonal hematopoiesis, one lost the RAS mutation that was present at diagnosis, suggesting that it was acquired after monoclonal hematopoiesis. Laurie et al\textsuperscript{80} retrospectively analyzed single nucleotide polymorphism arrays obtained for nonhematologic genome-wide association studies; because peripheral blood was used as the source of genetic information in these cases, they could be assessed for acquired, hematologic structural alterations if these occurred in >5% of blood cells. They identified mosaic hematopoiesis in multiple cases and noted an increasing incidence that was proportional to age. Furthermore, they observed recurrent deletions involving DNMT3A, TET2, and RB1. Likewise, Busque et al\textsuperscript{81} sequenced TET2 in elderly female patients with X-inactivation skewing and asymptomatic mosaic hematopoiesis. They identified 10 of 182 cases with clonal mutations in TET2.

Finally, single cells can be grown ex vivo in clonogenic assays, and individual colonies can be assessed for mutation combinations. Because each colony is derived from a single cell, this permits effective clustering of co-occurring variants. Price et al\textsuperscript{32} derived 26 colonies from a single patient and evaluated these for trisomy 8 and for an NRAS mutation. They found +8 in 25 of 26 colonies and an NRAS variant in only 19 colonies, consistent with sequential acquisition of the NRAS variant in a subclone that already carried +8. Likewise, Shouval et al\textsuperscript{83} used single-cell analysis to identify rare FLT3-mutated AML cells within 7 of 8 patients who were otherwise considered FLT3 wild type, and that loss of heterozygosity was
heterogeneously observed within populations of AML cells at relapse.

Additional data involving more cases will be required to better understand the frequency with which each variant occurs in subclones versus the founding clone. However, at this time, variants in FLT3, NRAS/KRAS, WT1, and KIT seem to be the most commonly occurring subclonal variants, with variants in NPM1, IDH1/2, and CEBPA occurring less frequently; mutations in DNMT3A and TET2 are nearly always associated with the founding clone.

THERAPEUTIC IMPLICATIONS OF SUBCLONAL MUTATIONS

As modeled in Figure 1A, application of a targeted drug to a patient whose mutation of interest exists in a subclone will apply selective pressure against the subclone and is unlikely to eradicate the founding leukemic clone. In contrast, the most successfully targeted drugs must affect an initiating event that exists in all malignant cells. Acute promyelocytic leukemia is an example of such a strategy. As described earlier, t(15;17) is likely the initiating event for this disease and is almost universally observed in the founding clone; all-trans retinoic acid and arsenic both target the resultant fusion oncoprotein PML-RARA. The efficacy of these agents may relate to the fact that they abrogate the initiating leukemic event.

Based on this model, one would predict that cases with low FLT3 mutant allelic burden (eg, cases in which the mutation is likely in a subclone) would be less susceptible to FLT3 inhibitors, and that resistance would emerge through the selection of the founding clone (or alternative subclones). Preliminary evidence for both of these outcomes has been observed. First, Pratzz et al correlated ex vivo cytotoxicity to 6 different FLT3 inhibitors and observed that samples with low allelic burden (which suggests that the mutation is in a subclone) were less sensitive to these inhibitors than cases with high allelic burden (which suggests that mutations may be in founding clones). Second, FLT3-mutated patients treated with AC220, sorafenib, or sunitinib commonly relapse with new, resistant FLT3 D835 or F691 mutations within the preexisting FLT3-ITD allele, and one third of the patients who discontinued therapy for any reason also have increased the risk or subsequent hematologic malignancy by only 10-fold. Likewise, clonal hematopoiesis occurs in nearly one third of women aged >60 years, but the incidence of AML in this population is only 15 per 100,000. Thus, such mutations may lead to clonal dominance, but they do not appear to lead inevitably to leukemia. Whether this is because the clone is eventually lost, or persists but fails to acquire additional mutations, is unknown. Whether this outcome is different during remission compared with a preleukemic state is also unknown.

CONCLUSIONS

Multiple lines of evidence now suggest that leukemogenesis involves a process of branching evolution, and that branch points can be delineated based on shared genomic mutations within each subclone. To date, the balanced translocations (eg, t[15;17], t[8;21], t[16;16], inv [16]) and nucleotide variants in DNMT3A and TET2 appear almost universally in the founding clone, and are likely to be initiation events. According to cytogenetic methods and VAF, these mutations are observed in all leukemic cells and are neither gained nor lost at relapse. In contrast, +8, +13, +22, −X, −Y, and variants in FLT3, NRAS/KRAS, WT1, and KIT appear frequently in subclones, are commonly gained or lost at relapse, and are therefore likely to be progression events. Although NPM1 and IDH1/2 mutations are rarely gained or lost at relapse, emerging evidence suggests that when they co-occur with DNMT3A mutations, they are commonly a secondary mutation (rather than the initiating mutation). This model of leukemogenesis, and the position of these mutations within the AML subclonal architecture, has important implications for the administration and interpretation of response to targeted agents, especially because many of the most promising small molecules in development target mutations that are commonly observed within evolutionary subclones.

REFERENCES

Subclonal architecture in AML


