Distinctive evolution and dynamics of epigenetic and genetic heterogeneity in acute myeloid leukemia

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Genetic heterogeneity contributes to clinical outcome and progression of most tumors, but little is known about allelic diversity for epigenetic compartments, and almost no data exist for acute myeloid leukemia (AML). We examined epigenetic heterogeneity as assessed by cytosine methylation within defined genomic loci with four CpGs (epialleles), somatic mutations, and transcriptomes of AML patient samples at serial time points. We observed that epigenetic allelic burden is linked to inferior outcome and varies considerably during disease progression. Epigenetic and genetic allelic burden and patterning followed different patterns and kinetics during disease progression. We observed a subset of AMLs with high epiallele and low somatic mutation burden at diagnosis, a subset with high somatic mutation and lower epiallele burdens at diagnosis, and a subset with a mixed profile, suggesting distinct modes of tumor heterogeneity. Genes linked to promoter-associated epiallele shifts during tumor progression showed increased single-cell transcriptional variance and differential expression, suggesting functional impact on gene regulation. Thus, genetic and epigenetic heterogeneity can occur with distinct kinetics likely to affect the biological and clinical features of tumors.

AML is a predominantly fatal hematopoietic malignancy1–3. Even when leukemia cells appear to have been eradicated from the bone marrow after chemotherapy treatment, most patients relapse eventually. Therefore, it is important to understand how subpopulations of AML cells are resilient to chemotherapy and can give rise to progressively more refractory disease. Several mechanisms have been hypothesized to endow subpopulations of cells within AML tumors with the capacity to survive exposure to therapeutic agents. These mechanisms include the presence of subsets of quiescent cancer stem cells with inherently greater self-renewal properties and reduced sensitivity to chemotherapy drugs4. Somatic mutations that can facilitate the expansion of putative leukemia stem cell populations can also emerge during disease establishment and progression5–8.

Genetic heterogeneity within tumors can also increase evolutionary fitness9,10. Genetic diversity among individual cells in a tumor presumably provides the greatest chance for a subset of cells with particular combinations of mutations (single-nucleotide variants (SNVs), insertions and deletions (indels), copy-number aberrations, and/or translocations) to survive when challenged by cytotoxic drugs. Genetic heterogeneity has been appreciated in AML since early karyotyping studies11,12, and tumor heterogeneity is relevant in solid tumors and lymphoid malignancies13–19, where several studies have linked the degree of genetic heterogeneity to clinical outcome16,20. However, cells from patients with AML have a paucity of genetic lesions compared to most solid tumors21,22, suggesting that other factors, such as epigenetic changes, may contribute to the aggressive behavior of AML.

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Indeed, aberrant epigenetic patterning is a hallmark of AML.8,9,20,21 Gain or loss of cytosine methylation at specific loci disrupts promoter activity and regulation of gene expression23,25,26. Epigenetic marks have great plasticity and may vary over time or through exposure to environmental stimuli. Thus, there is potential for epigenetic diversification to emerge in tumor cell populations and to change during disease progression. Sequence-based profiling can be used to determine quantitative cytosine methylation at base-pair resolution. DNA methylation at sequential cytosine–phosphate–guanine (CpG) dinucleotides can create a phased epigenetic pattern (epiallele) that represents a native ‘cellular barcode’27,28. Such data can be used to measure epigenetic heterogeneity, which has been associated with worse clinical outcomes in lymphoid malignancies52–54. But lymphoid malignancies, in contrast to AML, display a high burden of somatic mutations and can arise from cell types such as B cells that are naturally prone to epigenetic heterogeneity55.

It has been suggested that genetic mutations and/or cytogenetic abnormalities and epigenetic modifications diversify along similar lines during disease progression28,33. This may reflect a selection process of genetic and epigenetic changes, which enhance tumor fitness through expression of oncogenes and/or repression of tumor suppressors, or a process of genetic diversification and clonal selection34 in parallel with epigenetic modifications35 that leads to tumor progression. However, the interplay and/or independence of genetic and epigenetic heterogeneity in AML have not been thoroughly explored. Here we performed a large-scale comparative genomics and epigenomics study in serial specimens obtained from patients with AML. We assembled a cohort of 138 clinically annotated, paired AML patient samples (taken at diagnosis (diagnostic) and at relapse; Supplementary Tables 1 and 2).

Patient samples were characterized with genome-wide methylome sequencing using a modified version of reduced representation bisulfite sequencing36,37 called enhanced reduced representation of bisulfite sequencing (ERRBS)38,39. In a subset of this cohort, we conducted whole-exome sequencing (WES) in paired patient samples with matched germline control DNA, RNA-seq in paired patient samples, and single-cell RNA-seq (scRNA-seq) for the patient for whom we had samples from the most time points. This enabled assessment of epigenetic heterogeneity and comparison to genetic and transcriptional variance during disease progression. The epigenetic features explored in this study are conceptually different from previously reported epigenetic signatures and uniform patterns of differentially methylated genes in AML.23,24 Notably, we found that genetic and epigenetic allelic variations follow distinct and often independent kinetics and patterns. Moreover, shifts in epiallele composition are linked to transcriptional deregulation at the global and single-cell levels, and higher epiallele burden is linked to clinically more aggressive disease. Collectively, our data show that epigenetic allelic diversification occurs during AML establishment and also that disease progression and may be independent of the genetic landscape.

RESULTS
Epiallele burden is linked to poor clinical outcome
We calculated loci with epigenetic allelic variance in our patient cohort using the methylene compositional entropy equation.40 By definition, the epigenetic state of each locus is comprised of cytosine methylation at four consecutive CpG dinucleotides. Each of the possible 16 CpG methylation patterns at these loci is an epiallele (Supplementary Fig. 1). Epigenetically shifted loci (eloci) occur when the epiallele proportions at these sites undergo a statistically significant entropy shift (calculated as change in Boltzmann entropy AS < 90) in their composition when comparing two samples.40 The global metric eloci per million loci (EPM), which normalizes for the variable depth of coverage per specimen and the number of loci measured40, was used to determine the overall magnitude of epiallele shifting across the genome and epiallele burden. Shifting can include gain and/or loss of epialleles between two specimens. The epiallele and eloci measurements were determined using methylene data from ERRBS (Supplementary Table 3) and validated in a subset of specimens using orthogonal methylene sequencing methods on two different platforms (Agilent and Roche; Supplementary Fig. 2).

We divided patients into two cohorts on the basis of their EPM values (highest and lowest 50%). There was no difference in the burden of somatic mutations between the groups (Supplementary Fig. 3). We then plotted clinical outcome as time to relapse, and we observed that patients with high EPM at diagnosis (compared to normal bone marrow (NBM) controls) had a shorter time to relapse as compared to the low-EPM cohort (P = 0.0396, Mantel–Cox log-rank test; Fig. 1a). This association was most significant for EPM assessed on the basis of promoter-associated eloci (P = 0.0077, Mantel–Cox log-rank test; Fig. 1b).

We also performed a multivariate analysis using linear regression to determine whether EPM retained its association with clinical outcome independently of other clinical variables. We found that EPM maintained a significant association with clinical outcome (P = 0.024, Cox proportional-hazards regression model) when age, white blood cell (WBC) count at the time of diagnosis, and sex were taken into account. None of these criteria were independently linked to time to relapse in this cohort (Table 1). In contrast to the results with EPM, the burden of somatic mutations as determined by WES (n = 48; Supplementary Table 4) was not significantly associated with time to relapse (P = 0.272, Mantel–Cox log-rank test; Fig. 1c).

Relapsed AMLs show variable changes in epiallele burden
To further understand the nature of epiallele shifting in AML, we compared diagnostic and relapsed AML specimen methylomes to NBM controls (Fig. 2a). All AML patients showed substantial epiallele shifting at diagnosis and at relapse compared to NBM controls (Fig. 2b,c). Although the degree of EPM increase varied, it occurred across all patients and disease stages (Fig. 2d). We next compared the
**Table 1 Multivariate analysis of EPM association with time to relapse**

| Variable | P value | Hazard ratio |
|----------|---------|--------------|
| EPM      | 0.024   | 1.559        |
| Age      | 0.930   | 0.994        |
| Sex      | 0.303   | 1.223        |
| WBC count| 0.339   | 0.999        |

Multivariate Cox proportional-hazards regression model using relapse time as response variable to test EPM and clinical parameters as variables in the entire cohort (n = 127).

EPM values of individual patients at relapse and at diagnosis (Fig. 2a). We observed substantial intra-patient variation (Fig. 2d,e). Although most patients showed a considerable degree of epiallele shifting during disease progression (median \( \log_{10}(\text{EPM}) = 2.29 \)), the magnitude of this difference was highly variable. Indeed, a few patients (8%) showed no epigenetic changes (EPM = 0; Fig. 2e) between diagnosis and relapse. Overall, these data suggest that global epigenetic allele shifting is a universal feature of AML relative to NBM controls, but epiallele shifting is highly variable within individual patients during disease progression. Notably, the changes in epiallele burden were independent of patients’ age, percentage blast purity of specimens, disease French–American–British (FAB) subtype, and abundance of somatic mutations (Supplementary Fig. 4a,h).

We also noted that eloci were significantly overrepresented at CpG islands and promoters (\( P = 2.9 \times 10^{-3} \) and \( P = 0.027 \), respectively, compared to NBM controls; Wilcoxon signed-rank test) in AML patients at diagnosis (Supplementary Fig. 5a,f). Eloci were also significantly enriched at enhancers at diagnosis (active enhancers, \( P = 1.4 \times 10^{-4} \); poised enhancers, \( P = 7.5 \times 10^{-3} \); Wilcoxon signed-rank test; Supplementary Fig. 5j,k). At relapse enrichment for eloci was more significant at intronic and intergenic regions (\( P = 5.3 \times 10^{-3} \) and \( P = 7.6 \times 10^{-4} \), respectively; Wilcoxon signed-rank test; Supplementary Fig. 5h,i). These differences point to substantial disruption of regions with potential for gene-regulatory effects during AML progression.

**Independent patterns of epigenetic and genetic diversity**

The EPM measurement provides assessment of epigenetic allele shifting at the global level but does not provide information on how specific eloci vary during leukemia progression. The latter information would be similar to that from evaluation of individual genetic mutations in tumors. From the genetic perspective, AML progression has been examined in patients by next-generation sequencing and other techniques. These studies showed that although most genetic lesions did not change during disease progression, some were gained or lost at relapse. A subset of patients showed no new somatic mutations at relapse.

Our current data set enabled simultaneous comparison of epigenetic and genetic allele burdens to discern whether they are correlated and how their composition might vary during leukemia progression. To address each of these points, we called mutations at diagnosis and relapse for each patient sample with WES data, then defined three categories of eloci: (i) eloci unique to diagnosis (diagnosis-specific), (ii) eloci unique to relapse (relapse-specific), and (iii) eloci present both at diagnosis and at relapse (shared).

We examined the eloci in several ways. First, we performed K-means clustering based on the proportion of diagnosis-specific, relapse-specific, or shared eloci present in each patient (Supplementary Fig. 6a and Fig. 3a). This analysis segregated AML patients into three clusters: (i) those having a significant predominance of diagnosis-specific eloci (cluster 1; \( n = 57, P = 5.3 \times 10^{-11} \), Wilcoxon signed-rank test); (ii) those with mostly shared eloci and no predominance of diagnosis- or relapse-specific eloci (cluster 2; \( n = 39 \)); and (iii) those with a significant predominance of relapse-specific eloci (cluster 3; \( n = 42, P = 4.5 \times 10^{-13} \), Wilcoxon signed-rank test; Fig. 3a). None of these clusters was significantly linked with age, WBC count, or FAB classification (Supplementary Fig. 6b–d).

We next determined the abundance of somatic genetic mutated alleles (SNVs and indels) for patients for whom WES was available (\( n = 48 \)). We examined the abundance of somatic mutations at diagnosis and relapse in each epigenetic cluster. We annotated somatic mutations as diagnosis-specific, relapse-specific, or shared between the two time points, similarly to the classification used above. The proportions of mutations in these categories did not follow the distributions observed for eloci in clusters 1 and 3 (cluster 1, \( P = 0.89 \), cluster 3, \( P = 0.40 \); Wilcoxon signed-rank tests; Fig. 3b). For example, samples in epigenetic cluster 1 showed significantly lower frequencies of somatic mutation at diagnosis and relapse than samples in cluster 3 (Fig. 3c, \( P = 0.048 \); Fig. 3d, \( P = 0.008 \), Wilcoxon rank-sum tests; Supplementary Table 5). Concordantly, a reciprocal analysis in which we used the median mutation burden per subject to divide patients revealed enrichment for higher mutation rates in cluster 3 (Supplementary Fig. 6c). These data indicate distinct combinations of genetic and epigenetic subtypes among epigenetic clusters.

We next examined whether specific individual somatic mutations might be linked to the tendency to develop eloci. Such considerations are of interest given that many recurrent somatic mutations in AML occur in genes encoding epigenetic modifiers. However, there was no significant association between specific mutations in...
genes reported to be recurrent in AML\textsuperscript{11,45} and the abundance of eloci (all genes $P > 0.05$, chi-squared test with Monte Carlo simulation; Supplementary Fig. 6f and Supplementary Table 6). As in many tumors, somatic mutations in AML occur in a heterogeneous pattern, representing the presence of genetically distinct subclones. Thus, it is possible that the genetic clonal complexity of AML might mirror the abundance of eloci. Using sciClone\textsuperscript{46} we determined that 32.5% of patients showed increasing clonal complexity, 20% showed decreasing clonal complexity, and 47.5% showed no change in clonal complexity between diagnosis and relapse (Supplementary Fig. 7). Yet none of these clonal evolution patterns were enriched in epigenetically defined clusters ($P = 0.915$, chi-squared test; Supplementary Fig. 8), further indicating independent kinetics of genetic and epigenetic changes during AML evolution.

We also evaluated whether changes in variant allele frequency (VAF) of somatic mutations between diagnosis and relapse were linked to the pattern of eloci occurrence. Each patient exhibited variable numbers of alleles that decreased, increased, or remained stable in frequency between diagnosis and relapse samples. We focused our assessment on the patients in epigenetic clusters 1 and 3, which showed the greatest differences in eloci distributions between diagnosis and relapse samples. We assessed somatic mutations in diagnostic and relapse samples (compared to matched germline controls) for changes in VAFs during disease progression. An increase in VAF was defined as a minimum gain of 10% (Fig. 3e), and a decrease in VAF was defined by a minimum loss of 10% (Fig. 3f). Among patients in epigenetic clusters 1 and 3, there was no significant difference in the tendency to gain somatic mutation VAF at relapse (Fig. 3e and Supplementary Table 5). However, epigenetic cluster 3 patients showed a significantly lower proportion of somatic mutations with reduced VAF at relapse ($P = 0.0012$, Wilcoxon rank-sum test; n.s., not significant).

We next investigated whether the differences in epiallele shift patterns between clusters 1 and 3 were associated with perturbations in the transcriptional landscape. We conducted RNA-seq in paired diagnostic and relapse samples representing patients from each cluster ($n = 19$; Supplementary Table 7). A supervised analysis (Online Methods) revealed 114 genes differentially expressed between diagnostic samples in clusters 1 and 3 (Supplementary Table 8). These included genes encoding the cell cycle inhibitor CDKN1A and the transcription factor SPI-B, which were expressed more highly in cluster 3, and the gene encoding the transcription factor FOXC1, which was expressed more highly in cluster 1. Overall, we detected upregulation of genes encoding protein kinases and other signaling molecules in cluster 1, and enrichment of genes linked to inflammation and immune response in cluster 3 (Supplementary Table 9). Differential expression of transcription factors inflammatory and signal-transduction gene sets between the two groups suggested a potential for alternative mechanisms of oncogenesis between the patients in epigenetic clusters 1 and 3.

**Epiallele and mutation shifts throughout AML progression**

To create a higher-resolution view of the progression kinetics of genetic and epigenetic heterogeneity in AML, we performed whole-genome
sequencing (WGS) and ERRBS on leukemia cells from a patient (AML_130) from whom five serial specimens were available (T1 = diagnosis; T2–T5 = serial relapse time points; Supplementary Tables 2–4). We first evaluated the global epigenetic allele burden (EPM) for each time point compared to NBM controls (Fig. 4a). We observed a marked increase (150%) in epiallele burden occurring at T2. In contrast, the T2 sample showed little increase (2.7%) in the number of somatic mutations compared to germline DNA from the same patient (Fig. 4b). Increases in EPM at time points T3–T5 were relatively smaller; however, at T4 there was a larger increase (29%) in the abundance of somatic mutations than at other time points (5.4%), indicating that the initial epigenetic shift at T2 was antecedent to the later genetic evolution in AML cells from this patient at T4. Furthermore, genomic analysis did not yield evidence of acquisition or loss of a somatic mutation linked to known epigenetic modifier genes at T2 that might explain the jump in epiallele diversity at T2 (Supplementary Table 10).

We next examined the unique occurrences of individual eloci and somatic mutations at each time point. Analysis of eloci revealed a tendency of the epigenome to evolve continuously over time (Fig. 4c). A majority of eloci (65.79%) were unique to a single time point; far fewer eloci were shared among two, three, or four time points (18.78%, 10.04%, and 3.64%, respectively), and only 1.75% of eloci were shared across all five time points (Supplementary Fig. 9a). In sharp contrast, genetic alleles were much more stable; 24.6% of somatic alleles were detectable over all five time points (Fig. 4d, Supplementary Fig. 9b, and Supplementary Table 11). Hence, genetically defined, mutated alleles showed significantly greater stability over time than eloci \( (P < 2.2 \times 10^{-16}, \chi^2\text{-squared test}) \).

To better discern the relationship between epigenetic and genetic heterogeneity, we examined which of the 16 possible epialleles was dominant within each elocus at each time point. The proportion of these specific epialleles was used as a measure of epigenetic clonality. Reciprocally, to evaluate genetic clonal complexity, we examined the VAF of somatic mutations at each time point. Notably, we observed a shift in dominant epiallele frequency over time. At early time points, the dominant epialleles tended to occur at a frequency of >50% (Fig. 4e; median = 53%). A marked shift in epiallele frequency occurred at T3, when the dominant pattern shifted to a lower frequency (25%). By T5 the leukemia cells were again enriched for eloci with higher dominant epiallele frequency. However, the pattern was different when considering genetic VAF. At early time points (T1–T3), the median VAF was 44%. A significant shift to low-frequency genetic mutations (VAFs) occurred (Fig. 4f) only at T5. Genetic alleles were also distinct from dominant epialleles in that they exhibited a tighter allele frequency distribution \( (P = 0.008, \text{Wilcoxon rank-sum test}; \text{Fig. } 4e,f) \), perhaps because the epigenome is more plastic than the genome. Collectively, these data support the notion that epigenetic and genetic heterogeneity in AML are not necessarily linked and may follow distinct patterns and distributions during disease progression.

### Promoter epiallele shifts linked to transcription variance

Given that transcription is regulated through epigenetic marks, we next determined whether the presence of eloci was linked to alterations in gene expression. We focused on genes containing eloci within promoters, derived from the comparison of diagnostic to relapse specimens \( (n = 19 \text{ pairs}; \text{Supplementary Table 7}) \). Genes containing shifts in epiallele composition at promoters (promoter eloci) showed significantly greater variance in transcript abundance between relapse and diagnosis than genes without such epiallele shifts in their promoters \( (P < 0.001; \text{Wilcoxon rank-sum test}; \text{Fig. } 5a,b) \). We next focused our analysis on genes that were significantly differentially expressed in relapse versus diagnostic AML samples. Here again, a higher proportion of differentially expressed transcripts were significantly associated with promoters harboring eloci than with promoters not harboring eloci \( (P < 0.001; \text{Wilcoxon signed-rank test}; \text{Fig. } 5c) \).

Finally, we used an additional approach to examine potential links between promoter epigenetic heterogeneity and transcription. We performed scRNA-seq in 96 cells from the first relapse sample (T2) from patient AML_130. We found that the genes with higher epiallele heterogeneity within their promoters also had significantly higher levels of cell-to-cell transcriptional heterogeneity as measured by the coefficient of variation \( (P < 2.2 \times 10^{-16}, \text{ANOVA}; \text{Fig. } 5d) \). Hence the presence of eloci at gene promoters resulted in greater tendency of associated genes to show deregulated expression.
DISCUSSION

AML is well suited for examining questions about epigenetic heterogeneity, as this type of tumor generally manifests a relative paucity of genetic lesions but still shows genetic clonal complexity and clonal evolution during progression. Mazor et al. recently examined DNA methylation at individual CpG sites and somatic mutations during tumor progression in gliomas, where a phylogenetic analysis showed a co-dependency of change for the two features examined. However, an examination of overall DNA methylation at CpG sites with array-based measurements cannot fully address epigenetic heterogeneity as measured by epiallele diversification; thus, it has remained unclear whether epigenetic alleles and genetic alleles follow similar courses during disease evolution. Although others have documented epigenetic heterogeneity in B cell neoplasms, these tumors arise mostly from cells that display intrinsic epigenetic heterogeneity, and the studies did not explore the link between genetic and epigenetic allelic diversity during tumor progression. This is the first report of dynamic epiallele shifting in a human tumor. Previous reports have used DNA methylation heterogeneity metrics such as epipolymorphism approaches, which calculate diversity within individual specimens, rather than methclone compositional entropy, which measures shifting between samples and, hence, different properties of the epigenome.

Here we establish that tumor genetic and epigenetic heterogeneity in AML may arise as independent, biologically distinct phenomena, each presumably having a unique functional significance. The degree of leukemic epigenetic allelic burden was independent of age and other clinical parameters. There was no apparent association between the degree of epigenetic heterogeneity and the presence of somatic mutations affecting epigenetic modifier genes such as DNMT3A, TET2, and IDH1 or IDH2. We also explored whether dominant epigenetic alleles behaved in a similar or distinct manner from that of genetic alleles during clonal evolution. We found no association between the kinetics and pattern of genetic and epigenetic alleles during leukemic progression.

It is notable that AML patients can be classified on the basis of epiallele pattern kinetics and somatic mutation burdens during disease progression. Our results suggest that in at least some cases, AML patients may be divided at diagnosis as having high epiallele diversity and low somatic mutation burden (cluster 1, epigenetically driven) or as having lower epiallele diversity and higher mutation burden (cluster 3, genetically-driven) (Fig. 3a,c). The latter develop increasing epigenetic diversity during progression (Fig. 3a). In both cases, genetic clonal composition remains predominantly stable (Supplementary Table 5).

Most notably, epigenetic instability was not necessarily linked to genetic instability or specific somatic mutations. This observation may point to alternative modes of dominant heterogeneity in newly diagnosed patients: one genetic and one epigenetic. Tumor heterogeneity in AML can thus not be determined strictly by genetic or epigenetic analysis alone, and relying only on genetic mapping may underestimate the true tumor diversity. We speculate that de novo acquisition of eloci might represent the response of hematopoietic cells to environmental stresses at various points during the development and/or progression of disease. Conversely, exposure to chemotherapy may induce epigenetic plasticity, contributing to the increased number of eloci seen at relapse in some patients. Indeed, longitudinal profiling of a patient treated with four different regimens showed distinct patterns of epiallele shifting after each treatment. It is also possible that unrecognized mutations or alterations in coding or noncoding regions could destabilize the epigenome at specific loci and contribute to epigenetic heterogeneity. One example is the protein CTCF, which can regulate cytosine methylation patterning and boundaries. A CTCF haploinsufficiency mouse model results in the acquisition of epigenetic hypervariability hot spots and development of tumors. In the case of B cells, epigenetic heterogeneity may be linked to the actions of activation-induced cytosine deaminase. In the case of AML, biological differences among patients with different modes of eloci progression patterns may be mediated through differential expression of hematopoietic transcription factors and functional gene sets.

The acquisition of eloci can also lead to functional consequences, as we found that genes containing eloci at their promoters showed more pronounced transcriptional variability and differential expression during disease progression. A recent scRNA-seq study on newly diagnosed CLL patients also showed that sites of epigenetic variability had greater cell–to–cell transcriptional plasticity. A second indication of biological relevance is the inferior outcome of patients with higher EPM burdens in both univariate and multivariate analyses. The fact that the link to inferior outcome is even stronger for promoter epialleles suggests that eloci that perturb transcriptional regulation are particularly important in contributing to disease phenotype.

Using an epipolymorphism-based approach, we observed decreasing DNA methylation heterogeneity upon AML progression, which matches a previous report in diffuse large B cell lymphoma (DLBCL) (Supplementary Fig. 10a). However, unlike in DLBCL, the abundance...
of epipolymorphisms in AML did not segregate patients into distinct clinical groups on the basis of progression-free survival (Supplementary Fig. 10b); only the EPM separated the cohorts. Epiallele shifting as detected by methylene is thus a candidate biomarker in AML and may be indicative of more aggressive disease, perhaps reflecting greater epigenetic plasticity and adaptability in certain tumors. Given the heterogeneous nature of AML, our study was underpowered to detect specific eloci that could be used as outcome biomarkers, although there was a trend for a set of 21 promoter-annotated eloci to associate with shorter time to relapse (Supplementary Table 12).

To our knowledge, this study is the first to examine the longitudinal interplay of genetic and epigenetic heterogeneity in AML. It is possible that genetic and epigenetic heterogeneity are linked by other means in AML or in different manners in other types of tumors. Studies in larger cohorts using improved sequencing technologies are likely to yield additional insights. Collectively, our data suggest that epigenetic heterogeneity has important clinical and functional impacts in AML and does not fully follow the kinetics and patterns in the genetic compartment, thus creating additional means by which a tumor can evolve.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Database of Genotypes and Phenotypes (dbGaP): data from this study are available under the accession number phs001027.v1.p1.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.M.M. and C.E.M. conceived of the studies, designed analytical approaches, analyzed results, wrote the manuscript, and designed experiments with F.E.G.-B. S. Li conceived of computational analyses, wrote code and performed computational analysis, wrote the manuscript, and generated figures. E.F.G.-B. performed, coordinated and/or supervised all patient experimental procedures, performed bench experimental data management and analyses, and wrote manuscript. S.S.C. and R. Dillon performed experiments (flow cytometry and sorting of subject samples) and associated data analysis. T.H., F.R., and J.P. performed computational analyses. M.A.S. and P.J.M.V. performed experiments (esosome capture) and associated computational analysis. A.I.B., A.E.P., J.C., L.B., S. Lugger, M.B., I.D.L., L.B.T., B.L., H.D., K.D., P.J.M.V., J.D., and M.G. coordinated patient sample collection and analyzed clinical data. D.N. assisted with statistical analyses. P.V. performed single-cell RNA-seq library preparation. R. Delwel, M.L.G., D.C.H., G.J.R., D.G., C.Y.P., and R.L. helped with sample collection, writing, analysis, and patient annotation. All authors read, edited, and approved the manuscript.

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Patient characteristics. 138 clinically annotated, paired samples (diagnosis and relapse) from AML patients (86 males and 52 females) seen at medical centers in Australia, Germany, the Netherlands, and the United States were collected. Patients with acute promyelocytic leukemia were excluded. All patients were treated according to the protocols of the corresponding institutes and hospitals. The clinical and molecular characteristics of these patients are summarized in Supplementary Table 1, and detailed descriptions are provided in Supplementary Table 2. All patients were treated with combination chemotherapy (cytarabine arabinoside and an anthracycline) during induction phase followed by consolidation chemotherapy treatment with or without stem cell transplantation in first remission, per clinical center standards. Samples from further serial time points were available for patient AML_130, a male with previous high-dose radiation exposure who developed AML at age 57. Samples were available from the diagnostic time point (T1, leukapheresis sample) and during disease progression (T2T5) as follows: he underwent standard induction treatment (cytarabine + idarubicin) and achieved complete remission but developed relapse before initiation of consolidation treatment. The patient was then treated with high-dose cytarabine without response (sample T2; peripheral blood sample), combination chemotherapy (sirolimus, mitoxantrone, etoposide, and cytarabine) without response (sample T3; peripheral blood sample), combination chemotherapy without response (cytoplasm phosphamidase followed by clofarabine), hydroxyurea and external beam radiation to tonsillar site of extramedullary hematopoiesis (sample T4; bone marrow aspirate), and investigational FLT3 tyrosine kinase inhibitor (KW-2449) followed by an allogeneic stem cell transplant (sample T5; leukapheresis sample). No treatments beyond the chemotherapy administered immediately after diagnosis in AML_130 induced a complete remission.

Sample collection and processing. Donors (AML patients and individuals without known hematological malignancies) gave informed consent according to the Declaration of Helsinki for collection and use of sample materials in research protocols at the following clinical centers: Erasmus Medical Center (protocol number MEC-2015-155), Royal Adelaide Hospital and SA Pathology (Adelaide, South Australia; 1998–present), University of Pennsylvania (protocol number 703185), University of Rochester Medical Center (protocol number URCC ULEU07047), and the University of Hammersmith (protocol number 148/10). Study protocols were approved by the institutional review boards of corresponding institutes and hospitals (protocols noted above), and at Well Cornell Medicine (WCM; protocol number 08050509783). For cryopreserved specimens from AML patients at the Royal Adelaide Hospital and SA Pathology (Adelaide, South Australia) collected and stored before 1998, the requirement for informed consent was waived by the Royal Adelaide Hospital Human Research Ethics Committee (RAH HREC, protocol number 110304b), and use of the samples obtained in this specific research study was approved by the RAH HREC on 10 September 2010. Of 140 paired patient samples collected, 138 were successfully processed. Patient samples were collected at the time of diagnosis and within 3 months of clinically determined relapse. Samples were subjected to Ficol separation on the day of collection and viably frozen or immediately subjected to nucleic extractions using standard techniques. Deidentified samples were then provided to WCM. Viably frozen cells were available for further processing from patients AML_102 through AML_140, including serial samples from AML_130 (T1–T5). These samples were thawed and depleted of CD3+ and CD19+ cells using magnetic beads per the manufacturer’s recommendations (Miltenyi Biotec), yielding a blast percentage enrichment of 89 ± 9.5% as confirmed by post-separation flow cytometry. Lymphocytes were then isolated for germline controls using flow sorting for CD3+ and CD19+ cells using magnetic beads per the manufacturer’s recommendations (Miltenyi Biotec), yielding a blast percentage enrichment of 89 ± 9.5% as confirmed by post-sort flow cytometry analysis. Germline DNA was isolated from ex vivo–expanded lymphocytes for patient samples AML_074 through AML_101. Briefly, the procedure for ex vivo expansion was as follows: 1 million bone marrow cells were cultured in RPMI 1640 with l-glutamine and 10% FCS. T cell expansion was induced with T cell expander Dynabeads CD3/CD28 (Thermo Fisher). After 1–3 d 1RI2ML was added. Cells were subsequently purified to >98% with CD3 microBeads (MACS Miltenyi Biotec). Flow cytometry analysis and sorting was performed using CD19 PerCP–Cy5.5 (HIB19) and CD3 PerCP–Cy5.5 (HIT3a) from Biolegend (San Diego) and human CD45 APC–A780 (2D1) from eBioScience. Validation for each antibody is provided on the manufacturer’s website (http://www.biolegend.com/) and at Antibodypedia (http://www.antibodypedia.com/gene/4237/CD19/antibody/539284/302214; http://www.antibodypedia.com/gene/4581/CD3/antibody/539101/300437) and iDegreBio (1DB-DB-01-0001093758). Lymphocytes were defined as CD45+SSC−CD19+ or CD3+ cells, and leukemic blasts were defined as CD19−CD45−SSC−low cells49. DNA and RNA were extracted from the isolated cells using standard techniques. For patient samples AML_001 through AML_101, DNA and RNA were extracted from mononuclear cell layers using standard techniques. Normal bone marrow controls (NBM controls: CD34+ mononuclear cells; 7 males and 7 females) were purchased from AllCells (n = 5) or isolated using magnetic bead positive selection for CD34+ cells per the manufacturer’s recommendations (Miltenyi Biotec) from freshly collected bone marrow samples from volunteers without known hematological malignancies (n = 9). Purity was verified using CD34+ PE/CF594 (563) from BD Biosciences (validation provided on manufacturer’s website, http://wwwbdbiosciences.com/in/index.jsp) in flow cytometry post-separations to greater than 90%. All flow cytometry data were analyzed using FlowJo (TreeStar).

Enhanced reduced representation bisulfite sequencing. ERBRS is a modified version of RRBS56,57. Libraries were prepared by MspI restriction enzyme digestion of high-molecular-weight genomic DNA, followed by end repair, size selection, bisulfite conversion and library amplification as previously described38,39. Libraries were sequenced on a HiSeq 2000 Illumina machine using 75-bp single-end reads. Data alignment was performed to human genome hg19 as previously described38,39. Average reads per sample were 138,596,488 with a mean alignment rate of unique reads of 63.7%, covering on average 4,399,235 CpGps per sample at a minimum threshold coverage of 10x. Average coverage depth per CpG was 72.3, and average bisulfite conversion was 99.86%, determined as previously described18,39. A subset of patient samples were processed using SeqCap Epi 4M CG Enrichment kit (NimbleGen-Roche) and SureSelectXT Human Methyl-Seq (Agilent Technologies) per the manufacturer’s recommendations. These were sequenced using a paired-end 100-bp approach on a HiSeq 2000 (Illumina). See Supplementary Table 3 for detailed sequencing statistics.

Data analysis. The programming framework R59 version 3.2.1 was used for data analyses. Specific R packages and other tools and software used for analyses are described below and in Supplementary Methods.

Code availability. Analysis scripts used in this manuscript are included in Supplementary Methods and have been deposited at https://github.com/ShengLi/relapsed_AML.

ERBRS data analysis. We performed bisulfite–treated read alignment to hg19 genome and methylation calls as previously described38,39. Briefly, the adaptor sequences were removed by FAR software51. Preprocessed reads were then aligned to human genome reference hg19 using the bismark alignment software.

Epipalele shift analysis was performed using methclone40. Briefly, the epipalele patterns of compositional changes between different samples were evaluated using methclone to calculate the combinatorial entropy (ΔS) change of epipalleles at each locus. This analysis outputs the loci with a ranked list of epipalele changes defined by the entropy change. We first calculated the foreground combinatorial entropy using the epipalele composition detected, which is the sum of the entropy of each locus for two samples considered. We then calculated the background combinatorial entropy, using the epipalele composition after uniformly mixing all patterns of epipalleles between two samples. The sum entropy was determined, and the entropy changes were defined by the difference between foreground and background combinatorial entropy values. Epipalele shifts per million loci (EPM) is a normalized measure of the global epipalele changes with ΔS < −90 as the cutoff (loci). Comparisons of AML specimens to NBM controls report the average EPM determined from assessing each AML case against each NBM (n = 14). The diagnosis–specific eloci are eloci that are detectable only at diagnosis stage when compared to NBM controls. The relapse–specific eloci are eloci that are detectable only at relapse stage when compared to NBM controls. The shared
eloci are eloci that are detectable at both stages. The epigenetic clusters were determined using K-means clustering (K = 3, gap statistic for estimating the number of clusters) based on the proportion of eloci that are diagnosis specific, shared, or relapse specific. Within clusters 1 and 3, respectively, the proportion of eloci for diagnosis and relapse were compared using Wilcoxon signed-rank tests within each cluster to measure the significance of dominance. Finally, we assessed the distribution of patients from each clinical center (n = 5) in the epigenetically defined clusters. Using multinomial logistic regression analysis to model the clusters with clinical and demographic features, we determined that the center identity was not equally distributed among the three clusters. However, we confirmed no known technical or clinical etiology was related to this distribution for each center (age, WBC, gender, FAB classification, mutations, cytogenetics), and the sample sizes for the subcohorts were not powered to detect subclusters within each cohort.

Intratumor global methylation heterogeneity (MH) was assessed as previously described. Briefly, epipolymorphisms were determined using proportions of DNA methylation patterns at four adjacent CpGs (1 − 𝑝𝑖/2 ± 1), where 𝑝𝑖 is the fraction of each DNA methylation pattern i among the cell population. Epipolymorphism loci shared by at least 75% of the patients (n = 104) were considered for further analysis. Because epipolymorphism is dependent on DNA methylation levels, each locus was binned by the average DNA methylation levels from 0–100% (21 bins in total). Therefore, for each sample, all the loci were assigned to one of the 21 different groups on the basis of their mean DNA methylation levels. For each bin, the width was 5% (DNA methylation), except for the first and last bin (2.5% DNA methylation). The median epipolymorphism across all the loci within each bin were calculated. Then the overall epipolymorphism landscape was defined using the median epipolymorphism across the 21 bins spanning the DNA methylation levels from 0–100%. The intratumor overall MH was defined as the area under the median epipolymorphism across the spectrum of methylation percentages in 21 bins. The range of MH is 0–100. Higher MH represents higher overall intratumor heterogeneity. Each locus was covered by at least 60 sequencing reads.

Genomic annotation. Genomic annotation reference files (CpG islands and RefSeq genes) for eloci distribution analyses were obtained from UCSC (https://genome.ucsc.edu/) using the February 2009 (GRCh37/hg19) assembly. Promoters were defined as transcription start sites ± 1 kb. CpG shores were defined as 2 kb flanking CpG islands, subtracted by any regions overlapping with nearby CpG islands. CpG shelves were defined by 2 kb flanking CpG shores, subtracted by any regions overlapping with nearby CpG islands and shores. Enhancers were defined on the basis of the US National Institutes of Health Roadmap Epigenomics Project (CD34- and CD34+ mobilized primary cell active. Enhancers were defined as histone H3 monomethylated at K4 (H3K4me1) and histone H3 acetylated at K27 (H3K27ac) peaks without H3K4me3 marks, and poised enhancers were defined as H3K4me1 without H3K27ac or H3K4me3 marks. Wilcoxon signed-rank tests were used to compare the proportions of eloci falling into each of the genomic annotation regions.

RNA sequencing. RNA-seq libraries were prepared in two batches (Supplementary Table 7) using TruSeq RNA-Seq by poly(A) enrichment (Illumina) and sequenced on HiSeq2000 (Illumina) using a 50-bp paired-end approach per the manufacturer’s recommendations. All paired samples were prepared within the same batch. Alignment was performed using the STAR aligner (version 2.3.0e) and human genome hg19 as reference. Aligned results were annotated using the RefSeq gene model and HTSeq union mode. Gene expression data were normalized using RPMK. Blast-enriched patient samples from AML_130 (lymphocyte depleted as described above) were also used for single-cell isoforms. Single cells were captured and mRNA isolated using Clontech’s SMARTer chemistry (v2) on the Fluidigm C1 Single Cell Auto Prep system. Illumina’s Nextera XT kit was used for library preparation before 100-bp paired-end sequencing on the HiSeq 2500 (Illumina) platform. See Supplementary Table 8 for sequencing statistics. Data analysis was performed using r-make (http://physiology.med.cornell.edu/faculty/mason/lab/r-make/) for quality control, alignment, and gene expression quantification.

DESeq2 (ref. 56) was used for differential gene expression analysis. The significance of differentially expressed genes (DEGs) was determined using Wald significance test to compare cluster 1 compared to cluster 3. The design matrix includes batch information to control for any possible batch differences. For multiple-hypothesis testing, the significance cutoff used for optimizing the independent filtering was 0.05 (Benjamini–Hochberg). A log2 fold change greater than 1.2 or less than –1.2 was used for significance.

Transcriptional heterogeneity was assessed using single-cell RNA-seq data. Specifically, the coefficient of variations and transcriptional abundance per gene across cells was determined using log2 RPMK. Genes were included if the average log2 RPMK in cells was higher than 1.

Gene Ontology term enrichment analysis was performed using Gene Set Analysis Toolkit. A threshold of two overlapping genes was considered. Hypergeometric tests were used for significance determination, and Benjamini–Hochberg was used for multiple testing correction.

Whole-exome sequencing. Whole-exome capture was performed on DNA isolated from 48 paired diagnosis and relapse samples and patient-matched germ-line samples. Germline DNA (lymphocytes) was subjected to whole-genome amplification (repli-g kit; Qiagen) in 25 patients owing to limited materials (see Supplementary Table 2). To obtain sufficient quantities of DNA from the remaining samples, T cells were expanded ex vivo as described above. DNA was extracted using standard techniques. Libraries were prepared per the manufacturer’s recommendation using NimbleGen SeqCap EZ Human Exome Library v3.0, Agilent Human Exon V3 (exon 50 Mb), or Agilent SureSelect Human All Exon V4 (51 Mb; see Supplementary Table 2 for specification of kit use per patient tumor and germline samples) and sequenced at a minimum of 50-bp single-read sequencing on a HiSeq 2000 (Illumina) to a mean coverage per base of 73-fold. See Supplementary Table 4 for sequencing statistics.

Whole-genome sequencing. Illumina TruSeq Nano DNA Library Prep Kit (Illumina) was performed on DNA from time points T1–T5 and germline (CD19+CD3+ cells) isolated from the first relapse sample from patient AML_130 (T2). Libraries were prepared per the manufacturer’s recommendation and sequenced using a 101-bp paired-end sequencing approach on the HiSeq 2000 platform per the manufacturer’s recommendations to a mean coverage per base of 43-fold. See Supplementary Table 4 for sequencing statistics.

Next-generation sequencing data analysis. DNA sequencing data were analyzed to determine somatic mutations, copy-number aberrations, and clonal evolution patternning using publicly available tools (BWA59, GATK60–62, MuTect63, Varscan64, somaticSniper65, SnPEFF66, XHMM67, DNAcopy library68, and sciClone69). Tool versions; inclusion, exclusion, and significance tests; and specific commands used and implemented are described in Supplementary Methods and have been deposited into GitHub (https://github.com/Shengli/relapsed_AML).

Integrative analysis. The association between DEGs (derived from relapse versus diagnosis paired AML patient samples) and promoter-associated eloci was determined as follows. Eloci (maximum AS = 90) annotated to gene promoters (transcription start sites ± 1 kb) were considered for the integrative analyses. Patients with a minimum of 30 genes with promoter-associated eloci were included (n = 19). EpiData loci within gene promoters that exhibited a minimum AS greater than –2 were designated non-loci. RPMK log2 fold change was used to measure the transcriptome level dynamics. The variance of gene expression (RPMK log2 fold change) was calculated for genes with or without eloci within their proximal promoters and compared across all patients using a Wilcoxon signed-rank test. Genes with log2 fold change greater than 1 were defined as DEGs. The number of DEGs with or without eloci were compared by Wilcoxon signed-rank test.

Assessment for association between epigenetic clusters and mutations in genes recurrently affected in AML was performed. The variant calling pipeline (in-house-developed algorithm) used BAM files generated from the sequencing data of all paired diagnostic, relapse and germline samples to identify SNVs and small indels in driver genes associated with AML. For the identification of these somatic genetic aberrations we used a multi-variant-calling approach integrating the output of six different somatic mutation detection algorithms: MuTect (version 1.17), Indelocator, GATK UnifiedGenotyper, etc.
Clinical correlation analysis. The correlation of overall epiallele shift (log_{10} EPM) with clinical parameters was evaluated. The clinical correlation between EPM (log_{10}) and FAB classes was performed using ANOVA test. FAB classes with a minimum of seven patients were assessed. We used the Pearson correlation (r) between overall epiallele shift and age or blast purity post separation and Hoeffding’s D statistics for dependency test.

To determine which gene-associated epiallele shift loci (eloci) associated with clinical outcome, we performed the following analysis: (i) we determined eloci between diagnosis samples and NBM controls (we required that the epiallele region considered be covered by a minimum of 5 NBM controls); (ii) we annotated eloci to gene promoters (transcription start site ± 1 kb) and excluded epiallele loci which were covered in less than ten patients; (iii) we divided the patients into groups of longer (n = 69) or shorter (n = 68) relapse-free survival on the basis of the median value of time to relapse; (iv) we assessed the frequency of each elocus in the patient groups; (v) we used odds ratio to determine the association between early and late relapse group for each locus; (vi) we determined significance using Fisher's exact test for each locus; (vii) we applied the Benjamini–Hochberg correction to P values.

Survival analysis. Log-rank (Mantel–Cox) test was used for survival analysis. For relapse-free survival analysis, survival endpoints in this study were time from diagnosis until AML relapse. The patients were divided by the median EPM (low EPM versus high EPM), median number of somatic mutations (low MUT versus high MUT), or median MH (low MH versus high MH) for respective comparisons. Among 138 AML patients in the cohort, time to relapse was available for 137 patients, and WBC count was available for 127 patients. Multivariate Cox proportional hazards regression model used relapse time as response variable, and included log_{10} EPM, age, gender, and WBC as variables to be tested (n = 127). P value of each predictor was used to assess whether this clinical parameter was significantly associated with relapse time.

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