Integrated flow cytometry and sequencing to reconstruct evolutionary patterns from dysplasia to acute myeloid leukemia

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Clonal evolution in acute myeloid leukemia (AML) originates long before diagnosis and is a dynamic process that may affect survival. However, it remains uninvestigated during routine diagnostic workups. We hypothesized that the mutational status of bone marrow dysplastic cells and leukemic blasts, analyzed at the onset of AML using integrated multidimensional flow cytometry (MFC) immunophenotyping and fluorescence-activated cell sorting (FACS) with next-generation sequencing (NGS), could reconstruct leukemogenesis. Dysplastic cells were detected by MFC in 285 of 348 (82%) newly diagnosed patients with AML. Presence of dysplasia according to MFC and World Health Organization criteria had no prognostic value in older adults. NGS of dysplastic cells and blasts identified 3 evolutionary patterns: stable (n = 12 of 21), branching (n = 4 of 21), and clonal evolution (n = 5 of 21). In patients achieving complete response (CR), integrated MFC and FACS with NGS showed persistent measurable residual disease (MRD) in phenotypically normal cell types, as well as the acquisition of genetic traits associated with treatment resistance. Furthermore, whole-exome sequencing of dysplastic and leukemic cells at diagnosis and of MRD uncovered different clonal involvement in dysplastic myeloid-erythropoiesis, leukemic transformation, and chemoresistance. Altogether, we showed that it is possible to reconstruct leukemogenesis in ~80% of patients with newly diagnosed AML, using techniques other than single-cell multiomics.
Introduction

Clonal evolution in acute myeloid leukemia (AML) originates long before diagnosis and is a highly dynamic process that may affect survival.1 Thus, having the possibility of defining in each patient the genetic events causing AML would be a benefit to routine diagnostic workups. However, dissecting leukemic transformation at the onset of AML is challenging without single-cell sequencing.2,3 and most clinical laboratories do not have the infrastructure to perform single-cell studies routinely.

Patients with newly diagnosed AML may present with dysplasia regardless of their clinical ontogeny (ie, secondary AML [sAML] vs therapy-related vs de novo AML).4,6 If these residual, mature dysplastic cells were generated before the differentiation blockage of blasts preceding leukemic transformation, it could be hypothesized that studying the mutational profile of dysplastic cells and blasts could uncover the evolutionary process from dysplasia to AML. This hypothesis has never been investigated.

Methods

We aimed at reconstructing clonal evolution from dysplasia to AML based on the analysis of bone marrow (BM) dysplastic cells and leukemic blasts using integrated multidimensional flow cytometry (MFC) immunophenotyping and fluorescence-activated cell sorting (FACS) with next-generation sequencing (NGS) (Figure 1A).

In total, 358 patients with AML were studied after signing informed consent forms approved by the ethics committees, in accordance with the Declaration of Helsinki. The presence of dysplasia according to aberrant phenotypic differentiation of the neutrophil, monocytic, and erythroid lineages was investigated using EuroFlow protocols7,8 in 348 patients with newly diagnosed AML; 103 were eligible for intensive treatment, whereas 245 were older adults and enrolled in the phase 3 PETHEMA/FLUGAZA trial.9 Up to 168 cell types from a total of 38 patients with AML were isolated at diagnosis and in complete response for deep-targeted (supplementary Table 1) or exome sequencing. T cells were used as control. A description of methods is available in the supplemental Data.

Results and Discussion

We first assessed the applicability of our hypothesis by investigating how many patients show dysplasia by MFC at the onset of AML (supplemental Figure 1). Dysplastic cells were observed in 285 of 348 (82%) cases (supplemental Figure 2A). Phenotypic abnormalities were more frequent in neutrophils (45%), followed by monocytic (32%) and erythroid cells (23%) (supplemental Figure 2B). Lack of data on aberrant megakaryocytic differentiation is a limitation of the study. Multilineage dysplasia was detected in 186 (53%) patients. Only 35 (10%) cases had no signs of dysplasia, whereas the remaining 28 (8%) had nearly undetectable hematopoiesis due to massive blast infiltration. The presence of dysplasia was less frequent in patients eligible for intensive chemotherapy vs those ineligible (75% vs 96%, P < .001). Thus, the median age of the series (69 years) may help in explaining presence of dysplastic cells in most patients.

In the cohort of older patients enrolled in the PETHEMA/FLUGAZA trial, there were no differences in overall survival between those with or without dysplasia according to MFC (supplemental Figure 2C). The presence of dysplasia assessed by MFC and diagnosis of AML with morphological myelodysplasia-related changes were not significantly associated (supplemental Table 2). Diagnostic classification of AML with myelodysplasia-related changes vs other subtypes had no impact on overall survival (supplemental Figure 2D). These results in older patients treated with semi-intensive chemotherapy or hypomethylating agents support previous observations in other treatment scenarios of the limited prognostic value of assessing dysplasia in AML.10,11

Targeted sequencing of dysplastic cells and blasts isolated by FACS in 21 patients with newly diagnosed AML uncovered 3 evolutionary patterns (Figure 1B). Stable evolution in those displaying identical mutational profiles in blasts and dysplastic cells (n = 12 of 21), branching evolution in patients where blasts may have originated from leukemic stem cells other than the ones driving dysplasia due to mutations absent in blasts and present in dysplastic cells (n = 4 of 21), and clonal evolution in cases showing new mutations in blasts onto mutations shared between these and dysplastic cells (n = 5 of 21). These results are not novel but rather consistent with previous observations in whole BM samples sequenced before and after leukemic transformation14,12 and in FACS stem cell compartments in paired samples from 7 patients progressing from myelodysplastic syndrome to sAML.13 Furthermore, using single-cell DNA sequencing, Morita et al3 showed how single-cell data reveal cell-level mutation co-occurrence and enable reconstruction of mutational histories characterized by linear and branching patterns of clonal evolution. Accordingly, targeted sequencing of dysplastic cells and blasts at the onset of AML may help to recreate the history of the disease despite the notably lower resolution of this approach when compared with single-cell DNA sequencing.

The unique experimental design of this study yielded interesting observations (Figure 1C). Mutations in the cohesin complex and in transcription factors were never exclusive of dysplastic cells, whereas spliceosome mutations were never exclusive of blasts, which is consistent with their role in sAML.14,15 Mutations in genes involved in DNA methylation or tumor suppressors were systematically present in dysplastic cells and blasts. At the individual patient level, mutations in TP53 appeared to drive leukemogenesis in patients 1, 2, and 3 (Figure 1B). Conversely, leukemic transformation in patient 18 was associated with a new mutation in STAG2 onto a background of mutated DNMT3A and TP53 in dysplastic and leukemic cells. Other mutations observed exclusively in blasts were found in BCORL1, FLT3-ITD, and PTPN11; all 3 genes are associated with poor prognosis in AML16-18 FLT3-ITD is a therapeutic target,19 and PTPN11 may drive resistance to newer targeted therapies.20 Of note, subclonal mutations may be missed if whole BM samples are sequenced instead of isolated cell types (supplemental Figure 3).

The increased sensitivity in detecting mutations granted by prior FACS urged us to use the same experimental approach in 10 patients achieving CR after induction, stratified according to undetectable vs persistent MRD by MFC. Phenotypically normal CD34+ hematopoietic progenitor cells (HPCs) were isolated in the former, whereas MRD cells were sorted in the latter patients; cells of the neutrophil, monocyte, and erythroid lineages were isolated in both.
Newly diagnosed patients with AML

Identification of patient-specific aberrant immunophenotypes

NGS

Figure 1. Reconstructing clonal evolution from dysplasia to AML. (A) Study design. BM aspirates collected from 358 patients with newly diagnosed AML were analyzed using MFC. In 21 patients, leukemic cells were isolated using FACS according to patient-specific aberrant phenotypes and so were cells of the neutrophil (Neutro), monocytic (Mono), and erythroid (Erythro) lineages whenever dysplastic phenotypes were observed in 1 or more lineages. T cells were systematically isolated for germline DNA. NGS of genes frequently mutated in myeloid neoplasms was performed in all cell types available in each patient. (B) Mutational status of 33 genes in T cells (T), neutrophils (N), monocytes (M), erythroblasts (E), and blasts (B) isolated from 21 patients at the onset of AML; cell types not available for NGS are represented with gray lines. Mutated genes were colored in a gradient of red according to the variant allele frequency (VAF). Three models were identified: (1) stable transition according to identical mutational landscapes in blasts and dysplastic cells; (2) branching evolution with blasts originating from leukemic stem cells other than the ones driving dysplasia, due to mutations absent in blasts and present in dysplastic cells; and (3) clonal evolution with new mutations in blasts onto mutations shared between these and dysplastic cells. (C) Percentage of mutations grouped according to functional categories that were simultaneously mutated in mature dysplastic cells and blasts (gray), or that were private in the former (blue) and the latter (purple). Functional categories with significantly different distributions were highlighted in bold.
Among patients with undetectable MRD, most mutations present at diagnosis became undetectable after induction (Figure 2A). One exception was mutated DNMT3A in CD34+ HPCs from patient 23, which could represent postremission clonal hematopoiesis, and in erythroid cells from patient 24, which could represent the expansion of hematopoietic populations unrelated to the AML founding clone after induction therapy. Although the clinical expansion of hematopoietic populations unrelated to the AML in erythroid cells from patient 24, which could represent postremission clonal hematopoiesis, and the selection of genetic traits enabling treatment resistance or the selection of MRD but undetected in blasts at diagnosis (eg, MUC2 and KIR2DL3 in patients 33 and 35, respectively) (supplemental Figure 4; Figure 2C). These results uncover different clonal involvement in dysplastic myelo-erythropoiesis, leukemic transformation, and chemoresistance, in accordance with recent single-cell analysis suggesting that clones emerging during AML transformation and those selected by treatment may differ.

In conclusion, we showed for the first time that it is possible to reconstruct leukemogenesis in ~80% of patients with newly diagnosed AML, using techniques other than single-cell multimics. The possibility of identifying the genetic drivers of leukemic transformation and chemoresistance could be clinically meaningful to develop tailored treatment strategies aiming at the eradication of genetically diverse leukemic clones.

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Authorship

C.S., J.F.S.-M., P.M., and B.P. conceived and designed the study; C.S., M.-C.C., I.V., B.A., L.B., and B.P. analyzed and interpreted the data; C.S. and B.P. performed the statistical analysis; M.-C.-C., D.M.-C., M.-J.C., M.B.V., M.H.-R., P.A.-R., D.A., S.S., S. Villar, A.A.P., F.P., R.A., J.M.-L., J.M.B.B., S. Vives, J.A.P.-S., M.G.-F., T.B.d.C., M.C., M.O., J.R.-G., J.L., M.G., M.A.S., and P.M. provided the study material and/or patients; C.S. and B.P. drafted the manuscript; and all authors reviewed and approved the manuscript.
**Figure 2.** Different clonal involvement in dysplastic myelo-erythropoiesis, leukemic transformation, and chemoresistance. (A-B) Patients (Pat.) with undetectable (n = 5) and persistent (n = 5) MRD had phenotypically normal CD34+ HPCs and MRD cells, respectively isolated, whereas cells of the neutrophil, monocyte, and erythroid lineages were isolated in all patients. NGS of genes frequently mutated in myeloid neoplasms was performed in all cell types available in each patient. The VAF of mutated genes was colored in a gradient of gray. If 2 mutations in the same gene were detected, the 2 VAFs are indicated. (C) Representative patient with exome sequencing of dysplastic cell types, blasts at diagnosis, and persistent MRD. The fish plot illustrates different clonal compositions at different stages of disease progression. The blue arrow is pointing to a mutation present in dysplastic cells though absent in blasts at diagnosis and MRD, the olive arrow is pointing to mutations present in dysplastic cells and blasts at diagnosis though not at MRD, and the black arrow points to mutations present in MRD and dysplastic cells though not in blasts at diagnosis. The bar widths indicate the respective VAFs.
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**References**


**Figure 2** (continued)

![Individual dysplastic cell types](image_url)

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<th>Individual dysplastic cell types</th>
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101 mutations detected in all cell types

Mutations undetectable in one or more cell types


