Genetic diversity within leukemia-associated immunophenotype-defined subclones in AML

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Received: 25 August 2021 / Accepted: 14 December 2021 / Published online: 13 January 2022 © The Author(s) 2022

Abstract
Acute myeloid leukemia (AML) is a highly heterogeneous disease showing dynamic clonal evolution patterns over time. Various subclones may be present simultaneously and subclones may show a different expansion pattern and respond differently to applied therapies. It is already clear that immunophenotyping and genetic analyses may yield overlapping, but also complementary information. Detailed information on the genetic make-up of immunophenotypically defined subclones is however scarce. We performed error-corrected sequencing for 27 myeloid leukemia driver genes in 86, FACS-sorted immunophenotypically characterized normal and aberrant subfractions in 10 AML patients. We identified three main scenarios. In the first group of patients, the two techniques were equally well characterizing the malignancy. In the second group, most of the isolated populations did not express aberrant immunophenotypes but still harbored several genetic aberrancies, indicating that the information obtained only by immunophenotyping would be incomplete. Vice versa, one patient was identified in which genetic mutations were found only in a small fraction of the immunophenotypically defined malignant populations, indicating that the genetic analysis gave an incomplete picture of the disease. We conclude that currently, characterization of leukemic cells in AML by molecular and immunophenotypic techniques is complementary, and infer that both techniques should be used in parallel in order to obtain the most complete view on the disease.

Keywords AML · Immunophenotype · Molecular diagnostics · MRD

Introduction
Acute myeloid leukemia (AML) is a highly heterogeneous clonal disease. Heterogeneity is observed between and within patients morphologically, immunophenotypically, and genetically [1, 2] and different responses can be observed to the applied treatments. Immunophenotyping allows the detection of leukemic cell (sub)populations. When compared to healthy cells, malignant cells can be recognized by the presence of aberrant patterns of cluster of differentiation (CD) monoclonal antibodies (mAb), called leukemia-associated immunophenotypes (LAIPs) [3]. LAIPs can be used to follow the malignant cells in time, and enable the detection of measurable residual disease (MRD) after treatment. LAIPs are defined by the combination of markers directed against aberrant antigens on the AML cells. LAIPs defined by asynchronous expression of antigens or aberrant co-expression of cross-lineage antigens are often found. Less abundant and harder to follow up in time are LAIPs defined by the over- or under-expression of non-aberrant combinations of antigens and LAIPs defined by aberrant light scatter properties [4]. Multi-color flow cytometry currently allows the identification of patient-specific LAIPs in the vast majority of AML patients, and often several distinct LAIPs may be detected within an individual [3]. Next to LAIPs, different-from-normal (DfN) cell detection can also be used to identify the presence of MRD [5]. DfN cells are defined as cells which show aberrant markers or display an aberrant differentiation/maturation pattern that are new.
compared to diagnosis. DfN cell detection can be used in MRD settings, especially when there is no immunophenotypic data from the moment of diagnosis or when there were no LAIPs detected at diagnosis. In addition to immunophenotyping, genetic analyses by cytogenetics and sequencing of leukemia-associated genes may reveal pathogenic genetic aberrations in the majority of AML cases, which are used for prognostic risk categorization and the choice of personalized, targeted therapies [6–8].

Although almost 80% of AML patients may reach a complete remission after intensive treatment, in about 50% a relapse develops [9, 10]. Monitoring the persistence of malignant cells during and after treatment allows the early recognition of an impending relapse and tailoring of post-remission therapy [5, 11]. MRD is currently investigated by flow cytometry, which can be applied at a sensitivity between 1:10^3 and 10^4 for most patients as well as by molecular methods aiming to detect the disease-associated mutations. Molecular analysis using next-generation panel sequencing can be broadly applied, but generally has limited sensitivity (1–5%). In contrast, Q-PCR-based assays are highly sensitive allowing the detection of residual malignant cells with a sensitivity of 1:10^5–10^6. A drawback of the latter is that specific assays need to be optimized for each individual mutation. Currently, sensitive assays for nucleophosmin 1 (NPM1) and PML-RARA, RUNX1-RUNX1T1 and CBFB-MYH11 fusions are broadly used [10]. Previous studies revealed that genetically different subclones populating the bone marrow of myelodysplastic syndrome (MDS) and AML patients may co-exist at the same time. In addition, these subclones may show different evolutionary trajectories in time and respond differently to therapy [12].

Even though immunophenotyping and next-generation sequencing are widely used, their complementarity and overlap are still under investigation. The aim of our study was to compare how the two techniques characterize the malignancy, at the moment of diagnosis. In order to do that, we genetically investigate the different LAIPs which could be isolated from each patient, using the diagnostic panels.

Materials and methods

Patient samples

Bone marrow (BM) samples from AML patients were collected at diagnosis. Mononuclear cells were isolated using density gradient centrifugation (Ficoll) and cryopreserved. All selected patients had signed informed consent. The study was conducted in accordance with the Declaration of Helsinki and institutional guidelines and regulations from the Radboudumc Nijmegen (IRB number: CMO 2013/064).

The patient characteristics are listed in Supplementary Table 1.

Sorting of AML subclones

MNCs from BM were thawed and stained as reported in the supplementary material and methods. The monoclonal antibodies (Moabs) were selected based on the LAIPs found at diagnosis. For each patient, specific gating-strategies were used for identification of their LAIP populations. The specific gating-strategy as well as the used Moabs was first tested on a Navios Flow Cytometer (Beckman Coulter), and afterwards, the different subpopulations were sorted on a BD FACSAria II SORP cell sorter and represented in Figs. 2, 3 and 4. Only populations for which at least 5000 cells were sorted were further analyzed.

DNA isolation and amplification

DNA was isolated using NucleoSpin Blood QuickPure kit (Macherey Nagel, Düren, Germany) or NucleoSpin Tissue XS (Macherey Nagel, Düren, Germany), according to the manufacturer’s protocol. When the extraction yield was insufficient (< 5 µg), 5 µl of DNA was amplified using the Qiagen REPLI-g kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s protocol. All amplifications were performed in duplicate. The detailed protocol can be found in the supplementary materials and methods.

DNA sequencing

The full protocol’s descriptions can be found in the supplementary materials and methods. All the fractions were sequenced using a panel of single-molecule tagged molecular inversion probes (smMIPs) covering target regions in 27 myeloid and lymphoid malignancy-associated driver genes (Table S3). Libraries were prepared as previously described (Supplementary material [13]), and the sequencing was performed on the Illumina NovaSeq 6000 or Next-Seq500 platform (Illumina, San Diego, CA). Each sample was sequenced in duplicate to exclude artefacts caused by the amplification procedure. The indicated variant allele frequencies (VAFs) are the mean VAFs of the duplicates. The variability in VAFs between duplicates was calculated in 40 samples (comparing the VAFs detected for each SNP in
the different samples) and resulted to be ± 6.4%. To detect internal tandem duplication in fms like tyrosine kinase 3 (FLT3-ITD) gene, we used fragment length analysis by capillary electrophoresis. Wild type product size was 328 bp. FLT3-ITD relative mutant level was calculated using the area under the peak. The VAF was calculated dividing the area under the peak of the mutated signal to the sum of the area under the peak of the wild type and mutant signals.

**Results**

First, we compared the differences in characterizing the malignancy in 10 different AML cases, depending on the applied technique. The percentage of blast cells defined by morphological analysis ranged from 40 to 95%, whereas the percentage of LAIPs detectable ranged from 0 to 90% (Fig. 1). The number of distinct LAIPs found in individual patients ranged from 0 to 7 (Figs. 2, 3, and 4). For each patient, a personalized Moab panel was designed.
was immunophenotypically aberrant. Consequently, apart from the different LAIP-populations, only immunophenotypically normal T cells were isolated. In the other seven patients, the myeloid compartment also contained various immunophenotypically normal populations which were sorted as well. For the 10 patients, in total, 86 fractions were collected and sequenced using a next-generation panel for mutations (Figs. 2, 3, and 4).

We performed error-corrected targeted deep sequencing for 27 leukemia-associated driver genes using single-molecule molecular inversion probe (smMIP) technology (Table S3). In addition, CEBPA and PTPN11 mutations were assessed using PacBio sequencing and FLT3-ITD mutations were analyzed using quantitative fragment length analysis by capillary electrophoresis. In case of chromosomal translocations reported by cytogenetics, we also investigated the presence of gene fusions in the different subfractions using Q-PCRs. Cultured mesenchymal stem cells (MSCs) and sorted T cells were analyzed as non-tumor controls. Mutations in T cells were identified at low VAFs for some of the mutations (Figs. 2, 3, and 4). This might represent contamination occurring during the sorting procedure but, in some cases, the presence of mutated T cells was confirmed by sequencing cultured T cells (Supplementary table 4), indicating that part of the T cells were derived from leukemic progenitor cells.

When comparing the percentage of cells determined to be LAIPs by immunophenotyping to the percentage of cells that carried mutations, we could distinguish three scenarios, based on the data collected at diagnosis (Fig. 1). The first scenario (Figs. 1A and 2) is represented by four cases (AML2, AML3, AML7, and AML12). For these, the percentage of cells showing an aberrant immunophenotype approximately equaled the percentage of cells that carried one or more mutations. All the different LAIPs and immunophenotypically normal fractions from these cases were sorted and genotyped (Fig. 2). In 2 of the 4 patients (AML2 and AML3), multiple genetic clones were observed, showing that different LAIPs may have a different genetic make-up. The opposite was noticed in the other two cases (AML7 and AML12), where different LAIPs belonged to the same genetic clone. In the patients belonging to this category, the disease is equally well characterized by both techniques and could therefore be followed over time using either method, provided that sensitive assays for several of the mutations are available. Multiple mutations were only present in a small subclone of the entire leukemia population (NRAS mutation in AML2, 36 bp ins FLT3-ITD in AML12), in contrast to the former two cases. These subclones were derived from bigger subclones, the use of the other markers to keep track of the disease is more efficient for adequate MRD detection.

The second scenario (Figs. 1B and 3) is represented by five cases (AML4, AML6, AML8, AML9, and AML11) in whom the percentage of cells carrying a mutation was substantially higher than the percentage with an aberrant
immunophenotype. In one case, (AML4) no LAIP was identified, whereas more than 95% of the cells carried mutations. All the immunophenotypically non-aberrant populations were found, also in these cases, to carry genetic aberrancies. Also, within this category, heterogeneity could be observed. In AML4 and AML8, two genetically defined subclones could be observed, distinguished by the acquisition of a mutation in PTPN11 in AML4 and by a 68-bp insertion in FLT3 in AML8. In the rest of the patients, instead, multiple genetic subclones could be identified. For the cases in this group, molecular analyses would allow a more complete surveillance of MRD, as they cover a larger part of the leukemic population, at the moment of diagnosis. For each of the patient, one of the identified mutations which is present in all the cells could be used as marker in order to follow the disease in time. The third scenario (Figs. 1C and 4) is represented by one patient (AML10). While immunophenotyping detected aberrations in approximately 80% of the bone marrow cells of this patient, mutations were only seen in 20% of the cells. In this scenario, the malignancy is much better characterized by immunophenotyping than by the applied molecular technique, indicating that the panel of mutations that was analyzed was incomplete.

**Discussion**

In nine of the ten AML cases, at least one genetic mutation was present at a VAF of approximately 50% (and thus 100% of the cells) within the isolated LAIP clones, indicating a good correlation between the aberrant genotype and phenotype. In one case (AML10), however, the sorted LAIPs contained a mutation only in a fraction of the aberrant cells, indicating that in that case, the mutational screening likely failed to identify a major pathogenic mutation. This may be improved by broadening the screening panel of mutations analyzed at diagnosis and by increasing the depth of the detection limit of NGS, which at the moment is 1–5% for most of the commonly applied sequencing methods. In addition, our data showed that in seven cases, mutations were also clearly present in populations that were non-aberrant using the standard immunophenotyping panels. This implies that characterization of the malignancy at the moment of diagnosis by standard immunophenotyping may be incomplete, and that, potentially, relapses may be missed. Further expansion of the panel of monoclonal antibodies that are used may at least partially solve this issue [14]. Some mutations correlate with the presence of certain CD markers. One known correlation is NPM1 mutations that correlate with CD34- blasts [15]. The NPM1 mutated cases in our cohort (AML4, AML8, and AML9) were all myelo-monocytic leukemias (that indeed were CD34 negative). Currently, detection of MRD is commonly performed making use of the LAIPs that are defined at diagnosis. This can be broadly applied, as in most cases of AML, indeed, one or more LAIP populations can
be defined. The inclusion of DfN cells in the assessment of MRD can increase the number of patients in whom are suitable for MRD detection through IFT can be performed. MRD detection by genetic analysis is not yet commonly applied for all mutations. For many other molecular targets, standardized assays that allow the detection of malignant cells at the level of 1/10^4 to 1/10^5 are still lacking. Early therapeutic standardized assays that allow the detection of malignant cells at for all mutations. For many other molecular targets, stand-
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dardized assays that allow the detection of malignant cells at
the level of 1/10^4 to 1/10^5 are still lacking. Early therapeutic
intervention based on the detection of MRD after treatment
was clearly shown to be beneficial in several studies [16]. Our
data show that with the currently used standard immunophe-
notypic and molecular analysis, disease characterization at
diagnosis is incomplete. Although we did not measure this
in MRD samples, this creates the possibility that relapses
might be missed, even when both techniques are used simulta-
neously. Both techniques can further be ameliorated by the
inclusion of more immunophenotypic markers and further
expansion of gene panels. Furthermore, more precise data
need to be acquired on the prognostic value of MRD. This
is highlighted by the recent observation that in some cases,
a complete clinical remission after induction and consolidation
therapy was observed, while still very large (premalignant)
clones were present harboring TET2 or DNMT3A mutations
[11]. In addition, very low numbers of AML cells bearing an
(t;8;21) [17] or inv(16) [18] (detected by Q-PCR for the
corresponding fusion transcripts) may be present for many
years without leading to relapse of the disease. This is in
contrast to acute promyelocytic leukemia, in which the
presence of very low amounts of PML-RAR positive cells after
therapy almost invariably leads to relapse of the disease [19].
Therefore, the relevance of the presence of low numbers of
(pre)malignant cells in relation to the development of relapse
may be dependent on the mutations that are present and needs
to be further defined. We conclude that currently, detection
of the malignancy at the moment of diagnosis by molecular
and immunophenotypic techniques is complementary; we recommend that both should be used in parallel, also for
MRD detection during follow-up, in order to obtain the most
complete view on resistant disease and early detection of
relapse. At the same time, both techniques should be further
developed to enhance the prognostic value and justification
of clinical interventions.

Supplementary Information The online version contains supplemen-
tary material available at https://doi.org/10.1007/s00277-021-04747-x.

Funding This work was supported by a grant from the Dutch Cancer
Society (grant #10813).

Declarations

Ethical approval The study was conducted in accordance with the
Declaration of Helsinki and institutional guidelines and regulations
from the Radboudumc Nijmegen (IRB number: CMO 2013/064). All
selected patients had signed informed consent.

Conflict of interest The authors declare no competing interests.

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