Strategies for minimal residual disease detection: current perspectives

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Abstract: Currently, the post-remission treatment in acute leukemia is based on the genetic profile of leukemic cells at diagnosis (ie, FLT3 ITD positivity) and on the level of measurable residual disease (MRD) after induction and consolidation chemotherapy. Two methods are currently preferred for MRD evaluation in many centers: multiparameter flow cytometry and real-time quantitative PCR. Additional methods such as next-generation sequencing and digital PCR are under investigation, in an attempt to increase the sensitivity and thus allowing the detection of small clones. Many studies suggest that MRD positivity after chemotherapy is associated with negative prognosis, and the reappearance of MRD during follow-up allows impending relapse to be identified and consequently enables early intervention. Finally, MRD positivity before hematopoietic stem cell transplantation is predictive of the outcome. Although the significance of MRD in acute leukemia has been widely explored, the assessment of molecular MRD is not yet a routine practice. In this review, we describe the significance of MRD in different settings and the main markers and methods used for MRD detection.

Keywords: minimal residual disease, acute leukemia, multiparameter flow cytometry, real-time quantitative polymerase chain reaction, next-generation sequencing, digital PCR

Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of clonal hematopoietic stem cell disorders with different sensitivity to chemotherapy. The molecular pathogenesis of AML has been studied for years by cytogenetic and molecular analyses. Recurrent genetic abnormalities play an essential role in the pathogenesis of the disease and are well established prognostic markers. More recently, studies based on next-generation sequencing (NGS) clearly showed that leukemia is composed of different clones having different genetic profiles. Frequently, a founding clone can be identified at diagnosis and additional small subclones can be detected only by very sensitive methods. The small clones, undetectable at diagnosis, are capable of expansion over time and might be responsible for relapse. Furthermore, additional mutations may emerge in clones and subclones during chemotherapy and may change their sensitivity profile to different agents, thus favoring chemo-resistance and finally relapse. The so-called “clonal evolution” explains the different mutational profiles frequently observed from diagnosis to relapse.

Although the majority of patients with de novo AML can undergo morphological remission after chemotherapy treatment, the rate of relapse is still very high. A
number of studies clearly showed that the identification of persistence of a small amount of minimal residual disease, or more precisely measurable residual disease (MRD), is an important prognostic factor to establish the risk of relapse and, as a consequence, the post-remission therapy.\textsuperscript{6,7}

Currently, the post-remission treatment is based on the genetic profile of leukemic cells at diagnosis and on the level of minimal residual disease after induction and consolidation chemotherapy, detected by multiparameter flow cytometry (MFC) in the majority of the studies.\textsuperscript{8} In this review, an example is provided by the GIMEMA clinical trial AML1310 (ClinicalTrials.gov. Identifier NCT01452646) that stratified the post-remission therapy according to the presence of MRD. Intermediate-risk AML with positive MRD received allogeneic hematopoietic stem cell transplantation (allo-HSCT), while MRD-negative cases were treated only with consolidation chemotherapy. Although the results are still under analysis, it is reasonable that in the near future the decision between chemotherapy or chemotherapy plus allo-HSCT will be based not only on genetic risk at diagnosis but also on the presence of MRD.

The reappearance of MRD during follow-up allows identification of impending relapse and consequently enables early intervention.\textsuperscript{7}

Despite the significance of MRD in acute leukemia having been widely explored, with an increasing number of studies demonstrating the positive prognostic value of MRD negativity, the assessment of molecular minimal residual disease is not yet a routine practice. Many studies are based on few genetic markers and the agreement on the methods and targets is still far from being reached. An attempt to standardize the methodology and to provide suggestions on when and how to monitor MRD has been recently published under the aegis of the European Leukemia Net (ELN).\textsuperscript{9}

MRD indicates the presence of leukemia cells down to the levels of $10^6$ to $10^8$ white blood cells, compared with $1:20$ in morphology-based measurement.

Two methods are currently preferred for MRD evaluation in many centers: MFC and real-time quantitative PCR (qPCR). Additional methods such as NGS and droplet digital PCR (ddPCR) are under investigation, in an attempt to increase the sensitivity and thus allow the detection of small clones. Each methodology differs in the sensitivity to detect the presence of MRD and in the number of patients who can potentially be monitored.

When applying molecular monitoring in AML patients, an important aspect should be considered, ie, the age-related clonal hematopoiesis. In the last few years, at least three different large studies carried out on thousands of healthy subjects of different ages investigated the presence of mutations in hematopoietic cells by using NGS technique.\textsuperscript{10-12} The mutations detected in healthy subjects are typical of leukemic cells including $TET2$, $DNMT3A$, and $ASXL1$. These studies identified mutations with an allele frequency of more than 2% in an increasing proportion of subjects during aging. By the age of 70 years, more than 10% of the subjects have clonal hematopoiesis which is a risk factor for both the development of malignancies, which occur in 0.5%–1% of them per year, and cardiovascular events, with an increased risk of four times compared to age-matched controls.\textsuperscript{11,12} The detection of mutations in healthy people suggests the existence of pre-leukemic stem cells. When additional mutations are acquired, leukemia might develop. Intensive chemotherapy selectively targets the malignant clone, thus allowing the pre-leukemic clone to survive.

Recently, the persistence of these mutations has been demonstrated during remission after chemotherapy without an evident impact on relapse, suggesting the persistence of pre-leukemic stem cells.\textsuperscript{7} These data have an important impact on the significance of MRD and on the choice of the molecular marker to follow.

**Molecular MRD**

There are two main approaches for molecular MRD quantification: real-time PCR and DNA sequencing.\textsuperscript{8} The PCR approach includes real-time PCR, digital PCR, and chimerism analysis. Real-time PCR represents, so far, the gold standard for MRD detection, although its application is limited to a selected number of patients (about 40%), carrying one or more suitable molecular markers.\textsuperscript{13}

This limitation might be overcome by NGS that, at least in theory, can be applied to all leukemia-specific genetic markers. This approach can increase the percentage of patients who can be monitored by another 40%. However, this method still requires validation and standardization.\textsuperscript{9}

**Marker for molecular MRD**

Fusion transcript in core binding factor (CBF) leukemias: $CBFB-MYH11$ and $RUNX1-RUNX1T1$

Despite the negative impact of MRD positivity in core binding factor (CBF) leukemias on relapse, no effect was clearly demonstrated on overall survival (OS) in multivariate analysis. This could be ascribed to the relatively high response rates of CBF leukemias to salvage therapy. In inv(16) patients, Yin et al.\textsuperscript{14} demonstrated that after induction chemotherapy, more than ten $CBFB-MYH11$ copies /$10^5$ ABL copies in peripheral blood were
the most useful prognostic variables for relapse risk on multi-
variate analysis. MRD, after the end of therapy, was also infor-
mative with levels of transcript inferior to ten copies/10⁵ ABL
copies in peripheral blood (PB) associated with a cumulative
incidence of relapse at 5 years in 36% of cases compared to
78% for those not reaching this threshold. During follow-up,
the presence of less than 50 copies/10⁵ ABL copies of transcript
in bone marrow (BM) and ten copies in PB was associated with
an OS at 5 years of 100% and 91%, respectively. We should
therefore keep in mind that low and stable levels of transcripts
may be detectable by PCR for a relatively long period of time
after chemotherapy without evidence of relapse.

Similarly, for RUNX1-RUNXIT1, Yin et al⁰⁰ established
that a threshold of less than 100 copies/10⁵ ABL copies in PB
and less than 500 copies/10⁶ ABL copies in BM was associ-
ated with an OS at 5 years of 95% and 94%, respectively.
The French group⁰⁰ analyzed 94 RUNX1-RUNXIT1 positive
patients during follow-up and showed that the molecular
remission after the completion of consolidation therapy was
not predictive. By contrast, the negativity in PB at the same
time point predicts an OS at 4 years of 96% compared to
63% if positive. As for CBFB-MYH11, the MRD negativity
at earlier time was not prognostically relevant.

NPM1 mutation

The mutation of NPM1 can be target for MRD by qPCR.
NPM1 has been found to be mutated in 50%–60% of AML
patients with a normal karyotype.²¹

The presence of measurable NPM1 transcripts in PB or
in BM after at least two cycles of chemotherapy is associ-
ated with a high risk of relapse in many studies.²⁰ Ivey et al⁰⁷
reported a 3-year OS of 75% for patients with NPM1 negativ-
ity in PB vs 24% for those with positive NPM1. Shayegi et al⁰⁸
reported a 3-year OS of 84% for negative patients at the same
time point but measured in BM compared to 76% for those
with low NPM1 levels (NPM1/ABL <1%) and 45% with those
who maintain positive levels with a NPM1/ABL ratio >1%.
Similarly, Kronke et al⁰⁸ reported a 4-year OS of 90% for those
who reached NPM1 negativity in BM after two cycles of che-
motherapy compared to 56% for those with NPM1 positivity.

For patients who obtain a NPM1 negativity in PB but
remain positive in BM after the end of treatment, the ELN
recommendations suggest to closely monitor the mutation in
PB and BM every 4 weeks for at least 3 months.⁹

PML-RARA

In acute promyelocytic leukemia, the most significant MRD
end point is the achievement of PCR negativity for PML-
RARA at the end of consolidation treatment, independently
from the therapeutic strategy, all-trans retinoic acid (ATRA)
associated with chemotherapy, or ATRA and arsenic trioxide.
PML-RARA negativity at the end of consolidation treatment
is associated with a low risk of relapse and a high probability
of long-term survival.²²,²³

As for CBF leukemia, we should consider that measurable
levels of PML-RARA during active treatment should
not trigger a treatment change. The usefulness of serial
PCR-based MRD monitoring during treatment is still under
investigation.

The Wilms’ tumor gene (WT1)

The usefulness of WT1 quantitative assessment, using
q-PCR, as a marker for MRD detection in AML has been
demonstrated many years ago.²⁴-³⁰ WT1 is overexpressed
in about 80%–90% of the patients.²⁴,²⁵ The persistence of
WT1 overexpression after treatment is always indicative
of MRD.²⁴-³⁰ WT1 can thus be considered as the most universal
marker of AML. In a European study, it was shown that both
the level of WT1 reduction from baseline after induction or
consolidation therapy and the clearance of the transcript to
normal values are highly predictive of relapse.²⁵ Many stud-
ies suggested that the persistence of abnormal values of WT1
after induction or consolidation treatment has an impact on
the probability of relapse. An increase of WT1 levels during
follow-up always predicts the leukemia recurrence.

Importantly, WT1 is overexpressed independently of the
genetic lesion(s) in the leukemic cells. This could generate
suspicion because it is evident that WT1 is not specific for one
particular leukemic clone. By contrast, considering the recent
advances coming from NGS studies that clearly indicate that
there is a clonal selection in acute leukemias, the usefulness
of a molecular marker able to track all the leukemic clones
independently from all genetic lesions is unquestionable.

The main advantages of WT1 assay are that it can be
measured in PB, the method has been standardized,²⁵ the
assessment of the results is not dependent on human expertise,
in contrast to flow cytometric analysis of MRD, and it can
detect the emergence of leukemic clones that are genetically
or phenotypically different from those detected at diagnosis.

In the recent recommendations of the ELN by Schuurhuis
et al,⁹ the authors claimed that WT1 mRNA quantitation
should not be used as minimal residual disease marker in
AML, due to low sensitivity and specificity; unless no other
MRD markers, including flow cytometric ones, are available
in the patient. Despite these recommendations, in the last 15
years, many scientific papers have been published showing
that WT1 is a reliable marker of MRD that is able to predict relapse with a high level of accuracy.\textsuperscript{24–30}

The fear of the lack of WT1 sensitivity is mainly based on the fact that there is a background of expression in healthy subjects.\textsuperscript{25} In addition, the absolute value of WT1 in AML at diagnosis is not always two logs higher than in normal samples.\textsuperscript{28} This led to a sort of skepticism toward WT1 that, however, cannot be justified by the evidence provided by prospective and retrospective studies.\textsuperscript{24,25,28}

Additional molecular markers

\textbf{BCR-ABL}

The 2016 WHO diagnostic guidelines included BCR-ABL positive trait as a provisional entity. The vast majority of the patients are characterized by p190 transcript, which is uncommon in chronic myeloid leukemia patients.\textsuperscript{31}

Since BCR-ABL positive AML is a rare subtype of leukemia, very little data are available on the outcome and the prognostic value of BCR-ABL-based MRD detection.

\textbf{IDH1/IDH2}

Since the evidence that IDH1/IDH2 genes can be mutated in about 10\textperthousand–20\textperthousand of AML cases, many groups are investigating the possibility of using IDH1/2 as a marker for MRD detection.

Until now, it is not clear whether IDH1/IDH2 become negative during remission, and contrasting data are reported in literature.\textsuperscript{32,33} There are studies reporting the stability and suitability of IDH as a marker of MRD.\textsuperscript{32,34} Recently, Petrova et al\textsuperscript{35} published the evaluation of MRD in 90 patients, 22\textperthousand of them with IDH1/IDH2 mutations. They based the assessment on NGS and ddPCR. Many patients presented additional mutations such as NPM1 or MLL-PDT and this allowed clinicians to conclude that IDH1/2 correlated with the treatment response. Despite this, they found that the approach based on IDH1/2 is less sensitive than NPM1 in predicting relapse but more sensitive than MLL-partial tandem duplication (PTD).

Brambati et al explored the possibility of detecting IDH1/2 after transplantation to better identify the risk of relapse.\textsuperscript{34} They concluded that longitudinal monitoring of these mutations can be extremely useful in the allo-transplantation setting, a context in which these alterations can be considered markers of undesired residual pre-leukemic host hematopoiesis.

\textbf{Multiparameter flow cytometry}

Lots of effort has been devoted in an attempt to obtain the standardization of MFC.\textsuperscript{36} For years, the detection of the leukemic population has been based on a panel of antibodies targeting early markers such as CD34 and CD117, markers of myeloid-lineage such as CD33, and myeloid differentiation antigens like CD11b, CD13, CD14, CD15 or lymphoid antigens including CD2, CD7, CD19, or CD56.

Two different approaches have been used to measure MRD with MFC: the leukemia-associated immunophenotype (LAIP) approach which characterizes the LAIP at diagnosis and tracks the identified population of blast cells during follow-up.\textsuperscript{37} A second approach is based on “different from normal” approach (DfN), which is based on the identification of aberrant differentiation/maturation profiles at any time point. The DfN approach has the advantage that it can be applied even in the absence of the immunophenotype at diagnosis and can detect the immunophenotype shifts,\textsuperscript{38} which is caused by the appearance of new clones. Immunophenotype shifts may emerge from leukemia evolution or clonal selection.\textsuperscript{39}

The use of a large panel of antibodies can overcome the problem of using LAIP rather than DfN, as it is able to cover both the aspects.

Recently, the ELN recommendations suggest the term “LAIP-based DfN approach” for this combination strategy that can provide characterization of leukemic cells at diagnosis, MRD evaluation, and detection of new aberrancies not present at diagnosis.\textsuperscript{2} For all these reasons, the ELN researchers recommend to use at least eight colors.

Differently from what has been demonstrated for qPCR-based MRD assessment, the evaluation of MRD by MFC is not recommended in PB for the lower frequency of leukemic cells.

Another important limitation of MFC was the absence of a commonly accepted threshold of negativity that is able to distinguish between MRD-positive and -negative cases. As 0.1\textperthousand was found to be relevant in many published studies, the ELN recommends using this threshold. However, an MRD below 0.1\textperthousand can be consistent with the persistence of residual leukemic cells. Several studies demonstrated the prognostic value of MRD to be below 0.01\textperthousand,\textsuperscript{40,41} showing that this threshold can identify patients with a very good prognosis. Independent validation of these very low levels may be highly relevant in future.

\textbf{MRD in the setting of allogeneic stem cell transplantation}

Many published data support the notion that the presence of MRD immediately prior to allo-HSCT is a strong, independent predictor of post-transplant outcomes in AML.\textsuperscript{42}
The MRD positivity has been defined as one of the stronger predictive factors both in the ablative and non-myeloablative transplants.43

In a large study carried out in 279 AML patients, Zhou et al investigated MRD before and after HSCT.44 It was shown that patients with MRD positivity before transplantation have a high relapse risk regardless of whether or not they clear MRD with conditioning chemotherapy.44

Studies in NPM1-mutated patients confirmed the impact of MRD pre-transplantation on the OS.45 It was shown that only those patients who achieved at least a suboptimal reduction of NPM1 levels after chemotherapy showed an improved OS after HSCT.

Bill et al46 analyzed a cohort of 51 NPM1-mutated patients who received HSCT. Mutated NPM1 MRD-positive patients, measured by ddPCR, had higher cumulative incidence of relapse and shorter OS. They demonstrated that NPM1 MRD positivity, measured by ddPCR before allogeneic stem cell transplantation, is associated with worse prognosis independent of other known prognostic markers. Similar results have been described by Kayser et al47 in a series of 67 patients. More recently, to extend the evaluation of MRD to all the patients treated with HSCT, an NGS approach has been used in a cohort of 116 patients in complete remission who were treated with HSCT.48 The MRD was measured before transplantation, and it was demonstrated, in multivariate analysis, that MRD positivity was an independent negative predictor of relapse.

Finally, many papers reported the value of WT1 monitoring in the setting of HSCT, both before HSCT to assess the quality of the remission and therefore predict the outcome and after HSCT to predict relapse.48–52 The data reported in this setting are quite strong and led several centers to adopt WT1-based preemptive immunotherapy with cyclosporine discontinuation and/or donor lymphocyte infusion in patients with increasing WT1 values after transplantation.53,54

Finally, although many studies reported that the CBFB-MYH11 fusion transcript can persist at low level in patients during long-term remission after chemotherapy, only a few studies with small samples have addressed the detection of the CBFB-MYH11 fusion transcripts after allo-HSCT.55

More recently, Tang et al reported 53 high-risk adult AML patients with inv(16) who received allo-HSCT.56 During follow-up, seven patients experienced relapse. All relapses occurred in patients who either did not achieve major molecular response within the first 3 months or who lost major molecular response (MMR) in the first 3 months from transplantation.56

Digital PCR
New techniques are on the horizon for the detection of small leukemic clones. A promising approach is based on digital PCR. Digital PCR is a breakthrough technology designed to provide absolute nucleic acid quantification. It is particularly useful in detecting low amounts of target; therefore, it is highly sensitive in detecting MRD. It is estimated that ddPCR can detect up to 0.001% mutated allele frequency.57

This technique can overcome some difficulties faced by conventional PCR. With ddPCR, a sample is partitioned in single nucleic acid molecules. As a result of the partitioning of the sample into some sort of “bubbles”, each bubble will contain zero or one molecule. After PCR amplification, nucleic acids may be quantified by counting the bubbles containing PCR end products. The main disadvantages for now are the cost of the analysis, the limited availability of the instruments that are not routinely introduced into diagnostic laboratories, and the lack of standardization.

In conventional PCR, the starting copy number is proportional to the number of PCR amplification cycles. Digital PCR, however, is not dependent on the number of amplification cycles to determine the initial sample amount, eliminating the reliance on uncertain exponential data to quantify target nucleic acids and providing absolute quantification.

Few studies have been published on MRD by ddPCR. We have already mentioned the study by Petrova et al55 based on IDH1/2 mutation. This technique has also been explored by Brambati et al54 in the setting of allogeneic bone marrow transplantation to identify the reappearance of small mutated clones of the recipient. In the same setting, Bill et al56 reported the prognostic significance of NPM1 positivity by ddPCR. A new assay based on digital PCR technique composed of multiplex pools of insertion-specific primers that selectively detect mutated but not wild-type NPM1 has been described by Mencia-Trinchant et al.58

Next-generation sequencing
On the basis of the concept that MRD evaluation requires more than one technology and more than one marker, NGS is presently under investigation. Since there is a high degree of genotypic and phenotypic heterogeneity in AML, each patient should have a unique signature to be used to track MRD after therapy. Whole genome- or exome sequencing-based identification of clones and subclones in patients at diagnosis allows MRD to be followed by individualized monitoring. The usefulness of an NGS-based MRD assay is not only the prognostic stratification for relapse risk but also for the identification of different drugable targets for a
personalized therapy. Prospective studies of the use of NGS in AML as a marker of MRD in the pre- and post-transplant setting are ongoing.

The main advantages of NGS technology are the reduced DNA sequencing time and cost, and the remarkably increased data-production capacity. NGS technologies rely on different methods for DNA template preparation, massively parallel reading of sequenced millions of short DNAs, real-time image capturing, alignment of sequences, sequence assembly, and variant detection. Each method has specific advantages: read length, accuracy, run time, and throughput. The main disadvantages of using NGS are shorter read length and the fact that its ability to detect MRD depends on the depth of sequencing and on the type of computational algorithms used. These aspects can complicate the process of standardization of the method for MRD detection.

Recently Onecha et al explored the possibility of using deep sequencing MRD approach. They analyzed 190 patients affected by AML. A total of 211 (80%) single nucleotide variations and 46 (20%) indels were detected using the NGS custom panel. They followed the aberrations during follow-up with the same approach and were able to demonstrate that MRD status (MRD levels >0.1%) at post-induction was associated with a significantly lower rate of OS (33% vs 78%), while MRD-positive status after induction chemotherapy (MRD levels >0.025%) was associated with shorter OS (33% vs 81%) and significantly shorter DFS.

Levis et al recently published a sensitive and specific MRD assay for FLT3-ITD mutations using NGS. They demonstrated a relationship between the mutation burden, as detected by their assay, and OS.

Conclusion
In acute leukemias, the detection of MRD is highly informative of the outcome of therapies, including HSCTs. The majority of the studies on MRD reported data based on MFC and real-time qPCR. Many attempts are ongoing to improve the sensitivity and to standardize the currently available techniques. This aspect is important to reach a common agreement on the threshold of MRD that triggers therapeutic decisions. New molecular targets are under investigation with encouraging results. Furthermore, the availability of new molecular targeted drugs that can potentially fully eradicate the residual small clones stimulates the interest in the detection of residual leukemic cells. New promising approaches are on the horizon to enlarge the spectrum of patients who can be monitored for the persistence of leukemic clones, including NGS.

Disclosure
The authors report no conflicts of interest in this work.

References


