MINIMAL RESIDUAL DISEASE QUANTITATION IN ACUTE MYELOID LEUKEMIA

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Abstract

The prognosis for patients with acute myeloid leukemia (AML) is heterogeneous. A minority of patients has clinical and biologic features that are associated with a very high risk of relapse. For the remaining patients no clear prognostic factors can be identified at diagnosis. The degree of treatment response is likely to be an informative predictor of outcome for these patients. Modern assays to detect AML cells that are undetectable by conventional morphologic techniques, i.e. minimal residual disease (MRD), can potentially improve measurements of treatment response. It is plausible that modifications to treatment based on the results of these assays will improve clinical management and ultimately increase cure rates. Established MRD assays for AML are based on either polymerase chain reaction (PCR) amplification of genetic abnormalities or flow cytometric detection of abnormal immunophenotypes. Residual disease and treatment response can be measured by these assays in a manner that is much more sensitive and objective than that afforded by conventional morphologic examination. The expanding use of MRD testing is beginning to change the definition of treatment response and of remission. Other clinically informative uses of MRD testing include the detection of early relapse and the evaluation of the efficacy of new antileukemic agents.

Keywords

acute myeloid leukemia; minimal residual disease; flow cytometry; PCR

Introduction

Survival among patients with acute myeloid leukemia (AML) has failed to increase commensurately with our improved understanding of the disease biology. While the risk of relapse can be predicted with some accuracy in a minority of patients, based on presenting clinical and/or biologic features, risk assignment remains difficult for the majority of patients.¹,² Because of the increasing availability of novel agents and improvements in supportive therapy, it has become more urgent to identify patients who require more intensive therapy and experimental treatment, and those who have low-risk disease that is curable with conventional therapies. Patients who lack well-defined prognostic features at diagnosis ("standard risk")

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compose the majority of patients with AML; this subgroup has the greatest heterogeneity in response and potentially has the most to gain from improvements in definition of risk hazard.

Although still integral in following the effects of therapy on normal and abnormal cells, traditional morphologic examination of blood and marrow has limited sensitivity and specificity as a method for response evaluation. Therefore, by this approach, there is a risk of either over- or under-estimating disease burden. During the last 3 decades efforts to develop ways to measure the degree of residual leukemia better than morphology and to detect minimal residual disease (MRD) have progressively intensified resulting in clinically applicable methods. Efforts in the area of MRD testing for acute lymphoblastic leukemia and acute promyelocytic leukemia (APL) led the way. More recently, however, reliable methods to detect MRD in non-APL AML have been established and their clinical importance has been demonstrated. MRD testing replaces the inherent limitations of morphologic evaluation, with a one hundred-fold improved sensitivity and objective identification of leukemia-associated features. We will briefly review the clinical significance of MRD in AML, advantages and limitation of the available methods and future uses of MRD in clinical practice. The clinical utility of MRD monitoring in APL will not be discussed here.

Clinical significance of MRD

The potential clinical utility of monitoring MRD in AML has been demonstrated by several studies. Table 1 summarizes key findings in selected studies with the two methodologies that are most widely used: polymerase chain reaction (PCR) amplification of molecular abnormalities, and flow cytometric detection of abnormal immunophenotypes.

Quantitative PCR studies for molecular detection of AML1-ETO and CBβ/MYH11 in cases with t(8;21) and inv(16), respectively, have shown that these assays can identify patients at a higher risk of relapse.

While studies of MRD by PCR amplification of fusion transcripts are inherently limited to specific leukemic subtypes, studies of MRD by flow cytometry have a potentially much wider applicability. San Miguel et al. showed that in adult patients with AML in morphologic remission, MRD detected in the first bone marrow obtained after induction treatment was highly predictive of relapse. By MRD levels, patients could be subdivided into 4 groups: none of the 8 with fewer than 0.01% leukemic cells had relapsed at the time of publication, while the relapse rate for the 17 with more than 1% residual cells was 84%; patients with 0.01%-0.1% leukemic cells (n = 37) and those with 0.1%-1% (n = 64) had an intermediate relapse rate. Kern et al. monitored MRD in AML patients in complete remission after induction and consolidation therapy and found that the degree of reduction in cells expressing aberrant immunophenotypes was significantly and independently related to treatment outcome. Early studies by Venditti and colleagues also showed the clinical importance of MRD in adult patients with AML. More recently, this group reported a study that provided strong evidence supporting the clinical importance of this parameter. These authors studied 142 patients who achieved complete remission and found that a level of 0.035% MRD after consolidation therapy was very informative, identifying two groups of patients with a 5-year relapse-free survival of 60% and 16%, respectively. MRD-negative patients had a significantly more favorable outcome, regardless of whether they received autologous or allogeneic stem cell transplantation. A recent study by Al Mawali et al. suggested that the most informative MRD level after induction therapy was 0.15%, although this series included only 25 patients.

The Children's Oncology Group completed a prospective study of MRD in 252 children with AML. Occult leukemia was detected by flow cytometry in 41 (16%) of the patients who responded to initial induction therapy. While controlling for other disease and patient characteristics, patients with detectable disease were nearly five times more likely to relapse.
In a study performed with samples collected from children enrolled in the St. Jude Children's Research Hospital AML97 study, we found that 61 of 230 bone marrow samples from 46 patients during therapy had MRD levels of 0.1% or higher. Mean (± SE) 2-year overall survival was 33.1% ± 19.1% for patients with detectable MRD (≥ 0.1%) after induction therapy as compared to 72.1% ± 11.5% for those with undetectable MRD; AML relapse within the subsequent 6 months was significantly more likely in the MRD-positive group. MRD was the only significant prognostic indicator in this cohort. In a study by Langebrake et al. in 150 children with AML residual disease detected by flow cytometry was significantly associated with a lower event-free survival. However, when the prognostic value of the findings was assessed in combination with that of other parameters, residual disease lost statistical significance.

**Methods for MRD monitoring in AML**

**Polymerase chain reaction**

The fusion transcripts most extensively used to monitor MRD in AML (in addition to PML-RARA for APL) are AML1-ETO, CBFbeta-MYH11 and MLL-AF9 which are present in approximately one-third of non-APL AML cases. The detection of abnormal fusion transcripts by qualitative reverse transcriptase (RT)-PCR may persist long into clinical remission. Thus, the use of quantitative methods to estimate levels of target genes and by inference, levels of MRD at different phases of the disease, are preferred. The current standard method for molecular quantitative detection of MRD is real-time quantitative PCR (RQ-PCR). RQ-PCR rapidly quantifies PCR products by real-time fluorescent signals during exponential amplification. Moreover, it does so without additional post-PCR processing and associated risk of contamination.

The sensitivity of molecular detection of fusion transcripts ranges from 1 leukemic cell in 1,000 to 100,000 normal cells, i.e. 0.1% to 0.001%. Although RQ-PCR can precisely quantify PCR products, the relation between these and the number of cells from which they originate might be difficult to extrapolate. The transcript : cell ratio may vary among leukemias of the same genetic subtype, and perhaps also between cells collected at diagnosis and during therapy, and between cells at different maturation stages within the leukemic clone. Moreover, RNA is prone to degradation and the efficiency of its initial conversion to cDNA by reverse transcriptase can fluctuate. In sum, although very useful to determine presence or absence of MRD, this method has some limitations in regards to the precise assessment of MRD levels.

In addition to fusion transcripts, there are other potential targets for molecular studies of MRD in AML. The Wilms' tumor gene (WT1) is highly expressed in most acute leukemias, and its detection in bone marrow has been associated with the presence, persistence, or reappearance of leukemia, but the background of normal bone marrow cells may limit the reliability of this assay in many cases. In some cases of AML, cells have an internal tandem duplication of the FLT3 gene (FLT3/ITD), which is detected in approximately 25% of adult AML cases, and 15% childhood AML. Although methods for detecting FLT3/ITD for MRD monitoring purposes have been developed, the observation that this marker may not be stable during the course of the disease argues against its use as MRD target. The value of MRD studies using nucleophosmin mutations as targets is beginning to be explored but definitive data are not yet available. Immunoglobulin and T-cell receptor genes, extensively used as targets for molecular analysis of MRD in acute lymphoblastic leukemia, are rarely rearranged in AML and therefore their contribution to the arsenal of potential targets is negligible.
Flow Cytometry

Leukemic cells express abnormal patterns of cellular markers, and these aberrant immunophenotypes can be identified by flow cytometry. AML cells express unique cell marker and light scatter profiles that allow their identification among normal bone marrow myeloid cells.\textsuperscript{44,45} This phenotypic signature can include not only differential expression of myeloid markers but also expression of non-myeloid markers as well as a mixture of markers representing normally distinct maturation stages.\textsuperscript{46}

Published adult AML studies report identifying abnormal phenotypes in approximately 70%-75% of patients.\textsuperscript{17,19} In an early study, we found abnormal immunophenotypes in 46 of 54 (85.2%) children with newly diagnosed AML.\textsuperscript{23} In about half of the cases the immunophenotypes were sufficiently distinct from those of normal bone marrow cells to allow a sensitivity of detection of 1 leukemic cell among 10,000 normal cells. In the remaining cases, the immunophenotypes were aberrant but overlapped in part with those of normal myeloid cells, limiting the sensitivity of the assay to 1 in 1000. In the studies performed in our recently closed AML02 trial, we found aberrant immunophenotypes at diagnosis in 200 of 210 (95%) patients. As we previously noted, in approximately half of the patients, the antigenic expression was sufficiently distinct from that of normal cells to allow a sensitivity of 1 in 10,000. However, because this is not achievable in all patients, our standard definition of MRD positivity in AML is \( \geq 0.1\% \).

An advantage of flow cytometry-based studies of MRD is that they can accurately quantify residual leukemic cells. Moreover, they can distinguish viable from apoptotic cells and provide an overview of the status of normal hematopoietic cells in the sample. The issue of immunophenotypic changes that may occur during the course of the disease is often brought up as a potential cause of false-negative results. Indeed, immunophenotypic switches of markers or changes in signal intensity may occur but, in general, some or all the phenotypic abnormalities described at diagnosis reappear at the time of relapse.\textsuperscript{47-49} We strongly recommend the use of multiple antibody sets to maximize the chances of capturing the residual AML cells even when partial changes in phenotype have occurred and thus reduce the likelihood of false-negative findings. A second potential cause of erroneous findings is related to the fact that current flow cytometry-based MRD assays rely on the interpretative abilities of the investigator. Lack of extensive knowledge about the immunophenotypic features of normal bone marrow cells, and of the changes that may occur during bone marrow recovery after chemotherapy may lead to erroneous interpretation. Thus, an excess of highly proliferating immature myeloid cells may be mistakenly identified as residual myeloid cells. Therefore, in addition to specific training, it is important to use rigorous gating strategies based on objective findings in an extensive database of normal and regenerating bone marrow samples. In this regard, the development of software that allows automated analysis of flow cytometric data might be very helpful.\textsuperscript{50}

Future prospects

AML is a heterogeneous malignancy with a great need for improved therapeutic strategies. MRD assays are capable of producing information on treatment response with more sensitivity than microscopic analysis. Thus, MRD can be used to design risk-based therapies for AML in an effort to minimize toxicity and improve cure rates.\textsuperscript{7} The strategy that we used in our AML02 trial is shown in Figure 1. Quantitative RT-PCR and flow cytometry are currently the two most effective methods for MRD measurement. Because these are high-complexity tests, they need to be performed in laboratories with proven expertise to be beneficial.

There are other applications of MRD assays that are beginning to be implemented. MRD studies can be used to assess the effectiveness of novel antileukemic agents. Thus, changes in MRD
levels could represent an endpoint in phase II studies. A related application of MRD is to rapidly determine the effectiveness of remission induction chemotherapy regimens. In other words, prevalence and levels of MRD after a chemotherapy phase can be compared to data obtained in previous or parallel trials to decide whether the tested regimen is superior or not. In this context, MRD data can be part of stopping rules.

An increasing number of tyrosine kinase inhibitors are becoming available for clinical testing in AML. An exciting new possibility is to assess the status of cell signaling pathways targeted by these inhibitors in AML cells. These studies have become possible owing to the increasing battery of antibodies directed against phosphoproteins that can be visualized by flow cytometers designed to detect 9 or more fluorochromes. By using these techniques, one can determine if the targeted signaling pathway is affected in the residual leukemic cells.

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**REFERENCES**


FIGURE 1.
APPLICATION OF MRD TESTING FOR RISK STRATIFICATION IN THE ST JUDE AML02 STUDY FOR CHILDREN WITH AML (APL EXCLUDED). Abbreviations: GO, gemtuzumab ozogamicin; CR/PR, complete response, partial response; HR, high risk; SR, standard risk; MDS, myelodysplastic syndrome.
## TABLE 1

### RESULTS OF SELECTED MRD STUDIES IN AML

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Target gene</th>
<th>Patient number</th>
<th>Informative timepoint</th>
<th>Method</th>
<th>Informative MRD level</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults: t(8;21)</td>
<td>AMLI-ETO</td>
<td>21</td>
<td>Post-consolidation</td>
<td>RQ-PCR</td>
<td>Transcript control &gt;10^9</td>
<td>Leroy et al, 2005</td>
</tr>
<tr>
<td>Adults: inv(16)</td>
<td>WTI/ABL</td>
<td>116</td>
<td>61-120 days &amp; 121-180 days post start of chemotherapy</td>
<td>RQ-PCR</td>
<td>&gt;0.4 %</td>
<td>Weisser et al, 2005</td>
</tr>
<tr>
<td>Adults: inv(16)</td>
<td>CBFB-MYH11/ABL</td>
<td>21</td>
<td>Post-completion of chemotherapy</td>
<td>RQ-PCR</td>
<td>&gt;0.25 %</td>
<td>Buonamici et al, 2002</td>
</tr>
<tr>
<td>Adults: t(8;21)</td>
<td>AMLI-ETO/ABL</td>
<td>25</td>
<td>Post-consolidation &amp; intensification</td>
<td>RT-PCR</td>
<td>&gt;1000 mol/mg of RNA</td>
<td>Tobal et al, 2000</td>
</tr>
<tr>
<td>Adults: t(8;21)</td>
<td>CBFB-MYH11/ABL</td>
<td>28</td>
<td>Post-completion of chemotherapy</td>
<td>RQ-PCR</td>
<td>&gt;0.1 %</td>
<td>Perea et al, 2006</td>
</tr>
<tr>
<td>All adults</td>
<td>Aberrant phenotypes</td>
<td>126</td>
<td>Post-first induction</td>
<td>FC</td>
<td>&gt;0.01 %</td>
<td>San Miguel et al, 2003</td>
</tr>
<tr>
<td>All adults</td>
<td>Aberrant phenotypes</td>
<td>142</td>
<td>Post-remission</td>
<td>FC</td>
<td>≥0.035 %</td>
<td>Maurillo et al, 2008</td>
</tr>
<tr>
<td>All children</td>
<td>Aberrant phenotypes</td>
<td>252</td>
<td>Post-first induction</td>
<td>FC</td>
<td>≥0.5 %</td>
<td>Sievers et al, 2003</td>
</tr>
<tr>
<td>All children</td>
<td>Aberrant phenotypes</td>
<td>46</td>
<td>Post-first induction</td>
<td>FC</td>
<td>≥0.1 %</td>
<td>Coustan-Smith et al, 2003</td>
</tr>
<tr>
<td>All children</td>
<td>Aberrant phenotypes</td>
<td>150</td>
<td>Prior to second induction</td>
<td>FC</td>
<td>&gt;0.1 %</td>
<td>Langebrake et al, 2006</td>
</tr>
</tbody>
</table>

Abbreviations: RQ-PCR, real-time polymerase chain reaction; FC, flow cytometry