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Is Minimal Residual Disease Monitoring Clinically Relevant in Adults with Acute Myelogenous Leukemia?

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Abstract

In the past year, there has been increasing attention towards understanding the clinical relevance minimal residual disease (MRD) assessment. The monitoring of MRD levels at various stages of therapy has considerable potential to impact the guidance of treatment for AML patients and improve outcomes. Thus, efforts have increased to address important concerns regarding MRD measurements. These concerns include: (1) what should be monitored?; (2) what methodologies should be used?; (3) are such methodologies standardized across laboratories?; (4) how to define prognostic levels?; (5) when to monitor MRD?; and (6) what treatment options are available for MRD positive patients? In this review, we will discuss the methodologies available for MRD and the studies available to date aiming to address the concerns around the use of MRD measurements for AML patients

Keywords

Minimal residual disease (MRD); acute myelogenous leukemia (AML); Quantitative polymerase chain reaction (qPCR); leukemia-associated immunophenotypes (LAIPs); multiparameter flow cytometry (MFC); leukemia stem cells (LSCs)

Introduction

Acute myelogenous leukemia (AML) is a heterogeneous disease of early myeloid cell differentiation. Approximately 1 in 10,000 adults is diagnosed with AML every year. In 2012, the SEER database predicted that over 13,000 adults were diagnosed with AML in the United States, and over 6,000 adults died from this disease (Howlader N). AML is a disease more typical of older adults, with an average age of diagnosis of 66 (Howlader N). As the population ages, the frequency of AML is expected to increase. In addition to age, other contributing factors to development of AML include treatment for prior malignancies with chemotherapy (Ratain & Rowley, 1992, Smith *et al.*, 2003) or radiation, antecedent hematologic disease (i.e. myelodysplastic or myeloproliferative syndromes) and benzene exposure(Natelson, 2007). In a majority of individuals, the causative factor for development of leukemia is unknown.

The overall 5-year survival for adults with AML in the United States is a dismal 15% (Rowe, 2011). However, that number can vary widely based on an individual's age at diagnosis, comorbidities, and characteristics unique to each individual's AML. Good-risk

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features of non-M3 AML include the presence of cytogenetic changes such as inv(16;16), t(8;21) or mutations including biallelic CEBPA or NPM1 gene mutations (Marchesi *et al.*, 2011, Byrd *et al.*, 2002, Grimwade *et al.*, 1998). Poor-risk features include the karyotypic changes of monosomy 7, inv(3;3), MLL translocations (11q23), complex and monosomal karyotypes, and the presence of FLT-3 ITD mutation(Marchesi et al., 2011, Byrd et al., 2002, Grimwade et al., 1998). However, a majority of cases of AML have normal cytogenetics, and lack NPM1, CEBPA or FLT3-ITD mutation, placing them in the intermediate-risk category (Marchesi et al., 2011, Byrd et al., 2002, Grimwade et al., 1998).

Treatment of patients with AML includes chemotherapy and in many cases, allogenic bone marrow transplantation (Roboz, 2012). Chemotherapy for AML is divided into phases: induction of remission, consolidation and maintenance. Standard of care for all individuals with AML that does not exhibit good-risk characteristics is consolidation with allogenic stem cell transplantation in place of additional chemotherapy if: 1) an appropriate donor is available, and 2) the patient's comorbidities do not overwhelmingly limit the chance of survival during and immediately following transplantation.

Despite the aggressive measures taken to achieve and maintain remission from AML, relapse is frequent, even following allogenic-bone marrow transplantation. Depending on the duration of the antecedent remission, the likelihood of achieving each subsequent remission decreases with each attempt. There are multiple factors that limit overall survival for adults diagnosed with AML: 1) cytogenetic and molecular changes, 2) population heterogeneity, 3) differing response to chemotherapy, and 4) cell cycle state (i.e. leukemia stem cells) (Roboz & Guzman, 2009). Unlike acute promyelocytic leukemia (APL) and chronic myelogenous leukemia (CML), there is no common driving mutation that AML is completely 'addicted to'. This makes molecular targeted therapy as with all-trans-retinoic acid or specific tyrosine kinase inhibitors less effective for AML. Furthermore the bone marrow microenvironment is a protective niche, which provides resistance to a subset of leukemia cells to chemotherapy.

The difficulty in treating patients with AML is clearly linked to a residual subset of chemotherapy-resistant cells present during remission that eventually drive the leukemia relapse. For a patient to meet the formal hematologic definition of remission from AML the following three criteria must be met: 1) less than 5% myeloblasts present in the bone marrow, 2) peripheral blood absolute neutrophil count of greater than 1,000 cells/μL, and 3) peripheral blood platelet count of over 100,000 platelets/μL(Cheson *et al.*, 2003). This definition sets a maximum limit of myeloblasts in the bone marrow, and requirement for near complete hematopoietic reconstitution. However, these criteria still allow for the presence of a significant AML burden.

The detection and thus definition of residual AML is largely dependent on the methodology used to evaluate the bone marrow. Morphologic examination of bone marrow aspirate and biopsy has a sensitivity of 1–5% (Inaba *et al.*, 2012, Al-Mawali *et al.*, 2009). Although, it is the standard for determination of hematologic remission status, it is not suitable for detection of residual disease. Cytogenetic examination has also a sensitivity of 5% (Inaba et al., 2012, Al-Mawali et al., 2009). However, 40–45% of all AMLs have normal cytogenetics making cytogenetic evaluation inadequate for detection of residual disease for all AML patients. Detection of chromosomal changes by FISH has improved sensitivity of 0.3–0.5%, and cells in metaphase are not required (Inaba et al., 2012, Al-Mawali et al., 2009). However, as with cytogenetic examination, for individuals whose AML exhibits a normal karyotype, this methodology is of limited utility. Gene rearrangement studies by Southern blot can also be performed, but their sensitivity is low (1–5%), are labor-intensive and slow(Inaba et al., 2012, Al-Mawali et al., 2009).

NEED FOR MINIMAL RESIDUAL DISEASE (MRD) DETECTION ASSAYS

There are several reasons for developing increasingly sensitive techniques for detection of AML progenitors within the bone marrow and blood. For younger adult patients with high risk AML, the consensus is for rapid progression to allogenic-stem cell transplantation, and for patients with good risk AML the consensus is for consolidation chemotherapy and avoidance of stem cell transplantation in first remission. However, in good risk AML, there is still a relapse rate of 30–40% (Appelbaum *et al.*, 2006, Prebet *et al.*, 2009). Additional tools that can detect the presence of absence of MRD after induction of remission may help to identify patients at risk of relapse, even within a good-risk group. Clearly use of MRD measurement to guide intensification of therapy is helpful only when there are adjunctive treatment options that could be added for patients who are MRD-positive during periods of hematologic remission.

MRD Measurement by qPCR

Quantitative polymerase chain reaction (qPCR) is used to identify fusion genes, gene rearrangements, genetic alterations, and over-expressed genes. qPCR allows for the monitoring of genetic alterations that can be found in some cytogenetically normal AML patients. The PCR based assays used to monitor AML patients are to detect fusion genes that include RUNX1-RUNX1T1, CBFB-MYH11, and PML-RAR that result from t(8;21)(q22;q22), inv(16)(p13q22) or t(16;16)(p12;q22) and t(15;17)(q22;q12)(Rubnitz *et al.,* Corbacioglu *et al.*). A recent study from the United Kingdom (MRC 15 trial), evaluated MRD by qPCR monitoring core binding factor (CBF) in 278 patients AML patients [163 with t(8;21) and 115 with inv(16)]. They found MRD of predictive value during follow-up. MRD thresholds associated with a 100% relapse were of >500 copies (BM), and >100 copies (PB) in t(8;21) patients. For inv(16) patients, >50 copies (BM) and >10 copies (PB) (Yin *et al.*). The application of MRD monitoring of fusion genes is limited to ~25% of AML patients (Estey & Dohner, 2006). Furthermore, qPCR may be useful for patients that present genetic alteration that may be cytogenetically normal. Genetic alterations found in AML that have been found to be useful for MRD detection include: (1) insertions/duplications (e.g. FLT3-ITD, NPM1, MLL-PTD), (2) point mutations (e.g. KIT, RAS, RUNX1, CEBPA, IDH1, WT1, TP53, JAK2, MPL1), and (3) overexpression (e.g. EVI1, WT1, ERG) (Patel *et al.*). The RNA based qPCR assay for fusion genes have demonstrated sensitivities of 10^{-4} to 10^{-6}. Fusion genes can also be monitored by the assessment of patient specific DNA breakpoints with a sensitivity of 10^{-4} to 10^{-5} (Burmeister *et al.*, 2006). Another example of the utility of qPCR is the quantitative evaluation of NPM1A alterations has ben shown to be a reliable MRD marker in the BM post-transplant period, predicting relapse earlier than morphology assessment (Bacher *et al.*, 2009).

Although qPCR for MRD measurement has proven to have high sensitivity, this approach can only be applied in <50% of AML cases. In addition, it can miss therapy-related secondary AML if none of standardized measurements available. Furthermore, this approach will miss other genetic changes for which the probe set was not tested.

MRD measurement by flow cytometry

Even though, qPCR show the highest sensitivities to detect MRD, suitable markers for detection are only available for 60% of patients that present abnormal cytogenetics, rearrangements or mutations. Thus, other techniques that are suitable for most AML patients have been developed. Such technique is multiparameter flow cytometry (MFC). MFC techniques are based on the normal differentiation patterns of expression for antigens that characterize the diverse lineages of normal hematopoietic cells. AML blasts present a distinct immunophenotype patterns that are not detectable on the surface of bone marrow

cells from healthy donors (Reading *et al.*, 1993, Sievers *et al.*, 2003). For example, the patterns of expression for some antigens are not found in normal CD34+ bone marrow cells such as CD11b, CD14, CD56, and CD65. Thus, the aberrant patterns of antigen expression in leukemic patients are known as the leukemia-associated immunophenotypes (LAIPs). LAIPs in AML have shown to be heterogenous and thus several LAIPs have been observed within an AML patient. When this occurs, the LAIP with the major logarithmic difference relative to normal bone marrow is used to monitor MRD (Kern *et al.*, 2004). During MRD monitoring the residual levels of leukemic cells is evaluated by determining the log difference between the percent LAIP at diagnosis and at follow up(Buccisano *et al.*, 2009, Kern et al., 2004). MFC approaches can include three to ten-color antibody panels with the ability to detect population between 0.1 and 0.01 percent of bone marrow nucleated cells. However, as the number of parameters evaluated by MCF in a single tube increases, the complexity of analysis increases.

In addition to LAIP, LSC immunophenotype has been also tested for its utility at detecting MRD. LSCs can be identified in most patients by the cell presence or absence of the cell surface antigens that include: CD34+, CD38−, CD123+, CD96+, CD47dim, CD90−, CLL-1+, TIM3+, ALDH-1+, CD99+ (Jordan *et al.,* Bonnet & Dick, Hosen *et al.,* van Rhenen *et al.,* Gerber *et al.,* Jan *et al.,* Majeti *et al.*, 2009). Furthermore, it has been shown that patients presenting higher proportion of LSCs (defined as CD34+CD38−) demonstrate significantly lower relapse-free survival than patients with less LSCs(van Rhenen *et al.*, 2007a).

Prognostic implications

To date several clinical studies have use MFC to evaluate MRD and the ability to predict outcome. Studies have shown that patients in morphologic remission can be divided into four risk groups based in the levels of MRD evaluated by MFC: (1) very low risk ($<10^{-4}$ cells); (2) low risk (10^{-4} to 10^{-3} cells); (3) intermediate risk ($>10^{-3}$ to 10^{-2} cells) with a 50% relapse rate; and (4) high risk ($>10^{-2}$ cells) with an 84% relapse rate. These studies were performed in a total of 126 AML patients (San Miguel *et al.*, 2001). Thus this study demonstrated that further stratification was possible by evaluated the leukemic burden at remission using MFC parameters. Other studies have also shown the prognostic the value of MRD during consolidation (Buccisano *et al.*, 2006, Venditti *et al.*, 2000). These studies have shown that a threshold of 3.5×10^{-4} cells was able to separate risk groups (good and poor). Another use of LAIP detection has been to evaluate the MRD kinetics early during treatment (first 8 days). Studies have shown that patients that achieved CR at day 14, presented a decrease in MRD in the first 8 days of treatment (Gianfaldoni *et al.*, 2006). LSC phenotype has also been tested and shown that a higher proportion of LSCs at diagnosis was found to be highly predictive of MRD (van Rhenen et al., 2007a, van Rhenen *et al.*, 2005). It is important to mention that to achieve detection of such rare population of cells, large number of cells have to be evaluated. A more recent study showed that MRD detected during CR using LSC phenotypic markers ($CD34^{+}CD38^{-}ALDH^{int}$) highly correlated with relapse (Gerber et al.).

As this technique becomes part of standard clinical laboratory procedures, efforts increase for methodology standardization across laboratories (e.g. EuroFLow Consortium)(Kalina *et al.*). Factors important for standardization procedures include sample processing, instrument configuration, selection of fluorochromes, antigen selection (including manufacturer and clone number), number of events to be acquired (sensitivity of 0.01% necessitates 1,000,000 events). Additional factors to consider is the timing of evaluation (during induction, end of induction, end of consolidation) and the sample type (i.e. peripheral blood vs. bone marrow) However, there are factors that may diminish the enthusiasm for the use of MCF for MRD measurement. Such as, that at relapse, in some patients, LIAP represent only 10% of the

leukemic cells where the antigens may have changed(Oelschlagel *et al.*, 2000, Campana *et al.*, 1990).

Overall, the benefits of MCF for MRD measurement are: (a) its applicability to most patients with AML (>80%); (b) a relative rapid turn-around; (c) the technique allows single cell analysis; (d) provides information on normal cell population as well; (e) the technique allows for analysis of either live of fixed cells, thus it be combined with cell sorting if needed to study heterogeneous populations. To date this approach also has drawbacks: (a) standardization of antibody panels; (b) standardization for instrument configuration; (c) pathologist trained in over 8-color interpretation of the flow cytometric data generated; (d) difficulty collecting enough events to evaluate rare population that contribute to relapse such as LSCs; (e) difficulty identifying MRD in patients that present a different LAIP as they relapse; (f) the unavailability of the diagnostic sample which limits the determination of the LAIP to follow-up.

Use of MRD in AML for treatment decisions

There are few trials of adult AML that use MRD as a basis for treatment decisions. As such, one has to begin with the pediatric literature to begin exploration of this topic. The AML02 trial is one of the first multi-center trials to look at the role of MRD in risk-directed therapy in children with AML (Rubnitz *et al.*, 2010). The goal of this study was to combine standard criteria for risk-stratification in AML (cytogenetics, molecular markers) with MCF-MRD to direct therapy in children with AML. Between 2002–2008, 232 children (ages 2–21) with non-M3 AML were enrolled in one of eight centers. Patients were divided into: 1) low-risk (t(8;21), inv(16), t(9;11)), 2) high-risk (monosomy 7, FLT3-ITD, t(6;9) megakaryoblastic leukemia, treatment- or MDS-related AML), and 3) intermediate-risk (all other patients). LIAPs were characterized with the initial diagnostic bone marrow biopsy. On day 22 after the start of induction chemotherapy, bone marrow biopsies were examined. Detection of ≥1% leukemic blasts identified by MFC at that time point was used to determine whether patients proceeded immediately to a second induction therapy or were given time for count recovery prior to second induction, which was administered to all patients regardless of AML risk-classification. A third induction cycle was administered if there were >25% blasts after first induction or if patients had ≥0.1% leukemia cells detected by MCF at the end of their second induction. Consolidation with cytarabine-based chemotherapy was administered to patients with low-risk AML who had achieved MRD negativity. Patients with either poor-risk AML, >25% blasts after first induction, or persistent MRD-positivity after the third induction cycle underwent allogenic-stem cell transplantation if a bone-marrow donor was available,. Intermediate risk patients received allogenic stem cell transplantation if a matched sibling donor was available, otherwise they continued with consolidation chemotherapy.

MCF- MRD negativity after the first induction cycle coincided with standard risk factors. Patients with t(8;21) or inv(16) were far more likely to have negative-MRD after first induction, and patients with FLT3-ITD-positive leukemia were more likely to have positive-MRD. Furthermore, presence of MRD at that time point was predictive of overall outcome: the 3-year relapse/induction failure rate was 38% vs. 16.9% for MRD-negative versus MRD-positive patients ($p<0.0001$). MRD positivity after first induction was not predictive of relapse or induction failure in low- and intermediate-risk groups, but was highly predictive in higher-risk groups. Furthermore, the degree to which MRD was positive also correlated with risk of relapse/ induction failure. As can be anticipated, multivariate analysis identified MRD ≥1% after first induction and lack of good-risk karyotype as adverse risk factors for EFS.

The question of what is the best therapy for individuals who do not achieve MRD-negativity is a crucial one: if a patient cannot achieve MRD-negativity via chemotherapy, is there benefit to undergoing the rigors of SCT? For patients who remain MRD-positive despite multiple induction attempts, the standard of care has been allogeneic-SCT. However, a 2009 study of transplant in children with chemotherapy refractory AML (either overt or minimal residual disease) questioned the benefit of such an intensive therapy given the high relapse rate. To further explore this question using MCF-MRD, data from the AML02 and AML97 trials were pooled in order to determine the overall relapse rate was for patients who had either high-risk disease features (cytogenetic or molecular changes), or persistent MRD after first or second remission and then underwent HCT (Leung *et al.*, 2012). Patients going into transplant with lower levels of MRD had improved probability of survival than those with higher levels of MRD. However, there was still a clear survival benefit to undergoing HCT. In children with AML with persistent MRD (<5% leukemia cells in bone marrow aspirate), the 5-year overall survival was 66.7%. Even in those patients with >5% who meet criteria for residual AML, not in remission, there was still a survival rate of 58.3% at 5-years.

Based on the above trial, there is benefit in children to advancing therapy based on the presence of MRD or active AML. However, the significant rate of relapse, even post-transplant, does suggest that adjunctive therapies post-transplantation should be considered. The RELAZA trial was an open-label, single-center phase II trial that examined whether MRD as determined by CD34+ cell donor-chimerism in peripheral blood (LIAP-MRD measurements by MFC were not utilized) could be used to instruct use of post-transplantation use of azacitidine maintenance therapy in adult patients with MDS or AML(Platzbecker *et al.*, 2012). Patients (n=56) were treated with four-cycles of azacitidine when donor chimerism dropped below 80%. If patients had response of chimerism to >80%, azacitidine therapy was stopped and reinstituted only if chimerism dropped below 80%. If chimerism improved but did not reach a level above 80%, azacitidine therapy was continued for an additional four cycles. This trial suggested a delay in relapse based on MRD-directed therapy.

There are several questions that remain about use of MRD monitoring in adults with AML. As biochemical and MFC techniques are increasing in sensitivity, there is the potential for determination of increasingly small numbers of residual leukemia cells for patients in hematologic remission. What truly constitutes cure of AML? One would presume that there is a lower limit of cells that the innate immune system or that the Graft-versus-leukemia effect post transplantation can control to prevent relapse. This has yet to be determined.

Another key question is how molecular and cytogenetic risk factors can be combined with multichannel flow measurement of MRD for risk stratification. In 2012, Kohnke et al proposed a combined scoring system for MRD along with standard risk features that could be applied towards tailoring therapy for adults with AML(Thomas Köhnke, 2011). In their scoring system, points were assigned for ELN risk group (favorable versus other), age (60 years or older), and LAIP at time of aplasia during induction chemotherapy (>0.15% by 3-channel flow) (Mrozek *et al.*, 2012). For patients in their good risk group (0 points), the overall survival following standard induction and consolidation chemotherapy at 3-years was >80%. All other groups fell below 50% at this time point. The high overall survival trend was maintained as far out as 5-years in the good-risk group. This stratification system is helpful in younger patients who undergo upfront therapy with intensive regimens such as idarubicin and continuous infusion cytarabine. However, for older patients who are treated initially with hypomethylating agent, the role of MRD at the time of aplasia is more challenging to interpret as we know that a significant percentage of patients who achieve a remission with hypomethylating drugs such as decitabine have residual disease after the first induction cycle, but still go on to achieve remission after subsequent cycles (Blum *et al.*,

2010). Aplasia is may not resolved for months, and timing of count recovery is not as predictable as with other standard therapies. An alternative scoring system may be necessary for elderly AML patients who begin with hypomethylating therapy.

In pediatric AML, use of MRD to direct patients towards early SCT is increasingly clear. However, in adults it remains an open question. This is in part related to the increasing transplant-related-mortality with advancing age. Perhaps MRD will be most useful in adults with AML in order to guide post-consolidation maintenance therapy in older patients for whom allogeneic stem cell transplantation is not appropriate because of significant non-AML comorbidities. As the treatment of adult AML begins to incorporate MRD measurements into treatment decision making it may be worthwhile to also consider additional techniques to measurement of MRD such as next-generation sequencing. Use of AML-population screening as with next-generation sequencing may facilitate a more complete picture of disease relapse risk (Thol *et al.*, 2012).

Conclusion

Is MRD monitoring Clinically Relevant in Adults with AML? The answer to this question appears to be yes, but with caveats. As has been seen in APL and ALL, the presence of MRD after induction therapy for adults has prognostic implication. However, data for its use as a basis for treatment decisions in adults has yet to be explored, and extrapolation from pediatrics is not sufficient. Adults with AML cannot tolerate as intensive therapy as can children, and transplant-related mortality also differ between the two populations. Trials in adults with AML modeled after those that have been instituted in pediatric populations should be undertaken, with attention paid to differentiating between younger adults and the older elderly.

Another hurdle to regular incorporation of MRD into treatment of adults with AML is the limited number of institutions that can perform MFC or patient-specific qPCR measurements. Furthermore, there is not yet standardization of antigen combinations that are used among those institutions that can perform these assays. However, efforts towards multi-center standardization for MFC are advancing (Kalina et al.).

Before MRD for AML can be used as a standard for making treatment decisions for adults with AML, methods for MRD monitoring will need to be standardized and made readily available to all locations in which these patients are treated. Perhaps this can best be accomplished by cooperative group trials that include these measurements, and in which they can be performed at centralized locations. Use of risk-stratification schemes as by Kohnke et al may be helpful starting-points for developing decision-making tools that include all available data about an individuals' AML, and could perhaps be expanded by incorporating data from other techniques for measurement of MRD such as quantitative PCR. Standardization of the timing in which MRD measurements are made is also important. As cooperative trials are designed that include MRD monitoring, it will be important to consider the characterization of all LIAPs present in an individual's AML at the diagnostic bone marrow biopsy prior to initiation of chemotherapy. Furthermore, antibodies that detect LSC antigens should be added to the MFC panels as LSC-monitoring has shown relevance as part of MRD-assesment (Gerber et al.). For patients treated with standard induction therapy (idarubicin and cytarabine), high-sensitivity MFC examination for cells with LIAPs should be performed at the time of aplasia (i.e. day 14-after start of induction therapy), at the time of hematopoietic reconstitution, and at regular intervals post-remission. For those patients undergoing induction with hypomethylating agent, the timing of the most relevant MRD measurement for the purpose of prognosis is not yet clear. The other important consideration is what action to be taken when a new positive result is detected in a

patient who had achieved MRD negativity. The reliability of each institution's measurement of MRD should be considered along with the time to overt relapse for a given measurement (e.g. qPCR detection of NPM1 vs. MCF detection of LIAP >1%) and should prompt an appropriate repeat measurement prior to decisions regarding change in therapy.

We anticipate that future trials aimed at incorporation of MRD into the care of adults with AML will ask the following questions: for patients with good-risk and intermediate-risk AML, can MRD measurements throughout treatment and post-remission therapy be used to minimize therapy for those with decreased relapse risk? We would also hope that MRD monitoring may be able to drive post-remission therapy in patients with poor-risk disease but who are also poor-transplant candidates.

Going forward, MRD measurement throughout induction chemotherapy and post-remission should be encouraged. It will be helpful for determination of prognosis, but will ultimately help to drive decision-making in adults, but this will not become possible until these measurements become a standardized part of care for adults with AML.

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