Detection of minimal residual disease in acute lymphoblastic leukemia

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I. Introduction
In patients with acute lymphoblastic leukemia (ALL), the degree of treatment response guides clinical decisions, and information about this response is essential for selecting the optimal clinical management approach. Unfortunately, determining whether residual leukemia is present during treatment by traditional methods, i.e. the morphologic examination of cells in bone marrow smears, is typically a subjective and imprecise endeavor owing to the fact that the morphology of ALL cells is very similar to that of normal bone marrow cell subpopulations, such as immature B cells and activated mature lymphocytes. Hence, the remission status of patients with ALL often raises doubt in the mind of pathologists and clinicians; this uncertainty can lead to overtreatment (and excessive toxicities) or undertreatment (and increased risk of relapse). The advent of methods for detecting minimal residual disease (MRD) has revealed that many patients considered to be in "remission" by morphologic analysis still have substantial amounts of residual leukemia (Campana, 2008a). Because of the strong correlation between MRD levels and treatment outcome, MRD testing is increasingly being incorporated in clinical trials.

II. A brief review of methods for MRD detection

Polymerase chain reaction
Two main types of molecular targets can be used to identify leukemic cells. One is represented by clonally rearranged antigen-receptor genes, i.e., immunoglobulin (IG) and T-cell receptor (TCR) genes. The junctional regions of the rearranged genes are unique to the leukemic clone. Typically, the unique gene signature is identified at diagnosis in each case using PCR primers matched to the V and J regions of various IG and TCR genes. If a rearrangement is found, the PCR product is further analyzed to ensure its clonal origin by using heteroduplex analysis (van der Velden et al., 2007). The junctional regions of the IG/TCR gene rearrangements are then sequenced to design specific oligonucleotides which are then applied to monitor MRD (van der Velden et al., 2007).

Investigators have developed methods to detect clonal IG/TCR gene rearrangements without the need for patient-specific oligonucleotides. These efforts have relied on high-resolution electrophoresis, such as radioactive fingerprinting or fluorescent gene scanning, but this approach has a considerably lower sensitivity, usually not better than 0.1%, and date interpretation may be difficult (Delabesse et al., 2000; Knechtli et al., 1998).

Because the majority of B-lineage ALL cases have IG (Beishuizen et al., 1993) and cross-lineage TCR gene rearrangements (Szczechanski et al., 1999a), MRD monitoring by using these genes as targets is feasible in > 90% of cases of B-lineage ALL. Likewise, TCR genes are rearranged in most cases of T-lineage ALL and cross-lineage IG gene rearrangements occur in approximately 20% of T-ALL (Szczechanski et al., 2000; Kneba et al., 1995). In sum, the method can be used to monitor MRD in most cases of childhood and adult ALL (van der Velden et al., 2003; van der Velden et al., 2007; Bruggemann et al., 2006; Flohr et al., 2008).

Detection of MRD by PCR using IG/TCR gene rearrangements is most frequently performed by using "real-time" quantitative PCR (RQ-PCR) (van der Velden et al., 2003) and less commonly by limiting
dilution (Neale et al., 1999). Because rearranged IG and TCR genes are present in one copy per cells, very precise estimates of the MRD levels can be achieved. IG and TCR genes may be affected by continuing or secondary rearrangements (Szczepanski et al., 1999b), resulting in subclones with distinct clonal IG/TCR gene rearrangements, and minor clones at diagnosis may become predominant at relapse (Szczepanski et al., 2002; van der Velden et al., 2004). These possibilities have prompted the recommendation of targeting two or more different rearrangements during MRD studies (van der Velden et al., 2007). Multiple targets are identifiable in the majority of ALL cases although in approximately 30% of cases it is not possible to identify multiple targets that allow detection of MRD with a high sensitivity (e.g., 0.01%) (Pongers-Willems et al., 1999; Flohr et al., 2008).

The second type of gene target for MRD monitoring by PCR is represented by gene fusions, such as BCR-ABL1, MLL-AFF1, TCF3-PBX1, and ETV6-RUNX1, and their resulting aberrant mRNA transcripts (van Dongen et al., 1999; Gabert et al., 2003). Recurrent fusions are identified in less than half of patients with newly diagnosed ALL (Gabert et al., 2003), thus limiting the applicability of this approach. However, with the systematic use of novel whole-genome screening technologies (Mullighan et al., 2007; Mullighan et al., 2009), it is very likely that additional genetic targets will enrich the available array of gene targets for MRD studies.

One potential advantage of using fusion transcripts to monitor MRD is that it might be possible to detect pre-leukemic cells (Hong et al., 2008). If so, the clinical significance of such finding needs to be investigated. A clear disadvantage of using fusion transcripts as targets is an accurate estimate of the number of leukemic cells present in the sample is difficult. This is because that ratio between amount of PCR product and target cell number is uncertain, there may be interpatient variability in the number of transcripts per leukemic cell within the same genetic subtype of ALL, and this number could be altered by chemotherapy (Gabert et al., 2003).

**Flow cytometry**

Immunophenotypes characteristic of leukemic cells can be used to distinguish ALL from normal cells by flow cytometry (Campana, 2008). There are three main categories of leukemia-associate immunophenotypes. One is characterized by the expression of fusion proteins derived from fusion transcripts, such as BCR-ABL1, ETV6-RUNX1, or TCF3-PBX1. However, suitable antibodies for reliable flow cytometric analysis of these proteins are lacking. A second group is represented by the immunophenotype of T-lineage ALL cells, which is normally expressed only by a subset of thymocytes and it is not expressed by cells outside the thymus. Immature T-cell phenotypes can be effectively used to monitor MRD in T-lineage ALL (Coustan-Smith et al., 2002a), and also to detect disease dissemination in T-cell lymphoblastic lymphoma (Coustan-Smith et al., 2009a). The third group of leukemia-associated immunophenotype is constituted by multiple marker combinations that are found in B-lineage ALL cells but are normally not expressed during lympho-hematopoiesis. The use of these immunophenotypes, named "asynchronous" or "aberrant" (Hurwitz et al., 1988; Lucio et al., 1999; Campana and Coustan-Smith, 1999; Ciudad et al., 1998), requires a particularly good knowledge of the immunophenotypes expressed by normal hematopoietic cells, in both normal and recovering bone marrow.

Leukemia-associated immunophenotypes that are suitable for MRD studies and afford a sensitivity of at least 0.01% can be identified in nearly all patients with ALL (Coustan-Smith et al., 2002b; Campana and Coustan-Smith, 1999). Results obtained by flow cytometry are very similar to those obtained by PCR amplification of IG/TCR genes, if MRD is present at a ≥ 0.01% level (Neale et al., 1999; Neale et al., 2004; Kerst et al., 2005).

Current methods for MRD testing by flow cytometry typically require the use of extensive antibody panels and considerable interpretative expertise. We developed a simplified flow cytometric MRD test that can detect residual B-lineage ALL cells (which express CD19 plus CD10 and/or CD34) on day 15-26 of treatment with a minimum panel of antibodies (Coustan-Smith et al., 2006). The rationale for this strategy is that normal immature CD19+ cells, or those expressing CD10 and/or CD34, are consistently undetectable in bone marrow samples collected from children with T-lineage ALL after 2 weeks of remission induction chemotherapy, because of their high sensitivity to glucocorticoids and other antileukemic drugs. We therefore reasoned that any cell with this immunophenotype detected in patients with B-lineage ALL on day 19 of induction treatment would likely be residual leukemic cells. Indeed, our findings indicate that the results of the simplified test correlate very well with those of more complex flow cytometric assays or PCR amplification of IGH/TCR genes. It should be stressed that this test cannot be used beyond this early treatment interval because of the high risk of false-positive results in recovering marrow samples.

**III. Results of correlative studies with treatment outcome**

**Studies in pediatric ALL**

The clinical significance of MRD testing during the initial phases of treatment was definitively demonstrated by 3 prospective studies published in 1998 by the EORTC (Cave et al., 1998), St Jude (Coustan-Smith et al., 1998) and BFM groups (van Dongen et al., 1998). The results these studies consolidated those of many other previous reports of smaller series, and have been confirmed by several
Levels of MRD are directly proportional to the risk of subsequent relapse. Thus, MRD $\geq 1\%$ at the end of remission induction therapy predicted an extremely high rate of relapse in St Jude studies (Coustan-Smith et al., 2000), while MRD $\geq 0.1\%$ on both day 33 and day 78 of treatment had a very high risk of relapse in the I-BFM Study Group studies (van Dongen et al., 1998; Flohr et al., 2008). The threshold level commonly used to define MRD positivity is 0.01% of bone marrow mononuclear cells. Patients with $\geq 0.01\%$ MRD at any time point during treatment had a higher risk of relapse in earlier St Jude studies (Coustan-Smith et al., 1998; Coustan-Smith et al., 2000; Coustan-Smith et al., 2002b), as had those with $\geq 0.01\%$ MRD on day 29 of treatment in studies of the Children's Oncology Group (Borowitz et al., 2008). In other studies, however, a threshold of 0.1% appeared to be more informative (Cave et al., 1998; Dworzak et al., 2002; Zhou et al., 2007).

In addition to providing a parameter to identify patients at a higher risk of relapse, MRD can also identify patients with excellent early treatment response and undetectable ($< 0.01\%$) MRD after 2-3 weeks of therapy. We found that 183 of 402 (45.5%) B-lineage ALL patients were MRD $< 0.01\%$ on day 19 of treatment (Campana, 2008b), a feature that is associated with an excellent prognosis overall (Panzer-Grumayer et al., 2000; Coustan-Smith et al., 2002b). The prevalence of MRD differs among different genetic subtypes of childhood ALL (Pui et al., 2001; Borowitz et al., 2003). Thus, MRD is generally more prevalent among patients with BCR-ABL1 ALL and less prevalent among those with ETV6-RUNX1, hyperdiploid ($> 50$ chromosomes) and TCF3-PBX1 ALL (Campana, 2008c). More recently, it has been shown that patients with B-lineage ALL and mutations or deletions of the Ikaros (IHKZFI) gene had a higher prevalence of MRD during remission induction therapy than those without this abnormality (Mullighan et al., 2009). In addition, among patients with T-lineage ALL, MRD-positive findings were strikingly more frequent and levels higher in the subgroup of patients with early thymic precursor (ETP)-ALL (Coustan-Smith et al., 2009b).

MRD studies have now been included in clinical trials to guide therapy. Thus, the AIEOP-BFM group uses MRD to classify patients with newly diagnosed ALL into three risk groups: standard risk (MRD negative on days 33 and 78), intermediate risk (any MRD positivity on days 33 and 78 but $< 0.1\%$ on day 78) and high risk (MRD $\geq 0.1\%$ on day 78) (Flohr et al., 2008). In the AIEOP-BFM ALL 2000 trial, of the 3341 diagnostic samples examined, 88 (3%) lacked suitable gene rearrangements targets for PCR analysis, and an additional 217 (7%) had a target but not sufficient to reach a sensitivity of 0.01% (Flohr et al., 2008). At least two sensitive gene rearrangement targets could be identified in 71% of patients. Adequate data for MRD-based stratification were obtained in 2594 (78%) of the 3341 patients (78%).

In the St Jude Total XV trial for children with newly diagnosed ALL, our laboratory monitored MRD by using flow cytometric detection of aberrant immunophenotypes and/or PCR amplification of antigen-receptor genes (Pui et al., 2009). Overall, 482 of 492 patients (98%) were monitored by flow cytometry and 403 of 492 (82%) by PCR (applied only to patients with B-lineage ALL). As previously shown (Neale et al., 1999; Neale et al., 2004; Kerst et al., 2005), both methods yielded virtually identical results above the threshold level of 0.01%. The two methods in combination could be applied to study 491 of 492 patients (99.8%) (Pui et al., 2009). The single patient with no available immunophenotypic or antigen-receptor gene rearrangements had a MLL-AF9 fusion transcript and was monitored by RQ-PCR using that marker. In our current Total XVI trial, patients with MRD $\geq 1\%$ on day 15 receive intensified remission induction therapy; further intensification is reserved for patients with $\geq 5\%$ leukemic cells. By contrast, patients with MRD $< 0.01\%$ on day 15 receive less intensive reinduction therapy and lower cumulative doses of anthracyclin. Patients with standard-risk ALL who have MRD of $\geq 0.01\%$ on day 42 are reclassified as high-risk; patients with MRD $\geq 1\%$ are eligible for transplant in first remission. Because in patients with T-lineage ALL MRD levels in peripheral blood are similar to those in bone marrow (Coustan-Smith et al., 2002a; van der Velden et al., 2002), it is our current practice to use blood instead of marrow to monitor MRD after day 42 in these patients.

**Studies in adult ALL**

Several studies have also demonstrated the prognostic importance of MRD in adult ALL patients (Mortuza et al., 2002; Bruggemann et al., 2006; Raff et al., 2007; Holowiecki et al., 2008; Bassan et al., 2009). Bruggeman et al. (Bruggemann et al., 2006) studied MRD in 196 standard-risk patients using PCR amplification of antigen-receptor genes and segregated three groups: 10% of patients had $< 0.01\%$ MRD on days 11 and 24 of treatment and 23% had persistent MRD $\geq 0.01\%$ until week 16. The 3-year relapse rates were 0% and 94%; for the remaining patients, the relapse rate was 47%. The same group subsequently studied post-consolidation samples from 105 patients
who were in hematologic remission, had completed the first-year chemotherapy, and were MRD-negative before enrolling in the study. MRD was detected in 28 patients, 17 of whom relapsed. By contrast, 77 patients remained MRD-negative and only 5 relapsed (Raff et al., 2007). Using IG/TCR gene rearrangements or fusion transcripts as targets, Bassan et al. (Bassan et al., 2009) measured MRD at the end of consolidation. Five-year overall disease-free survival estimates were 72% among 58 MRD negative patients and 14% among the 54 patients with positive MRD. In a study using flow cytometry, Holowiecki et al. (Holowiecki et al., 2008) measured MRD in 116 patients with Philadelphia-negative ALL and found that MRD ≥ 0.1% after remission induction therapy was an independent predictor for relapse. Together, the results of these studies provide convincing evidence of the clinical significance of MRD in adult ALL, although the strengths of the correlations with outcome depend on the subgroup of patients studied and the type of treatment.

Monitoring of MRD in adult patients with Philadelphia-positive ALL receiving transplant and/or imatinib therapy has been shown to predict treatment outcome (Radich et al., 1997; Wassmann et al., 2005; Pane et al., 2005). It has been shown that MRD detected before initiation of conditioning is a significant predictor of failure post-transplant (Sanchez et al., 2002; Spinelli et al., 2007).

Areas for further research

Measuring MRD provides unprecedented insights into the kinetics of treatment response in patients with acute leukemia which not only have prognostic ramifications but can also provide novel endpoints for correlative studies with cellular and biologic features. For example, the correlation between MRD and gene expression of leukemic lymphoblasts at diagnosis revealed genes associated with treatment response (Cario et al., 2005; Flotho et al., 2006; Flotho et al., 2007), while correlations with gene polymorphisms has pointed to drug-metabolizing molecules which may have a direct impact on leukemia response to treatment (Rocha et al., 2005; Yang et al., 2009). These areas are clearly worthy of further research, which may lead to the identification of new prognostic factors and provide clues about targets for molecular therapies.

Although MRD can be studied in virtually all patients with ALL using molecular and/or flow cytometric methods, MRD assays require considerable expertise and can be performed well only in specialized centers. Simplification of the methodologies to widen the applicability of MRD testing should be an objective for future research. At the same time, increasingly sophisticated methodologies provide new opportunities for investigation. To this end, the availability of reliable flow cytometers that can detect 6 or more fluorochromes together with the a wide array of commercial antibodies open the possibility to investigate the biologic features of the leukemic cells that contribute to MRD in extraordinary detail. In turn, such studies should help unearthing some of the biologic roots of drug resistance in ALL and ultimately lead to more effective and less toxic treatment.

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