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Evaluation of minimal residual disease in chronic lymphocytic leukemia

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Chronic lymphocytic leukemia (CLL) is a malignancy with a variable prognosis. Poor-risk cases are characterized by advanced clinical stage, short lymphocyte doubling time, unmutated immunoglobulin heavy gene (IgVH) status, distinct genomic aberrations, ZAP70 and CD38 expression and elevated serum thymidine kinase levels.[1-6] During the last decade, effective chemotherapy combinations have been developed, making high complete remission rates in this disease feasible. However, after conventional chemotherapy and autologous stem cell transplantation (SCT), all patients will eventually relapse. Nevertheless, time to progression is highly variable in patients who achieve complete clinical remissions.[7–10] Therefore, minimal residual disease (MRD) undetectable by clinical means must have been present in all of these cases.

This situation has driven the development of more sensitive techniques to quantify CLL in patients who achieve clinical remission. Ideally, the method for MRD detection should be independent from the above mentioned pretreatment risk factors that predict time to progression.

MRD detection – methodological approaches

Polymerase chain reaction (PCR)-based and flow cytometry-based assays are used to assess MRD in CLL. With the development of the real-time PCR method, quantification by PCR has become common. Both methods share high sensitivity but show different advantages, which will be reviewed below (Figure 1).

PCR analysis of MRD

Each B cell can be characterized by its rearranged IgVH complementarity determining region 3 gene (CDR3). PCR with consensus primers, directed to conserved framework regions and IgH joining genes flanking the CDR3, can be used to amplify the CDR3 of all B cells in the sample. Malignant B cells of a single clone share identical IgH gene sequences, resulting in CDR3 PCR fragments of identical sequence and size, whereas PCR products from benign B cells differ in size and sequence with a range of about 30–60 base pairs. CDR3 PCR products can be visualized by automated fluorescence fragment analysis (gene scanning)[11] allowing the detection of a single tumor cell in the background of 100–1000 polyclonal B cells (Figure 2). Sensitivity and specificity of this consensus approach depends on the size, and probably the mutational status, of the clone-specific PCR product in relation to the polyclonal background pattern.

To circumvent the above limitations, the initial PCR products can be sequenced to generate allele-specific oligonucleotides (ASO) that can be used as primers for the specific gene rearrangements within individual patients. These clone-specific detection methods, using either allele-specific primers for PCR (ASO-IgH-PCR) or allele-specific probes[12] matching the previously sequenced CDR3, can increase sensitivity to 1 in 10,000.

ASO-PCR is not usually performed as a quantitative procedure[13] but its use in real-time PCR is quantitative and real-time PCR can be used to quantify the expansion of specific B-cell clones, i.e. MRD in CLL in comparison with a pretherapeutic sample with a sensitivity of 1:10,000 to 1:100,000. ASO real-time PCR employs an ASO primer matching the clone-specific CDR3 sequence.[14,15] The requirement for patient-specific primers makes the design of ASO-PCR expensive and labor intensive.[14] However, Bruggeman *et al.* have devised a highly standardized real-time PCR strategy that permits quantification of about 90% of IgH rearrangements, making MRD detection by PCR the standard detection method, at least in acute lymphocytic leukemia. MRD detection by ASO-PCR is more sensitive when performed on bone marrow cells than on peripheral blood cells.[16] A negative result from peripheral blood should, therefore, be confirmed by bone marrow testing. Conversely, a positive result from peripheral

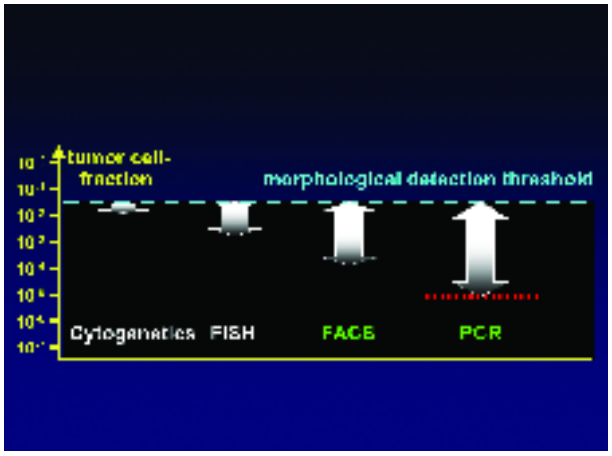

Figure 1. Detection thresholds for different MRD assay methods.

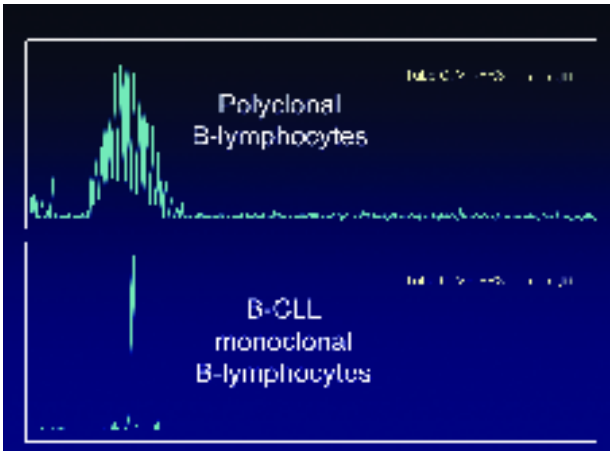

Figure 2. Consensus PCR on polyclonal and monoclonal lymphocytes.

blood, the expected result for most patients, will obviate the need for bone marrow testing.

Flow MRD

The advent of multicolor flow cytometry in combination with the identification of the unique CLL immunophenotype improved the sensitivity and specifity of flow cytometric methods. Using specific gating procedures, by which populations of cells with specific characteristics are identified and then subjected to further analysis, high degrees of discrimination can be obtained. *Conventional* flow fluorocytometric analysis for the detection of residual CLL cells has utilized CD19/CD5 double staining. CLL cells are presumed to be CD19-positive cells, of which more than 25% are also CD5-positive.[13,17] This approach may also be enhanced by detection of excess κ or λ expression.[18] However, CD5 can be expressed by a significant proportion of normal B cells, which can even be increased after high-dose chemotherapy.[19] Rawstron *et al.* have developed a sequential gating method using CD5, CD19, CD20, and CD79b antibodies that can discriminate CLL cells in peripheral blood. Analysis with three separate antibody combinations may provide discrimination in bone marrow cells. Other combinations may be used, but to date, all published approaches identify CLL cells by their typical $CD19^{+}/CD5^{+}/CD20^{low}$ immunophenotype in combination with at least one additional marker such as $CD79b^{low}$[13] or CD43.[19] Sensitivities almost as high as those achieved by ASO-PCR have been reported.[13,19] The sensitivity of flow cytometry can be substantially reduced by the normal precursor B cells in the sample displaying a similar phenotype to CLL cells or by the use of the anti-CD20 antibody rituximab. Quantification of MRD by flow cytometry is fast and reliable over a broad range.

MRD flow vs PCR

Most reported sensitivities for MRD flow and PCR relied upon dilution experiments of CLL cells in normal mononuclear blood or bone marrow cells.[13] This comparison might result in higher sensitivities than can be achieved in real samples containing reactive B cells. The immunophenotype of these B cells partially resembles the immunophenotype of CLL cells. This holds especially true in the bone marrow. In contrast to flow cytometry, ASO-IgH PCR is not disturbed by reactive B cells or B-cell precursors but is, to some extent, dependent on the number of normal B cells in the sample and is therefore more robust to reactive changes and independent from the starting material. An additional advantage of ASO-PCR is the possibility of calculating the maximum sensitivity of the assay in every individual sample, which is impossible for MRD flow.

To date there is only one published direct comparison of clinical samples assessed by both methods in parallel.[19] In this prospective comparison of 158 blood samples from patients after autologous or allogeneic SCT and assessed by MRD flow, consensus PCR, and ASO real-time PCR, the MRD flow assay was more sensitive than consensus PCR but less sensitive than the ASO real-time PCR assay. However, within the com-

Table 1. Sensitivity comparison of real-time PCR vs modified MRD flow assays.[19]

n=92		ASO real-time PCR	
		Positive	Negative
Modified MRD flow	Positive	47	0
	Negative	14	31

mon sensitivity range of both methods (1–0.02% MRD level) the quantitative results correlated very well (Table 1). Our preliminary data show that sensitivity of MRD flow might be further improved by analyzing more cells (Böttcher, Ritgen, *unpublished data*, 2004).

A major disadvantage of MRD flow is that this method requires analysis of more leukocytes. While MRD flow requires at least 5,000,000 blood or bone marrow leukocytes, 500,000–1,000,000 cells are sufficient for real-time ASO-IgH PCR.

MRD flow is a very fast method that produces results within a working day, compared with an initial requirement of a few weeks to establish an individual ASO-PCR for each patient. Thus, MRD flow is the more appropriate method for rapid guidance of clinical decisions. However, samples for flow cytometry must be processed within 48 hours to avoid loss of sensitivity. Given the stability of DNA and the possibility of freezing DNA, ASO-PCR is more suitable for retrospective analysis. It is hoped that this, and similar studies, will lead to the development and acceptance of standardized methods for monitoring MRD in CLL.

Clinical relevance of MRD detection

Published data have shown an association between MRD negativity and improved outcome after various therapies.[12,13,20-23] However, it is essential to relate the attainment of MRD negativity to the methods used for MRD assessment and the applied therapy. After alemtuzumab treatment, blood or bone marrow may be rapidly cleared of CLL cells, but these cells may persist in other compartments and subsequently cause clinical relapse.[20,24] This assumption is supported by the observation that lymphadenopathy is an adverse prognostic factor after alemtuzumab.[23,25] On the other hand, after allogeneic SCT, early MRD positivity is common, at least after non-myeloablative conditioning.[26] This is probably because the onset of graft-versus-leukemia is not immediate and is not necessarily related to final outcome. In contrast to auto-SCT, after which MRD positivity always precedes eventual clinical relapse, MRD negativity can be achieved several months after allogeneic SCT.

Currently, there are no convincing data demonstrating that MRD assessment has additional prognostic value over pretherapeutic prognostic factors. However, in the early phase after autologous SCT, MRD levels are comparable and independent from VH mutational status, cytogenetic aberrations or other risk factors, implying that CLL cells might have similar chemosensitivity, regardless of mutational status.[21] Although low MRD levels or MRD negativity, especially after alemtuzumab treatment, seem to correlate with prolonged progression-free survival, overall survival data are still lacking: MRD detection remains a diagnostic tool only in clinical research and should not be used to guide further treatment intensification, except in controlled clinical trials.

Conclusions

MRD detection has added significantly to our understanding of relapse mechanisms in CLL. Two basic methods are currenty used for MRD detection. While flow cytometry has the advantage of speed and high accuracy combined with good sensitivity, real-time ASO-PCR is more sensitive and can be retrospectively performed using frozen blood, bone marrow or DNA samples. Whether the higher sensitivity of PCR is of clinical significance remains to be determined, especially with regard to different therapies. Nevertheless, available data demonstrate that identical MRD levels after different therapies can be of different significance to outcome. This is especially true in the setting of allogeneic and autologous SCT. Moreover, when comparing MRD data, extra caution needs to be taken regarding the term *MRD negativity* because sensitivity may vary by three orders of magnitude between the established detection methods.

From the available data it is clear that the current National Cancer Institute (NCI) remission criteria may be considerably improved by the incorporation of standardized MRD detection. International consensus would be highly desirable in this regard.[27] Future directions in MRD might include MRD-guided therapy intensification or modification.

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