Clinical implications and prognostic role of minimal residual disease detection in follicular lymphoma

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Abstract: The identification of patients at high risk of relapse is a critical goal of modern translational research in oncohematology. Minimal residual disease (MRD) detection by polymerase chain reaction-based methods is routinely employed in the management of patients with acute lymphoblastic leukemia. Current knowledge indicates that it is also a useful prognostic tool in several mature lymphoproliferative disorders and particularly in follicular lymphoma (FL). Based on this evidence clinical trials employing MRD-based risk stratification are currently ongoing in FL. In this review the ‘state of the art’ of MRD evaluation in FL is discussed. A short description of technical issues and recent methodological advances is provided. Then, the bulk of the review focuses on critical take-home messages for clinicians working in the field. Finally, we discuss future perspectives of MRD detection and more generally outcome prediction in FL.

Keywords: Bcl-2, follicular lymphoma, minimal residual disease, next-generation sequencing, polymerase chain reaction

Introduction

In the past 15 years many advances have been made in the treatment of follicular lymphoma (FL). Since the introduction of rituximab most patients achieve complete remission (CR), and overall survival (OS) rates are improved. Nevertheless most patients still have relapsing disease, often after several years of CR, and frequently die [Freedman, 2011; Fisher *et al.* 2005; Liu *et al.* 2006; van Oers and Kersten, 2011]. Treatment options are becoming increasingly complex. Patients with FL usually receive several therapeutic lines during the natural history of their disease. Every treatment line consists of multiple agents and modalities, including induction, consolidation and maintenance, and in the near future possibly also a preemptive treatment. Early identification of patients with FL at high risk of having relapsing disease is critical for treatment optimization and might allow risk-adapted treatments for FL to be designed in the near future. For this, both effective predictors at diagnosis and reliable tools for post-treatment assessment of treatment efficacy are required. At present, a large number of biological predictors

available at diagnosis have been tested for their clinical effectiveness. These have been excellently revised by Relander and colleagues [Relander *et al.* 2010]. Although many interesting predictors have been identified, their broad applicability and reproducibility are still a matter of debate. Thus clinical prognostic scores, most notably Follicular lymphoma International Prognostic Index and Follicular lymphoma International Prognostic Index 2 (FLIPI and FLIPI2), are still the most reliable tools for pre-treatment prognostic discrimination in FL [Federico *et al.* 2000, 2009; Solal-Céligny *et al.* 2004].

Imaging techniques such as positron emission tomography (PET) scanning and minimal residual disease (MRD) are the most important tools for post-treatment outcome discrimination (Figure 1). PET scanning proved to be effective in at least four major studies on large series of patients included in prospective clinical trials [Le Dortz *et al.* 2010; Trotman *et al.* 2011; Lopci *et al.* 2012; Dupuis *et al.* 2012]. MRD monitoring also proved to be a powerful outcome predictor in FL in a

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Figure 1. Tools for prognostic stratification in lymphomas.

The treatment of follicular lymphoma (FL) and other mature lymphoid tumors is getting increasingly complex, requiring effective baseline and post-treatment predictors. Several prognosticators are useful to predict response and risk of relapse in patients with lymphoma. The most useful in the FL field are represented here. Treatment options which are still experimental are represented with dotted lines. Prognostic tools which are still not validated are indicated in *italic*. MRD, minimal residual disease; PCR, polymerase chain reaction; PET, positron emission tomography; SNP, single nucleotide polymorphism.

large series of studies [Gribben *et al.* 1991a, 1991b, 1992; Hardingham *et al.* 1995; Corradini *et al.* 1997; Moos *et al.* 1998; Freedman *et al.* 1999; López-Guillermo *et al.* 1998; Apostolidis *et al.* 2000; Rambaldi *et al.* 2002, 2005; Ladetto *et al.* 2002, 2006, 2008; Corradini *et al.* 2004; Ghielmini *et al.* 2004; Brown *et al.* 2007; Hirt *et al.* 2008; Goff *et al.* 2009; Morschhauser *et al.* 2012]. MRD can be detected using different methods, including cytogenetics, flow cytometry, PCR-based tools and potentially also with high-throughput output sequencing methods [Faham *et al.* 2012; Ladetto *et al.* 2012a]. Currently most of the available information on FL derives from PCR-based MRD detection. This subject will thus represent the bulk of the present review, discussing the role of MRD analysis in FL. First a brief description of methodological issues will be provided, including the potential use of novel next-generation sequencing (NGS) approaches for MRD detection. Then the predictive value of MRD in different clinical settings will be discussed. Finally future challenges and perspectives will be addressed, including the development of MRD-based clinical trials.

Polymerase chain reaction based minimal residual disease detection in follicular lymphoma: methodological issues

Whenever a patient has a CR, a number of different scenarios may take place, including eradication of the neoplastic clone, long-term persistence of quiescent (nonclonogenic or immunologically regulated) tumor cells, or persistence of clonogenic cells capable of giving rise to a full clinical relapse within months or years. MRD is defined as any approach aimed at detecting and possibly quantifying residual tumor cells beyond the sensitivity level of routine imaging and laboratory techniques. Cytogenetics and more frequently flow cytometry proved to be effective instruments for MRD detection in several hematologic cancers [Morice *et al.* 2007; de Tute *et al.* 2007; Rawstron *et al.* 2008; Böttcher *et al.* 2008; Campana *et al.* 2012]. However, in FL the vast majority of MRD studies were based on PCR amplification of the t(14;18) translocation due to its broad applicability, high sensitivity and predictive value.

MRD analysis by PCR-based methods investigates the persistence of residual tumor cells through the

amplification of a tumor-specific molecular marker. This consists of a DNA sequence that is ideally always detectable in tumor cells and always absent in normal cells. From these sequences, primers and probes are designed and used for qualitative or quantitative PCR. In mature lymphoid tumors two categories of molecular markers are available: tumor-specific translocations and antigen-receptor rearrangements. In FL the t(14;18) tumor translocation is particularly suitable for MRD detection. In this lesion the BCL-2 gene is juxtaposed to the immunoglobulin heavy chain (IGH) gene. This translocation is relevant to the pathogenesis of FL as it induces the overexpression of the antiapoptotic Bcl-2 protein [Bakhshi *et al.* 1985; Shaffer *et al.* 2002; Tsujimoto *et al.* 1985]. The t(14;18) occurs in four clusters, namely the major breakpoint region (MBR), the minor clustering region (mcr), the 3'-BCL-2 region and the 5'-mcr region. However, only MBR and to a lesser extent mcr have been extensively used for MRD analysis. Thus, at present only a subset of patients with FL ranging from 55% to 70% can be assessed using the t(14;18) [Ladetto *et al.* 2008; Gribben *et al.* 1993]. Mainly epidemiological reasons are responsible for the shortage of adequate development of MRD detection protocols for the uncommon rearrangements. However, there are no technical reasons precluding the diffusion of such protocols and the development of novel PCR assays aimed at detecting 'minor' BCL-2 breakpoints.

From a technical point of view tumor translocations have two major advantages: first, MRD detection is relatively simple to perform, as it is not based on patient-specific identification of clonal sequences; second, tumor translocations are directly linked to lymphoma genesis resulting in a highly stable marker, whose loss would clearly be a disadvantage for tumor cell proliferation or survival.

The use of the IGH rearrangement as a marker of clonality has broader applicability in B-cell tumors. Using the IGH rearrangement for PCR-based MRD detection is more complex than using chromosomal translocations because it requires sequencing analysis of the hypervariable region of the IGH rearrangement in each patient to design clone-specific primers and probes [Corradini *et al.* 1995; Voena *et al.* 1997]. The use of the IGH rearrangement is particularly effective in tumors such as mantle cell lymphoma (MCL) and multiple myeloma (MM), which arise from mature B cells that may harbor stable

somatic mutations. However, it has a number of limitations in FL, mostly due to the high rate of somatic hypermutation which makes the identification of the tumor marker particularly complex. Moreover the presence of an ongoing somatic hypermutation process in FL might introduce a degree of variation in IGH sequences during the natural history of the disease, resulting in a less reliable tumor marker compared with MCL and MM [Bahler and Levy, 1992; Tonegawa, 1983].

Detection strategies used for PCR-based MRD can be broadly classified into two subgroups: qualitative and quantitative strategies. Qualitative strategies usually consist of nested or semi-nested PCR approaches. These involve two primer sets, used to amplify a specific DNA fragment, in two separate rounds of amplification in which the second reaction employs a primers set whose function is to amplify a smaller specific DNA fragment located within the first PCR product. These approaches have optimal sensitivity (in the order of 10^{-5}), can be performed using affordable amplification devices, and proved highly informative in different clinical contexts. However, they have some drawbacks, particularly the lack of quantitative power and increased risk of contamination due to reamplification of PCR products. Moreover, no efforts have been made so far to standardize these strategies in a multilaboratory setting.

A major advance in MRD detection in lymphoid tumors using both tumor-specific translocations and the IGH rearrangement has been the development of quantitative PCR methods. Preliminary efforts were made in the 1990s, using limiting dilution and endpoint product quantification tools [Billadeau *et al.* 1992; Vescio *et al.* 1996]. Later the development of TaqMan-based approaches [Holland *et al.* 1991; Gibson *et al.* 1996; Donovan *et al.* 2000; van der Velden *et al.* 2003; Ladetto *et al.* 2000] allowed the growth of extremely sensitive and reproducible quantitative PCR analysis systems. Real-time quantitative PCR (RQ-PCR) assays have a sensitivity close to 10^{-5} [Gibson *et al.* 1996; Donovan *et al.* 2000] and generate highly reproducible quantitative MRD data. RQ-PCR is considered the gold standard for MRD assessment in hematological malignancies and its use has become widespread in the context of large clinical trials in FL. It is still a matter of debate whether older qualitative approaches, usually based on nested PCR, should be considered obsolete or if they might still have a

role together with RQ-PCR. Nested PCR is probably more sensitive because higher amounts of DNA are tested in each reaction; furthermore, it is less expensive [Voena *et al.* 1997]. However, RQ-PCR has the advantage not only of being able to quantify the amount of residual tumor cells, but also of ensuring a lower risk of contamination and is definitely more suitable for multilaboratory standardization programs [Ladetto *et al.* 2000; van der Velden *et al.* 2007].

Major progress on standardization of RQ-PCR, including data interpretation and reporting, has been made by the European network project EURO-MRD, formerly called the European Study Group on MRD Detection in Acute Lymphoblastic Leukemia [van der Velden *et al.* 2003]. The group is part of the European Scientific Foundation for Laboratory Hemato-Oncology and plays a major role in the harmonization of MRD methods and development of guidelines for the interpretation of RQ-PCR-based MRD data. In the past 5 years strategies for MRD assessment in malignant lymphoma have also been refined within the consortium.

Recent evidence indicates that NGS tools can provide an alternative approach to PCR-based MRD detection in mature lymphoid tumors [Ladetto *et al.* 2012a]. This method basically involves the identification of tumor-related sequences at diagnosis based on specific algorithms that take into consideration the increased frequency and the structural features of tumor-related antigen receptor rearrangements. Clonotypic sequences are then subsequently identified and quantified in follow-up samples. This approach has been currently applied to ALL, MCL and MCL using the IGH genes as targets. The results indicate an excellent potential for this novel tool, which currently provides results which are superior to flow cytometry (FC) and comparable to RQ-PCR, although no correlation with clinical outcome has been provided so far. It is reasonable to hypothesize that NGS could also be applied to FL, targeting either the tumor translocation or the immunoglobulin rearrangement, although no data are currently available in this specific setting.

MRD detection is more informative on bone marrow (BM) cells than on peripheral blood (PB) cells [Gribben *et al.* 1994] as the tumor infiltration is higher. Moreover in the rituximab era [Ghielmini *et al.* 2004], the extremely effective clearance of FL cells from the PB ensured by rituximab further

suggest that BM might represent a more reliable tissue for MRD analysis in FL.

Clinical role of minimal residual disease detection in follicular lymphoma

MRD detection by PCR-based methods is currently part of the routine clinical management of patients with ALL. Its role in the management of mature lymphoid tumors has been debated for nearly two decades. However, the prognostic role of MRD is now well established in MCL, MM, and FL.

FL was the first mature B-cell tumor for which MRD was used and it is still the disease for which MRD detection is most frequently employed. The original experiences dating back to the 1990s were performed at the Dana Farber Cancer Institute: Gribben and colleagues evaluated the effects of immunologic purging of BM on MRD status before autologous transplantation [Gribben *et al.* 1991b]. Patients obtaining PCR-negative BM had a superior outcome compared with those with persistently detectable residual lymphoma cells. This study was recently updated at a median follow up of 12 years [Brown *et al.* 2007]: patients receiving a PCR-negative graft had a progression-free survival (PFS) at 67% with a clear plateau, while those receiving a PCR-positive marrow had a PFS of 26%. These results indicate that MRD status at the time of marrow infusion has a long-term impact on the natural history of FL.

Since then, most studies investigating this issue have further emphasized the major predictive value of MRD detection in FL [Hardingham *et al.* 1995; Freedman *et al.* 1999; Corradini *et al.* 2004; Ladetto *et al.* 2008], which is now considered a major independent outcome predictor, whenever multivariate analysis was used [Corradini *et al.* 2004; López-Guillermo *et al.* 1998; Apostolidis *et al.* 2000; Ladetto *et al.* 2008]. The bulk of the experience accumulated thus far allows us to draw a number of clinically relevant conclusions:

- (1) In the absence of rituximab, PCR negativity can be achieved in a minority of patients receiving cyclophosphamide, hydration, oncovirine, prednisone (CHOP)-like chemotherapy at diagnosis (25–30%) [Rambaldi *et al.* 2002]. In contrast, conventional treatment does not lead to any molecular remission (MR) if applied at the time of relapse [Gribben *et al.* 1991a].

- (2) Autologous stem cell transplantation (ASCT)-based programs, even in the absence of rituximab, allowed a large proportion of patients with FL to obtain MR (up to 70%) [Corradini *et al.* 2004; Apostolidis *et al.* 2000; Ladetto *et al.* 2002]. This contrasts with the nearly constant failure in eradicating MRD observed in MCL and small lymphocytic lymphoma, clearly indicating the superior chemosensitivity of FL compared with these entities [López-Guillermo *et al.* 1998; Rambaldi *et al.* 2005].
- (3) Allogeneic transplantation induces a high rate of persistent MRs in FL, providing direct evidence of the potent graft *versus* leukemia effect observed in this neoplasm [Corradini *et al.* 2007; Ghielmini *et al.* 2004].
- (4) The addition of rituximab to conventional chemotherapy significantly increased the rate of MR in FL. Modern chemo-immunotherapy leads to MR rates similar to those observed with ASCT in the pre-rituximab age (50–60%) [Rambaldi *et al.* 2002].
- (5) Even in the rituximab era, ASCT-based programs induce more MRs compared with conventional chemotherapy (70–80% *versus* 50–60%) [Rambaldi *et al.* 2002; Ladetto *et al.* 2008]. Nevertheless, for patients achieving MR, the outcome is similar regardless of the treatment received [Ladetto *et al.* 2008].
- (6) Patients not achieving MR following conventional chemotherapy can achieve PCR negativity following consolidation with yttrium-90 (90Y) ibritumomab tiuxetan: the results from the randomized First-Line Indolent Trial (FIT) indicate that radio immunotherapy increases the rate of PCR-negative patients from 24% to 71% (assessed on PB samples). Moreover, patients achieving PCR negativity are those who show the greatest benefit from consolidation (with a median PFS of 38 *versus* 8 months in the PCR-positive control group) [Goff *et al.* 2009].
- (7) Quantification of tumor burden at diagnosis by RQ-PCR provides an additional prognostic tool: an Italian study showed that patients with a BM tumor load at diagnosis greater than 10^{-2} BCL-2/IGHV variable (IGHV)-positive BM cells have a worse clinical response and 5-year event-free survival compared with those with

less than 10^{-2} BCL-2/IGHV-positive BM cells (PFS 32% *versus* 59%, $p = 0.02$) [Rambaldi *et al.* 2005].

Only a minority of reports failed to demonstrate a predictive value for PCR-based MRD detection. Some of them are based on very small patient series and used heterogeneous tissue sources [Mandigers *et al.* 2001; Schmitt *et al.* 2006]. The largest study failing to find a predictive value for MRD detection was based on patients enrolled in the European Organization for Research and Treatment of Cancer 20981 trial, assessing the benefit of rituximab both in combination with chemotherapy and as maintenance therapy [van Oers *et al.* 2010]. The authors confirmed the predictive value of tumor burden at study entry and confirmed the predictive value of MRD detection at the end of maintenance treatment. However, they failed to observe any prognostic impact of MRD detection at the end of chemotherapy. This unexpected negative result can be easily explained by some specific features in the study design and technical conduction of the analysis. First, all of the patients who were scored as PCR negative after treatment were included in the outcome analysis regardless of their BCL-2/IGHV status at diagnosis. This choice contrasts with previous studies and is not based on a strong biological rationale, as a prognostic role for the absence of BCL-2/IGHV MBR-positive cells in patients with FL who are *ab origine* lacking this rearrangement is unlikely. Second, the series is heterogeneous and poorly characterized in terms of tissue source. The number of patients whose BM was assessed after treatment is not reported. As PB is less reliable than BM for MRD analysis in FL, particularly after heavy rituximab exposure [van Oers *et al.* 2010; Gribben *et al.* 1994], this might have contributed to the lack of predictive value in this analysis. Third, the timing of tissue collection is not clearly specified, suggesting that some samples could have been taken very close to the time of rituximab exposure. Moreover, technical definitions of MR are not specified and no methodological discussion is provided, which is somewhat unusual in a study that contradicts the bulk of previous experience. Apart from these limitations, the paper by van Oers and colleagues has the merit of raising concerns about the role of MRD detection in the context of maintenance regimens, an issue that was not previously considered in FL [van Oers *et al.* 2010].

Additional findings on the value of MRD detection in the context of rituximab intensive maintenance based programs have been reported by our group in abstract form at the 2012 American Society of Hematology meeting [Ladetto *et al.* 2012b]. We investigated the prognostic role of MRD in the context of the ML17638 trial [ClinicalTrials.gov identifier: NCT01144364], a randomized phase III trial from Fondazione Italiana Linfomi [Vitolo *et al.* 2011]. An extensive MRD monitoring program was performed on BM cells that included eight molecular time-points. The achievement of PCR negativity by both nested PCR and RQ-PCR at most time-points predicted for a better PFS in patients receiving maintenance treatment and those under observation. Moreover, MR emerged as an independent outcome predictor, documenting the predictive value of MRD in FL even in the context of rituximab-intensive programs.

Another molecular study has recently been conducted by Fondazione Italiana Linfomi in a large series of 223 patients with FL in the context of the multicenter randomized phase III trial FOLL5 [ClinicalTrials.gov identifier: NCT00774826]. In this study the most prominent impact on PFS is exerted by the molecular status detected at least 6 months after the end of therapy. This observation is in line with the hypothesis of a quite slow, but long-term sustained, MRD clearance exerted by rituximab-based therapies [Galimberti *et al.* 2012].

Future perspectives

Over the last two decades, MRD detection has had an impact on clinical research in FL. Its inclusion as a secondary endpoint in clinical trials has allowed a better understanding of response patterns in different clinical settings and provided a powerful independent prognostic indicator [Liu *et al.* 2006; van Oers and Kersten, 2011; Federico *et al.* 2000]. In the near future, we expect a further increase in the use of MRD detection in the context of multicenter clinical trials as a secondary endpoint for outcome. Moreover, we are aware of at least three clinical trials in MCL and FL that plan to modulate treatment based on PCR results in FL [ClinicalTrials.gov identifiers: NCT00772655, NCT00317060, NCT001676-11].

It is clearly more difficult to draw any hypotheses regarding the future of MRD detection in the medium and long term as therapeutic paradigms

in FL are evolving very rapidly. In the long run, MRD detection in mature B-cell disorders will have a future if the following requirements are met: full standardization of methods; demonstration that the predictive value of MRD is not influenced by the use of different therapeutic programs; and ability to survive competition from alternative prognostic tools.

Achieving successful standardization is not an easy task, but considerable work has been done in the field. Several MRD laboratories are performing quality control rounds in the context of the EURO-MRD group. The ultimate aim of this effort is the full standardization of MRD tools in FL and other mature lymphoid disorders, similarly to what has already been achieved in the ALL field [van der Velden *et al.* 2007].

The second point is more critical: biomarkers and clinical scores which proved highly predictive in a specific therapeutic context lost their prognostic value in different scenarios [Sehn, 2006]. Due to its *a posteriori* nature, MRD detection is probably less susceptible to treatment-related variability compared with biomarkers that are measured at diagnosis as shown by the disparate type of treatments employed in positive MRD studies. Further integrations of NGS tools are expected to increase sensitivity, specificity and predictive value of MRD determination, although these tools will need to undergo a full multilaboratory standardization process similar to that performed for PCR-based MRD approaches.

Finally, the number of prognostic markers that are becoming available for clinical use is rapidly growing. Clinical scores are simple but highly effective tools that can be easily employed in different settings. Imaging tools such as PET are also highly predictive methods and are expected to play a major role in the future, although they seem more effective in detecting nodal disease as opposed to PCR-based MRD, which monitors disease at the BM and PB level [Seam *et al.* 2007]. It is conceivable that some of the basic knowledge that is becoming available thanks to postgenomic cancer discovery will be translated in future years into clinically applicable prognostic markers [Relander *et al.* 2010]. Clinical management of patients with FL requires only a few simple prognosticators. Thus many current and upcoming candidate biomarkers will undergo rapid obsolescence during future years. It is unknown whether MRD will still be among this

restricted subgroup of surviving prognostic factors in the next decade.

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Conflict of interest statement

The authors have no conflict of interest to declare.

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