Recurrence of Bcl-2/IgH polymerase chain reaction positivity following a prolonged molecular remission can be unrelated to the original follicular lymphoma clone

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Objective. The aim of this study was to evaluate whether reappearance of polymerase chain reaction (PCR) positivity for the Bcl-2/IgH translocation following a phase of molecular remission in autografted follicular lymphoma (FL) patients is always associated with reappearance of the original neoplastic clone.

Patients and methods. The molecular follow-up of 119 autografted Bcl-2/IgH positive patients was evaluated by nested PCR. In case of molecular recurrence, direct sequencing of involved rearrangements has been performed both at diagnosis and at the time of recurrence. The two sequences then were compared in terms of breakpoints, N insertions, and JH usage.

Results. Seventy-five patients achieving molecular remission were identified in our patient sample (63%). Of these patients, eight (10.6%) experienced molecular recurrence. Direct sequencing of the Bcl-2/IgH translocation performed at diagnosis and recurrence showed identical rearrangements in six subjects and unrelated rearrangements in two. As opposed to most true molecular relapses, unrelated rearrangements always occurred several years after transplantation. To date, the two subjects carrying unrelated rearrangements show no signs of active lymphoproliferative disease.

Conclusions. This report is the first evidence that Bcl-2/IgH rearrangements unrelated to the original tumor clone can lead to false-positive results during the molecular follow-up of autografted FL patients. Based on these results, we recommend confirmation by direct sequencing, at least for patients experiencing molecular relapse 2 or more years after the end of treatment. This will be particularly important for patients enrolled in clinical trials that schedule additional treatment in case of molecular evidence of persistent disease activity. © 2003 International Society for Experimental Hematology. Published by Elsevier Inc.
subjects and cancer patients at diagnosis [6–12]. These results suggest only a minor confounding role of NLABRs in the post-treatment molecular follow-up of FL patients. However, it cannot be excluded that at least a proportion of molecular relapses might be associated to NLABRs and not to reappearance of the original FL clone, particularly in molecular relapses occurring several years after the end of chemotherapy.

To verify whether molecular relapses are constantly associated with reappearance of the original FL clone, we performed direct sequencing analysis on all patients reverting to PCR positivity following a molecular remission persisting for at least 3 months. Bcl-2/IgH sequences detected at diagnosis and at molecular relapse then were compared in terms of breakpoint sites, N insertions, and JH region usage in order to verify whether these sequences were identical, similar, or unrelated. In addition, samples reverting to PCR positivity were assessed by real-time PCR in order to quantify the number of Bcl-2/IgH carrying cells.

**Patients and methods**

**Patient characteristics**

During the period 1990 to 2001, 119 Bcl-2/IgH positive FL patients treated with an autografting-containing regimen were evaluated at our institution for the presence of minimal residual disease using the Bcl-2/IgH translocation. The treatment plan consisted of the intensified high-dose sequential regimen with (R-HDS) or without (i-HDS) rituximab supplementation (16 vs 103) [13,14]. All patients were included in prospective consecutive or randomized trials and gave written informed consent for both chemotherapy and molecular follow-up analysis. All trials were approved by our Institutional Review Board and local Ethical Committee.

**Samples and DNA extraction**

Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. Genomic DNA was extracted with the DNAzol reagent (Gibco BRL Life Technologies, Grand Island, NY, USA) according to the manufacturer’s recommendations.

**Nested PCR for the Bcl-2/IgH translocation**

The Bcl-2/IgH major breakpoint region (MBR) was amplified by nested PCR on genomic DNA using oligonucleotide primers and amplification conditions as previously described [1,13]. Amplified DNAs were analyzed by electrophoresis on 2% agarose gel containing ethidium bromide and visualized with ultraviolet light. Sensitivity was one or more Bcl-2/IgH rearrangements in 100,000 normal cells on repeated dilution assays. Samples were tested twice on separate amplifications performed on different days, and both negative and weakly positive controls were always included. Standard procedures to avoid contamination of PCR samples were carried out as previously described [15].

**Real-time PCR for the Bcl-2/IgH MBR translocation**

Real-time PCR for the Bcl-2 MBR translocation was carried out as previously described [6,16]. Reactions were performed in an AbiPrism 7700 sequence detector system (PE Applied Biosystems, Foster City, CA, USA). The GAPDH gene was chosen as reference standard in order to normalize patient samples for DNA quality and quantity, as previously described [17].

**Sequencing analysis of Bcl-2/IgH rearrangements**

The presence of Bcl-2/IgH rearrangements was confirmed by direct sequencing of both forward and reverse DNA strands of PCR products obtained from nested PCR. PCR products were run on 2% low-melting-point agarose gels. Bands were excised and purified using the QIAquick Gel Extraction Kit (QIAGEN S.p.A., Milan, Italy). Samples were sent to the Genenco M-Medical Sequencing Facility Laboratories (Rome, Italy) for sequencing reactions and runs [6]. Sequences were compared to the published germine Bcl-2 and IgH gene sequences [18,19] to identify breakpoints, JH usage, and N insertions.

**Results**

Of 119 patients in whom minimal residual disease (MRD) analysis was performed following R-HDS or i-HDS at our laboratory, 75 (63%) achieved a molecular remission lasting at least 3 months. Of these patients, 8 (10.6%) experienced molecular recurrence. One of the patients was treated with R-HDS and seven with i-HDS. Follow-up of patients who experienced molecular relapse is given in Fig. 1. Molecular recurrence took place at a median time from transplant of 15 months (range 7–106). Median duration of molecular remission was 12 months (range 6–100). Three of eight patients who reverted to Bcl-2/IgH PCR positivity subsequently also experienced clinical relapse at 3, 4, and 6 months, respectively. Patient no. 360 decided to undergo treatment with rituximab at molecular relapse and reverted to a stable PCR-negative status. Patient no. 379 developed secondary acute myeloid leukemia (s-AML) at 5 months from molecular recurrence, while her FL was clinically silent. After an aggressive regimen for s-AML, the patient reverted to PCR negativity. Patient no. 245 still is in clinical remission at 5 months from molecular relapse. Unfortunately, patient no. 864 was lost to both clinical and molecular follow-up.

The Bcl-2/IgH rearrangement was successfully sequenced at diagnosis and at molecular recurrence in all patients. Amplification and sequencing at diagnosis were always performed starting with heavily infiltrated bone marrow or lymph node samples. In six patients, the rearrangement detected at relapse was identical to that observed at diagnosis (data not shown). All three patients who experienced both molecular and clinical relapse were in this group.

In two patients, molecular recurrence was associated with a Bcl-2/IgH rearrangement different from that detected at diagnosis (Fig. 2). In both patients, breakpoint sites and N insertions differed substantially from those observed at diagnosis (Fig. 2). In patient no. 360, two different JH regions were used (JH4 at diagnosis and JH6 at repositivization). Notably, in both patients novel rearrangements occurred 3′ from the breakpoint site observed at diagnosis (16 and 25
bp, respectively) in an area that was deleted in the original FL clone. Thus, the novel rearrangement had to occur in cells unrelated to the original neoplastic clone. Of note, these two patients reverted to PCR positivity several years after the end of treatment (38 and 106 months, respectively), as opposed to most of the patients who relapsed with the original FL-related rearrangement (median 10 months, range 7–87) (Fig. 1). Despite these novel rearrangements had small differences in size compared to the original FL clone (12 and 39 bp, respectively), routine agarose gel electrophoresis did not allow clear discrimination (data not shown). Novel rearrangements also were sequenced in subsequent available follow-up analyses and proved identical to those observed at molecular relapse. Importantly, the two patients with unrelated rearrangements showed no evidence of lymphoproliferative disease by radiology, histology, and flow cytometry.

**Figure 1.** Molecular and clinical follow-up of the eight patients who experienced molecular relapse. Yellow circles represent PCR positivity associated with the original tumor clone; purple circles represent PCR positivity associated with NLABR; blue circles represent PCR negativity; black square and arrow represent clinical relapse. When performed, real-time PCR results are indicated by the green rectangles. Results are expressed as bcl-2/IgH rearrangements per 1,000,000 diploid genomes. “bst” (below sensitivity threshold) indicates a sample that is PCR positive by nested PCR and PCR negative by real-time PCR. This means a very low level of contamination (<5e⁰ rearrangements per 1,000,000 diploid genomes) [16]. Additional treatments for molecular relapse or second malignancy also are indicated.

**Figure 2.** Comparison of Bcl-2/IgH rearrangement at diagnosis and at the time of PCR positivity recurrence in the two patients who showed evidence of NLABRs.
Real-time PCR analysis was performed at molecular relapse on all samples in which sufficient DNA was available. Results are shown in Fig. 1. The number of rearrangements per 1,000,000 diploid genomes at molecular recurrence assessed by real-time PCR was always low (median 12 rearrangements per 1,000,000 diploid genomes, range 11–15) in patients reverting to PCR positivity due to unrelated rearrangements. A more heterogeneous pattern was observed in patients relapsing with the original FL clone (median 11 rearrangements per 1,000,000 diploid genomes, range from undetectable by real time PCR to 28,000). Of note, in subjects with unrelated rearrangements, real-time PCR showed no evidence of a sharp increase in the number of clonal cells on follow-up samples at 2 (patient no. 47) and 20 (patient no. 360) months (Fig. 1).

Discussion

This study describes clinical and molecular features of a group of FL patients reverting to PCR positivity for the Bcl-2/IgH rearrangement after at least 3 months of clinical and molecular remission. Molecular recurrence of the original FL clone accounted for the majority of cases. These “true” molecular relapses usually occurred during the first 2 years after transplant. In contrast, unrelated rearrangements explained only a minority of molecular recurrences. Unrelated rearrangements usually occurred only after several years from the end of treatment. Clinical relapse followed molecular recurrence only in subjects reverting to PCR positivity due to the original FL clone.

The observation that most molecular relapses are associated with recurrence of the original FL clone further supports the role of PCR analysis for prognostic evaluation of FL patients, particularly after intensified chemotherapy. Although most subjects at high risk for relapse never achieve PCR negativity, there is a non-negligible subset of patients who revert to PCR positivity following a phase of molecular remission. Interestingly, in one patient (no. 245) molecular recurrence due to the original FL clone took place after 8 years of molecular remission. Although in our series the number of molecular recurrences was fairly low, their incidence is expected to increase as novel treatments such as rituximab, which are particularly effective in clearing disease in the bone marrow and peripheral blood, become more widely used [14,20–22]. Based on these results, we recommend long-term molecular monitoring of FL patients achieving molecular remission.

Appearance of unrelated rearrangements had already been observed by Price et al. [23] in FL patients who were in clinical but not molecular remission for their original neoplastic clone. However, our report shows for the first time that unrelated rearrangements can complicate the interpretation of MRD analysis, leading to false-positive results. This had a considerable impact on the treatment plan of one of our patients. Patient no. 360 probably was overtreated with rituximab, given that his molecular recurrence was unrelated to the original FL clone. Indeed, the overall incidence of unrelated rearrangements appeared to be very low in our patient population (1.6%). This is in accordance with our previous observation on chemotreated patients with malignancies other than lymphoma [6]. Based on our results, it should be concluded that the predictive value of MRD probably would not suffer considerably due to the presence of unrelated rearrangements. However, because unrelated rearrangements seem to occur with fairly specific features (i.e., after prolonged periods of molecular remission and long periods after the end of therapy), their identification probably represents a cost-effective effort. At our laboratory, we have decided to perform direct-sequencing analysis on every molecular relapse occurring 2 or more years after the end of treatment. This will spare a few patients from expensive and/or risky procedures by means of a relatively simple laboratory procedure.

The appearance of unrelated rearrangements, only after a prolonged chemotherapy-free interval, further points attention to the yet unexplained phenomenon of the reduction of NLABR incidence following chemotherapy. In our previous work, we observed that NLABR incidence is low in patients treated with chemotherapy; however, the median time from the end of chemotherapy was only 11 months [6]. It is possible that NLABR incidence might increase again after a prolonged chemotherapy-free interval. The results of the current study showing that unrelated rearrangements in FL patients occur only after a prolonged chemotherapy-free period (38 and 106 months, respectively) is suggestive of this hypothesis, although investigation of larger series is required to demonstrate this pattern.

Our results provide additional information on the natural history of FL. Reappearance of a cell population carrying a novel unrelated Bcl-2/IgH rearrangement that appeared to persist for nearly 2 years is an intriguing finding. It suggests that some specific host-related factor exists in these patients [24]. Genetic or acquired factors might operate in order to generate a permissive environment for the long-term survival of Bcl-2/IgH positive clones, as opposed to the general population in which these clones often persist only for very limited periods of time [6]. Careful prospective follow-up of these subjects will allow verification of whether Bcl-2/IgH positive clones are prone to progress toward a clinically evident second lymphoproliferative disease in FL patients apparently cured of their first neoplasm.

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References


