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Usefulness of real-time PCR in long-term follow-up of follicular lymphoma patients★

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The aim of this study was to evaluate the usefulness of quantitative real-time PCR (RQ-PCR) for the monitoring of molecular remission in follicular lymphoma (FL) patients during long-term follow-up. RQ-PCR by the use of TaqMan® detection system is a sensitive tool to monitor minimal residual disease (MRD) in FL through amplification of the t(14;18) fusion gene during and post-therapy. In most cases the breakpoint region occurs within the major breakpoint region (MBR). Among 75 patients diagnosed with FL, cells harboring the fusion gene *BCL2/JH* were found in peripheral blood of 31 patients (41%). We further monitored 30 of these patients in a period varying from 6 months to 5 years by RQ-PCR. In our study the level indicating the possibility of the presence of MRD was established at more than five t(14;18)-positive cells in the background of 83000 normal cells. The results of this work also confirmed that the presence of MRD detected by RQ-PCR is an indication for careful observation of patients because of a higher risk of disease recurrence.

Keywords: molecular remission, minimal residual disease, real-time PCR, follicular lymphoma

INTRODUCTION

Follicular lymphoma (FL) is the most common type of indolent lymphoma and accounts for about 30% of newly diagnosed non-Hodgkin's lymphomas (NHLs) (Federico *et al.*, 2000). The course of the disease is characterized by clinical remissions after initial treatment but almost all patients will finally relapse (Peterson, 1999). These relapses are caused by residual lymphoma cells which persist despite a complete clinical remission and are detectable only by very sensitive methods. PCR is a method which allows identification of a few tumor cells in 10^4–10^6 normal cells (Horsman *et al.*, 1995). Therefore PCR may be used to monitor the disease progress and minimal residual disease (MRD) in FL patients (Gribben *et al.*, 1994; Drexler *et al.*, 1995; Rambaldi *et al.*, 2002).

In FL the neoplastic clone is characterized by the chromosomal translocation t(14;18)(q32;q21) in about 85% of cases. This translocation is the result of juxtaposition of the *BCL2* oncogene located on chromosome 18 to the immunoglobulin heavy chain (*IgH*) locus on chromosome 14q32. This leads to a constitutive expression of the *BCL2* gene and overproduction of the apoptotic BCL2 protein (Yunis *et al.*, 1982; Tsujimoto *et al.*, 1984; 1985). Since the breakpoints

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Abbreviations: BM, bone marrow; CHOP, chemotherapy (cyclophosphamide, adriamycin, vincristine, prednisone); COP, chemotherapy (cyclophosphamide, vincristine, prednisone); CR, complete remission; CT, computed tomography; FL, follicular lymphoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; icr, intermediate cluster region; MBR, major breakpoint region; mcr, minor cluster region; NHL, non-Hodgkin lymphoma; MRD, minimal residual disease; PB, peripheral blood; PD, progressive disease; PR, partial remission; RQ-PCR, real-time quantitative PCR.

on chromosome 14 are mainly located 5′ of the J_H genes and 70% of the *BCL2* breakpoints on chromosome 18 cluster within the major breakpoint region (MBR), the t(14;18) translocation is a good marker for PCR monitoring of MRD. According to numerous data, the incidence of *BCL2* translocation varies between North America, Western Europe and Asia (Aster & Longtine, 2002; Biagi & Seymour, 2002). It is detected the most frequently in the United States (almost 90% of FL cases) and very rarely in Japan – below 50%. European studies also show a lower frequency of *BCL2* rearrangements compared with American studies (Biagi & Seymour, 2002). Previous studies have shown that detection of t(14;18) in post-autologous bone marrow (BM) transplantation patient samples is correlated with relapse (Zwicky *et al.,* 1996). Patients with PCR-negative BM had a significantly better progression-free survival than those positive by PCR. Studies by McLaughlin and coworkers (1998) showed that patients initially t(14;18)-PCR-positive in peripheral blood (PB) responded to rituximab better than PCR-negative patients. Recent studies have demonstrated that quantitative PCR of bone marrow BCL2/IgH-positive cells at diagnosis predicts treatment response and long-term outcome in FL (Rambaldi *et al.*, 2006).

Nested PCR, used commonly for the detection of the t(14;18) translocation, determines only the presence of tumor cells in the sample. Rapid and easy assessment of the number of t(14;18) cells in the BM or PB is possible by quantitative real-time PCR (RQ-PCR) (Dolken *et al.*, 1998). It allows better molecular monitoring of MRD after various therapeutic protocols. Studies by Summers *et al.* (2002) suggested that RQ-PCR may be used for monitoring the molecular evidence of disease in PB and BM of FL patients. However, the copy number detected in BM generally was higher than in PB. In this work we present results of monitoring of MRD in patients with FL by RQ-PCR during long-term follow-up (up to 5 years). We tried to ascertain whether quantitative PCR analysis provides useful data for the management of FL patients.

PATIENTS, MATERIALS AND METHODS

The study was undertaken on a group of 75 patients with recognized FL who had been under observation or medical treatment in the Department of Lymphoproliferative Diseases of the Cancer Center and Institute of Oncology in Warsaw since 1999. The median age of patients was 53, male/female ratio: 30/45. Sixty-five (87%) patients during the observation (median time 3 years, 0.5 to 5) required beginning of treatment. All individuals had FL recognized by histopathological examination. The first sample of each patient was collected during the diagnosis of the disease. Next samples were taken at different time points after MRD had been detected by nested PCR targeting the major breakpoint region (MBR). Clinical recognition may be classified into three categories: complete remission (CR) defined as the complete disappearance of all clinically detectable disease and/or lowering of the size of all lymph nodes visible on a computed tomography (CT) scan to less than 1×1 cm; partial remission (PR) defined as a higher than 50% reduction in two sequential measurements for all measurable lesions; progressive disease (PD) is considered to be a grow in size of more than 25% than previously documented during disease, or the appearance of the disease at any point.

Nine patients are under clinical observation because of the absence of clinical symptoms (watch and wait strategy). In the first line treatment 24 patients received COP or CHOP-like chemotherapy combined with rituximab (Mabthera®; F. Hoffmann-La Roche Ltd., Switzerland), a chimeric anti-CD20 monoclonal antibody with a high therapeutic efficacy against B-cells lymphomas. Seven patients underwent bone marrow transplantation. All patients gave written consent for this study.

DNA extraction. DNA was isolated from fresh peripheral blood (PB) or bone marrow (BM) by standard phenol/chloroform extraction or by NucleoSpin Blood L (Macherey-Nagel, Düren, Germany) isolation kit. The concentration of DNA was measured spectrophotometrically. Purity of DNA was checked by control PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Fabisiewicz *et al.*, 2004).

Real-time PCR. To estimate the number of cells harboring the t(14;18) translocation a real-time PCR was used. PCR conditions were according to a protocol supplied with the quantification kit — RoboGene MBR fusion transcript Quantification Module (Roboscreen, Leipzig, Germany). The MBR primers and probes were part of the kit and their sequences are not available. The reaction was optimized for the Abi Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The main advantage of this kit is eight points standard curve ready to use. The range of the standard points varies from 5 copies (detection limit) to 100 000 copies of the MBR target. The final volume of the reaction mix was 25 μl with 500 ng of DNA. PCR condition: 45 cycles with the following steps: 95°C for 30 s and 59°C for 2 min without 9600 emulation. All unknown samples were amplified in triplicate. Results were taken into consideration only if all three samples had a similar amplification, i.e. the differences between samples C_t were <1. A reference albumin gene was amplified in duplicate for each

patient sample to monitor the quality of DNA and exclude the presence of PCR inhibitors. DNA isolated from DOHH-2 cell line (Deutsche Sammlung für Microorganizmen und Zellkulturen (DSMZ), Braunschweig, Germany) was used as a positive control. The MCF-7 breast cancer cell line (American Type Culture Collection, Rockville, MD, USA) and DNA isolated from healthy individuals served as negative controls.

The sensitivity of the assay was tested by analyzing serial dilutions of DNA derived from the DOHH-2 cell line. Our assay can detect 5 lymphoma cells in a background of 83 000 normal cells (each sample contains 500 ng target DNA, 1 cell = 6 pg DNA (Rambaldi *et al.*, 2005, Dessars *et al.*, 2006) so 500 ng DNA was obtained from 83 000 cells).

Additionally, we investigated 44 individuals who were negative for MBR but they were known to have follicular lymphoma, for mcr (minor cluster region) and icr (intermediate cluster region) rearrangement. PCR conditions for mcr detection were previously described by Gribben *et al.* (1991) and for icr by Batstone and Goodlad (2005). A mcr-positive standard was made by Invivoscribe (Carlsbad, CA, USA), unfortunately no icr-positive standard was available.

RESULTS

From 75 patients diagnosed with FL, cells harboring t(14;18) were found by nested PCR in the PB of 31 patients (41%). In the negative control group (n = 20) we did not find any *BCL2* rearrangement. Clinical and pathological features were recorded for each patient as shown in Table 1. The median time of observation was 3.0 years (0.5 to 5.0) with 10 deaths (13%) in 9 cases connected with progression of lymphoma. There was no difference in the median time of observation in patients with the presence or absence of t(14;18). Samples from 30 patients were further analyzed by RQ-PCR.

Estimation of t(14;18) positive cells in PB

We monitored 30 patients for a period varying from 6 months to 5 years. The most remarkable examples of clinical and molecular clearance of neoplastic clones for representative patients are shown in Fig. 1. These are detailed data for 10 patients. The results obtained for the rest of the monitored patients (20 persons) were classified as very similar, therefore are not described in details. Figure 1 shows the molecular follow-up based on the evaluation of t(14;18) cells in PB.

DNA analysis of patients no. 1, 2, 5, 8 and 9 (Fig. 1) and thirteen other patients (data not included) showed the presence of t(14;18)-positive cells correlating with the clinical diagnosis in each monitored sample. The observed clinical CR was supported by the lack of t(14;18) cells or their number below the detection limit. In the case of patient no. 3 CR was in concordance with a lack of t(14;18) cells except for one sample when the number of positive cells was 56. However, the next sample taken from this patient again showed that molecular observation is in agreement with patient's clinical condition (no t(14;18)-positive cells).

All monitored patients treated with chemotherapy and rituximab showed that this therapy consequently leads to a decrease and further eradication of t(14;18)-positive cells from the PB and BM (Fig. 1, patients no. 1, 2, 4, 8, 9 and six other patients (not shown)).

Patients no. 6, 7 (Fig. 1) and other four (not shown) represent cases when no lymphoma cells were detected in PB by RQ-PCR despite a clinically detectable disease and medical treatment. Patient no. 6 was diagnosed in 1986. Up to 2001 she was treated with many courses of chemotherapy and achieved only clinical PR, which does not correlate properly with molecular remission established by RQ-PCR.

Table 1. Patients' characteristics

	n=75
Age	
Range (y)	25–82
Median (y)	53
Sex	
Female	45 (60%)
Male	30 (40%)
Architecture	
Entirely follicular	47 (67%)
Partly diffuse	22 (31%)
Diffuse	1 (1%)
Unclassified	5
Histological subtype	
G1	39 (55%)
G2	16 (23%)
G3	16 (23%)
Unclassified	4
Site of diagnosis	
Lymph nodes	62 (83%)
Extranodal involvement	13 (17%)
Systemic symptoms	9 (12%)
Ann Arbor stage	
I	8 (11%)
II	17 (23%)
III	21 (28%)
IV	29 (39%)
FLIPI	
1	33 (44%)
2	27 (36%)
3	15 (20%)

Abbreviations: FLIPI, Follicular Lymphoma International Prognostic Index; IPI, International Prognostic Index.

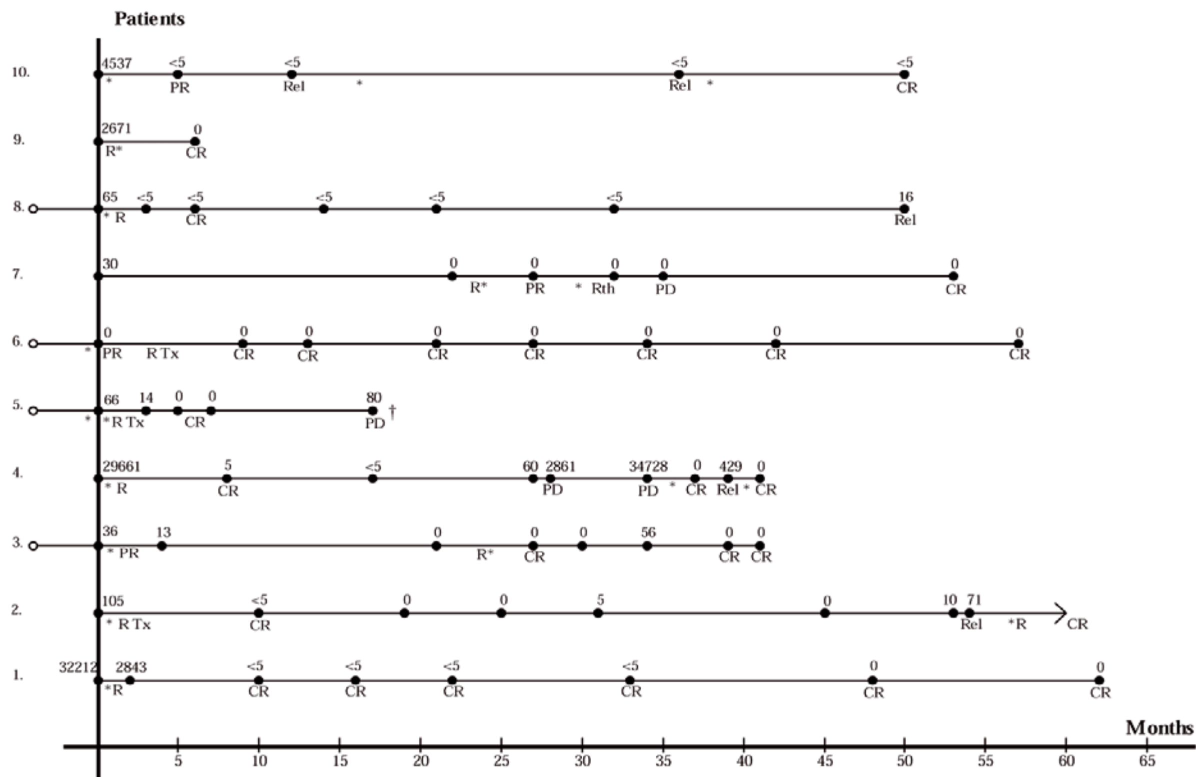

Figure 1. Clinical and molecular analyses of patients with t(14;18)-positive follicular lymphoma.
Point 0 on X axis is the time of first blood sample estimation by RQ-PCR. Numbers above solid symbols represent the number of t(14;18) cells detected in the sample, i.e. in the background of 83 000 cells. PR, partial remission; CR, complete remission; PD, progressive disease; Rel., relapse; †, death; Tx, transplantation; *, chemotherapy; R, rituximab; Rth, radiotherapy.

She was then treated with chemotherapy and autotransplantation, achieved clinical CR and stable molecular remission. Patient no. 7 received chemotherapy and rituximab after 22 months of observation which caused complete molecular remission but clinically only PR. Unfortunately, progression begun 4 months later (no detection in RQ-PCR), therefore he underwent two courses of chemotherapy. After short recovery, relapse of the disease occurred with a spinal cord infiltration. Radiotherapy and chemotherapy were applied with short clinical PR followed by progression of FL and complete molecular remission.

Patients no. 8 and 10 (Fig. 1) display permanent, at low level but detectable, presence of t(14;18) cells in PB. From the medical point of view this explains the reason of relapse in case no. 8 after 50 months of observation. Also in case no. 10 the small number of lymphoma cells found in PB correlated with the clinical status, except the last measurement.

Patient no. 9 (Fig. 1) and four other (not shown) belong to a group diagnosed for a short period of time and will be further investigated.

Patient no. 5 (Fig. 1) after treatment with chemotherapy, rituximab and autotransplantation achieved complete clinical and molecular remission. However, the relapse was fast and severe and despite further treatment the patient died. The last sample showed 80 positive cells.

Search for mcr and icr rearrangements

In the case of patients with recognized FL but lacking t(14;18)-positive cells in PCR for MBR, we investigated whether another rearrangement was present. Therefore we examined 44 patients for the mcr and icr rearrangements. None of the examined patients showed the presence of either of these rearrangements.

Comparison of the detection of t(14;18) positive cells in PB and BM

The lack of positive cells correlated with clinical CR for most of the patients with the exception of patients no. 6, 7 and 10 (Fig. 1). The question arose whether the number of t(14;18)-positive cells present in BM correlates with the clinical condition of the patient better than the number present in PB. BM is a reservoir of neoplastic clones which are less accessible to immune-mediated or comple-

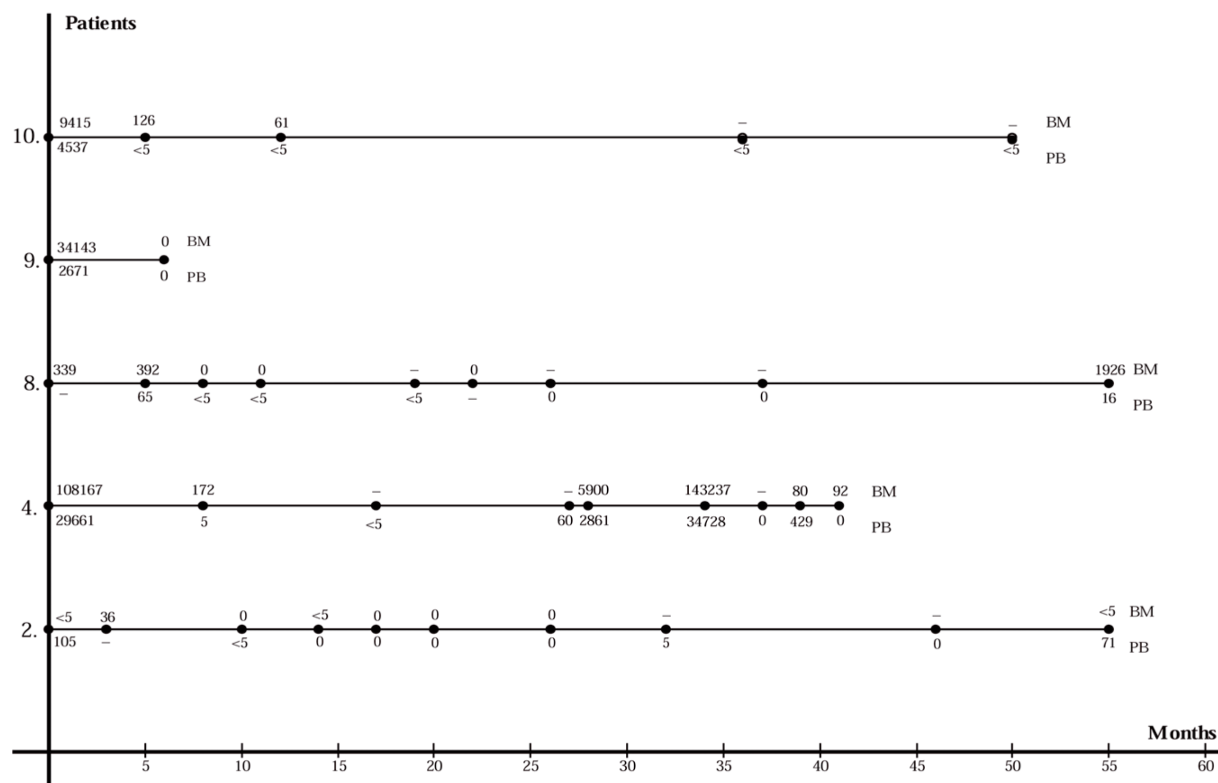

Figure 2. Molecular detection of t(14;18)-positive cells in PB and BM of FL patients.
Numbers above and below solid symbols represent the number of t(14;18) cells detected by RQ-PCR in BM and PB, respectively. (–), not determined.

ment lysis than the clones present in PB. As sampling of BM is more invasive than PB sampling, it was performed only when required for other clinical diagnosis.

We were able to obtain BM of 5 out of 10 patients described above (no. 2, 4, 8, 9 and 10). The results are shown in Fig. 2. The numbers above and below the solid symbols represent the numbers of t(14;18)-positive cells detected in BM and PB, respectively.

In most cases the number of positive cells detected in BM was higher than in PB. The lack of positive cells in BM always correlated with clinical CR, except for patient no. 4. This patient, with a very high level of t(14;18)-positive cells in PB and BM, achieved clinical CR but showed a detectable number of t(14;18)-positive cells in BM (172 cells after 8 months of observation). The situation was repeated in the last sample taken, when there were no positive cells in PB but still 92 cells in BM. The presence of positive cells in BM indicates that despite the observed clinical remission this patient is in risk of recurrence.

For patient no. 2 the observed number of t(14;18)-positive cells in BM was lower than in PB. Seventy-one cells present in PB after 55 months indicated the presence of MRD which was consistent with the observed clinical relapse of this patient.

A BM sample from patient no. 7 was not available. We were not able to estimate the number of t(14;18)-positive cells in BM, which would be helpful in explaining the observed situation of a lack of positive cells in PB but the presence of MRD and spinal cord infiltration.

In the case of patients no. 8 and 10 the high number of positive cells in BM correlated with the observed relapse.

Patient no. 9 had only two samples taken. The first, at the beginning of observation with a high amount of t(14;18)-positive cells in PB and BM, and the second, when he achieved clinical CR. At that moment no positive cells were present in PB and BM.

DISCUSSION

Studies have been undertaken for many years to estimate the usefulness of quantitative measurement of tumor burden in MRD. However, only limited data are available and the usefulness of RQ-PCR in managing FL patients has not been clearly supported or refuted. We have shown in this study that quantitative evaluation of the t(14;18) translocation during long-term treatment of FL patients may be helpful for clinician in monitoring the MRD.

The use of nested PCR leads only to detection of the presence of t(14;18)-positive cells in the sample. Quantitative real-time PCR allows estimation of the number of cells in PB or BM and, more significantly, assessment of how the pool of neoplastic clones changes during the patient's treatment or follow-up. This method is therefore optimal for the assessment of MRD in complete clinical remission.

In our study we detected t(14;18) in 31 cases (41%). It is known that the incidence of *BCL2* translocation varies between North America, Western Europe and Asia. It is detected the most frequently in the United States (up to 90% of cases) and very rarely in Japan — below 50% (Aster & Longtine, 2002; Biagi & Seymour, 2002). The reason for that phenomenon is uncertain, but it can be associated with alternative etiology mechanisms in follicular lymphomas in Asian countries or with different techniques used for t(14;18) detection. European studies also show a lower frequency of *BCL2* rearrangements than that in American population. (Biagi & Seymour, 2002).

The main goal of this work was to monitor the MRD during treatment and/or long-term follow-up. We have followed a group of 30 patients. The data obtained for 10 representative patients from this group are presented here in details.

In the case of 18 patients the presence of t(14;18)-positive cells correlated with the clinical diagnosis and outcome during the consecutive sampling (follow-up). Quantitative analysis showed that treatment with a combination of chemotherapy and rituximab consequently leads to a decrease and further eradication of t(14;18) positive cells from the patient's PB and BM. In our studies it was observed for all monitored patients except patient no. 10. Similar results were achieved by Mandigers *et al.* (1999), Rambaldi *et al.* (2005), Hirt *et al.* (2003), and Galimberti *et al.* (2003).

The data presented for patient no. 10 show that results obtained from BM are more reliable than those obtained from PB. Our results confirm the results of several investigators. Mandigers and coworkers (2001), who determined molecular response rates in PB before and after conventional chemotherapy, found that a decrease of circulating t(14;18) cells after chemotherapy does not correlate with the clinical response. Other studies showed that RQ-PCR evaluation in BM before and after autologous transplantation might predict the clinical course of FL patients (Hirt *et al.*, 2003; Ladetto *et al.*, 2001; Galimberti *et al.*, 2003). Rambaldi and coworkers (2005) provided evidence that low level of BCL2/IgH$^+$ cells in BM at diagnosis was the best predictor for the achievement of a complete clinical and molecular response. Thus, those studies provide evidence that the results obtained from PB only may be inaccurate. A partial explanation why BM sampling has a better predictive value than PB sampling is the higher BM infiltration by lymphoma cells. Therefore, clearance of BCL2/IgH$^+$ cells from PB seems to be much easier and faster than from BM or lymph nodes. Taking those circumstances into account it may be concluded that for MRD assessments longer follow-up with serial determinations of t(14;18)-positive cells in PB is recommended. Such a procedure would provide a more precise information and better correlation with the clinical response than single assessment before and after treatment. It should be stressed that determination of t(14;18)-positive cells in BM is less accessible than in PB because the method of BM sampling is more invasive for the patient.

The presence of MRD is clearly shown on the example of patient no. 4. Despite the high number of lymphoma cells, the patient's condition was comparable to that of patients with a lower number of t(14;18) cells. This example shows that the stage of the disease is not reflected by the molecular assessment. A similar observation was made by Hosler *et al.* (1999).

An example of a high correlation between the clinical status and molecular monitoring is the case of patient no. 6. Complete remission observed clinically throughout a period of 4.5 years was confirmed by the lack of lymphoma cells in the PCR assay of every sample.

In some cases no detection of lymphoma cells does not mean that those cells are absent in PB. The false negative results might be a consequence of clonal relatedness. This is caused by molecular creation of a translocation which may vary from patient to patient. The breakpoints may appear in different regions of chromosome 18 and 14, which results in products of sizes which may vary by hundreds of base pairs (Hosler *et al.*, 1999; Albinger-Hegyi *et al.*, 2002; Dolken *et al.*, 2002; Sanchez-Vega *et al.*, 2002). Additionally, the junction point contains the N-region where small insertions are observed (Cleary & Sklar, 1985; Albinger-Hegyi *et al.*, 2002). Moreover, in rare cases the 5′ region of the *BCL2* gene may be involved in the t(14;18) translocation (Tsujimoto *et al.*, 1986; Albinger-Hegyi *et al.*, 2002). In such cases, the commonly used primers and probes may be unspecific. This is probably the situation observed for patient no. 10. Therefore, the most effective way is to sequence the fusion breakpoint individually in each case and than set specific primers and probes. Such an approach is time-consuming and expensive. This was also underlined by Donovan *et al.* (2000) and Sanchez-Vega *et al.* (2002). Additionally, we can not exclude that the t(14;18) junction changes during clinical treatments as a consequence of chemo- and radiotherapy.

In this study we also investigated 20 healthy individuals for the t(14;18) translocation. None of them were positive but there are some reports in the literature that claim about the presence of lymphoma cells in patients without follicular lymphoma (Summers *et al.*, 2001; Tsimberidou *et al.*, 2002; Schuler *et al.*, 2003).

In conclusion, our studies show that quantitative real-time PCR allows determination of changes in the population of lymphoma cells during treatment and follow up of FL patients. The level indicating the possibility of the presence of MRD was established at more than five t(14;18)-positive cells in the background of 83 000 normal cells. This number is lower than assessed by other groups (Mandigers *et al.*, 2001; Summers *et al.*, 2001). However, a lack of t(14;18)-positive cells in peripheral blood does not mean that the patient is in complete clinical remission. As mentioned above, those cells may still be present in bone marrow, lymph nodes or cerebrospinal fluid.

In this work we showed a direct association between an increase of the number of lymphoma cells and clinically observed disease recurrence. Our results also confirm that monitoring of MRD by RQ-PCR allows identification of patients who should be under careful observation because of a higher risk of disease recurrence.

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