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Commercial LightCycler®-based quantitative real-time PCR compared to nested PCR for monitoring of Bcl-2/IgH rearrangement in patients with follicular lymphoma

M. Kornacker · B. Kornacker · C. Schmitt · E. Leo ·
A. D. Ho · M. Hensel

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Abstract Translocation of chromosomes 14 and 18 [t(14;18)] for detection of minimal residual disease in follicular lymphoma patients can be analyzed by nested polymerase chain reaction (PCR) or by quantitative PCR like LightCycler®-based assays. We have compared both methods in blood and bone marrow samples of 28 patients enrolled in a clinical study on immunochemotherapy. In 42% of samples, the bcl2–IgH rearrangement was detectable by nested PCR, but not by LightCycler® PCR. Nested PCR was able to reveal a significant drop in positive bone marrow or peripheral blood samples after therapy. In contrast, with LightCycler® PCR, the detected drop in t(14;18)-positive cells did not reach statistical significance. The majority of patients showed positive results with nested PCR of peripheral blood or bone marrow without any associations to presence or absence of histological bone marrow (BM) infiltration by lymphoma cells. With LightCycler® PCR, the numbers of positive cells were higher in samples from patients with BM infiltration of lymphoma cells (1.9×10^{-2}) compared to samples from patients without involvement (4.08×10^{-5}). A similar trend was seen in samples derived from the peripheral blood. Positivity for t(14;18) after therapy in two patients correlated with clinical relapse 6 months later. The data shown here demonstrate a lower sensitivity of LightCycler® vs. nested PCR for detection of t(14;18). The usefulness of nested PCR for

t(14;18) for risk stratification after primary therapy has to be validated in larger trials.

Keywords t(14;18) · Quantitative PCR · Nested PCR · Follicular lymphoma · Molecular monitoring · Minimal residual disease

Introduction

The translocation (t) (14;18) (q32;q21), juxtaposing the B cell lymphoma 2 (Bcl-2) oncogene to the immunoglobulin heavy chain (IgH) locus is a common genetic event in follicular lymphoma (FL). It can be detected in 70% of FL cases by polymerase chain reaction (PCR) spanning the major breakpoint region (mbr) of this translocation. The amplification of t(14;18) by PCR has been employed as a marker for minimal residual disease (MRD) [1] and is more sensitive than morphology, flow cytometry, or Southern blotting [2]. It could be demonstrated that high-dose therapy [3] as well as conventionally dosed treatment [4, 5] was able to achieve complete molecular responses in a significant number of patients. In these as well as in other reports [6–9], MRD negativity after treatment correlates with prolonged overall and progression-free survival. Usually, semi-nested or nested PCR approaches have been used to detect t(14;18) [1]. In nested PCR, two sets of primers are used in two successive runs. The second primer set is intended to amplify a secondary target within the product of the first run. This prevents the amplification of sequences resulting from unexpected primer binding sites, thereby reducing contaminations in the PCR product and increasing sensitivity. Nested PCR is an end-point PCR, relying on the detection of the PCR product at the end of the run. In contrast, LightCycler® PCR is a real-time PCR

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M. Kornacker (✉) · B. Kornacker · C. Schmitt · E. Leo ·
A. D. Ho · M. Hensel
Department of Hematology, Oncology and Rheumatology,
University of Heidelberg,
Im Neuenheimer Feld 410,
69120 Heidelberg, Germany
e-mail: martin.kornacker@arcormail.de

which allows for the quantification of the amplified DNA during the linear-log phase of PCR. The PCR product is detected by fluorescence using two short oligonucleotides which are each labelled with a different fluorescent dye. They hybridise to an internal sequence of the amplified fragment. After hybridisation to the template DNA, the two probes come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorescent dyes. FRET causes them to emit fluorescence at a specific wavelength which correlates to the amount of PCR product and is measured by the LightCycler® apparatus. Results are expressed as the target/reference ratio of each sample normalised by the target/reference ratio of a standardised DNA sample (calibrator). Recently, a LightCycler®-based commercial assay to quantify cells harbouring t(14;18) has become available. The target concentration in each sample is calculated relative to the housekeeping gene tissue plasminogen activator (tPA). In this paper, we report on the comparison of a nested PCR assay with the LightCycler® t(14;18) quantification kit (mbr; Roche Diagnostics) in a homogeneous population of patients with FL positive for t(14;18).

Materials and methods

Patients and patient samples

Samples analysed in this study represent a subpopulation of patients enrolled in the randomised phase II multicenter study HD2000 on whom both nested PCR as well as quantitative real-time LightCycler® PCR were performed and at least one of the samples collected was positive for t(14;18) by nested PCR. Thus, all patients reported here were considered to bear t(14;18) within their lymphoma cells. Blood and bone marrow samples were collected prospectively from follicular lymphoma patients treated within this trial with CHOP chemotherapy and rituximab (Mabthera, Roche, Grenzach-Wyhlen, Germany). The aim of the clinical study was to determine the influence of combined administration of CHOP chemotherapy with varying numbers of rituximab infusions as first-line treatment on the remission quality/duration of patients with FL. All patients investigated in this study have given written informed consent before entering the study. The study was conducted according to good clinical and laboratory practice rules and the principles of the Declaration of Helsinki. To be eligible, patients were required to have proven diagnosis of CD20 positive FL (grades I, II and III) according to the REAL classification and to be older than 18 years of age with clinical stages III–IV (according to Ann Arbor staging system). Characteristics of

patients are summarised in Table 1. Patients received 6 cycles of CHOP (cyclophosphamide 750 mg/m², doxorubicin 50 mg/m², vincristine 1.4 mg/m² and prednisone 100 mg) chemotherapy every 21 days. Furthermore, patients were randomly assigned to receive rituximab (375 mg/m²) administered by one, three or six intravenous infusions either with the first, the first to third or the first to sixth cycle of CHOP, respectively. Rituximab was always administered 1 day prior to chemotherapy. Samples were taken before therapy and after 3 and 6 cycles of therapy. Overall, 249 PCRs were performed on samples from blood and from bone marrow collected from 28 patients. Fifty-six bone marrow samples were analysed with a nested PCR and 54 with a quantitative real-time LightCycler® PCR. Seventy-five samples derived from the peripheral blood of the patients were analysed with the nested and 64 with the LightCycler® PCR.

Polymerase chain reaction

Isolation of DNA

Samples from peripheral blood and bone marrow aspirations were taken before therapy and after three and six treatment cycles. Total cellular DNA was extracted using the QIAamp DNA blood maxi kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

For quantitative analysis, a real-time detection of t(14;18) using the commercially available "LightCycler® t(14;18) quantification kit (mbr)" (Roche Diagnostics) was performed according to the manufacturer's recommendations. Final reaction volume was 20 µl containing 5 µl of DNA and 15 µl of the reaction mix which includes FastStart Taq DNA polymerase, Mg⁺⁺, a forward primer binding to exon 3 of mbr, a reverse primer binding to JH segment and fluorescently labelled hybridisation probes. The amount of DNA added to the reaction ranged from 100 to 7180 ng (mean 1,290 ng, SD±1,054 ng). The mixture was transferred into

Table 1 Patients' characteristics

Patient number	28
Age (median and range)	54 (34–69)
Sex (m/f)	(16/12)
Clinical stage	
III	11
IV	17
Histological grade	
I	12
II	9
III	3
No data	4

glass capillaries, denatured at 95°C for 8 min and then amplified with 45 cycles [denaturation at 95°C (10 s), annealing at 54°C (10 s) and extension at 72°C (20 s)] on a LightCycler® device. Its software calculates the crossing point, which indicates the cycle number at which exponential DNA amplification begins. In order to allow a relative quantification, a PCR for tPA, which occurs as a single DNA copy in human cells, was performed in parallel. In addition, a calibrator sample of DNA from the cell line DoHH2, which expresses t(14;18) constitutively, is included in each run. All samples were tested as duplicates. Water instead of DNA served as negative control.

Results are quantified with the LightCycler® relative quantification software (Roche Diagnostics), and the final results are expressed as the ratio of target copies [t(14;18)] to reference copies (tPA) in the sample relative to the ratio of target to reference copies in the calibrator. Thus, a value of 1 would indicate that each cell of the sample contained t(14;18), and a value of, e.g. 10^{-3} would indicate that one cell in 1,000 cells would contain the translocation, i.e. one t(14;18) positive follicular lymphoma cell would be found within 1,000 normal blood or bone marrow cells.

For nested PCR, the mbr of the t(14;18) translocation was amplified by a two-step nested PCR. The first PCR reaction was performed with 500 ng of DNA in a final volume of 50 µl using primers Bcl-2NFO (5'-GAC CAG CAG ATT CAA ATC TAT GGT GGT-3') and JHNRO (5'-GGA CTC ACC TGA GGA GAC GGT GA-3') in a concentration of 0.36 µM each, 54 °C annealing temperature and 25 cycles. Three microlitres of the PCR product was subjected to the second PCR reaction containing the inner primers Bcl-2NFI (5'-CCT TTA GAGAGT TGCTTTACGTGGCC-3') and JHNRI (5'-GGAGACGGT GAC CAG GGT-3') in a concentration of 0.36 µM each. The second PCR reaction was performed using 55°C annealing temperature and repetitions of 30 cycles. Samples were tested as duplicates in three independent runs. They were scored as PCR-positive when at least one of six reactions showed a specific amplification product. Nested PCR was shown to detect one positive cell in 10^5 normal cells [10].

Statistics

Values of p to compare the percentage of positive results between different sample groups were calculated using Fisher's exact t test. Differences in LightCycler® PCR results were measured using the Wilcoxon two-sample test. McNemar's test was used to compare nested PCR and LightCycler® PCR results for *Bcl-2/JH* rearrangement detection on the same samples.

Results

Serial tenfold dilutions of the t(14;18) harbouring cell line K422 in peripheral blood mononuclear cells from normal donors established a sensitivity of the LightCycler® PCR assay between one t(14;18) positive cell in 10^5 – 10^4 normal cells and a linear measuring range at dilutions of K422 cells between 10^0 and 10^{-4} . These results are similar to data provided by the manufacturer and to data described before [11] and are therefore not shown.

Sensitivity of quantitative vs. nested PCR

To assess sensitivity of nested and LightCycler® PCR, all 117 samples on which both PCR methods had been performed were evaluated regarding PCR status and displayed in a 2×2 contingency table (Table 2). The bcl2–IgH rearrangement was detectable in 42% of samples by nested but not by LightCycler PCR. Samples with positive results in LightCycler PCR were always positive with nested PCR ($p < 0.0001$, Table 2).

Molecular disease status before and after therapy

To further compare nested PCR with LightCycler® PCR, results from bone marrow and peripheral blood samples taken before start of therapy and after 3 and 6 cycles of immunochemotherapy were analysed (Figs. 1 and 2). A summary of results can be found as [Electronic Supplementary Material](#).

Table 2 Negative and positive results with nested and LightCycler® PCR: 2×2 contingency table summarizing results on all 117 BM and PB samples of which nested as well as quant. LightCycler® PCR results were available

		Nested PCR		Total
		Negative	Positive	
LightCycler® PCR	Negative	44	49	93
	Positive	0	24	24
	Total	44	73	117

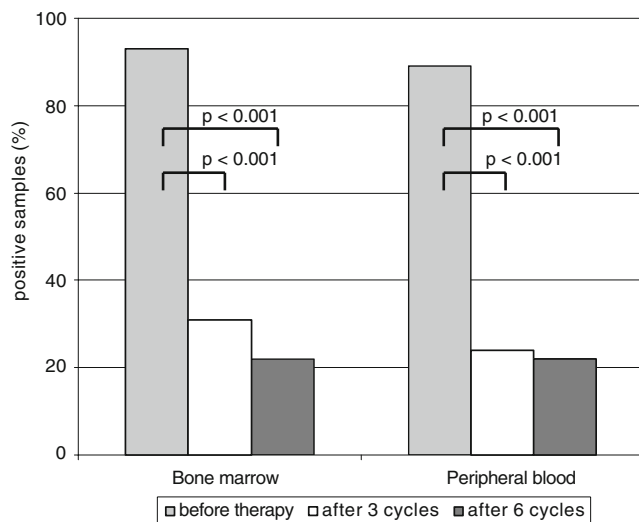


Fig. 1 Nested PCR for t(14;18) in bone marrow and peripheral blood samples of FL patients taken before start of therapy and after 3 and 6 cycles of immunochemotherapy. Twenty-three of 25 (93%) of bone marrow samples were positive before therapy, four of 13 (31%) after 3 cycles and four of 18 (22%) after 6 cycles of chemotherapy. With peripheral blood samples, 25 of 29 (86%) were positive before therapy, five of 22 (23%) after 3 cycles and five of 24 (21%) after 6 cycles of therapy

With nested PCR, 23 of 25 (93%) bone marrow samples were positive before therapy, four of 13 (31%) after 3 cycles and four of 18 (22%) after 6 cycles of chemotherapy. With peripheral blood samples, 25 of 28 (89%) were positive before therapy, five of 21 (24%) after 3 cycles and five of 23 (22%) after 6 cycles of therapy. Differences between samples

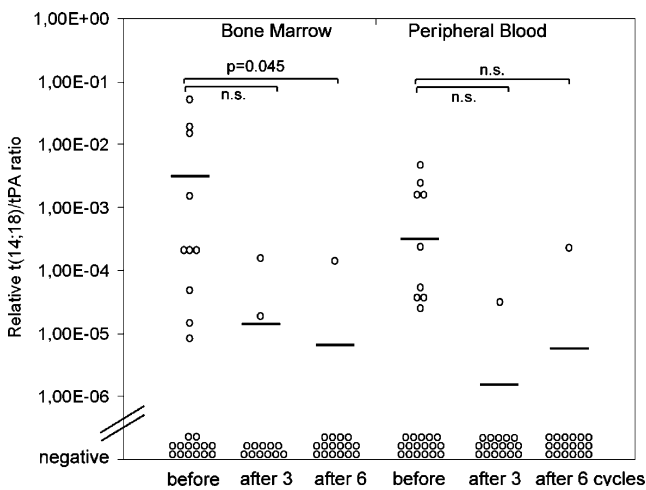


Fig. 2 LightCycler® PCR for t(14;18) in bone marrow and peripheral blood samples of FL patients taken before start of therapy and after 3 and 6 cycles of immunochemotherapy. The mean relative t(14;18)/tPA ratio before therapy (4.1×10^{-3}) dropped to 2.17×10^{-5} after 3 cycles and 8.12×10^{-6} after 6 cycles of therapy. In peripheral blood, the mean t(14;18)/tPA ratio before therapy was lower (4.08×10^{-4}) than in bone marrow samples and dropped to 1.59×10^{-6} after 3 cycles and to 7.89×10^{-6} after 6 cycles of therapy

obtained before therapy and after 3 or 6 cycles were highly significant (Fig. 1).

With LightCycler® PCR for bone marrow samples, the mean relative t(14;18)/tPA ratio before therapy (4.1×10^{-3}) dropped to 2.17×10^{-5} after 3 cycles and to 8.12×10^{-6} after 6 cycles of therapy. In peripheral blood, the mean t(14;18)/tPA ratio before therapy was lower (4.08×10^{-4}) than in bone marrow samples and dropped to 1.59×10^{-6} after 3 cycles and to 7.89×10^{-6} after 6 cycles of therapy (Fig. 2). In bone marrow as well as in peripheral blood, only few samples remained positive after therapy, but the observed reduction in lymphoma cells during treatment was only significant for bone marrow when results before were compared to results after 6 cycles of therapy.

Molecular disease status dependent on bone marrow involvement

To correlate PCR results to histomorphological measures of tumour involvement, we performed nested as well as LightCycler® PCR on bone marrow and peripheral blood samples from patients with or without histological proven BM infiltration. With nested PCR, the majority of samples from BM as well as from PB proved to be positive whether bone marrow was infiltrated by lymphoma cells or not (Fig. 3). In BM, 100% of samples were positive in cases with and 87% were positive in samples without lymphoma involvement. Similarly, 92% of PB samples were positive when BM was infiltrated by FL and 87% were positive in cases without BM infiltration. With LightCycler® PCR, the mean t(14;18)/tPA ratio was higher in the samples from patients with histological proven BM infiltration of lym-

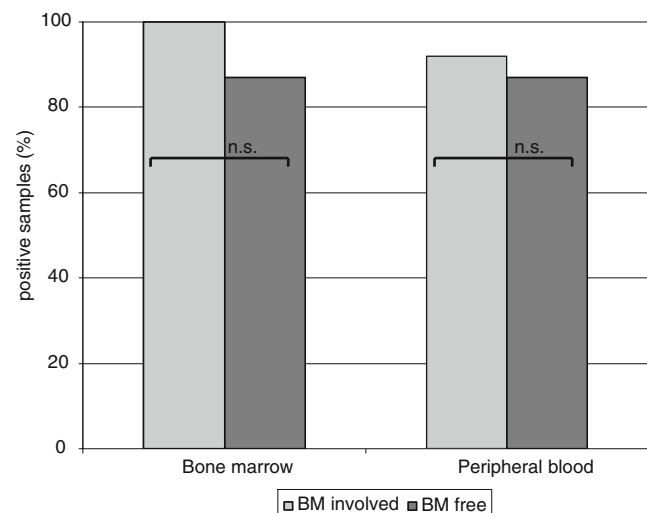


Fig. 3 Nested PCR for t(14;18) on bone marrow and peripheral blood samples from patients with or without histological proven BM infiltration by FL. With nested PCR, the majority of samples from BM as well as from PB proved to be positive whether bone marrow was histologically infiltrated by lymphoma cells or not

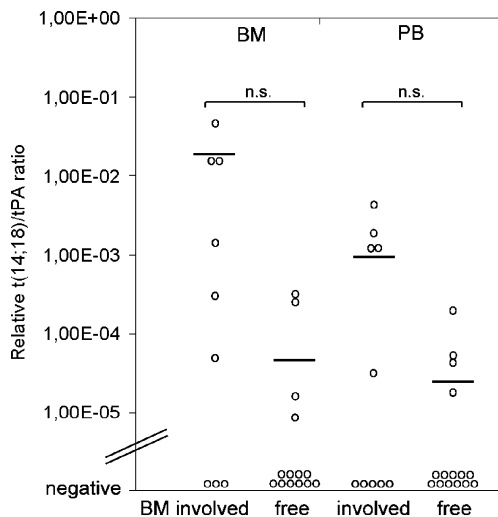


Fig. 4 LightCycler® PCR for t(14;18) on bone marrow and peripheral blood samples from patients with or without histologically proven BM infiltration by FL. With LightCycler® PCR, the mean t(14;18)/tPA ratio was higher in the samples from patients with histologically proven BM infiltration of lymphoma cells (1.9×10^{-2}) compared to samples from patients without BM involvement (4.08×10^{-5}). A similar trend was seen in samples derived from the peripheral blood, albeit at lower overall values (1.03×10^{-3} with, 2.1×10^{-5} without BM involvement)

phoma cells (1.9×10^{-2}) compared to samples from patients without BM involvement (4.08×10^{-5} , Fig. 4). A similar trend was seen in samples derived from the peripheral blood, albeit at lower overall values (1.03×10^{-3} with, 2.1×10^{-5} without BM involvement). These correlations were not statistically significant.

Correlation between MRD after therapy and relapse

We were interested if PCR results at the end of therapy would be able to predict if these patients will suffer from early relapse. We were able to obtain PCR results after

6 cycles of therapy as well as information on the remission status 6 months later on 13 patients. Eleven of these stayed in remission (CR=6, PR=4, SD=1), whereas two suffered from PD. The two patients with PD had achieved a PR after therapy, and with nested PCR, they had a positive result in their BM as well as in their PB samples at that time (Fig. 4). Of the 11 patients remaining in remission, one was positive in BM and two in PB with nested PCR. With LightCycler® PCR, the two patients with PD had divergent results in BM and PB after therapy. None of the 11 patients in remission had a positive LightCycler® result at the end of therapy. The PCR results before, during and after therapy of the two relapsing patients are shown in Table 3.

Discussion

We have compared nested and quantitative LightCycler®-based PCR for detection of t(14;18) in patients with FL. Sensitivity was one t(14;18) positive cell in 10^5 negative cells for nested and one in 10^5 to one in 10^4 for LightCycler® PCR. This difference resulted in a significant larger number of t(14;18)-positive blood and bone marrow samples obtained before therapy with nested compared to LightCycler® PCR. A likely explanation is that at this time point, a significant proportion of patients harbour between 1×10^{-4} and 1×10^{-5} FL cells in their circulation and in their bone marrow, and these positive cases are missed when the less sensitive LightCycler® PCR method is utilised. A lesser sensitivity of real-time vs. nested PCR has also been reported in patients with chronic myeloid leukemia. With real-time PCR, 12 of 94 bone marrow samples were negative for the disease marker BCR-ABL. With nested RT-PCR, in six of these 12 patients, BCR-ABL transcripts could be detected after the second round of amplification [12].

Table 3 Time course of nested and LightCycler® PCR results in the two patients having relapsed 6 months after therapy

	Before	After 3 cycles	After 6 cycles	6 months after therapy
Patient A				
Remission status	NA	PR	PR	PD
Nested PCR—BM	Positive	Positive	Positive	
LightCycler® PCR—BM	6.12×10^{-2}	2.6×10^{-4}	1.38×10^{-4}	
Nested PCR—PB	Positive	Positive	Positive	
LightCycler® PCR—PB	ND	Negative	Negative	
Patient B				
Remission status	NA	PR	PR	PD
Nested PCR—BM	Positive	Positive	Positive	
LightCycler® PCR—BM	1.39×10^{-5}	Negative	Negative	
Nested PCR—PB	Positive	Positive	Positive	
LightCycler® PCR—PB	5.49×10^{-5}	ND	1.5×10^{-4}	

A hint for the clinical relevance of PCR testing would be if a therapeutic intervention is reflected by PCR results. Indeed, this is the case for nested PCR: Three or 6 cycles of immunochemotherapy resulted in a significant drop in PCR-positive samples in blood and in bone marrow, demonstrating that t(14;18)-positive cells drop below 1×10^{-5} in these patients after therapy. LightCycler[®]-based PCR also showed a reduction of t(14;18) bearing cells and an increase in negative samples, but without reaching statistical significance. This may have been due to the low sensitivity of the assay, with 14 of 24 and 17 of 26 pre-therapy samples being negative with LightCycler[®] PCR in bone marrow and peripheral blood, respectively.

Similar to the study reported here, Martin et al. [11] have used the commercially available LightCycler[®] t(14;18) quantification kit (mbr) in patients with follicular lymphoma. Contrary to our finding, they were able to describe a significant correlation between LightCycler[®] PCR results and lymphoma activity [t(14;18)-positive cells in BM or PB before vs. after therapy]. Several factors may be responsible for this discrepancy:

- We did observe a higher number of samples being negative at initial diagnosis (58% in blood and 65% in bone marrow samples) compared to the samples analysed by Martin et al. (46% and 52%, respectively) with LightCycler PCR. Correspondingly, median t(14;18)/tPA ratio was lower at initial diagnosis with zero as the median in blood and bone marrow samples in our patients compared to a median of 5×10^{-5} and 6×10^{-5} , respectively, in the series presented by Martin et al.. This may have been caused by differences in sample handling and DNA isolation. Even when similar protocols are used, quantitative results may vary: A two log variation in the number of positive cells has been reported in an international study on 12 laboratories performing quantitative PCR for t(14;18) on aliquots of blood spiked with defined numbers of lymphoma cells [13].
- Almost one quarter of the patients analysed by Martin et al. had relapsed FL, which may have resulted in more aggressive disease with more lymphoma cells disseminating into PB or BM compartments.
- Patient numbers were smaller in our study.

t(14;18) is not specific for malignant disease. This translocation has been detected in lymph nodes and tonsils with benign follicular hyperplasia [14] and in blood and spleen cells of healthy volunteers [10, 15–17]. We have reported previously that 24% of healthy blood donors carried the translocation when analysed with nested PCR and 19% were positive with a real-time quantitative PCR. In that study, detection limit for both assays was one t(14;18) copy in 10^5 cells [10]. Thus, we cannot exclude

that part of the positive results reported here are derived from t(14;18) in non-malignant lymphocytes unrelated to follicular lymphoma. The majority of healthy individuals being positive for t(14;18) carry the translocation at a frequency below 10^{-4} [18]. In another study with 25% of healthy volunteers being positive for t(14;18), only 3% were positive at frequencies of more than one in 10^4 cells [17]. A clear correlation between PCR sensitivity and the prevalence of circulating t(14;18)-positive cells has been demonstrated in a meta-analysis of 13 studies with 1,908 healthy individuals [19]. These findings suggest that nested PCR will detect t(14;18) unrelated to follicular lymphoma more often than LightCycler[®] PCR, resulting in false positive results. A comparison of DNA sequences between PCR products and the initial lymphoma sample will be important to elucidate the origin of the translocated genes in future studies.

Nested PCR was not able to differentiate between bone marrow samples with or without histological proven involvement: All samples with lymphoma involvement and 87% of samples without involvement were t(14;18)-positive. In contrast, LightCycler[®] PCR showed lower number of cells with t(14;18) and a higher percentage of negative samples in bone marrow not infiltrated with follicular lymphoma, albeit missing statistical significance. This does suggest that the number of t(14;18)-positive cells is above 10^{-5} in the majority of BM samples independent of lymphoma infiltration, preventing nested PCR to differentiate between samples with or without histological evidence of involvement.

Interestingly, with LightCycler PCR, there was also a correlation between BM involvement and t(14;18) cell numbers in the peripheral blood. This could point towards a spill-out phenomenon, allowing FL cells to enter circulation from the BM compartment. On the other hand, this correlation could merely reflect increased tumour load or biologically more aggressive disease in patients with BM involvement, leading to higher numbers of circulating lymphoma cells. A correlation between histological marrow infiltration and higher levels of BLC2/JH fusion sequences with real-time PCR in BM [20] and PB [20, 21] has been demonstrated before.

The data shown here demonstrate a lower sensitivity of LightCycler[®] vs. nested PCR for detection of t(14;18). This has implications for the selection of the most suitable PCR method when analysing clinical samples: In samples where the ratio of target to reference gene is approaching the limit of detection of LightCycler[®] PCR, e.g. after immunochemotherapy, only nested PCR may be able to demonstrate the effect of a therapeutic intervention on residual lymphoma cells. On the other hand, with numbers of positive cells well above the limit of detection in the majority of samples, as in the case of stage IV disease before therapy, LightCycler[®] PCR may be well able to quantitate t(14;18) in clinical samples.

Most, if not all, patients will ultimately relapse after immunochemotherapy for follicular lymphoma. Consolidation or maintenance therapy, e.g. with rituximab, may help to prevent or delay clinical relapses [22–25]. If it would be possible to predict disease control after first-line therapy, these therapies could be selected for patients with a high likelihood of early relapse. With qualitative PCR, several studies were able to document a relationship between molecular response and time to relapse in patients treated with standard-dose chemotherapy [4, 5, 26], with high-dose chemotherapy [7, 27–30] and with rituximab-containing regimen [8, 31, 32]. Due to the low number of patients and the limited follow-up time of 6 months, we can only speculate on the role of molecular diagnostics for the prediction of relapsed disease. The two patients in our study relapsing within 6 months after therapy were positive in BM and PB with nested PCR at the end of therapy and could have been chosen to receive maintenance treatment. At the same time, positive results in patients who did not relapse occurred, albeit at low rates. With LightCycler® PCR, BM or PB samples of the two relapsing patients were not invariably positive at the end of therapy.

Quantitative PCR may offer additional benefits as it may (a) allow for the identification of patients with an increase in MRD after therapy, being at an increased risk of relapse [33–35] and (b) stratify patients according to the initial tumour load in BM, thereby predicting response to therapy and long-term outcome [36]. Our results suggest that the relatively low sensitivity of the LightCycler assay may be disadvantageous for the prediction of early relapse after therapy. This may be particularly relevant for rituximab-containing regimen where a single antibody infusion added to 6 cycles of CHOP chemotherapy resulted in MRD negativity in the majority of patients [37]. In contrast, nested PCR for the bcl2–IgH rearrangement might be better suited for predicting early relapse after primary therapy. Its use for decision making regarding maintenance therapy has to be validated in larger trials.

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