Combined polymerase chain reaction methods to detect *c-myc*/IgH rearrangement in childhood Burkitt´s lymphoma for minimal residual disease analysis

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KERSTIN BUSCH
ARNDT BORKHARDT
WILHELM WÖßMANN
ALFRED REITER
JOCHEN HARBOTT

A B S T R A C T

Background and Objectives. The translocation t(8;14)(q24;q32), involving the *c-myc* gene (8q24) and the immunoglobulin heavy chain (IgH) locus (14q32), represents about 75% of all chromosomal translocations in Burkitt´s lymphoma (BL). Due to the large variability of the breakpoint region, only the recently improved long-distance LD-polymerase chain reaction (PCR) allows the specific *c-myc*/IgH fusion to be identified at the genomic level. The sensitivity of the LD-PCR is only 10^{-2} to 10^{-3} due to the relatively large size of the amplification products (1 to 10 kbp). We, therefore, established a more sensitive nested PCR with a specific primer combination for each patient based on sequence analysis of the variant breakpoint regions.

Design and Methods. Using the combined PCR methods, we analyzed bone marrow and peripheral blood without visible blasts at diagnosis from 18 patients with t(8;14)-positive BL.

Results. In tests employing dilutions of genomic DNA from the BL cell line CA-46 in the T-cell lymphoma cell line KARPAS-299, which lacks the t(8;14), the sensitivity increased 100-fold, to 10^{-5}. However, the investigation of 18 *c-myc*/IgH-positive BL patients with each breakpoint-specific nested PCR showed an inter-patient variability of sensitivity between 10^{-3} and 10^{-5}. Using this assay, the rearrangement was detected in 4/16 bone marrow samples and in 6/15 peripheral blood samples without visible blasts at diagnosis.

Interpretation and Conclusions. Using the combined PCR methods the detection of *c-myc*/IgH reaches a level of sensitivity required for the evaluation of minimal residual disease (MRD) in BL patients. Furthermore, the results highlight the importance of verifying the patient-specific sensitivity level for individual MRD monitoring.

Key words: Burkitt´s lymphoma, *c-myc*/IgH translocation, PCR, MRD.

From the Dept. of Pediatric Hematology and Oncology, Children´s University Hospital, Giessen, Germany (KB, WW, AR, JH), Pediatric Hematology and Oncology, Dr. v. Haunersches Kinderspital, LMU, Munich, Germany (AB).

Correspondence: Kerstin Busch, MD, PhD, Children´s University Hospital, Dept. Pediatric Hematology/ Oncology, Feulgenstr. 12 D-35385 Giessen, Germany.
E-mail: kerstin.busch@paediat.med.uni-giessen.de

Burkitt`s lymphoma (BL)/ leukemia, mature high grade B-cell neoplasms, are the most frequent subtype of non-Hodgkin's Lymphoma (NHL) of childhood in western countries.[1,2] Recent multicenter therapeutic studies showed that the probability of event-free survival has increased to 90% even among patients with advanced stage disease.[3,4] However, the prognosis of the remaining patients who suffer a relapse is still very poor.[5,6] Strongly predictive prognostic parameters are needed in order to identify patients with a high risk of relapse early enough to allow therapy to be altered. Minimal residual disease (MRD) is a highly predictive prognostic parameter in childhood acute lymphoblastic leukemia (ALL).[7-9] Three types of techniques have been described for MRD analysis: flow-cytometric immunophenotyping, polymerase chain reaction (PCR) analysis of transcripts from fusion genes resulting from chromosomal translocations, and detection of clone-specific immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements by PCR amplification.[7-12] A sensitive and specific marker is needed to detect MRD; the marker should not alter during chemotherapy and relapses.

In 75% of BL/ leukemia cases characteristic translocations involving the *c-myc* gene on chromosome 8 and the Ig heavy or light chain, κ or λ genes on chromosome 14, 2, or 22 can be detected.[13-15] The t(8;14)(q24;q32) translocation is the most common genetic lesion (80% of all translocations), in which the *c-myc* gene is juxtaposed to the immunoglobulin heavy chain (IgH) gene on chromosome 14 in a divergent orientation,[16] whereas the t(2;8)(p11;q24) (15%) and t(8;22)(q24;q11) (5%) occur much more rarely. Recombinations

take place only at the DNA level and no fusion m-RNA or fusion-protein is generated.

The positions of the chromosomal breakpoints in the *c-myc* gene and various Ig genes are widely dispersed and individual for each patient; thus the *c-myc* /IgH translocation is a patient-specific molecular marker.

The *c-myc* /IgH rearrangement was detected at the genomic DNA level by the recently improved long distance (LD)-PCR, amplifying PCR products ranging from 1-10 kbp.[17-20] The sensitivity of this technique for the detection of MRD is limited, however, because of the varying size of the amplification products. In dilution experiments with a BL cell line, BL41, in Jurkat leukemic cells, a modified LD-PCR showed a sensitivity of 10^{-4}.[21] The sensitivity level might differ between patients because of the large variation of the amplification products in the LD-PCR. We, therefore, established a PCR assay combining the LD-PCR with a nested PCR using breakpoint-specific primer pairs generated after sequencing the LD-PCR products.

Design and Methods

Patients

Tumor biopsy material, bone marrow, peripheral blood, lymph nodes or ascites from 118 children suffering from BL/ leukemia (collected from April 1996 until December 2002) and enrolled into the NHL-BFM 95 multicenter study were examined at diagnosis.

Isolated tumor cells were obtained by scraping the fresh tumor tissue with a scalpel onto a cell strainer with a pore size of 70 μm. Mononuclear cells were isolated from the tumor material using Lymphoprep (Axis Shield, Oslo, Norway). After centrifugation, the mononuclear cells were removed from the interface, washed twice with 5 mL phosphate-buffered saline (PBS) pH 7.2, resuspended in completed RPMI-medium supplemented with 10% DMSO or PBS and stored in liquid nitrogen prior to use.

Cell lines

The human BL cell line, CA-46, and the human T-cell lymphoma cell line, KARPAS-299, were purchased from the cell bank of the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany). Cell lines were maintained in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum (Gibco BRL, Karlsruhe, Germany) in a humidified atmosphere with 5% CO_2 at 37°C.

DNA preparation

High molecular weight genomic DNA was prepared from cultured cell lines and frozen mononuclear cells by washing them twice with PBS before using the QIAamp DNA or DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the instructions provided by the manufacturer. The concentration and purity of the DNA were determined by measuring the absorbance at 260 and 280 nm.

Primers for LD-PCR

To detect the rearrangement involving the *c-myc* gene on chromosome 8q24 and the IgH locus on chromosome 14q32, one primer for the *c-myc* gene (*c-myc*/M6 at position 4885 in exon 2) and four primers for the IgH locus were combined: three primers for the constant region (Cμ03, Cγ02, Cα01) and one for the joining region (JH) as previously described (Table 1).[20] Primers for both genes represent the antisense strands in reverse direction, due to the head-to-head orientation of *c-myc* and IgH genes (indicated by arrows in Figure 1).

The primers *c-myc*/M6 and *c-myc*/M9 (at position 8151 in intron 3) are positioned downstream (Table 1 and Figure 1). The quality of the genomic DNA and adequacy of sample for the amplification of long DNA fragments were tested in each sample by using *c-myc*/M6 and *c-myc*/M9 primers together with an upstream primer, *c-myc* up (at position 1), which yielded PCR products of 4.9 kbp and 8.2 kbp, respectively.

Long-distance PCR

Each reaction mixture (50 μL) contained 250 ng of genomic DNA, 300 nM of downstream and upstream primer, 500 μM of each dNTP, buffer III with 22.5 nM $MgCl_2$ and 2.6 units of a polymerase mix as indicated in the Expand Long Template PCR System Kit (Roche, Mannheim, Germany). Reaction conditions were as follows: denaturation at 94°C for 2 minutes followed by 10 cycles of denaturation at 94°C for 30 seconds, annealing at 68°C for 30 seconds, extension at 68°C for 4 minutes, followed by 20 identical cycles with gradual increments of the extension time (15 s/cycle) and a final extension for 10 minutes at 68°C. PCR reactions were performed in a Thermal Cycler 9600 (Applied Biosystems, Darmstadt, Germany). PCR products were analyzed by agarose gel electrophoresis (1% agarose in TAE buffer stained with ethidium bromide) and visualized under UV illumination.

PCR product purification and sequencing

For sequence analysis further LD-PCR reactions were performed using *c-myc* primers upstream of *c-myc*/M6 (Table 1). For these tests five *c-myc* primers (*cmyc*/M5, *c-myc*/M4, *c-myc*/M2, *c-myc*/M1, *c-myc*/M0) were combined with the IgH primer detecting the *c-myc*/IgH translocation. These primers were combined with *c-myc* up primer (at position 1) as amplification controls of the normal *c-myc* allele. The PCR products

Table 1. Primers used for LD-PCR and *narrowing* the breakpoint region.

	Position	*c-myc – primer - downstream*
c-myc/M9	8151	5´- GAG ATC CTC TGG GGT TTG CGA GAT AAC CCA TGG – 3´
c-myc/M6	4885	5´- ACA GTC CTG GAT GAT GAT GTT TTT GAT GAA GG TCT – 3´
c-myc/M5	4458	5´- AGC GGG AGG CAG TCT TGA GTT AAA GGG GTC – 3´
c-myc/M4	3250	5´- TAC TCC AAG GAG CTC AGG ATG CAA GGG GC – 3´
c-myc/M2	2410	5´- CTC CGT TCT TTT TCC CGC CAA GCC TCT GAG AAG – 3´
c-myc/M1	1611	5´- GCT ATC TGG ATT GGA TAC CTT CCA CCC AGA CTG – 3´
c-myc/M0	829	5´- CCC AGC CCC ACA CAT GAT TTG TTT GCT CCC TG – 3
	Position	*c-myc – primer – upstream*
c-myc up	1	5´- AGC TTG TTT GGC CGT TTT AGG GTT TGT TGG – 3´
		IgH – Primer
Cμ/03		5´- TGC TGC TGA TGT CAG AGT TGT TCT TGT ATT TCC AG – 3`
Cγ/02		5´- AGG GCA CGG TCA CCA CGC TGC TGA GGG AGT AGA GT – 3´
Cα/01		5´- TCG TGT AGT GCT TCA CGT GGC ATG TCA CGG ACT TG – 3´
JH		5´- ACC TGA GGA GAC GGT GAC CAG GGT – 3´

The location of the c-myc primers in the c-myc gene are indicated as positions. The c-myc/M6 and IgH primers are as described by Basso et al.[20]

containing the *c-myc*/IgH breakpoint were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Concentrations of the purified PCR products were determined by measuring the absorbance at 260 and 280 nm, whereas the quality was determined by a 1% agarose gel. Sequence analysis of the PCR products was performed with *c-myc*-specific primers (*c-myc*1 to *c-myc*16) upstream of the primer *c-myc*/M6 to obtain the specific *c-myc*/IgH breakpoint region (Table 2) (commercially done by GATC Biotech, Konstanz, Germany). The sequences thus obtained were analyzed by BLAST homology search (*www.ncbi.nlm.nih.gov/BLAST/*) for human *c-myc* proto-oncogene (ACC.No. J00120) as well as for the DNA sequence of the human immunoglobulin D segment locus (ACC.No. X97051), human IgA C α1 switch region (Sα1) (ACC.No. L19121), Igαγ heavy-chain, membrane-bound-type and secrete-type (ACC.No. D78345) and human Mu switch DNA of the immunoglobulin heavy-chain gene locus (ACC.No. X54713) to identify the breakpoint region. The sequences of the identified *c-myc*/IgH rearrange-

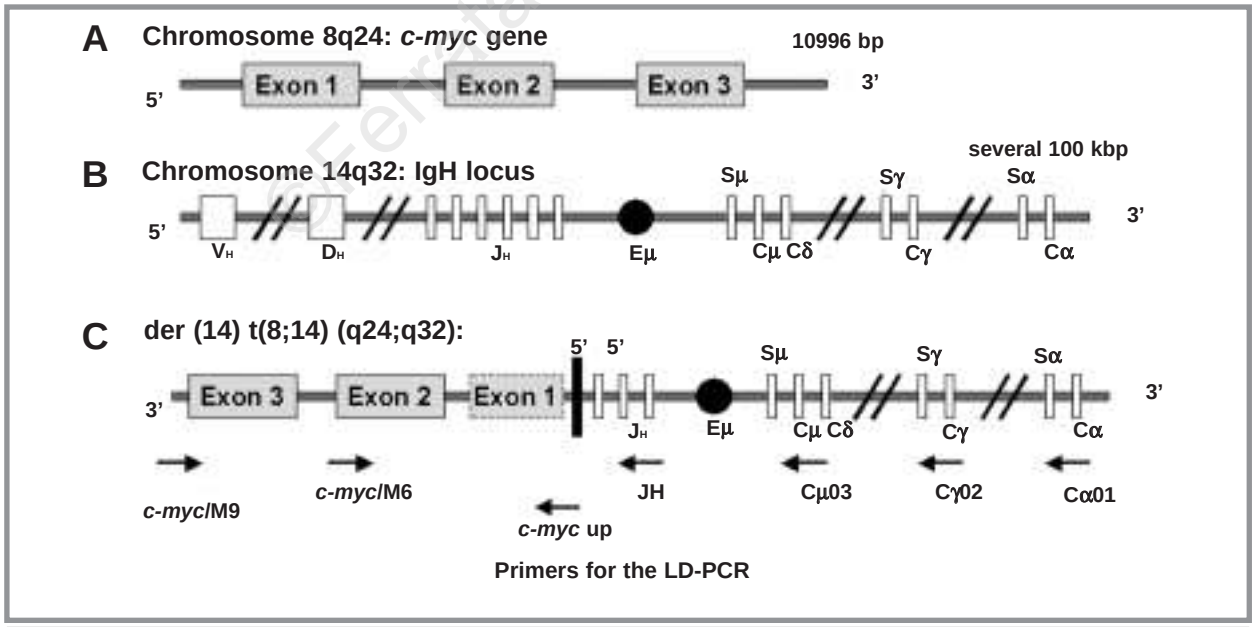

Figure 1. Model of the *c-myc*/IgH rearrangement and detection by LD-PCR (A) Map of the human *c-myc* gene on chromosome 8 and (B) of the IgH locus on chromosome 14. (C) Example of recombination involving the immunoglobulin JH region and the 5´ region of the *c-myc* gene. The translocation occurs head to head (5´-5´) between the two loci. The primers in both segments are indicated by arrows. The combination of *c-myc*/M6 or *c-myc*/M9 with *c-myc* up primer at position 1 was used to check the quality of the genomic DNA and amplification.

Table 3. Detection of *c-myc*/IgH fusion at diagnosis in BL patients without microsocpic evidence of FAB-L3 blasts in bone marrow and peripheral blood.

Patient No.	Sex/Age at Diagnosis (years)	Stage/LDH prior to therapy (U/L)	c-myc/IgH breakpoint			c-myc/IgH fusion detected by nested PCR		Sensitivity level	Clinical outcome (weeks after diagnosis)
			Position in the c-myc gene	Region in the IgH locus	Accession No.	Bone marrow	Peripheral blood		
1	m/ 7.7	III/ 969	2806	switch α	AJ586971	neg.	positive	10^{-3}	relapse (16)
2	m/ 8.6	III/ 1408	2872	JH	AJ601405	positive	positive	10^{-4}	87 (FOP)
3	m/ 14.2	III/ 469	2365	switch μ	AJ601398	neg.	positive	10^{-3}	208 (FOP)
4	m/ 8.9	III/ 2240	2775	switch μ	AJ601396	positive	n.r.	10^{-4}	188 (FOP)
5	m/ 9.0	IV/ 1390	2452	JH	AJ601397	n.r.	positive	10^{-3}	relapse (22)
6	m/ 14.4	III/ 1962	2398	switch μ	AJ601399	neg.	positive	10^{-4}	10 (FOP)
7	m/ 10.1	IV/ 291	3281	JH	AJ586974	positive	positive	10^{-3}	25 (FOP)
8	m/ 12.4	III/ 280	2066	switch γ	AJ601403	positive	n.r.	10^{-4}	66 (FOP)
9	m/ 4.3	III/ 546	3140	switch α	AJ601404	neg.	neg.	10^{-3}	208 (FOP)
10	f/ 15.4	III/ 327	2889	switch α	AJ601400	neg.	neg.	10^{-3}	relapse (14)
11	m/ 5.3	III/ 996	2365	JH	AJ586975	neg.	neg.	10^{-4}	58 (FOP)
12	m/ 4.1	III/ 525	2037	switch α	AJ586976	neg.	neg.	10^{-5}	15 (FOP)
13	m/ 7.3	III/ 185	2977	switch μ	AJ601402	n.r.	neg.	10^{-4}	188 (FOP)
14	m/ 5.8	II/ 434	2156	switch α	AJ586973	neg.	n.r.	10^{-4}	92 (FOP)
15	m/ 12.2	II/ 140	3467	switch μ	AJ586978	neg.	neg.	10^{-4}	relapse (26)
16	m/ 6.3	III/ 300	3879	switch α	AJ586977	neg.	neg.	10^{-3}	96 (FOP)
17	m/ 3.8	II/ 204	1937	switch γ	AJ601401	neg.	neg.	10^{-3}	13 (FOP)
18	m/ 9.7	II/ 226	2838	switch α	AJ586972	neg.	neg.	10^{-4}	12 (FOP)

Sex ratio: m (17), f (1): median age: 8.75; range 4.1-15.4; median LDH : 451.5 U/L; range 185-2240 U/L; m: male; f: female; LDH: lactate dehydrogenase; U/L: units per liter; staging according to Murphy;[23] neg. : negative; n.r.:no result; FOP: freedom from progression at latest examination.

ments were submitted to the EMBL Nucleotide Sequence Databank (*www.ebi.ac.uk/submission/webin.html*). The assigned accession numbers are listed in Table 3 according to the *c-myc*/IgH breakpoint.

Nested PCR

Selection of different primers close to the individual *c-myc*/IgH breakpoint allowed a sensitive nested PCR (Figure 2). In this case four primers were selected to amplify PCR products ranging in size from 80 to 400 bp: two primers specific for the *c-myc* gene (1st and 2nd run), one primer specific for the IgH gene (1st run) and another one overlapping the breakpoint region (2nd run). For BL patients with *c-myc* breakpoints very close to each other, the same primers within the *c-myc* gene could be used for the breakpoint-specific nested PCR, in contrast to the necessity of having the breakpoint-specific primers located within the IgH regions for each patient individually. The PCR reaction mixture (20 μL) contained 100 ng genomic DNA template, 1.6 pmol of each outer primer, 16 nmol $MgCl_2$, PCR reaction buffer and 1 unit Taq polymerase (Invitrogen, Karlsruhe, Germany) during the first run. After an initial melting step (1.5 minutes at 94°C), PCR products were amplified in 35 cycles of 15 seconds at 94°C for denaturation, 45 seconds at 64°C for annealing and 45 seconds at 72°C for elongation. PCR product (1 μL) of the first run was passed through the second run of PCR, containing 8 pmol of each internal primer and reducing the number of cycles to 25 under the same conditions. In order to check the quality of DNA, the *c-myc* gene was also amplified in a separate single PCR.

PCR reactions were performed in a Thermal Cycler 9600 (Applied Biosystems, Darmstadt, Germany) and analyzed on a 1% agarose gel.

Table 2. Primers for sequencing LD-PCR products.

	Position	c-myc – primer - downstream
c-myc 1	321	5´-GCG CCT GGA TGT CAA CGA GG-3´
c-myc 2	616	5´- GCA TGT ACG CTG TTC AAG ATG G-3´
c-myc 3	901	5´- CAC TGT ATG TAA CCC GCA AAC G-3´
c-myc 4	1215	5´- GCA AGT GGA GAG CTT GTG GAC-3´
c-myc 5	1516	5´- GCG GGA GGC GTC TGT TTA G-3´
c-myc 6	1829	5´- GAA GGG TAT TAA TGG GCG CG-3´
c-myc 7	2142	5´- CGA GCA CTC TAG CTC TAG G-3´
c-myc 8	2491	5´- CGC TGG AAT TAC TAC AGC GAG-3´
c-myc 9	2724	5´- CCT TGC TCG GGT GTT GTA AGT-3´
c-myc 10	3061	5´- CCT CTC CCA TCT TGA CAA GTC AC-3´
c-myc 11	3359	5´- GTC AGA AAT GCG GTG AGC CG-3´
c-myc 12	3656	5´- CAT CTC GGG CGC GAG GAC-3´
c-myc 13	3958	5´- CCA ACC TTC CCT CTC CAC CG-3´
c-myc 14	4279	5´- CTT AGT GAA CCA GCG GCT TG-3´
c-myc 15	4559	5´- TGC ACC GAG TCG TAG TCG AGG-3´
c-myc 16	4856	5´- GGT CGC AGA TGA AAC TCT GG-3´

The positions of the primers c-myc 1 – c-myc 16 in the c-myc gene are indicated.

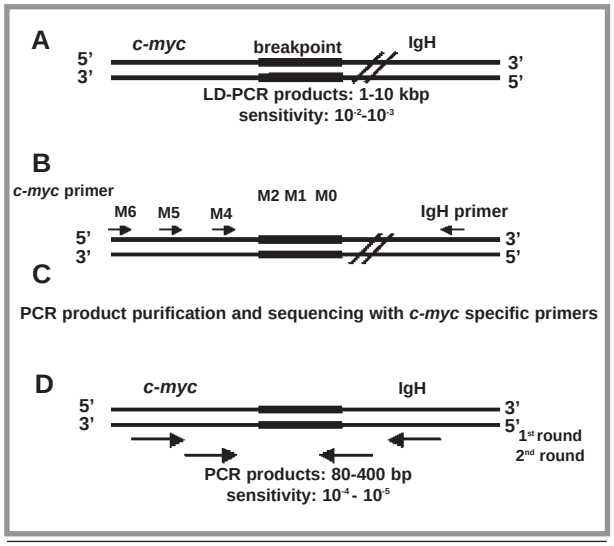

Figure 2. Schematic presentation of the combined PCR methods for the detection of *c-myc*/IgH fusions. (A) LD-PCR. (B) *Narrowing* of the breakpoint region. (C). PCR products were purified, then sequenced with *c-myc* specific primers. (D). Nested PCR with specific primer combinations flanking the breakpoint region.

Sensitivity and specificity testing

In order to evaluate the sensitivity of the described PCR methods, genomic DNA from the BL cell line CA-46 was diluted 10^{-1} to 10^{-6} in genomic DNA from KARPAS-299 cells, which lack the *c-myc*/IgH fusion. For comparison, dilutions of CA-46 cells in KARPAS-299 cells were made in the same way, followed by genomic DNA isolation. All dilution steps were tested with the LD-PCR as well as the nested PCR with breakpoint-specific primers (Figure 3). The sensitivity level of the patient-specific primer combination was tested for each *c-myc*/IgH-positive patient in the nested PCR by dilution of the genomic DNA from the tumor biopsy by 10^{-1} to 10^{-6} in genomic DNA from patients lacking the *c-myc*/IgH fusion (Table 3).

Additional PCR reactions were always performed for each breakpoint-specific primer combination with genomic DNA from six patients lacking the *c-myc*/IgH fusion, to determine the specificity of the nested PCR.

Results

Detection of c-myc/IgH rearrangements

In 78 of 118 tumor probes (66%) from childhood BL/leukemia patients a *c-myc*/IgH rearrangement was detected using the *c-myc*/M6 primer (positioned in exon 2) in the described LD-PCR. The PCR products thus revealed ranged in size from 1 to 10 kbp. The 40 LD-PCR-negative patients were tested with *c-myc*/M9 primer (positioned in intron 3) in combination with the four IgH primers to establish whether breakpoints could be located downstream of exon 2 in the *c-myc* gene. No PCR product could be obtained with these primer combinations, indicating that the LD-PCR-negative patients had no detectable breakpoints between exon 2 and intron 3 in the *c-myc* gene. The DNA quality control was checked using the *c-myc*/M6 primer and the *c-myc*/M9 primer combined with *c-myc* up, revealing PCR products of 4.9 kbp and 8.2 kbp, respectively. The BL cell line CA-46 was also tested with *c-myc*/M9 combined with Cα01 as well as with *c-myc* up as a positive control.

Verification of the sensitivity level

For the analysis of MRD, we performed a test to verify the sensitivity level. Dilutions of genomic DNA from the BL cell line CA-46 in the genomic DNA from KARPAS-299 cells, lacking the *c-myc*/IgH fusion, were tested by LD-PCR using the primer *c-myc*/M6 combined with Cα01. The PCR product representing the *c-myc*/IgH translocation of CA-46 cells was 4.2 kbp and was detectable at a sensitivity level of 10^{-2} - 10^{-3} (Figure 3A, lanes 5 and 6). A very faint signal was visible for the 10^{-4} dilution (Figure 3A, lane 7). The same result was obtained for the comparative test using dilutions of CA-46 cells in KARPAS-299 cells followed by genomic DNA isolation.

Further PCR reactions with *c-myc* primers upstream of exon 2 combined with the respective IgH primer (Cα01) of the CA-46 *c-myc*/IgH rearrangement were performed for sequence analysis. The resulting PCR products narrowed the individual breakpoint region as closely as possible on the *c-myc* gene (Figure 3). PCR products were obtained with the *c-myc* primers M6, M5 and M4 (lanes 8, 9 and 10), whereas no amplification was found for *c-myc* primers M2, M1 and M0 (lanes 11, 12 and 13). The PCR products from lanes 9 and 10 were purified and sequenced with *c-myc* 11 and 12, revealing the breakpoint at position 3183 in the *c-myc* gene, as described in 1985 by Showe *et al.*[22] A specific primer combination was created, flanking the breakpoint of CA-46, for the nested PCR. The detection level of *c-myc*/IgH rearrangement increased to 10^{-5} as shown in Figure 3C, lane 8. When the same experiments were performed with the dilution of CA-46 cells in KARPAS-299 cells, a sensitivity level of 10^{-5} was also obtained.

The dilution of genomic DNA containing t(8;14)(q24;q32), from 18 BL patients (Table 3), in genomic DNA from patients lacking the *c-myc*/IgH fusion revealed a sensitivity level of 10^{-3} - 10^{-5} in the nested PCR (Table 3). For the verification of specificity, each specific primer combination was always tested in six patients who lacked the t(8;14)(q24;q32), as negative controls.

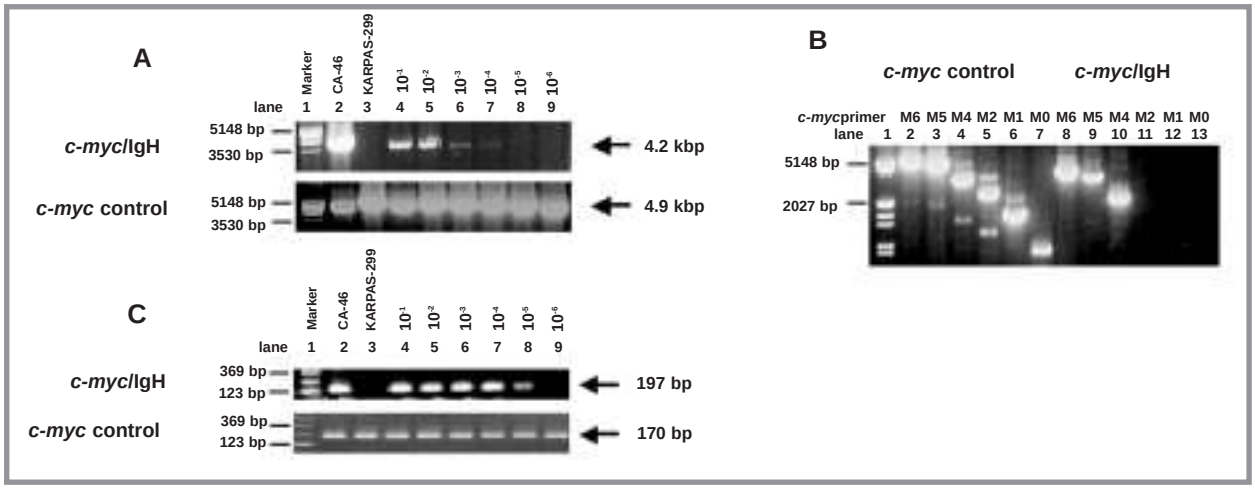

Figure 3. Detection of the sensitivity level of the combined PCR methods. Genomic DNA from the BL cell line CA-46 was diluted from 10^{-1} to 10^{-6} in genomic DNA of the cell line KARPAS-299, which lacks the *c-myc*/IgH rearrangement. Internal primers of the *c-myc* gene combined with the *c-myc* up primer at position 1 were used for amplification controls and to check the quality of the DNA. (A). Sensitivity of the LD-PCR (positive until dilution 10^{-3}, lane 6). (B). *Narrowing* of the breakpoint region (positive with *c-myc* primers M6, M5 and M4 in lanes 8-10). (C). Nested PCR with specific primer combinations flanking the breakpoint region (positive until dilution 10^{-5}, lane 8).

Detection of c-myc/IgH fusions in BL patients at diagnosis

The bone marrow and peripheral blood from 18 BL patients with a detectable *c-myc*/IgH rearrangement were investigated at diagnosis with the combined PCR methods described above. Staging was performed according to Murphy.[23] Stage IV disease was diagnosed in patients #5 and #7 because of initial central nervous system (CNS) involvement. Cytogenetic data were only available for 10 of the 18 patients. A translocation t(8;14)(q24;q32) was detected in 6 patients by karyotyping and in 4 patients by fluorescent *in situ* hybridization (FISH) analysis.

No FAB-L3 blasts were detectable at the microscopic level in either the bone marrow or in the peripheral blood of any of these 18 BL patients. The positions of the specific *c-myc*/IgH breakpoints within the *c-myc* gene and the region in the IgH locus are presented in Table 3 according to the assigned accession numbers of the breakpoint sequences. The analysis of each *c-myc*/IgH rearrangement was performed with the individual breakpoint-specific primer combinations. Table 3 shows the results of the nested PCR in comparison with the clinical outcome of these BL patients.

Tumor cells with the specific *c-myc*/IgH rearrangement were detectable in 4/16 samples of bone marrow and 6/15 samples of peripheral blood without visible blasts (Table 3). Two patients were *c-myc*/IgH positive in the bone marrow as well as in the peripheral blood. For 5 patients (#4, 5, 8, 13 and 14) we could only evaluate either bone marrow or peripheral blood due to poor quality DNA or unavailability of samples. Nevertheless, in these patients the *c-myc*/IgH translocation was identified in 2 bone marrow samples, and 1 peripheral blood sample (Table 3).

Two of the 8 PCR-positive patients relapsed, while 2 out of 10 patients without detectable *c-myc*/IgH fusion at diagnosis also relapsed. Bone marrow and/or peripheral blood was available from all the relapsed patients at the time of their relapse (patients #1, 5, 10 and 15). The investigation with the breakpoint-specific primer combinations confirmed the same PCR product for each patient at diagnosis as well as at the time of relapse. It follows that no genetic rearrangement had occurred in the individual *c-myc*/IgH breakpoint between first diagnosis and relapse.

Discussion

The translocation t(8;14)(q24;q32) is the most common chromosomal abnormality in BL/ leukemia. In this translocation the *c-myc* gene on chromosome 8 is juxtaposed to the Ig heavy chain gene on chromosome 14 and this rearrangement represents a specific marker of the disease. The genomic analysis of *c-myc*/IgH rearrangement at diagnosis may serve as a disease- and patient-specific marker for MRD during follow-up. The *c-myc*/IgH fusions are located over a wide range on both chromosomes and can be detected by means of the previously described LD-PCR.[20,24] In 78 of 118 (66%) pediatric patients from the NHL-BFM 95 trial the t(8;14)(q24;q32) was detected with LD-PCR using the primer combinations described by Basso *et al.*[20]

The breakpoints in the *c-myc* gene of all 78 positive patients were located upstream of exon 2. Further investigation of the LD-PCR-negative patients with primer combinations of *c-myc*/M9 with the four IgH

primers revealed no PCR products, indicating no breakpoints between exon 2 and intron 3 in the *c-myc* gene. In view of the variability of the breakpoint regions on both chromosomes, *c-myc*/IgH rearrangements could be localized outside the range of the described primer combinations. The use of more primers from different regions of the IgH gene, as well as from the Igκ and Igλ genes, will be required in further LD-PCR experiments to extend the range of verifiable c-myc/IgH fusions and the variant translocations, t(2;8) and t(8;22).[25]

Therefore, cytogenetics and FISH analysis are still important tools to demonstrate the t(8;14)(q24;q32) and the variant translocations t(2;8)(p11;q24) and t(8;22)(q24;q11), as well as secondary aberrations. However, the sensitivity of these techniques is not sufficient for MRD studies. The LD-PCR is not only an additional method to verify the *c-myc* gene fused to one of the Ig genes, but in combination with a nested PCR it is an appropriate assay for MRD analysis.

After sequencing the LD-PCR products, we were able to produce primers flanking the patient-specific breakpoint and to use them for a nested PCR. Using this assay, the sensitivity level was increased up to 10^{-5} for a dilution of the BL cell line CA-46 in the T-cell lymphoma cell line, KARPAS-299 (Figure 3). The variability of the detection level, which was between 10^{-3}-10^{-5} for different patients, might be caused by the amount of infiltrated malignant cells within the initial lymph node samples (Table 3). Nevertheless, these results reach the required sensitivity for MRD analysis of being able to detect one malignant cell in 10^{3}-10^{6} normal hematopoetic cells.[7,10] In a recent study, the modification of the LD-PCR had a detection sensitivity of 10^{-4}, although shown with dilutions of t(8;14)-positive BL41 cells in the leukemic Jurkat cell line.[21] However, due to the large variability of the amplification products within the LD-PCR, the sensitivity level might be lower *in vivo* and differ between patients.

We are, therefore, convinced that the sensitivity level must be defined for each breakpoint-specific primer combination by the amplification of small PCR fragments in a nested PCR. Thus, the patient-specific sensitivity must be determined for MRD monitoring. The great importance of this procedure for the prognostic value of MRD in ALL in childhood has already been shown by van Dongen *et al.*[7]

To clarify the significance of these combined PCR methods for detecting residual malignant cells, samples of bone marrow and/ or peripheral blood from 18 t(8;14)-positive BL patients without visible blasts at diagnosis were investigated. In 8 of the 18 patients the specific *c-myc*/IgH rearrangement was detected not only in bone marrow (4/16) but also in peripheral blood samples (6/15). Interestingly, in 2 of the 4 bone marrow-positive cases the peripheral blood was also positive, whereas in the remaining 2 cases no peripheral blood was available. In contrast, in 3 of the 6 peripheral blood-positive cases the bone marrow was negative. Thus, it seems to be very important to analyze the peripheral blood in comparison to the bone marrow as follow-up controls for monitoring of residual malignant cells. In 10 patients no *c-myc*/IgH translocation could be detected either in bone marrow or in peripheral blood. The clinical relevance for patients with an identified molecular involvement in the bone marrow or peripheral blood at diagnosis remains to be established in a larger series of patients.

The sensitive method for detecting *c-myc*/IgH rearrangement described here should make it possible to evaluate a minimal number of residual lymphoma cells during therapy, which could be predictive of relapse. Further investigations with a quantitative real-time PCR assay will be necessary to determine the kinetics of minimal residual lymphoma cells. The impact of information from quantitative real-time PCR has recently been shown for MRD analyses in adult patients with follicular lymphoma (FL) in whom the *bcl-2*/IgH fusion gene represents the tumor-specific chromosomal translocation t(14;18).[26-28] and in adult patients with multiple myeloma (MM) in whom the allele-specific oligonucleotide PCR for IgH has been used.[29-31]

Molecular detection of disease has been shown to be a strong predictor of relapse in childhood ALL by the International BFM Study Group,[7-9] using quantitative real-time PCR analysis of patient-specific Ig and TCR rearrangements.[32,33] Patient-specific Ig and TCR rearrangements in ALL are clone-specific and might be rearranged during the course of disease.[12,34,35] Genetic markers for MRD analysis should be stable in relapses and resistant to chemo- and radiotherapy, as has been shown for the BCR/ABL fusion gene in chronic myelogenous leukemia and for AML1/ETO in acute myeloid leukemia.[36,37] In our study, investigation of 4 *c-myc*/IgH positive BL patients at diagnosis and at relapse with the breakpoint-specific primer combination confirmed the same PCR product for each patient, demonstrating the stability of c-myc/IgH.

In summary, the additional nested PCR is a valuable tool, sensitive enough to detect minimal residual lymphoma cells with patient-specific sensitivity and to address two clinically important questions: (i) do the kinetics of the initial disappearance of MRD predict outcome of patients, as is the case for ALL, and (ii) can relapses be detected at a molecular level, possibly increasing the chance for successful rescue therapy.

Finally, the ability to detect *c-myc*/IgH fusions with a high level of sensitivity makes it possible for lymphoma cell contamination to be evaluated in autologous stem cells collected for autologous transplantation.

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