Technical Advance

Improved Long-Distance Polymerase Chain Reaction for the Detection of t(8;14)(q24;q32) in Burkitt's Lymphomas

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The t(8;14)(q24;q32), involving MYC gene (8q24) and the immunoglobulin heavy chain (IgH) locus (14q32), represents about 75% of all translocations in Burkitt's lymphoma (BL). Due to the great variability of the breakpoint region, a standard polymerase chain reaction assay is not sufficient for the detection of this chromosomal translocation. The availability of new and more efficient DNA polymerases that allow the amplification of genomic fragments many kilobase-pairs long, makes it possible to identify the t(8;14) in BL cells by long-distance polymerase chain reaction (LD-PCR). We have established a simplified and efficient LD-PCR for the detection of t(8;14)(q24;q32) that relies on the use of one primer specific for MYC exon II combined, in different reactions, with four primers for the IgH locus: three for the constant regions C μ , C γ , and C α , and one for the joining region (JH). We first studied seven BL cell lines and optimized LD-PCR reaction for analysis of tumor specimens. Five of seven cell lines were positive for the t(8;14), whereas two lines derived from endemic BL were negative, as expected. Of 15 biopsies obtained from pediatric BL and subsequently analyzed, 13 (87%) were positive for the translocation detected by LD-PCR and showed a product ranging in size from 2.0 to 9.5 kb. C μ region was involved in 6 cases, C γ and C α in 2 cases each, and JH in 3 cases. Interestingly, 2 of the tumors positive for JH showed a second, larger PCR product with the C α - and C γ -specific primer, respectively. We established that our LD-PCR method could detect 10⁻³ BL cells within a population of hematopoietic cells lacking the translocation. In conclusion, our LD-PCR method represents a fast, highly sensitive, and specific tool to study sporadic BL and to detect minimal disease and residual disease in patients affected by

t(8;14)-positive lymphomas. (*Am J Pathol* 1999, 155:1479-1485)

Burkitt's lymphoma (BL), the most frequent non-Hodgkin's lymphoma of childhood,¹ is characterized by specific chromosomal translocations involving the MYC gene on chromosome 8 and the immunoglobulin (Ig) heavy or light chain genes on chromosome 14, 22, or 2.²⁻⁴ The most common translocation, accounting for 75% of the total, is t(8;14)(q24;q32), in which the MYC gene is juxtaposed to the immunoglobulin heavy chain (IgH) gene on chromosome 14 in divergent orientation.

Molecular analysis of the genomic sequences involved in this translocation has demonstrated that the breakpoints occur in different regions within both the MYC and the IgH genes and give rise to tumor-specific MYC/IgH rearrangements. On chromosome 8, breakpoints are usually located upstream of the MYC gene in the endemic (African) BL and within exon 1 or intron 1 in the sporadic (Caucasian) BL.⁵ The breakpoints within the IgH locus are distributed over a region of at least 100 kb, but they are preferentially located in the Ig joining region (JH) or in the switch regions (S μ or S γ) in the endemic and in the sporadic BL, respectively.^{6,7}

The MYC/IgH rearrangement is detectable only at the genomic DNA level, because no fusion transcript originates from the chromosomal translocation. Differently, the relevant molecular event generated by the t(8;14) is the deregulation of the translocated MYC gene, which results in the enhancement of its oncogenic potential.^{4,8-10}

Regardless of the endemic or sporadic origin of the BL, the MYC and IgH genes are juxtaposed in divergent

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orientation on the derivative chromosome 14q+ and the breakpoints on the partner chromosomes are distributed over a large region.¹ This is the main reason why, in the past, molecular analysis of t(8;14) was performed by Southern blot technique, whereas polymerase chain reaction (PCR) was limited to selected cases in which the breakpoint could have been well characterized previously.¹¹

The recent availability of more efficient DNA polymerases, which can yield products several kilobasepairs long from genomic DNA target sequences, has allowed the detection of a higher percentage of junctional sequences by using a limited number of primer combinations.^{12,13} In a series of pediatric BL and B-cell acute leukemia (B-ALL), 11 of 20 cases were positive for the long-distance PCR (LD-PCR) product combining 7 primers for the MYC locus and 5 for the IgH locus in different reactions.¹³

Because the t(8;14)(q24;q32) represents a unique characteristic of BL and can be used as a marker for the detection of Burkitt's cells, we established a LD-PCR based on the use of one MYC-specific primer, recognizing a region in exon 2, three primers for the constant IgH regions C μ , C γ , and C α , and one for the JH region. The assay was first used to study 7 Burkitt's cell lines and then applied to the analysis of 15 tumor samples obtained from children affected by BL.

We demonstrated that our LD-PCR method, which relies on a limited number of primers, has a high sensitivity and can be used efficaciously for the diagnosis and for the study of bone marrow micrometastases, as well as minimal residual disease.

Materials and Methods

Cell Lines and Tumor Samples

The BL cell lines BL41, DAUDI, DG75, P3HR1, RAJI, and RAMOS were kindly provided by Dr. R. Dolcetti (CRO, Aviano, Italy), and CA46 cells were a gift of Dr. I. Magrath (National Cancer Institute, Bethesda, MD). They have been previously described.^{11,14-18} The T-cell leukemia cell line Jurkat and the rhabdomyosarcoma line RH30 were purchased from the American Type Culture Collection (Manassas, VA); the anaplastic large cell lymphoma cell lines SUDHL-1 and KARPAS-299 were purchased from the DSMZ cell bank (Braunschweig, Germany). Cell lines were maintained in RPMI-1640 medium (Life Technologies, Milan, Italy) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal calf serum (Seromed, Berlin, Germany) in humidified atmosphere with 5% CO₂ at 37°C. *Mycoplasma* test performed on each cell line was negative.

Tumor specimens were obtained at diagnosis from unselected children with BL enrolled in the non-Hodgkin's lymphoma trial of the Italian Association of Pediatric Hematology and Oncology (AIEOP). Each patient was identified by a number to protect confidentiality. Tissue was frozen and stored at -80°C until used.

Table 1. Sequence of the Primers Used for LD-PCR

Primer	Sequence 5'-3'
MYC/04	ACAGTCCTGGATGATGATGTTTTGATGAAGGTCT
C μ /03	TGCTGCTGATGTCAGAGTTGTTCTTGTATTTCAG
C γ /02	AGGGCACGGTCACCACGCTGCTGAGGGAGTAGAGT
C α /01	TCGTGTAGTGCTTCACGTGGCATGTCACGGACTTG
JH	ACCTGAGGAGACGGTGACCAGGGT

DNA Extraction

High molecular weight genomic DNA was prepared from cultured cell lines and frozen tissue using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) following instructions of the manufacturer.

LD-PCR

Quality of genomic DNA and adequacy of sample for the amplification of long DNA fragments were tested in each case by using a set of primers of the Human tPA Control Primer Set (Boehringer Mannheim, Mannheim, Germany), which yield PCR products of 4.8, 9.3, and 15 kb, respectively. To detect the translocation involving the MYC and the IgH genes we used 1 primer for the MYC gene (MYC/04)¹² combined, alternatively, with 4 primers for the IgH locus: 3 primers for the constant regions (C μ /03, C γ /02, C α /01)¹² and 1 for the joining region (JH).¹³ Primers for both genes represent the antisense strands in reverse direction, due to the head-to-head orientation of MYC and IgH genes. Primer sequences are reported in Table 1.

Each reaction mixture (50 μ l) contained 250 ng of genomic DNA, 60 pmol of each primer, and amounts of dNTPs, buffer III, and a mix of *Taq* and Pwo polymerases as indicated in the Expand Long Template PCR System Kit (Boehringer Mannheim). PCR was performed in a Thermal Cycler 9600 (Perkin Elmer, Norwalk, CT). Reaction conditions were as follows: denaturation at 94°C for 2 minutes followed by 10 cycles of denaturation at 94°C for 10 seconds, annealing at 65°C for 30 seconds, extension at 68°C for 4 minutes, and then 20 identical cycles with gradual increment of extension time (20 seconds/cycle) and a final extension for 10 minutes at 68°C. PCR products were analyzed by agarose gel electrophoresis (0.8% agarose) and visualized under UV illumination after ethidium bromide staining.

Southern Blotting

After gel electrophoresis, PCR products were transferred onto positively charged nylon membrane (Boehringer Mannheim) and subsequently hybridized to a MYC-specific oligonucleotide probe (5'-TCGCTCTGCTGCTGCTGCTGG-3') recognizing a sequence of MYC exon 2 and previously described as MYC/06.¹² It was labeled with digoxigenin by using the Dig oligonucleotide 3'-end Labeling Kit (Boehringer Mannheim) following the manufacturer's instructions. Hybridization signals were detected by chemiluminescence (CDP-Star, Boehringer Mann-

Chromosome 8q24: MYC gene

Chromosome 14q32: IgH locus

Sporadic BL: derivative chromosome 14q+

Figure 1. Schematic representation of t(8;14)(q24;q32) in sporadic BL. The breakpoint on chromosome 8q24 usually occurs in the region of exon 1 or intron 1 of the MYC gene, which is juxtaposed to the immunoglobulin heavy-chain (IgH) gene on chromosome 14q32, in opposite orientation. In the chromosome 14q+ scheme, **arrows** indicate the regions recognized by each primer used in our study.

heim) using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Antidigoxigenin-AP Fab fragments, Boehringer Mannheim), according to the suggestions of the manufacturer. Membrane was exposed to Hyperfilm (Amersham, Milan, Italy) for 30 seconds to 5 minutes.

Results

LD-PCR in BL Cell Lines

Based on the structure of the genes involved in the t(8;14)(q24;q32) translocation and on data in the literature,^{12,13} we selected a limited number of primers bracketing the breakpoints and specific for exon 2 of the MYC gene and for the joining and constant regions of the IgH gene (Table 1). Figure 1 is a schematic representation of the location of each of the 5 primers with respect to the MYC and the IgH genes. Primers and reaction conditions were first tested in 7 Burkitt's cell lines with cytogenetically proven t(8;14), using in different reactions the same MYC-specific primer in combination with each of the primers recognizing a selected IgH region. In 5 of the cell lines (BL41, CA46, DG75, RAJI, RAMOS) we obtained a PCR product ranging in size from 1.5 to 7.6 kb (Table 2 and Figure 2A). The cell lines DAUDI and P3HR1, obtained from endemic BL and known to have the breakpoint on chromosome 8 about 170–190 kb upstream of MYC gene,¹⁹ were negative, as expected. Among the 5 BL cell lines positive for the LD-PCR product, we found that BL41 and CA46 cells were positive with the C α /01 primer and DG75 cells were positive with the primer specific for the JH region, whereas RAJI and RAMOS were positive when using the C γ /02 and C μ /03 primer, respectively. In this manner we not only identified the

Table 2. LD-PCR for Detection of t(8;14) in BL Cell Lines and Tumor Samples

Tissue source	LD-PCR result	Region involved [†]	LD-PCR fragment size (kb)
Cell Line			
BL41	+	C α	4.0
CA46	+	C α	4.2
DAUDI	—	—	—
DG75	+	JH	1.5
P3HR1	—	—	—
RAJI	+	C γ	7.6
RAMOS	+	C μ	4.9
Patient*			
B01	+	C α	2.4
B02	+	C μ	5.5
B03	—	—	—
B04	+	C μ	8.5
B05	+	C μ	6.0
B06	—	—	—
B07	+	C α	6.0
B08	+	JH	5.0
B09 [‡]	+	JH	2.5
		C α	8.0
B10	+	C μ	9.5
B11	+	C μ	6.0
B12	+	C μ	6.0
B13	+	C γ	6.0
B14 [‡]	+	JH	2.0
		C γ	9.0
B15	+	C γ	5.5

*Patients were identified by a number to protect their identity.

[†]C γ , C μ , C α , and JH refer to the region of the IgH locus (C, constant; J, joining) recognized by the primer which, in combination with MYC/04 primer, obtained the PCR product.

[‡]Two different LD-PCR products were obtained in cases B09 and B14.

region near the IgH gene breakpoint in each cell line, but at the same time established a positive control for each pair of primers to be used in the study of pediatric BL specimens.

We also tested the T-cell leukemia cell line Jurkat, the rhabdomyosarcoma cell line RH30, the anaplastic lymphoma cell lines SUDHL-1 and KARPAS-299, two specimens of soft tissue sarcomas, two of T-cell lymphoblastic lymphomas, and one of Hodgkin's disease. In none of these cases was LD-PCR product obtained (data not shown).

Southern blot analysis was performed on all of the positive cell lines using an oligonucleotide probe complementary to a MYC exon 2 sequence,¹² which should be contained in all of the amplified genomic sequences. All of the products of the LD-PCR were recognized by the probe, thus demonstrating the specificity of the assay (Figure 2B).

Sensitivity of LD-PCR

To determine the sensitivity of the method, increasing dilutions of BL41 cells were prepared in T-lymphoblastic leukemia cells (Jurkat) followed by DNA extraction. LD-PCR was then performed using the MYC and C α primers. The PCR product was detectable in ethidium bromide-

Figure 2. LD-PCR analysis of BL cell lines. DNA from 7 BL cell lines was processed in the LD-PCR assay for the detection of t(8;14). The cell lines BL41, CA46, DG75, RAJI, and RAMOS showed a PCR product ranging from 1.5 to 7.6 kb in an ethidium bromide-stained agarose gel (A). They represent the positive controls for all four of the primer combinations selected for our study. Hybridization to a MYC-specific digoxigenin-labeled oligonucleotide probe (MYC/06) demonstrates the specificity of the reaction (B).

stained gel at BL41 cell concentration as low as 10^{-3} , although the signal was weak (Figure 3A). In an attempt to increase the sensitivity of the method, PCR products were transferred to a nylon membrane and hybridized to the digoxigenin-labeled MYC/06 oligonucleotide probe. The signal, which appeared better defined than in the ethidium-stained gel, was clearly visible at the same dilution of 10^{-3} BL41 cells (Figure 3B).

LD-PCR in BL Specimens

Tumor specimens obtained from 15 unselected children with BL were studied. DNA extracted from the samples was suitable for LD-PCR analysis because amplification of a 4.8-kb fragment of the tPA gene was achieved in all cases.

In 13 tumors (87%) we detected the t(8;14)(q24;q32) by LD-PCR, whereas 2 specimens were negative. The PCR fragments ranged in size between 2.0 and 9.5 kb (Figure 4A and Table 2). The IgH region involved in the

Figure 3. Sensitivity of the t(8;14) LD-PCR for the detection of BL cells. Increasing dilution of BL41 cells were prepared in acute T lymphoblastic leukemia cells (Jurkat). DNA was extracted and used as a template for LD-PCR. The PCR product was visible in ethidium bromide-stained gels at a ratio of BL41/Jurkat cells as low as 10^{-3} (A). Southern blot and subsequent hybridization to a MYC specific digoxigenin labeled oligonucleotide probe (MYC/06) increased the detectability of the signal (B).

translocation was C α in 2 cases (B01, B07), C γ in 2 cases (B13, B15), C μ in 6 cases (B02, B04, B05, B10, B11, B12) and JH in 1 case (B08). In cases B09 and B14 we obtained two different PCR products for each sample, one using the JH primer and a larger one using the C α /01 and C γ /02, respectively (Table 2 and Figure 5).

Specificity of the PCR products was confirmed by Southern blot analysis followed by hybridization to the digoxigenin labeled MYC/06 oligonucleotide, which also increased the detectability of the signal (Figure 4B).

Discussion

The most frequent genetic alteration in BL is t(8;14)(q24;q32). In this translocation the MYC gene is juxtaposed to the Ig heavy chain gene and this rearrangement represents a specific marker of the disease and, at the same time, is considered a relevant event in its pathogenesis.¹ Differently from endemic BL, in which the breakpoint in the MYC locus occurs often at a distance of up to 300 kb from the coding regions of the gene,^{19,20} in the sporadic BL the breakpoint is located preferentially within intron 1 or exon 1 of the MYC gene.⁵ These characteristics, together with recent advances in technology, have allowed the establishment of LD-PCR-based approaches to study the chromosomal translocations in sporadic BL,^{12,13} whereas it is still impossible to study endemic BL by this technique because the region involved is too wide.

Figure 4. LD-PCR analysis of BL tumor samples. Fifteen pediatric BL tumors were studied by LD-PCR and the products were analyzed by gel electrophoresis on agarose gels and subsequently stained with ethidium bromide. The results for a representative group of patients are illustrated in **A**. Specificity of the PCR products was demonstrated by hybridization to a MYC-specific oligonucleotide probe (MYC/06) after Southern blotting (**B**).

In particular, by using 7 primers specific for the MYC gene and 5 for the IgH locus, zur Stadt et al could detect the t(8;14) in approximately 50% of children with BL or B-ALL. Their method implied that, on average, 20 different primer pairs were used to study each sample.¹³

In this work we established a simplified and highly efficient LD-PCR approach for the detection of the t(8;14)(q24;q32) using fewer primers and focused our attention exclusively on BL. The MYC/04 primer was chosen because it recognizes a sequence in exon 2 that is external to the region of the gene where the breakpoints occur more often (exon 1 and intron 1). C μ /03, C γ /02, and C α /01 primers were selected because they recognize constant regions of the IgH locus in the proximity of the switch regions most often involved in the translocation. Furthermore, because it was reported that in European BL, similarly to the endemic BL, the joining region could be often involved,²¹ we decided to use also the JH primer. The decision to use only four pairs of primers was supported by the preliminary observation that our LD-PCR method allowed the amplification of large fragments of genomic DNA, up to 12 to 15 kb.

Initially we characterized 7 BL cell lines cytogenetically positive for the t(8;14). The CA46 cell line was positive with the C α /01 primer, as expected by the reported location of the breakpoint within S α region;²² RAJI was positive for the C γ /02 primer confirming the breakpoint in

Figure 5. LD-PCR analysis of case B14. The t(8;14) translocation was studied by LD-PCR combining, in different reactions, the MYC/04 primer with the C γ /02, C μ /03, C α /01, and JH primers, respectively. A PCR product was obtained with two different primer couples. The tPA primers were used to confirm the amplifiability of the DNA.

the region S γ ^{23,24} and RAMOS cell line was positive with the C μ /03 primer, as expected by the description of the breakpoint within S μ region.¹¹ BL41 and DG75, for which no detailed information existed in the literature regarding the exact region of the IgH breakpoint, were characterized by an involvement of C α and JH regions, respectively. The analysis of BL cell lines allowed us also to establish a positive control for each pair of primers, which we used on clinical specimens (Table 2), and to optimize reaction conditions. The cell lines DAUDI and P3HR1 originated from endemic BL were negative, as expected, since the breakpoint on chromosome 8 is far 5' of the MYC gene in this group of BL.¹⁹

LD-PCR analysis of 15 tumor samples obtained from children with BL showed that 13 (87%) were positive for the t(8;14): although obtained on a small series of patients, this represents the highest sensitivity of such an approach compared with the data for other approaches reported in the literature.¹³ Whether the absence of patients with B-ALL in our series, compared with previous reports,¹³ might have any relevance for the results of our study, although unlikely, remains to be demonstrated.

In 6 of 15 cases cytogenetic analysis was performed successfully. For the remaining patients we do not have cytogenetic data, either because only fixed tumor tissue was available or because the quality of the fresh tissue was inadequate for cytogenetic studies.

One of the two tumors negative by LD-PCR was cytogenetically positive for the translocation; in this case, failure to detect the recombined sequences might be due to an unusual breakpoint in the MYC gene (ie, 3' of the

exon 2 region recognized by the MYC primer), to a distant breakpoint in the IgH region, or to a loss of the IgH recognition site of the IgH primers used. Unfortunately, because of the limited amount of tissue available, a Southern blot analysis was not possible. The second negative case was that of a patient with histologically typical BL localized to the Waldeyer ring who had surgery for a suspect benign tonsillar hypertrophy and for whom no cytogenetic analysis was planned.

Interestingly, the C μ region was the most frequently involved in our patients, as previously reported for BL occurring in North America.⁷ Moreover, 3 of 13 positive cases had a breakpoint within or close to the JH region.

This region is frequently involved in endemic BL, but was also reported in European BL. In a series of Italian and Spanish BL, 6 of 12 cases had the involvement of the JH region, but almost all of them were adults.²¹ In the pediatric series of zur Stadt, 3 of 11 positive children had the breakpoint in the JH region.¹³

With regard to the 3 cases positive with the JH primer, case B09 and B14 also showed a PCR product with C α /01 and C γ /02, respectively. This was not reported in previous studies and has different possible explanations. The PCR products obtained with the JH primer, in both cases, were smaller (2.5 and 2.0 kb) than those obtained with the C α /01 and C γ /02 primers (8 and 9 kb, respectively). This observation, together with the knowledge that the DNA region between the JH and the C α /01 or the C γ /02 primers is far above the size limit of amplification for the current LD-PCR techniques, suggest that there might be a deletion between the joining and the C α regions in case B09 or between the joining and C γ regions in case B14. This hypothesis is supported by the absence of any PCR product using primers for regions located between JH and C α or C γ , respectively (Figure 5). Alternatively, one could speculate that two different clones, harboring a different t(8;14) translocation, might coexist in the same tumor or that two different t(8;14) translocations affected both alleles within the same cell clone. Because it is unlikely that in a tumor cell there might be more than one t(8;14) and because the rearrangements of the IgH locus with DNA deletion is a normal event in the process leading to immunoglobulin production in B lymphocytes, the first hypothesis appears the more suitable to explain our findings.

Given the fact that the LD-PCR product detecting the t(8;14) is specific for each individual tumor, it represents a marker that could be used to monitor micrometastases at diagnosis or during treatment in patients with BL. In our hands this technique was capable of detecting 10⁻³ BL cells when mixed *in vitro* with other leukemic cells (Figure 3, A and B). Thus, it has the potential to be applied for the determination of minimal residual disease in the bone marrow, as well as for the discrimination of tumor cells inside a residual mass during or after treatment. Although validation of these methods and of their clinical relevance for BL of childhood requires a larger series of patients (particularly in the setting of the current high-dose-intensity chemotherapy regimens, which can cure the great majority of pediatric BL), we believe their use could improve the management of this disease.

As a general consideration regarding the sensitivity and applicability of the LD-PCR technique for the detection of t(8;14) in BL, it should be clear that other methods, such as cytogenetic and FISH analyses, can be used. Cytogenetics has certainly the advantage of detecting not only the t(8;14), but also the variant translocations t(2;8) and t(8;22) typical of BL⁴ and any other chromosomal aberrations. Classic cytogenetics is hampered by a low mitotic index or by a poor quality of metaphase spreads in approximately 20% of the cases even in very experienced laboratories.²⁵ FISH analysis, on the other hand, is suitable to demonstrate the t(8;14)(q24;q32), it can give some information on the distribution of the translocation within a tumor cell population on a cell-by-cell basis and, most importantly, it can be performed on fresh as well as on fixed tissue. It has the limitation, though, that the breakpoints on the two chromosomes cannot be characterized so precisely as by LD-PCR, and it might suffer from excess false positive or false negative results, depending on the criteria used to interpret the results.²⁶ A disadvantage common to both cytogenetic and FISH analyses, compared to LD-PCR techniques, is that they are not suitable for minimal residual disease studies.

Ideally, any BL case should be studied by means of classic and molecular cytogenetics and LD-PCR, which represent two complementary methods in the genetic characterization of the tumor.

In conclusion, we have shown that using LD-PCR for the detection of t(8;14)(q24;q32) based on a limited number of primer combinations can be useful in the study of sporadic BL and has the potential for application to the assessment of micrometastases and minimal residual disease.

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