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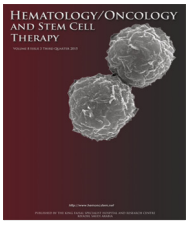

ORIGINAL RESEARCH REPORT

Detection of t(8;14) *c-myc/IgH* gene rearrangement by long-distance polymerase chain reaction in patients with diffuse large B-cell lymphoma

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Abstract
Objective/background: Specific chromosomal translocations are found in human leukemias and lymphomas. These translocations are closely related to particular histological and immunological phenotypes. In Burkitt's lymphoma, translocation t(8;14)(q24;q32), which involves the *c-myc* gene (8q24) and the immunoglobulin heavy-chain (IgH) locus (14q32), accounts for 90–95% of all chromosomal translocations. This translocation can be found in 2–5% of diffuse large B-cell lymphoma (DLBCL). Long-distance polymerase chain reaction (LD-PCR) assays, which can identify oncogene/Ig gene rearrangement, can detect these fusion genes. The objective of this study was to detect t(8;14) *c-myc/IgH* gene rearrangement by LD-PCR in patients with DLBCL.
Methods: In this study, 54 DLBCL cases were tested by LD-PCR with specific primers. LD-PCR was used for two breakpoints in both the *IgH* gene (joining region and γ switch region) and the *myc* gene (Exons 2 and 3).

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Results: As much as 1.85% of the samples were positive for the γ constant region and Exon 2 of the *myc* gene.
Conclusion: LD-PCR can be used for the detection of t(8;14) *c-myc/IgH* gene rearrangement in patients with DLBCL.

Introduction

Non-Hodgkin's lymphoma (NHL) is the eighth most commonly diagnosed cancer in men and the eleventh in women [1]. The diffuse large B-cell lymphoma (DLBCL) type is the most common histological subtype of NHL. Although the treatment options for different subtypes of NHL are almost similar, DLBCL includes a heterogeneous group of NHL with different prognoses and molecular and clinical characteristics [2]. Moreover, other mature B-cell neoplasms with aggressive behavior exhibit changes in the *myc* gene [3]. Most patients with Burkitt's lymphomas and some of those with DLBCL display chromosomal translocation t(8;14)(q24;q32) accompanied by juxtaposition of the *myc* gene with enhancer elements in the immunoglobulin heavy-chain (*IgH*) gene locus. Approximately 3% of adult patients with B-lineage acute lymphoblastic leukemia show such aberration [4].

As a pleiotropic transcription factor, *myc* contributes to cellular development. It not only regulates target genes related to growth and proliferation directly, but also controls complex networks of microRNAs and apoptosis mediators. Increased expression of *myc* is seen in ~70% of all human malignancies [5]. Recombination takes place only at the DNA level and no fusion messenger RNA or fusion protein is generated. The positions of the chromosomal breakpoints in the *c-myc* gene and various Ig genes are widely distributed and these breaks have specific characteristics in every patient [6]. Balanced chromosomal translocations are mostly seen in mature B-cell lymphomas often involving the Ig genes and a variety of partner genes [7]. Because the translocated partner gene is transpositioned into the Ig locus, it frequently becomes transcriptionally deregulated. The frequent involvement of Ig loci in chromosomal translocations indicates the remodeling of Ig genes. This remodeling occurs during B-cell development due to *V* gene segment assembly, somatic hypermutation, and class-switch recombination [8]. Translocations occur head to head (5′—5′) between the two loci of *c-myc* and IgH genes [5]. The enhancer affects the transcription of *myc* when it is juxtaposed with enhancer elements of Ig gene, resulting in *myc* overexpression (Fig. 1) [5,9].

Many techniques are used for detecting t(8;14) such as Southern blotting [10], fluorescent *in situ* hybridization [11], microarray [12], and long-distance polymerase chain reaction (LD-PCR) [13,14]. Many traditional techniques such as Southern blot tests have been replaced by PCR-based methods. The advantages of PCR are the low need for DNA, relatively fast process, and detection of abnormalities at a very low amount [15].

To detect the *myc/IgH* fusion in DLBCL cases, LD-PCR and touch-down PCR were performed. In the LD-PCR assay, PCR products ranging from 1 kb to 10 kb are amplified with two forward primers specific for *myc* Exons 2 and 3 combined, in different reactions, with two reverse primers for the IgH locus IGHG constant (Cγ) and IGHJ joining (JH) regions. The touch-down PCR assay was also used for the detection of t(8;14)(q24;q32).

Materials and methods

Patients

In this investigation, we analyzed 54 cases of suspected lymphomas collected from the Department of Pathology, Cancer Institute of Imam Khomeini Hospital, Tehran, Iran, and Imam Reza Hospital, Tabriz, Iran, between November 2008 and August 2011. We used formalin-fixed, paraffin-embedded (FFPE) blocks. The pathological board re-evaluated all the samples and confirmed the diagnosis according to the World Health Organization classification criteria [16].

Cell lines

The human BL cell lines, RAJI (IGHG breakpoint) and DG-75 (IGHJ breakpoint), were purchased from the cell bank of Pasteur Institute of Iran (Tehran, Iran) and maintained in Roswell Park Memorial Institute-1,640 medium with 10% fetal calf serum. The genomic DNA, which was used as positive control in the PCRs, was then isolated from cultured cell lines.

Template DNA extraction

From each FFPE tissue, 4- or 5-μm sections were obtained, and transferred into sterile containers and stored at room temperature until their use in DNA extraction. DNA was extracted from the FFPE specimens using the FFPE DNA extraction kit as described by the manufacturer (Quick Extract FFPE DNA Extraction; Epicentre, Madison, WI, USA).

To evaluate the quality and quantity of the extracted DNA [15], it was amplified by PCR and UV spectrophotometry at 260/280 nm using the NanoDrop ND-1,000 spectrophotometer (NanoDrop Technology, Wilmington, DE, USA). The average concentration of the DNA samples was 155 ng/μL (range 120—560 ng/μL) and its $OD_{260/280}$ ratio was 1.87.

Primers

To detect the rearrangement involving the *c-myc* gene on Chromosome 8q24 and the *IgH* locus on Chromosome

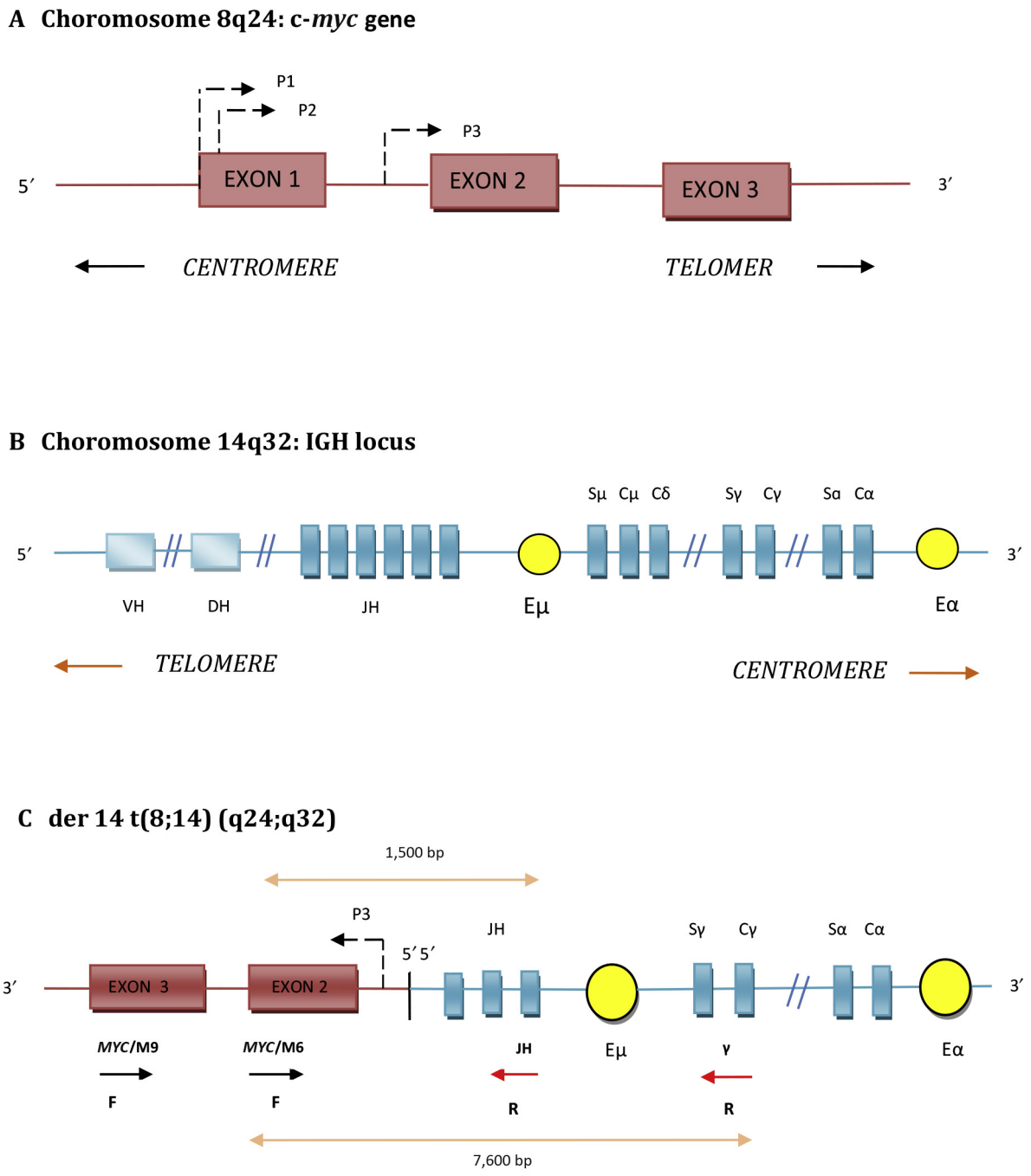

Fig. 1 Positions of *c-myc*/*IgH* breakpoints in DLBCL and detection by LD-PCR. (A) Map of the human *c-myc* gene on Chromosome 8; (B) the IgH locus on Chromosome 14. (C) Prototypical *c-myc*/*IgH* fusion gene in DLBCL showing position breakpoints, and forward and reverse primers that are used for LD-PCR. LD-PCR performed for the JH and γ constant regions. *Note*. bp = base pair; DLBCL = diffuse large B-cell lymphoma; LD-PCR = long-distance polymerase chain reaction.

14q32, two primers for the *c-myc* gene and two primers for the *IgH* locus were combined. One primer for the IGHG constant region (Cγ) and another one for IGHJ of the joining region (JH) were used (Table 1).

Long-distance polymerase chain reaction

LD-PCR refers to the amplification of DNA lengths that cannot typically be amplified using routine PCR methods or reagents. For simple DNA templates, polymerases optimized for long-range PCR can amplify ~30 kb. Each reaction mixture (25 μL) contained 300 ng of DNA genome, 10 pM downstream and upstream primers, 500 μm of each deoxyribose nucleoside triphosphate, 10× buffer with 4 mM $MgCl_2$, and 2 units of KBC Extend long polymerase enzyme mix as indicated in the KBC Extend long PCR kit (Kawsar Biotech Company, Tehran, Iran).

LD-PCR was performed in two steps. In the first step, the reaction condition was as follows: an initial denaturation at 94 °C for 4 min followed by 15 cycles of denaturation at 94 °

Table 1 Primers used for LD-PCR.

FORWARD	REVERSE
MYC/M6:	jH:
5'ACAGTCCTGGATGATGATGTTTTTGATGAAGGTCT3'	5'CTTACCTGAGGAGACGGTGACCGTGGTCCC3'
MYC/M9:	C:
5'GAGATCCTCTGGGGTTTGCGAGATAACCCATGG3	5'GGTCACCACGCTGCTGAGGGAGTAGAGT3'

C for 20 s, annealing at 60 °C for 50 s, extension at 68 °C for 4 min, and a final extension for 10 min at 68 °C. In the second step, the reaction condition was as follows: an initial denaturation at 94 °C for 4 min followed by 12 cycles of denaturation at 94 °C for 10 s, annealing at 65 °C for 30 s, extension at 68 °C for 4 min with gradual increments of the extension time (20 s/cycle), and a final extension for 10 min at 68 °C.

PCR products were analyzed by agarose gel electrophoresis (0.8% agarose in Tris base—acetic acid—EDTA buffer stained with ethidium bromide) and visualized under a UV transilluminator.

Results

Based on the structure of the genes involved in the t(8;14) (q24;q32) translocation, 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. In the PCRs, an antisense primer in *myc* Exon 2 (*myc/M6*) was combined with an antisense primer in the CH1 exons of IGHG or in the sixth joining segment of IGHJ. Each investigation included two positive and negative controls. PCR conditions were optimized to fit a set of primers for reproducing the translocation. Primers and reaction conditions were first tested in two Burkitt's cell lines with cytogenetically proven t(8;14), using the same myc-specific primer in different reactions in combination with each primer recognizing a selected IgH region.

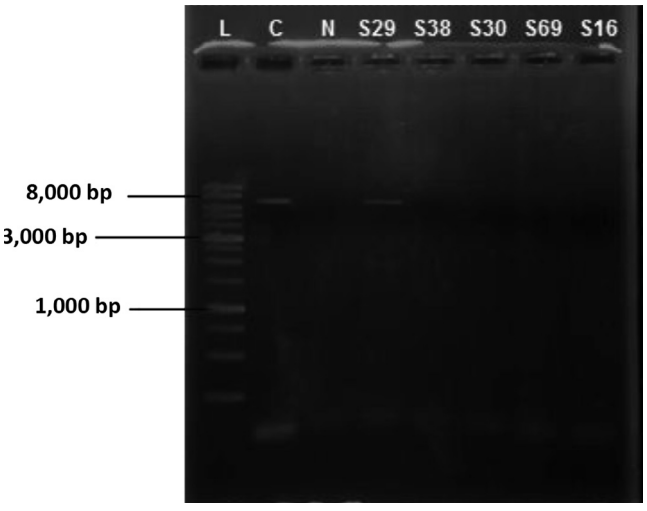

Fig. 2 Ethidium bromide-stained gel electrophoresis of long-distance polymerase chain reaction. L, C, and N, respectively, are ladder, positive control (RAJI cell line), and negative control. S29—S16 are samples showing the *myc/IgH* translocation in the γ breakpoint. The size of the product is 7,600 bp. Others were negative for this translocation. *Note*. bp = base pair; *IgH* = immunoglobulin heavy-chain locus.

We obtained a PCR product with sizes of 1.5 kb and 7.6 kb in DG75 and RAJI cell lines, respectively. DG75 cells were positive for the JH breakpoint with the primer specific for the JH region and RAJI cells were positive for the Cγ breakpoint when using the Cγ primer. The LD-PCR was performed for 54 cases to detect t(8;14). One patient (a 55-year-old man) was positive for the myc/M6 and Cγ primer pair (Fig. 2). The 53 LD-PCR-negative patients were tested with the myc-9 primer (positioned in Intron 3) in combination with the Cγ primer to establish whether breakpoints could be located downstream of Exon 2 in the *c-myc* gene. The results showed all of them to be negative for these breakpoints. Existence of a PCR product was investigated in the cases using LD-PCR for the myc-6 and JH primer pair. However, none of them showed any PCR product. Patients negative for the joining region (JH) breakpoint were re-examined with touch-down PCR. No PCR product was produced with this method either.

Discussion

DLBCL is a heterogeneous disease comprising 30% of adult NHLs. It has different subtypes and clinical outcomes [17]. Considering the curable nature of the disease by suitable treatment, it is crucial to identify infiltration of lymphomatous for determination of the state of the disease accurately as well as evaluation of the therapy [18]. Most DLBCLs are accurately diagnosed using immunohistochemistry or flow cytometric immunophenotyping. However, diagnosis becomes complicated in ~5—10% of the patients [19,20]. In these patients, molecular gene rearrangement studies have been found to be useful as a complementary diagnostic tool [21]. The t(8;14) translocation is common in Burkitt's lymphoma and a subset of DLBCL [4]. The myc oncogene on Chromosome 8 and the *IgH* locus on Chromosome 14 are involved in this translocation. The *c-myc* gene consists of three exons, among which Exons 2 and 3 include the coding sequence for the c-myc protein. Because of sequences that block transcriptional elongation, Exon 1 encodes an untranslated sequence acting as a negative regulatory sequence. The negative regulatory Exon 1 and Intron 1 sequences are removed in some lymphomas because the t (8:14) breakpoints tend to be in the first intron [22].

The Ig-enhancer elements that contribute to *c-myc* dysregulation specifically stimulate transcription in B cells normally. The *IgH* Eμ or Eα enhancer in the *c-myc* coding sequences can potentially induce B-cell tumors, suggesting that in tumors with translocation t(8;14), *IgH* elements *per se* might increase oncogenic levels of *c-myc* expression

[23]. The LD-PCR can identify t(8;14) at the genomic level, and is therefore useful for this translocation considering that it does not produce a fusion gene [24]. Moreover, it can be applied to translocations in which the DNA sequences of the regions immediately adjacent to the breakpoint have not yet been identified. The other advantage is that amplified fragments have sequences unique to individual patients. Thus, this PCR technique could be applied to analyze translocations in leukemia. PCR using DNA is considerably easier than RT-PCR, which requires RNA isolation and complementary DNA synthesis.

DLBCL of B-cell phenotype comprises 30—40% of adult NHL [25] and is a good candidate for such an aggressive treatment. Therefore, sensitive genetic markers of this particular lymphoma subtype are required for assessing minimal residual disease [17]. Translocations involving *myc* are also found in other hematological malignancies in addition to Burkitt's lymphoma, most importantly in the subsets of DLBCL. In this tumor, a chromosomal translocation at the myc locus shows a more aggressive tumor phenotype and a lower survival rate [26]. Two to five percent of DLBCLs are affected by the t(8;14) translocation in several breakpoints in the *myc* (Exons 2 and 3) and *IgH* genes (Jh, Cμ, Cγ, Cδ, Cα, and Cε). We performed LD-PCR only for the JH region and one constant region (γ) with Exon 2 of the *myc* gene in 54 samples. In lymphoma patients with JH break, the positions of the *IgH* μ enhancer affects transcription of the *myc* gene. The breaks in the *IgH* gene occur during attempted V(D)J recombination, leading to the juxtaposition of the IgH enhancer with *c-myc*. However, with respect to the γ switch region, the breakpoints in the *IgH* gene occur during attempted Ig switching, leading to the juxtaposition of 3′ *IgH*-enhancer elements (such as Eα) with sequences of *c-myc*. These enhancers affect *myc*'s promoter and expression of myc. Only one patient was positive (1.85%) for the γ breakpoint. However, in negative samples, it is suggested that translocation happened in other breakpoints. DLBCL can affect any age group, but mostly occurs in elderly patients (normally in people in the mid-60s). Therefore, LD-PCR can be used for the detection of t(8;14) *c-myc*/*IgH* gene rearrangement in patients with DLBCL.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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