

# Alternative Translocation Breakpoint Cluster Region 5' to *BCL-6* in B-cell Non-Hodgkin's Lymphoma<sup>1</sup>

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## ABSTRACT

Chromosomal translocations involving band 3q27 with various different partner chromosomes represent a recurrent cytogenetic abnormality in B-cell non-Hodgkin's lymphoma. In a fraction of these translocations, the chromosomal breakpoint is located within the 5' noncoding region of the *BCL-6* proto-oncogene where the *BCL-6* major breakpoint region (MBR) maps. As a result of the translocation, *BCL-6* expression is deregulated by promoter substitution. However, between 30 and 50% of lymphomas with cytogenetically detectable translocations affecting band 3q27 retain a germ-line configuration at the *BCL-6* locus. To identify possible additional breakpoint clusters within 3q27, we cloned a t(3;14)(q27;q32) lymphoma without MBR rearrangement and found a novel breakpoint site located between 245 and 285 kb 5' to *BCL-6*. Breakpoints within this newly described region, which we called the alternative breakpoint region (ABR), were found to be recurrent in lymphomas carrying t(3q27) chromosomal translocations but devoid of *BCL-6* MBR rearrangements. Comparative analysis of multiple lymphomas carrying rearrangements within the ABR showed that the breakpoints cluster within a 20-kb distance. Translocations involving the ABR may juxtapose *BCL-6* to distantly acting, heterologous transcriptional regulatory elements which cause deregulation of the proto-oncogene. The identification of *BCL-6* ABR provides new tools for the diagnosis of lymphomas carrying aberrations at 3q27 and deregulated *BCL-6* genes.

## INTRODUCTION

B-cell NHL<sup>3</sup> form a heterogeneous group of neoplasms that arise as a result of the clonal expansion of a single cell arrested at a specific stage of B-cell differentiation (1). The uncovering of the genetic elements involved in lymphomagenesis has been aided by cytogenetic analysis, which has revealed that nonrandom chromosomal translocations are found in association with specific subtypes of NHL (1–2). The molecular cloning of the breakpoint junctions of these translocations has led to the identification of several proto-oncogenes that undergo transcriptional deregulation because of their juxtaposition to regulatory elements of genes constitutively expressed in mature B

cells, most often represented by Ig genes (1). The B-cell NHL-associated oncogenes discovered to date include *BCL-1* at 11q13 involved in ~95% of mantle cell lymphomas, *BCL-2* at 18q21 involved in 70–90% of FLs (1), *c-MYC* at 8q24, which is found to be rearranged in 100% of Burkitt's lymphoma (1), *PAX-5* at 9p13 involved in 50% of lymphoplasmacytoid lymphomas (1), and *BCL-6* at 3q27 rearranged in 30–40% of DLBCLs and in 4–15% of FL cases (3–5).

The *BCL-6* gene encodes a nuclear phosphoprotein characterized by six COOH-terminal Kruppel-type zinc finger motifs, and an NH<sub>2</sub>-terminal POZ motif, shared by several zinc finger molecules, including the *Drosophila* developmental regulators *Tramtrak* and *Broad-Complex*, as well as the human *KUP*, *ZID*, and *PLZF* proteins (3). *BCL-6* has been shown to function as a potent transcriptional repressor of promoters linked to its DNA target sequence (6–8). The *BCL-6* protein is expressed in mature B-cells within germinal centers but not in immature B-cell precursors or in differentiated plasma cells (9). Targeted disruption of the *BCL-6* gene shows that *BCL-6* is essential for germinal center formation, being involved in the control of Th2-type immune responses (10, 11).

Rearrangements involving the *BCL-6* gene at 3q27 cluster mainly in a 4-kb genomic region, termed the MBR, which spans the first noncoding exon of the gene (3–5). Translocations affecting band 3q27 in NHL are not limited to the Ig loci but may involve numerous different partner chromosomes. The molecular characterization of several 3q27 chromosomal translocations has shown that the coding domain of the translocated *BCL-6* gene becomes fused downstream to heterologous promoters. These include the  $E\mu$  and  $I\gamma$  promoters of Ig genes in the case of t(3;14) as well as promoters of other genes, namely *TFF*, *BOB-1*, *H4*, in the case of t(3;4), t(3;11), and t(3;6), respectively (12–15). A general feature of promoters juxtaposed to translocated *BCL-6* is their constitutive expression in B-cells, which leads to the inappropriate expression of *BCL-6* (12–15).

Between 30 and 50% of the 3q27 chromosomal breakpoints found in lymphomas do not occur within the MBR of *BCL-6*, suggesting the presence of alternative breakpoints at 3q27 (3). To address this question, we cloned a t(3;14)(q27;q32) occurring in a FL case shown to be negative for rearrangement at the MBR of *BCL-6*. We detected a novel breakpoint cluster located 245–285 kb upstream of the first exon of the *BCL-6* gene and termed ABR. This breakpoint site was shown to be recurrent in several lymphomas with 3q27 chromosomal translocations that lacked *BCL-6* MBR rearrangement.

## MATERIALS AND METHODS

**Tumor Biopsies and Cell Lines.** Lymph node biopsies from NHL cases were obtained from the Memorial Sloan-Kettering Hospital. Tumors were classified histologically according to the Revised European-American Lymphoma Classification (16). The t(3;14)(q27;q32)-positive NHL case 1952, which served for *BCL-6* ABR cloning, was diagnosed as having a FL demonstrating an add(8)(q24) but no karyotypic evidence of a t(14;18)(q32;q21)

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<sup>3</sup> The abbreviations used are: NHL, non-Hodgkin's lymphoma; Ig, immunoglobulin; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; MBR, major breakpoint region; ABR, alternative breakpoint region; FISH, fluorescent *in situ* hybridization; PFGE, pulsed-field gel electrophoresis.



Fig. 1. Molecular characterization of the t(3;14) lymphoma case 1952. In A, the figure shows mapping of the recombinant phage clones λ1952-23 and λ1952-2, representing der(3) and der(14) respectively, in addition to the normal germ-line phages λ1952-15/11 at the *BCL-6* ABR. Structural elements include the Ig enhancer element (*E<sub>μ</sub>*), as well as the switch region (*S<sub>μ</sub>*) and coding segments (*C<sub>μ</sub>* and *J<sub>H</sub>*). Arrows indicate the position of the breakpoint in case 1952. Probes 2.1 B/H and 1.3 X are represented as thick horizontal lines and were used to confirm chromosome 3 derivation by hybridization to somatic cell hybrid DNA filters. In B, the figure shows the nucleotide sequence analysis of the breakpoint junctions of case 1952 and alignment to the corresponding germ-line regions. Sequence identities are indicated by the vertical lines. The sequences absent in der(3) and der(14), but present in germ-line chromosome 3, are the result of a deletion during translocation. *S<sub>μ</sub>* motifs are underlined. Restriction enzyme abbreviations are: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; and Bg, *Bgl*II.

translocation. Immunohistochemical analysis demonstrated that this tumor expressed surface IgM.

**Southern and Northern Blot Analysis.** High molecular weight DNA was extracted by the SDS/proteinase K method followed by “salting out” and ethanol precipitation. Southern blot analysis was performed following standard procedures. The following probes were used for Southern blot analysis of Ig gene rearrangements: *J<sub>H</sub>* probe, represented by a 6.6-kb *Bam*HI-*Hind*III fragment from the *Ig<sub>H</sub>* locus (17); and *C<sub>μ</sub>* probe, represented by a 1.3-kb *Eco*RI fragment from the *Ig<sub>H</sub>* locus (17). Some of the *BCL-6* ABR probes used in this study have been used in a previous study (18). For Northern blot analysis, RNA was extracted from cell lines and patient samples using the guanidinium thiocyanate/cesium chloride method, electrophoresed on a 1.0% agarose gel containing 2.2 mM formaldehyde and transferred to a nylon membrane (Hybond N; Stratagene).

**Cloning of Chromosomal Breakpoints.** High molecular weight DNA from NHL case 1952 was digested with *Bam*HI and size fractionated by running on a 0.7% low-melting agarose gel. Fragments ranging from 12 to 23 kb were gel-purified and ligated into the λDash-II vector (Stratagene, La Jolla, CA) and subsequently *in vitro* packaged using Gigapack III Gold packaging extract (Stratagene, La Jolla, CA). Recombinant phage clones ( $1 \times 10^6$ ) were screened by plaque hybridization using the *J<sub>H</sub>* and *C<sub>μ</sub>* Ig probes. Isolation of germ-line 3q27 sequences was achieved by screening a human placenta library (Stratagene) with probe 2.1 B/H derived from phage λ1952-2 {corresponding to der(14)} (Fig. 1).

**Isolation of P1/PAC and YAC Clones and Construction of a 3q27 Contig.** To establish a clone contig encompassing the *BCL-6* gene, the Sac 4.0 probe (intron 1 of *BCL-6*) was used to screen a gridded P1 and PAC library (Genome Systems, St. Louis, MO). P1 and PAC clone ends were isolated by a vectorette-bubble PCR method and then used for genomic “walking” by additional screening of P1 and PAC libraries (19). The single YAC clone 19GA10 described in the contig, isolated from the Zeneca (formerly ICI) human YAC library, was obtained from the United Kingdom Human Genome Mapping Project Resource Centre, Hinxton, United Kingdom. Confirmation that the isolated P1 and PAC clones were from chromosome 3 was achieved

through the hybridization of PCR-derived end clone fragments to somatic cell hybrid filters (panel 2, National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Coriell Institute, Camden, NJ), as well as FISH analysis to metaphase spreads of mitogen-stimulated normal human lymphocytes. Insert sizing of P1 and PAC clones was achieved through their digestion with *Not*I/*Sal*I and *Not*I restriction digests, respectively. For YAC clones, yeast cells were embedded in agarose plugs by a standard method and analyzed by PFGE on the CHEF-DR II (Bio-Rad, Richmond, CA) after restriction digestion.

**FISH.** Briefly, probes were labeled by nick translation with biotin-16-dUTP or digoxigenin-11-dUTP and detected by indirect immunofluorescence using fluorescein isothiocyanate-conjugated avidin and rhodamine-conjugated anti-digoxigenin, respectively. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole. Images were captured with a cooled charge-coupled device camera (Photometrics, Tucson AZ) attached to a Nikon Microphot-SA microscope and processed using Smart capture imaging system (Vysis, Downers Grove, IL).

**DNA Sequencing.** DNA sequencing was performed by the dideoxy chain termination method using the ABI 373A automated sequencing system (Applied Biosystems, Foster City, CA).

**RESULTS**

**Cloning of t(3;14) from a FL.** Cloning of t(3;14)(q27;q32) was performed on case 1952, represented by an IgM-producing FL. Southern blot analysis of Ig genes using *Bam*HI and *Hind*III digestions and probes for *J*, *C<sub>μ</sub>*, *C<sub>γ</sub>*, and *C<sub>α</sub>* sequences showed the presence of three rearranged J fragments, two of which did not comigrate with any constant gene region probe (data not shown) as observed previously in several cases of chromosomal translocations involving the *Ig<sub>H</sub>* locus (20). We then proceeded to clone the rearranged *Bam*HI fragments containing *J<sub>H</sub>* and *C<sub>μ</sub>* sequences from a recombinant phage library constructed from *Bam*HI-digested DNA from case 1952, which was



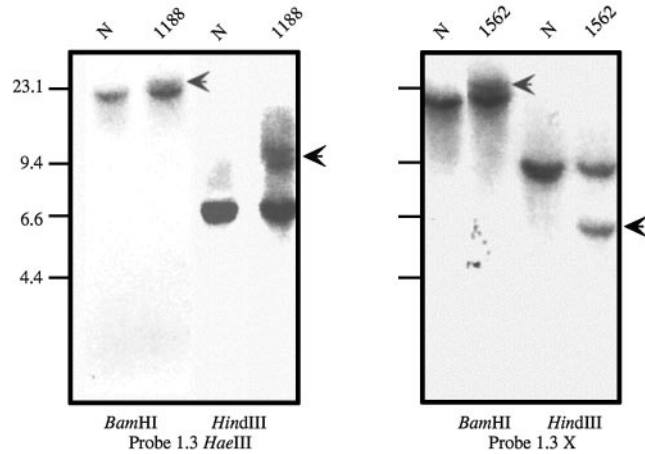
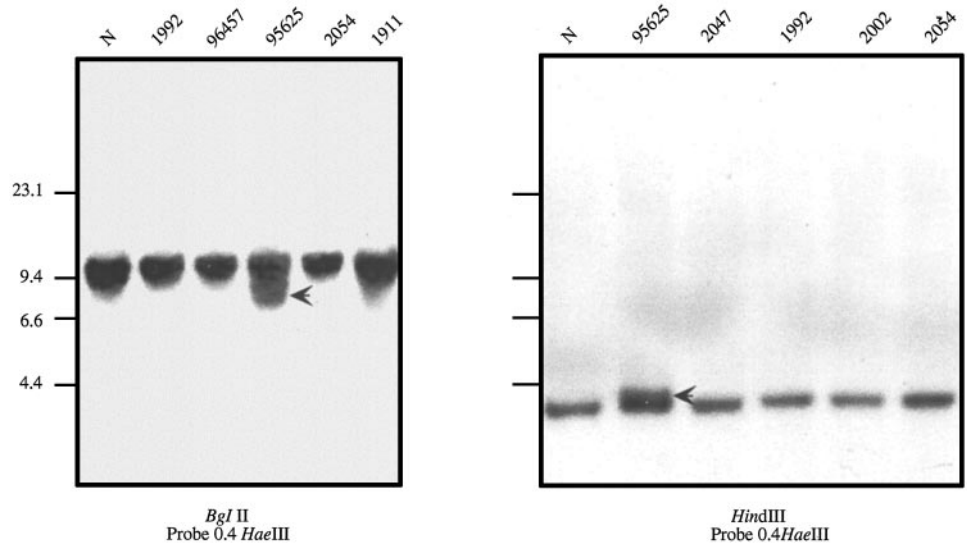


Fig. 3. Southern blots showing rearrangement at the *BCL-6* ABR in three representative lymphoma cases. Rearrangements are depicted in lymphoma cases 1188, 1562, and 95-625, confirmed by two DNA digests.



**Recurrency of the *BCL-6* ABR in Lymphomas.** To assess the frequency of ABR, we collected a series of lymphomas ( $n = 12$ ) with cytogenetic evidence of  $t(3q27)$  translocations but absence of rearrangement involving the MBR of *BCL-6*. The histological and partial cytogenetic profiles of these NHL cases are presented in Table 1. Southern blots of *HindIII*-, *BglII*-, and *BamHI*-digested tumor DNAs were prepared and hybridized sequentially with probes derived from phages  $\lambda$ 1952-15/11, exploring a genomic distance of  $\sim 40$  kb surrounding the breakpoint of case 1952. All rearrangements reported were confirmed by analysis with a second enzyme digest. Representative results of hybridization analyses

with probes from the ABR on Southern blots of  $t(3q27)$  NHL cases are shown in Fig. 3. Using probes 1.3 X and 0.4 *HaeIII* on *HindIII*, *BglII*, and *BamHI* Southern blot digests of lymphoma cases (Fig. 3), rearrangements were seen in 42% (5 of 12) of NHL cases that had cytogenetic evidence of  $t(3q27)$  chromosomal translocations and that showed germ-line configuration of the MBR (Table 1).

Subsequently, we investigated a cytogenetically uncharacterized series of FL ( $n = 20$ ) and DLBCL ( $n = 84$ ) that were known to be devoid of *BCL-6* MBR rearrangements. Rearrangements of the *BCL-6* ABR were detected in 10% (2 of 20) FL and 2.4% (2 of 84) DLBCL, including one case arising in an immunocompetent host and one case associated with AIDS. Mapping studies showed that all ABR rearrangements of *BCL-6* clustered within a 20-kb region.

***BCL-6* Expression Levels in NHL Cases with ABR Rearrangement.** To analyze the effect of ABR rearrangement on *BCL-6* gene expression, we examined the levels of *BCL-6* expression in lymphoma cases 1952 (FL) and 1188 (DLBCL) (both rearranged at ABR), as compared with two control FLs (cases 2065 and 1992) showing no rearrangement at the ABR or MBR of *BCL-6* (Fig. 4). *BCL-6* RNA levels were similar, independent of the *BCL-6* genomic status. In addition, *BCL-6* expression levels of case 1952 were also comparable with those of DLBCL cell line LY18 with germ-line *BCL-6*, as well as of immunoblastic lymphoma LY8 carrying a MBR rearrangement. Overall, these results indicate that

Table 1 Rearrangement analysis at ABR in NHL cases with 3q27 chromosomal translocations

Case	Histology	3q27 aberration	ABR	MBR
1952	FL	t(3;14)(q27;q32)	R	G
1562	DLBCL	t(2;3)(q21;q27) t(3;8)(q27;q13)	R	G
591	DLBCL	t(3;22)(q27;q11)	R	G
95-625	DLBCL	t(3;14)(q27;q32)	R	G
1188	DLBCL	add(3q27)	R	G
498	DLBCL	t(3;9)(q27;p13)	G	G
1187	FL	t(2;3)(q21;q27)	G	G
770	DLBCL	add(3q27)	G	G
1879	FL	t(3;14)(q27;q32)	G	G
95-517	DLBCL	t(2;3)(q12;q27)	G	G
95-89	DLBCL	t(3;6;14)(q27;p35;q32)	G	G
91-153	DLBCL	t(3;14)(q27;q32)	G	G

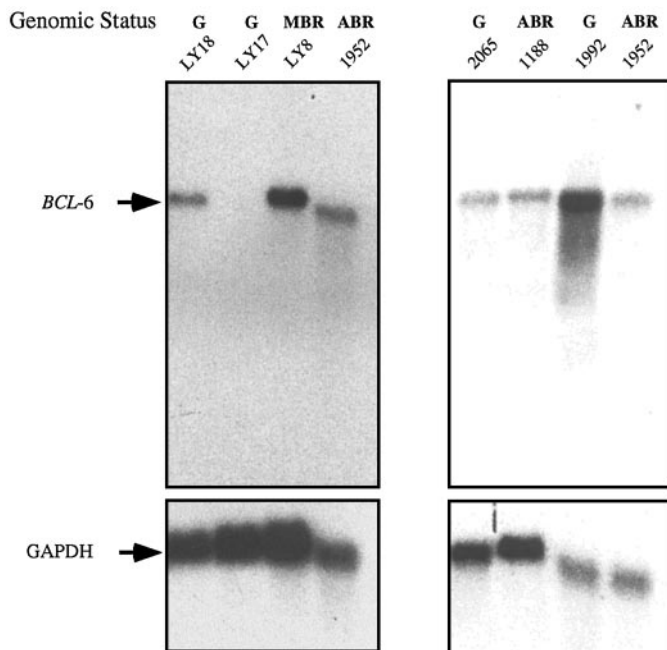


Fig. 4. Northern blot analysis of the t(3;14) positive lymphoma case 1952 hybridized with the *BCL-6* probe. Other lymphoma cases are also included for comparison. A 10- $\mu$ g aliquot of total RNA was loaded onto each lane, blotted, and hybridized with the full-length *BCL-6* cDNA probe together with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to control for the amount of RNA loading, as well as RNA degradation for the tumor RNAs. LY18 and LY17 are DLBCL cell lines; LY8 is an immunoblastic lymphoma cell line; primary cases 2065, 1992, and 1952 are FL; case 1188 is a DLBCL. The genomic status of each sample is indicated as ABR; MBR; G (for germ-line configuration at the ABR and MBR of *BCL-6*).

cases with ABR rearrangement express *BCL-6* at levels comparable with phenotypically similar cases with MBR rearrangement or lacking 3q27 abnormalities.

## DISCUSSION

B-cell NHL are typically characterized by recurrent chromosomal translocations, which deregulate proto-oncogenes found at or in close proximity to the chromosomal breakpoint junction areas (1). In some translocations, exemplified by the *BCL-1* and *BCL-2* translocations, the proto-oncogene may be involved by multiple, distinct breakpoint clusters that are recurrent in different patients (21, 22). In the case of 3q27 translocations involving the *BCL-6* proto-oncogene, only one breakpoint cluster has been identified to date (3). This breakpoint cluster, termed *BCL-6* MBR, is detectable at the molecular level in ~50–60% of lymphoma cases carrying the 3q27 translocation (3–5). The occurrence of lymphomas harboring 3q27 breaks but devoid of *BCL-6* MBR rearrangements point to the putative existence of other *BCL-6* breakpoint clusters. Here we report the identification of a novel ABR, which, together with the *BCL-6* MBR, accounts for ~70% of the total number of 3q27 chromosomal breakpoints detectable in NHL cases.

The *BCL-6* ABR is located at a distance of ~280 kb telomeric to *BCL-6*, raising the question of how the translocation induces *BCL-6* deregulation. On the basis of the model of several other lymphoma translocations, the ABR breaks may exert several possible modes of *BCL-6* deregulation. These include substitution of enhancer regions, a mechanism which has been well demonstrated for many B-cell-associated oncogenes, including *BCL-1*, *BCL-2*, *c-MYC*, *PAX-5*, *BCL-8*, and *BCL-9* (1). Enhancer substitution results from the insertion of various Ig<sub>H</sub> transcriptional control elements, such as the potent J<sub>H</sub>-C<sub>H</sub> intron enhancer E $\mu$ , the 3'  $\alpha$ E, or the 3'  $\alpha$ -hs4, at the junction

region of the translocation breakpoint (23). An alternative mode of *BCL-6* deregulation by ABR translocations consists in the removal of *cis*-acting regulatory domains in a fashion similar to that suggested for some rearrangements involving the *c-MYC* locus (24). In the case of NHL cases rearranged at the ABR but not involving the Ig genes, it is conceivable that *BCL-6* is deregulated by enhancer substitution of genes other than Ig genes. Finally, it cannot be formally excluded that ABR breaks affect genes mapping to 3q27 but unrelated to *BCL-6*. However, the genomic region surrounding ABR analyzed to date failed to reveal any functionally proven gene.

Our survey of *BCL-6* ABR breakpoints in B-cell lymphoma defines that the frequency of ABR rearrangements is substantially lower than that of *BCL-6* MBR. In fact, MBR rearrangements seem to be involved in ~75% of DLBCL cases with cytogenetically detectable 327 breakpoints (3q27+) (3), whereas ABR rearrangements account for only a fraction (4 of 7, >50% in this study) of the remaining MBR-negative 3q27+ cases. Thus, ABR rearrangements are expected to account for ~12% of all 3q27 rearrangements, corresponding to an expected frequency of 4% in the overall DLBCL populations and consistent with the actual frequency observed in this initial study (2 of 84). Conversely, it is curious that the frequency of ABR rearrangements in FL appears to be similar, or possibly higher, than that of MBR rearrangements. Future studies on large panels of cases are needed to define whether *BCL-6* ABR rearrangements preferentially associate with FL.

From a diagnostic standpoint, the results of this study point to the need of long range DNA analysis for the correct identification of all *BCL-6* breakpoints occurring in lymphomas. In this respect, FISH provides a powerful tool to detect both MBR and ABR *BCL-6* breakpoints, thereby surpassing the capabilities of conventional Southern blot hybridization analysis. In particular, highly predictive analysis of *BCL-6* rearrangements can be performed by FISH using as a probe a 650-kb clone contig designed to detect breakpoints occurring both at the MBR and ABR, as well as those which scatter around the *BCL-6* locus. Because a fraction of 3q27 breaks cannot be currently explained by *BCL-6* breaks at MBR or ABR, FISH studies may also lead to the discovery of additional *BCL-6* breakpoint clusters that, together with MBR and ABR, will recapitulate the molecular pathology of 3q27 breaks in B-cell lymphoma.

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