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Heterogeneous Breakpoints on the Immunoglobulin Genes Are Involved in Fusion with the 5′ Region of *BCL2* in B-Cell Tumors

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The 5′ flanking region of the *BCL2* gene (5′-*BCL2*) is a breakpoint cluster of rearrangements with immunoglobulin genes (*IG*s). In contrast to t(14;18)(q32;q21) affecting the 3′ region of *BCL2*, 5′-*BCL2* can fuse to not only the heavy chain gene (*IGH*), but also two light chain gene (*IGL*) loci. We report here cloning and sequencing of a total of eleven 5′-*BCL2*/*IG*s junctional areas of B-cell tumors, which were amplified by long-distance polymerase chain reaction-based assays. The breakpoints on 5′-*BCL2* were distributed from 378 to 2312 bp upstream of the translational initiation site and, reflecting the alteration of regulatory sequences of *BCL2*, 5′-*BCL2*/*IG*s-positive cells showed markedly higher levels of *BCL2* expression than those of t(14;18)-positive cells. In contrast, the breakpoints on the *IG*s were variable. Two 5′-*BCL2*/*IGH* and two 5′-*BCL2*/*IGL*κ junctions occurred 5′ of the joining (*J*) segments, suggesting operation of an erroneous variable (*V*)/diversity (*D*)/*J* and *V*/*J* rearrangement mechanism. However, two other 5′-*BCL2*/*IGH* junctions affected switch regions, and the κ-deleting element, which is located 24 kb downstream of the constant region of *IGL*κ, followed the 5′-*BCL2* in another case. One 5′-*BCL2*/*IGL*κ and two 5′-*BCL2*/*IGL*λ junctions involved intronic regions where the normal recombination process does not occur. In the remaining one case, the 5′-*BCL2* fused 3′ of a *V*λ gene that was upstream of another *V*λ/*J*λ complex carrying a non-producing configuration, indicating that the receptor editing mechanism was likely involved in this rearrangement. Our study revealed heterogeneous anatomy of the 5′-*BCL2*/*IG*s fusion gene leading to transcriptional activation of *BCL2*, and suggested that the mechanisms underlying the formation of this particular oncogene/*IG*s recombination are not identical to those of t(14;18).

Key words: 5′ region of *BCL2* — Immunoglobulin genes — *V/D/J* recombination — Receptor editing — κ-Deleting element

Rearrangement of the *BCL2* gene with the immunoglobulin (*IG*) heavy chain gene (*IGH*) is one of the most frequent genetic abnormalities in B-cell tumors.[1, 2] We showed that breakpoints on *BCL2* involved in t(14;18)(q32;q21) translocation are not only clustered within two common sites, i.e., major breakpoint cluster region (MBR) and minor cluster region (mcr), but also distributed over a large region from the MBR through mcr.[3] As a result of t(14;18), the coding exons of *BCL2* are juxtaposed 5′ to a joining (*J*) segment of *IGH* (*JH*) in the same transcriptional orientation. Therefore, an erroneous variable (*V*)/diversity (*D*)/*J* recombination process at the stage of B-cell precursor is likely responsible for the formation of this translocation, although this issue is still controversial.[4–6]

The 5′ flanking region of *BCL2* (5′-*BCL2*) is another breakpoint cluster of the translocation between *BCL2* and *IG*s. In contrast to t(14;18), 5′-*BCL2*/*IG*s translocation can involve not only *IGH*, but also two light chain gene (*IGL*) loci as partners,[7–16] and fusion between the relevant genes occurs in divergent orientation. However, the precise anatomy of the 5′-*BCL2*/*IG*s fusion gene, as well as its influence on the expression of *BCL2* in large numbers of cases, has not been described. Another particular feature of 5′-*BCL2*/*IG*s translocations is that they are observed in various types of B-cell tumors, including chronic lymphocytic leukemia (CLL),[13, 16] follicular lymphoma (FL)[8, 11, 12] and diffuse large cell lymphoma (DLCL).[7, 8] Thus, it is possible that the role of 5′-*BCL2*/*IG*s translocation in the pathogenesis of B-cell tumors may not be identical to that of t(14;18), which is closely associated with FL.

We have developed a long-distance (LD-) PCR method, which is capable of amplifying oncogene/*IG* junctions of up to 30 kb in size.[3, 17] Here, we cloned and sequenced a total of eleven 5′-*BCL2*/*IG*s fusion genes of B-cell tumors using an LD-PCR-based approach. We next studied levels of transcription of the *BCL2* gene in 5′-*BCL2*/*IG*-carrying cells and compared them with those in t(14;18) cells.

MATERIALS AND METHODS

Patients and established cell lines The patient popula-

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tion consisted of 9 patients with various types of B-cell tumor, which were confirmed to have rearrangements of 5′-*BCL2* by Southern blot hybridization. Of the 9 patients, one had CLL (no. 491), one had FL (no. 61), five had DLCL and the immunoblastic variant (nos. 472, 517, 585, 1001 and 1077), and the remaining two lacked lymphadenopathy but presented with leukemic manifestations (nos. 605 and 1122). More clinical details of 7 of those patients were described previously.[8] The Boar88 cell line was established from leukemic cells of a patient with FL. YM[7] and ShK cell lines were derived from clinical materials of nos. 1001 and 1077, respectively. KIS-1, FL-18, FL-218, FL-318 and FL-418 cell lines were described elsewhere.[18] These cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum under the standard culture conditions.

Southern and northern blot hybridization Genomic DNA was digested with restriction enzymes, electrophoresed through 0.8% agarose gels, and transferred onto nylon membrane filters (GeneScreen Plus, NEN Research Products, Boston, MA). Total cellular RNA was prepared using an RNeasy Total RNA Kit (Qiagen, Hilden, Germany), electrophoresed on 1.0% agarose gels containing 0.66 *M* formaldehyde and transferred onto membranes. The membranes were hybridized with probes labeled with ^{32}P-dCTP (Amersham Pharmacia Biotech, Buckinghamshire, England) using a random primer labeling system (Amersham Pharmacia Biotech). Hybridization and washing conditions were as recommended by the manufacturer. pB16 probe was a cDNA fragment corresponding to the second exon of *BCL2* and was used to detect rearrangements within 5′-*BCL2*.[15] The MBR probe was 'probe b'[19] provided by Dr. Y. Tsujimoto.

LD- and LD-inverse (LDI-) PCR Oligonucleotide primers and PCR cycling conditions for LD-PCR to detect 5′-*BCL2*/*IG*s fusion genes were described in our previous papers.[7, 17] Cλ/04 primer was newly designed for efficient amplification; the sequence was 5′-CTGTCTTCTCCACGGTGCTCCCTTCATGCGTGACCY-3′. LDI-PCR was developed to amplify unknown sequences adjacent to a known DNA segment.[20, 21] Briefly, genomic DNA was digested with appropriate restriction enzymes and purified by standard methods. The DNA was diluted to a concentration of 1 μg/ml and incubated at 16°C overnight in the presence of *T4* DNA ligase to facilitate intramolecular ligation. The self-ligated circular DNA was used as a template for nested PCR. The primers designed for the known 5′-*BCL2* sequences were: YAB/01, 5′-CCACCCCACGGCCCCCAGAGAAAGAAGAGGAGTTA-3′; YAB/02, 5′-AATCCAAGGTGGTAACTGGTATCTGTCTATCAAAT-3′; and YAB/03, 5′-GCATACAGACAGATTTTCTTCACATTTACTACCAA-3′.

Molecular cloning and nucleotide sequencing The PCR products were cloned into the plasmid pCR2.1 (Invitrogen, San Diego, CA) and DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen). Nucleotide sequencing was performed with a *BigDye* Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA), and the sequencing reactions were resolved on an ABI 310 automated sequencer (PE Applied Biosystems). The GenBank sequence database at the National Center of Biotechnology Information was accessed on the World Wide Web. The Basic Local Alignment Search Tool (BLAST) was used to identify nucleotide homology.

RESULTS

Amplification of 5′-*BCL2*/*IG*s fusion genes by LD-PCR-based assays We and others have cloned and characterized fusion genes consisting of 5′-*BCL2* and three *IG*s loci; i.e. 5′-*BCL2*/*IGH*, 5′-*BCL2*/*IGL*κ and 5′-*BCL2*/*IGL*λ fusions.[7–11, 13–16] To encompass the previously recognized breakpoints of these three types of fusion, we designed oligonucleotide primers for the second exon of *BCL2*, the *E*μ enhancer of *IGH*, the *C*κ constant gene of *IGL*κ and the consensus sequence of the *C*λ constant genes of *IGL*λ. Since the 5′-*BCL2*/*IG*s fusions reported previously occurred in divergent orientation, these primers corresponded to the anti-sense strand in the reverse direction.[17] Genomic DNA from clinical materials of 9 patients and Boar88 was subjected to LD-PCR, and 7 were positive for amplification. Two cases had a 5′-*BCL2*/*IGH* fusion, three had a 5′-*BCL2*/*IGL*κ fusion and three had a 5′-*BCL2*/*IGL*λ fusion gene; one case (no. 472) had two independent fusion genes. The amplified fragments ranged in size from 4.8 to 17.3 kb and were readily identifiable on ethidium bromide-stained gels (Fig. 1A).

We next applied the LDI-PCR strategy for cases in which LD-PCR was negative (nos. 61 and 1122, and Boar88). Genomic DNA was digested with *Hin*dIII and the circularized DNA was subjected to LDI-PCR. The positions and orientations of nested primers are presented in Fig. 2. As shown in Fig. 1B, the three cases had a unique LDI-PCR product, which was the expected size from the results of Southern blot analysis using the pB16 probe and the distance of the primers used.

Distribution of breakpoints on 5′-*BCL2* The LD- and LDI-PCR products were cloned into plasmids for sequencing analysis. Using a set of primers designed for 5′-*BCL2* in reverse orientation, we determined the positions of breakpoint on the 5′-*BCL2*. As illustrated in Fig. 2, the breakpoints were distributed from 378 to 2312 bp upstream of the translational initiation site, confirming that the coding region of *BCL2* was not disrupted by this type of translocation. Five breakpoints were close to or within the alternating purine-pyrimidine stretch reported previously.[22] Two were associated with the upstream P1 promoter, which is rich in G and C and contains multiple SP1

sites.[23] The other four breakpoints had no apparent association with characteristic sequences that were previously recognized within this area.

Heterogeneous breakpoints on *IG*s We next determined the sequences of each *IG* partner (Fig. 3). Two 5′-*BCL2*/*IGH* and two 5′-*BCL2*/*IGL*κ fusions involved the 5′

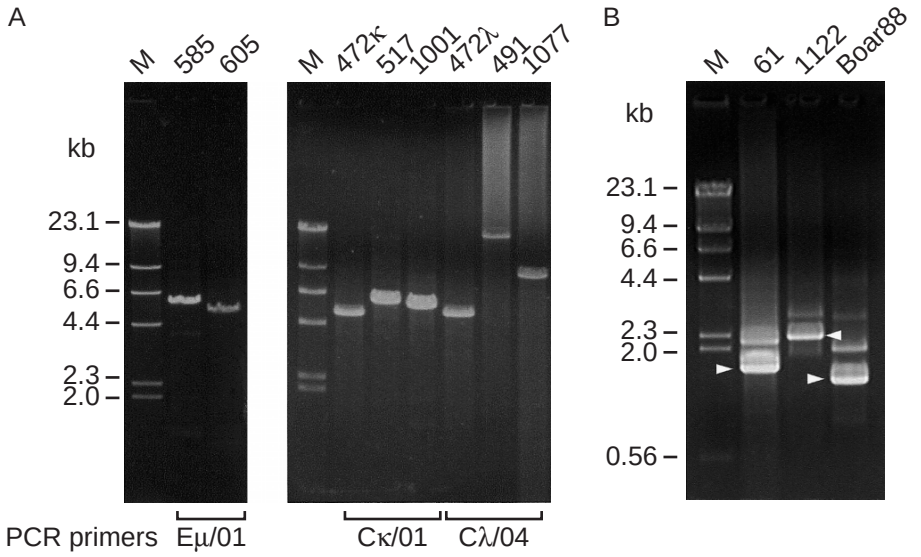

Fig. 1. Ethidium bromide-stained gel electrophoresis of LD- (A) and LDI- (B) PCR showing the 5′-*BCL2*/*IG*s fusion genes. The LD-PCR primers corresponding to the *IG* partners are indicated at the bottom. Arrowheads indicate the LDI-PCR products that contained the fusion genes. Aliquots of 10 μl were loaded in each lane and electrophoresed through a 0.7% agarose gel. *Hin*dIII-digested λDNA was used as a molecular weight marker.

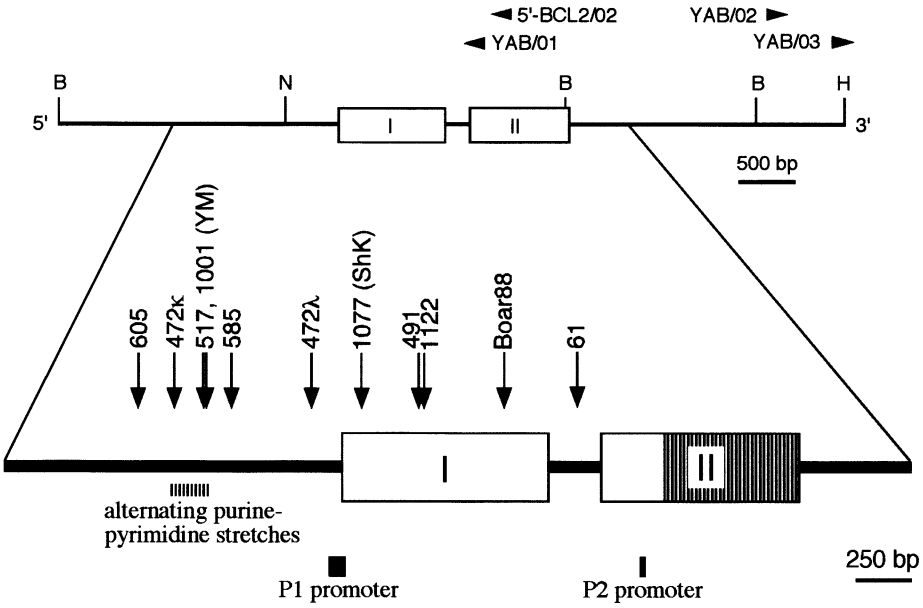

Fig. 2. Schematic representation of the 5′-*BCL2* and distribution of the breakpoints of 5′-*BCL2*/*IG*s fusion genes. The sequences including this area are registered in the database (GenBank accession no. AC009267). Exons I and II, and two promoters (P1 and P2) were determined by Seto *et al.*[23] The hatched area within exon II is the protein-coding region. Alternating purine-pyrimidine stretches are clustered 5′ to the P1 promoter.[22] Vertical arrows indicate the positions of breakpoints, and arrowheads indicate the approximate positions and orientations of primers for LD- and/or LDI-PCR. Restriction sites are: B, *Bam*HI; H, *Hin*dIII; and N, *Not*I.

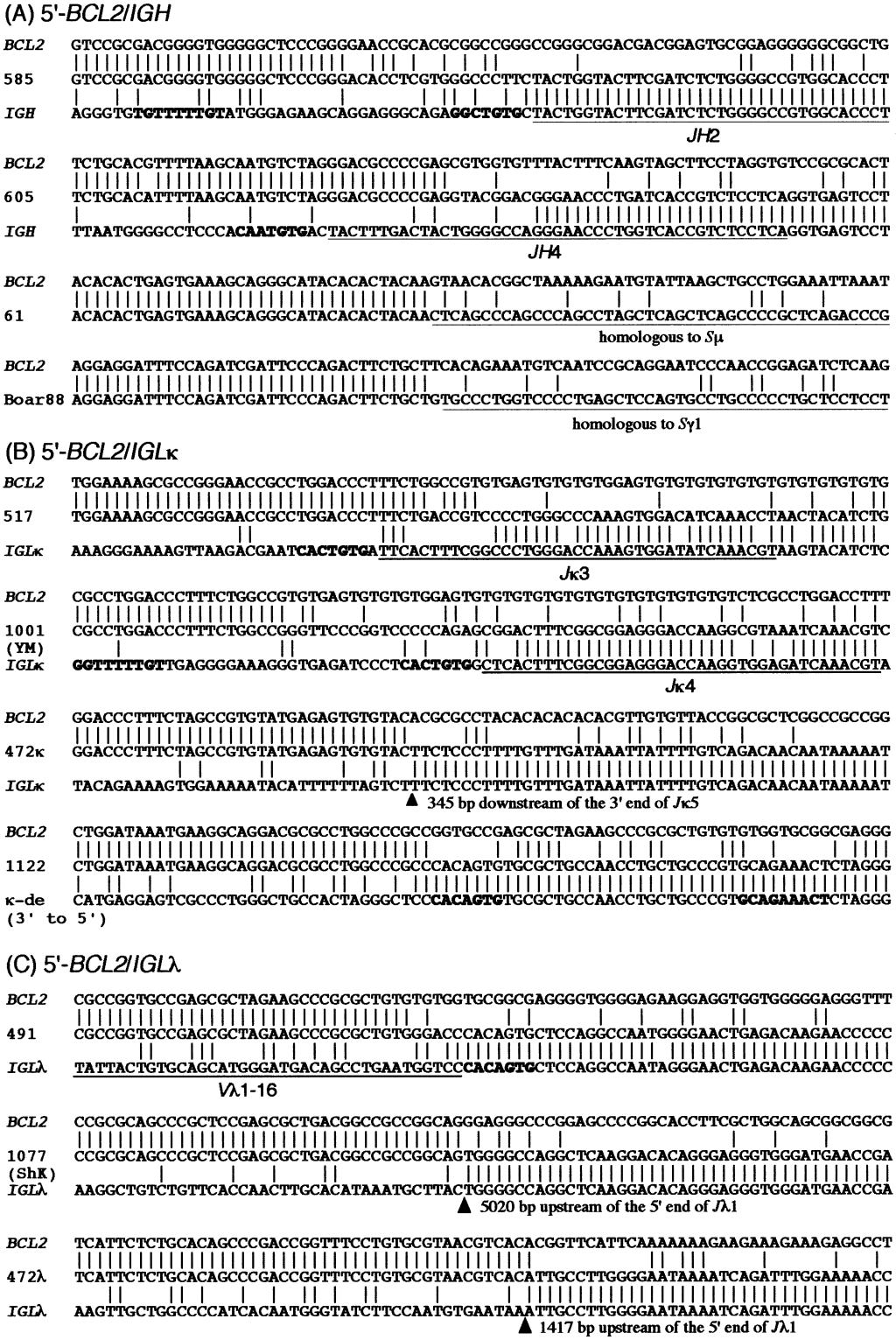

Fig. 3. Nucleotide sequences of the 5′-*BCL2*/*IG*s fusion genes. The sequences were oriented 3′ to 5′ for the 5′-*BCL2*. The germ line sequences of relevant *IG* segments refer to accession nos. X97051 for *IGH*, X67858 for *IGL*κ, and D86999 and D87023 for *IGL*λ genes. Vertical lines indicate nucleotide identity, and the heptamer and nonamer signal sequences are in boldface. The *IG* sequences of no. 61 showed the highest homology to an *Sμ* switch region registered in the database.

region of *J* segments, and variable numbers of nucleotides of unknown origin were inserted at the breakpoints, most likely reflecting errors in *V*/(*D*)/*J* recombination. As compared with the germ line sequences of the *J*κ3 and *J*κ4 segments, there was considerable nucleotide diversity. In contrast, the breakpoint of one 5′-*BCL2*/*IGL*κ was localized downstream of the 3′ boundary of the *J*κ segments, and two 5′-*BCL2*/*IGL*λ fusions occurred at points between the *V*λ2-1, which is the most 3′ *V* gene of *IGL*λ, and the *J*λ1 segment. There was no addition or deletion of nucleotides at these three 5′-*BCL2*/*IGL* fusions that occurred within the intronic sequences of *IG*s.

Three breakpoints recognized by LDI-PCR were not localized within the regions enclosed by primer sets for LD-PCR. The 5′-*BCL2* sequences of no. 61 were contiguous to a series of regular pentamer repeats with the consensus CCCAG or CTCAG, which are complementary to the repeated motifs of *IGH* switch regions.[24] The sequences juxtaposed to the 5′-*BCL2* in the Boar88 cell line matched those of the switch region associated with the *C*γ1 constant gene. On the other hand, the *IG* partner of no. 1122 was the κ-deleting element (κ-de),[25, 26] which is localized 24 kb downstream of *C*κ, and the joining point was immediately 3′ of the palindromic heptamer sequence. These three fusions occurred in the head-to-tail orientation.

Fig. 4A shows comparative structures between the 17.3 kb LD-PCR product from no. 491, 5′-*BCL2* and corresponding germ line *IGL*λ locus. The LD-PCR product included a 14.2 kb fragment that encompassed the *V*λ1-16 and *V*λ3-2 variable genes, localized ~0.5 megabases upstream of the *J*λ1 segment.[27] Fusion with the 5′-*BCL2* occurred immediately 3′ of the *V*λ1-16, while the downstream *V*λ3-2 joined to a *J*λ segment. The *V*λ3-2 gene of no. 491 carried multiple mutations in addition to a nucleotide insertion, resulting in a frame shift and therefore a non-producing configuration (Fig. 4B).

Expression of *BCL2* in lymphoma cells associated with 5′-*BCL2*/*IG*s fusion genes We established three cell lines that carried 5′-*BCL2*/*IG*s fusion genes. Cytogenetic analysis of ShK (5′-*BCL2*/*IGL*λ) and Boar88 (5′-*BCL2*/*IGH*) revealed that both cell lines had complex karyotypes including abnormalities of chromosomes 18 and 22 in ShK, and 14 and 18 in Boar88. The karyotype of YM (5′-*BCL2*/*IGL*κ) was described elsewhere.[7]

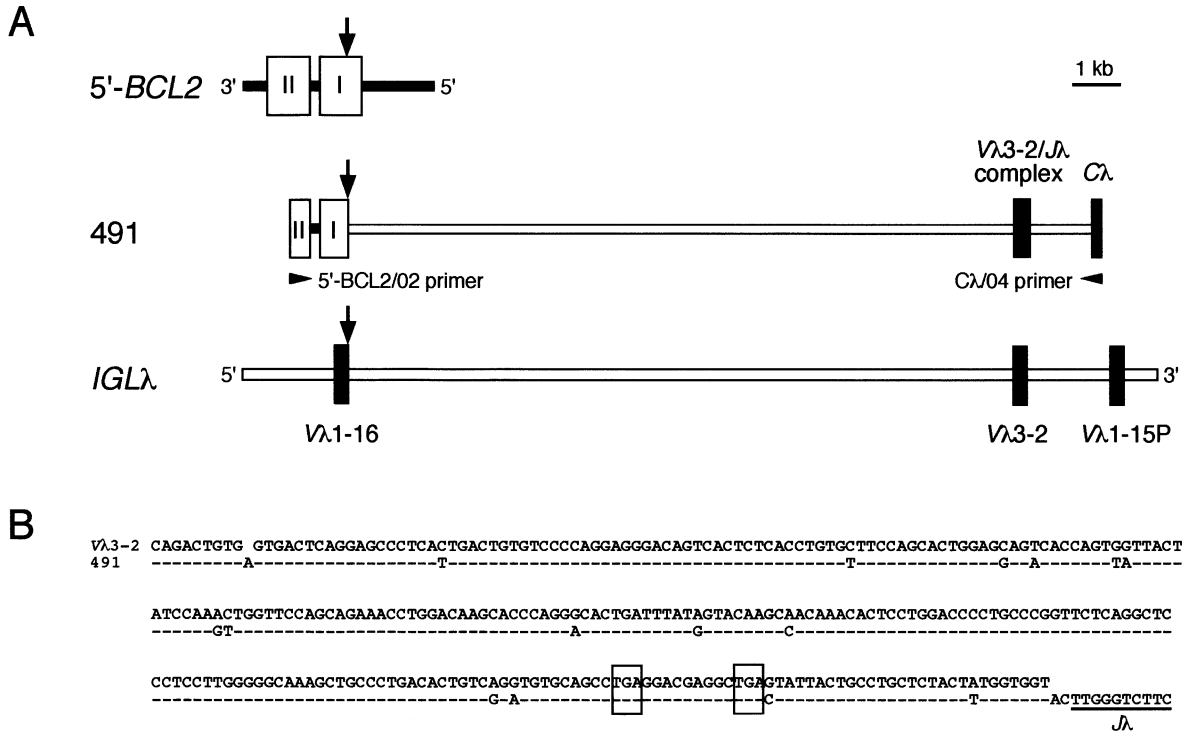

Fig. 4. (A) Comparative diagram of the 5′-*BCL2*/*IGL*λ fusion gene of no. 491 with the corresponding germ line structures. The two *V* genes (*V*λ1-16 and *V*λ3-2) and one pseudo-gene (*V*λ1-15P) were determined by Kawasaki *et al.* (accession no. D86999).[27] Vertical arrows indicate the breakpoints of the fusion and arrowheads are the approximate positions of the primers. (B) Comparison between the sequences of the germline *V*λ1-16 gene and those of no. 491. Dashes show nucleotide identity. Two potential stop codons resulting from the frame shift mutation are boxed.

To study whether these genetic lesions altered the expression of *BCL2* that was juxtaposed to each *IG* partner, we performed northern blot analysis using an MBR probe. Total cellular RNA isolated from leukemic cells of no. 605 was also subjected to this analysis. As indicated in Fig. 5, YM, ShK and Boar88 cells as well as no. 605 showed markedly higher levels of *BCL2* transcripts than those of t(14;18)-carrying cell lines; the latter cell lines carried *BCL2*/*IGH* fusion genes with breakpoints at the MBR or 3′-MBR.[3] As compared with the size of transcripts of a cell line with the germ line configuration of *BCL2*, 5′-*BCL2*/*IG*s-carrying cells expressed aberrantly-sized *BCL2* mRNA, indicating that these transcripts represent transcription from the rearranged allele.

DISCUSSION

In the present study, we cloned and sequenced a total of eleven 5′-*BCL2*/*IG*s fusion genes, and showed that heterogeneous breakpoints on *IG*s were involved in this type of oncogene/*IG* recombination. These included 5′ of *J* segments, 3′ of a *V* gene, κ-de, switch regions, and intronic sequences. Thus, it is apparent that errors in the *V*/(*D*)/*J* recombination process alone cannot account for the generation of 5′-*BCL2*/*IG*s fusion genes.

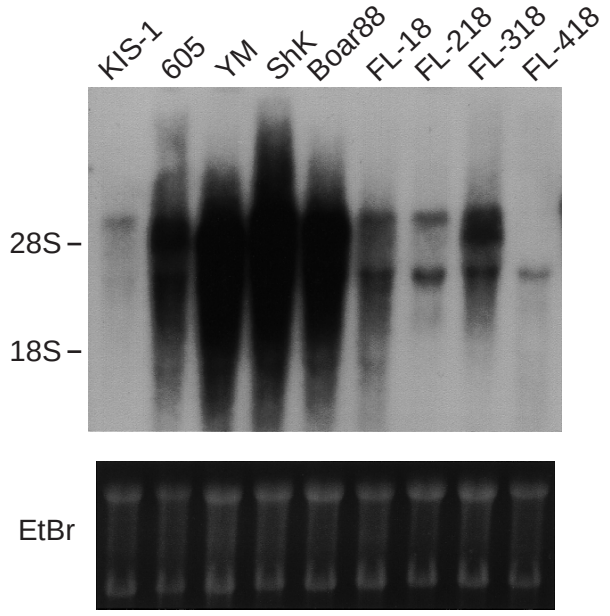

Fig. 5. Northern blot hybridization showing expression of *BCL2* in 5′-*BCL2*/*IG*s-carrying cells as compared with that in t(14;18) cell lines. Total cellular RNA (10 μg each lane) was hybridized with 'probe b' MBR probe. The positions of 28S and 18S rRNA are indicated. The ethidium bromide (EtBr) gel prior to transfer is shown at the bottom. KIS-1 is a diffuse large cell lymphoma cell line with the germ line *BCL2*; FL-18, FL-218, FL-318 and FL-418 are follicular lymphoma cell lines with a t(14;18)(q32;q21) translocation involving the MBR or 3′-MBR breakpoint.[3]

Recombination of *IGL* segments during B-cell differentiation occurs in an ordered fashion, such that recombination of *IGL*κ initiates prior to that of *IGL*λ. In λ-producing B-cells, a part of *IGL*κ is frequently deleted.[25] The κ-de, which is downstream of the *C*κ, joins to a point within the *J*κ-*C*κ intron, resulting in deletion of the enhancer and *C*κ elements. Since the κ-de is flanked by heptamer and nonamer signal sequences and a heptamer motif lies within the *J*κ-*C*κ intron, it is likely that this process of deletion of intervening sequences is mediated by the *V*/*J* recombination machinery.[26] The breakpoint of no. 1122 was localized immediately 3′ of the heptamer signal sequence; therefore, the 5′-*BCL2*/*IGL*κ fusion of this particular case was likely mediated by this deletional recombination. Indeed, the lymphoma cells of this case expressed λ light chain on their cell surface.

There are three types of molecular processes that modify the gene encoding antibody molecules; i.e. receptor editing, class switching and somatic hypermutation.[28] Receptor editing is a process by which the originally expressed antibody polypeptide chain is replaced by another.[28, 29] In the *IGL* loci, receptor editing is mediated by secondary rearrangements of the variable region gene, usually involving upstream *V* segments, and downstream *J* segments. In this process, the gene encoding the originally expressed light chain is deleted from the chromosome. Once this process leads to the expression of an 'innocent' immunoglobulin molecule, the B-cell leaves the bone marrow to become a naive B-cell.[28] The structure of 5′-*BCL2*/*IGL*λ of no. 491 seems to match a theoretical intermediate of the receptor editing of *IGL*λ loci; the *V*λ3-2/*J*λ recombination occurred first and then secondary *V*/*J* recombination occurred erroneously between *V*λ1-16 and 5′-*BCL2*. However, multiple mutations as well as a nucleotide insertion[30] leading to the out-of-frame rearrangement were introduced into the downstream *V*λ3-2, suggesting that the cell was at the stage of germinal center (GC) or post-GC B-cell, when the 5′-*BCL2*/*IGL*κ recombination developed. These observations together with the presence of two 5′-*BCL2*/*IGH* cases involving switch regions suggest that a proportion of 5′-*BCL2*/*IG*s recombination events do not occur in the bone marrow, but in the peripheral lymphoid organs. The reactivation of recombination activation genes *RAG1* and *RAG2* in GC[31] may in part support the hypothesis that oncogene/*IG* translocation mediated by *V*/(*D*)/*J* recombination is not restricted to B-cell precursors in the bone marrow.[5, 28]

The mechanisms responsible for the intronic breakpoints remain to be determined. We recently reported two DLCL cases with c-*MYC*/*IGH* translocation having *JH*-*Eμ* and four with *BCL6*/*IGL*λ having *V*λ-*J*λ1 and *J*λ-*C*λ

intronic breakpoints.[20, 32] There is evidence that c-*MYC*/*IGH* and *BCL6*/*IG*s translocations are related to somatic hypermutation, which targets not only *V* genes of *IG*s, but also particular hot spots of these two oncogenes.[4, 6] Thus, it is possible that somatic mutations are also introduced within the introns in addition to the relevant oncogenes, thereby playing a role in the development of translocations. Indeed, small deletions and nucleotide substitutions were observed in 5′-*BCL2*.[33, 34] In summary, the results of the present study suggested that multiple molecular mechanisms are involved in the generation of 5′-*BCL2*/*IG*s translocations and that B-cells at heterogeneous stages of differentiation are targeted by this particular type of translocation, potentially accounting for the diverse clinicopathological features of B-cell tumors carrying rearrangement of 5′-*BCL2*.

The *BCL2* gene is composed of three exons, and two potential promoter regions have been identified.[23] The P1 promoter is associated with exon 1, and is rich in G and C with multiple SP1 sites. In contrast, P2 is located 5′ to the open reading frame in exon 2, and has classic TATA and CAAT boxes. In lymphoma cells carrying t(14;18), breakpoints of the translocation are ~370 kb downstream of this promoter area, and the coding region of *BCL2* separated by the extremely large intron is placed 5′ to a *J* segment of *IGH*. As the result of translocation, multiple chimeric transcripts that contain both the *BCL2* and *JH-CH* messages are generated. [23] Thus, one possible mechanism for the deregulated expression of Bcl-2 protein is that replacement of the A and U-rich 3′ untranslated region of *BCL2* mRNA with *IGH* components can alter the stability of *BCL2* mRNA,[35] although an earlier study demonstrated comparable half-lives of *BCL2* and *BCL2*/*IGH* fusion mRNA.[23] In the present study, we examined the level of *BCL2* expression, the promoter of which is placed in close proximity to *IG*s. The results showed invariably high levels of *BCL2* mRNA in 5′-*BCL2*/*IG*s-carrying cells irrespective of the diversity of the *IG* partners and juxtaposed segments. Thus, it was suggested that transcriptional activation of the *BCL2* gene under the control of *IG* enhancer/promoter activity is a particular feature of 5′-*BCL2*/*IG*s rearrangements. Our study suggested that the mechanisms underlying the formation of 5′-*BCL2*/*IG*s translocations and their pathogenetic role in B-cell tumors are not identical to those of t(14;18).

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