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bcl-2 Translocation in Japanese B Cell Lymphoma: Novel *bcl-2* Translocation with Immunoglobulin Heavy Chain Diversity Segment

Masao Seto,[1,6] Hirotaka Osada,[2] Ryuzo Ueda,[1] Chie Ito,[2] Osamu Iwaki,[3] Atushi Oyama,[4] Taizan Suchi[5] and Toshitada Takahashi[2]

Laboratory of [1]Chemotherapy and [2]Immunology, Aichi Cancer Center Research Institute, [5]Clinical Laboratory, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya 464, [3]Department of Internal Medicine, Nagoya City University Medical School, Mizuho-cho, Mizuho-ku, Nagoya 467 and [4]Department of Internal Medicine, Aichi Hospital, Kakemachi, Okazaki 444

Breakpoints of a lymphoma case with *bcl-2* gene rearrangement that did not show comigration of immunoglobulin (Ig) heavy chain joining (J_H) fragment were cloned. Sequence analysis revealed that the translocation broke the 3′ side of the Ig heavy chain diversity (D_H) segment at the heptamer recombination signal and each end was ligated to the *bcl-2* locus. Since Southern blot demonstrated that both alleles of J_H were rearranged, this translocation was suggested to have occurred at the step of V_H-D_H, or D_H-D_HJ_H recombination, one step later than that of D_H-J_H recombination where the common pattern of *bcl-2* rearrangement generally occurs. Cases that showed comigration with J_H fragment were also studied by polymerase chain reaction with 5′ bcl-2 oligomer and 3′ J_H consensus anti-sense oligomer since it has been demonstrated that *bcl-2* translocation at the major breakpoint clustering region (mbr) in American cases clusters within an about 150 bp region in the mbr. The results demonstrated that four out of five cases studied were amplified, indicating that the same clustering mechanism exists for Japanese cases. The present study, together with our previous report on *Igκ-bcl-2*, indicated that *bcl-2* translocation in Japanese B cell lymphomas might occur at a later stage of B cell development, as compared with that in American cases. Less involvement of *bcl-2* in Japanese B cell lymphoma may also be in part explainable by low susceptibility to *bcl-2* rearrangement at the step of D_H-J_H recombination.

Key words: *bcl-2* — Lymphoma — Translocation — Immunoglobulin — Diversity segment

Chromosomal translocation, t(14;18)(q32;q21), is one of the most common chromosomal abnormalities in lymphoma.[1-3] Molecular analysis revealed that the *bcl-2* gene at 18q21 is juxtaposed to Ig heavy chain locus (14q32),[4-6] resulting in deregulation of the *bcl-2* gene.[7] A major breakpoint clustering region (mbr) and a minor breakpoint clustering region (mcr) at the *bcl-2* locus were demonstrated and the ratio of rearrangement at each region has been reported to be two to one.[8] Pathological study has revealed that the incidence of lymphoid malignancy is quite different between Japan and the USA for each histological type. For example, the incidence of follicular lymphoma has been reported to be 3.8 per 100,000 in the USA, but 0.5 in Japan.[9] Diffuse B cell lymphoma was also reported less frequently in Japan than in the USA.[9,10] We and others have studied *bcl-2* gene rearrangement in Japanese B cell lymphoma and have found that, in contrast to 80 to 90% involvement in American cases, only 30% of follicular lymphoma showed *bcl-2* gene rearrangement.[11,12] In diffuse B cell lymphoma, involvement of *bcl-2* gene rearrangement was also less in Japanese cases.[8,12] These studies suggested that lower frequency of B cell lymphoma, particularly follicular lymphoma, in Japan is partly due to lower frequency of *bcl-2* involvement.

To study the molecular basis for less *bcl-2* involvement in Japanese B cell lymphoma, we have attempted to study the cases in which rearranged *bcl-2* fragment does not show comigration with J_H fragment, since most *bcl-2* rearrangements reported in the USA showed comigration with J_H fragment.[4-6,8,13] The previous study showed that two out of 10 cases with *bcl-2* rearrangement did not exhibit comigration.[12] One of these two cases possessed translocation with Igκ (Case HN, follicular lymphoma),[12] being similar to the variant form of *myc* translocation in Burkitt's lymphoma,[14] suggesting that the translocation in this case had occurred at the stage of Igκ rearrangement. The present study of the other case (Case JA, diffuse small cleaved cell lymphoma) demonstrated that *bcl-2* gene had rearranged with D_H and suggested that *bcl-2* rearrangement had occurred after the step of D_H-J_H rearrangement at which *bcl-2* translocation takes place in most American cases.[15,16] Five cases showing comigration with J_H fragments were also studied by polymerase chain reaction (PCR) to examine whether the breakpoints in the mbr cluster within an about 150 bp region, similarly to the American cases,[5,15,17,18] and to study whether breakpoints involve the D_H segment. Four

[6] To whom all correspondence should be addressed.

cases were found to be clustered in the same region, suggesting that a common mechanism for clustering in the mbr existed for Japanese cases as well. Involvement of known D_H, however, was not demonstrated.

MATERIALS AND METHODS

Southern blot analysis Southern blot analysis was carried out as described previously.[12] Patients studied in this paper were those studied previously for *bcl-2* rearrangements.[12] Case JA was diagnosed as diffuse small cleaved cell lymphoma. Probes used in this study were 1) *bcl-2* cDNA#58 which detects exons I, II and III,[7] 2) *Eco*RI/*Hin*dIII 2.8 kb mbr,[4] 3) 3′ *bcl-2* of *Bgl*II/*Bam*HI 1.2 kb (shown in Fig. 2), 4) *Hin*dIII/*Bam*HI 1.2 kb fragment derived from phage#37, a region introduced at 3′ *bcl-2* (Fig. 2), 5) J_H *Bam*HI/*Hin*dIII 6.0 kb.

Genomic library DNA of Case JA was digested to completion with either *Bam*HI (for EMBL3 vector) or *Eco*RI (for λ dash vector; Strategene, La Jolla, CA), and size separated on low melting point (LMP) agarose. The 22 kb to 12 kb region was cut out. DNA was extracted with phenol, ligated with the respective vector, and packaged *in vitro*.

Polymerase chain reaction (PCR) The modified 5′bcl-2 oligomer 5′-TAGAGATCTGCTTTACGTGGCCTG-TTT-3′ and the 3′ anti-sense J_H consensus oligomer 5′-ACCTGAGGAGACGGTGACC-3′ were synthesized.[18] PCR was carried out for 35 cycles of denaturation at 92°C for 1 min, annealing at 62°C for 2 min and extension at 72°C for 3 min using a GeneAmp kit (Cetus, Norwalk, CT). One-tenth of the 100 μl reaction mixture was electrophoresed on 2% agarose gel, and Southern blot analysis was done with an mbr probe. The remaining solutions was applied to LMP agarose and the amplified fragment was purified, kinased with polynucleotide kinase (Pharmacia, Tokyo), digested with *Bgl*II and ligated into pBluescript (Strategene) at the *Bam*HI/*Sma*I site.

DNA sequencing DNA fragments were cloned into pBluescript and double-stranded DNA was sequenced with T3 or T7 primer by the dideoxy method (Sequenase, USB, Cleveland, OH) with some modification.[19]

RESULTS

Southern blot analysis A representative Southern blot of Case JA is shown in Fig. 1. With *Bam*HI digestion, cDNA#58 probe demonstrated one rearranged fragment of 18.5 kb and two germline fragments of 20.5 kb containing exon III and of 4.4 kb containing exons I and II (Fig. 1a, left panel). The same blot was rehybridized with 3′ bcl-2 probe and demonstrated a 4.3 kb rearranged

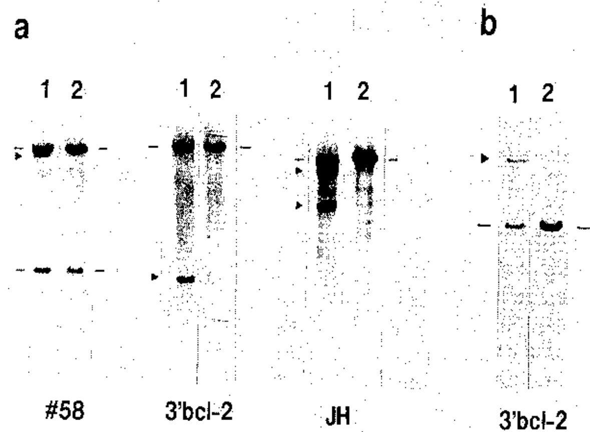

Fig. 1. Southern blot analysis of Case JA. DNA from Case JA (lane 1) and normal liver (lane 2) was digested either with *Bam*HI (a) or *Eco*RI (b). a) cDNA#58 probe revealed one rearranged fragment of 18.5 kb and two germline fragments of 20.5 kb and 4.4 kb (left panel). The same blot was rehybridized with 3′ bcl-2 probe (shown in Fig. 2) and revealed a 4.3 kb rearranged fragment (middle panel). The two rearranged fragments of *bcl-2* did not show comigration with J_H fragments (rearrangement fragments of 11 kb and 8.4 kb as well as 13 kb germline fragment were seen with J_H probe; right panel). b) 3′ bcl-2 probe revealed a 19 kb rearranged fragment as well as a 7 kb germline *bcl-2* fragment. Germline fragments are marked with dashes, and rearranged fragments with triangles.

fragment (Fig. 1a, middle panel). Rearranged fragments of *bcl-2* detected by #58 or 3′bcl-2 probe did not show comigration with rearranged J_H (Fig. la, right panel), J_κ, or C_λ fragment (data not shown). Thus, it was suggested that *bcl-2* is translocated to a unique region that has never been described before. It should be noted that both J_H alleles showed rearrangements and neither of the J_H regions was involved in the *bcl-2* translocation.

Genomic cloning Since *Bam*HI digestion of Case JA showed an 18.5 kb rearranged fragment with cDNA#58 probe, a genomic library with complete *Bam*HI digestion was constructed with size-selected DNA and screened with an mbr probe. Consequently, two phages were obtained out of 5×10^5 recombinant clones. The size of the insert was 20.5 kb and 18.5 kb, respectively, suggesting the former (#20) to be germline *bcl-2*, and the latter (#37) to be rearranged *bcl-2* (Fig. 2). A unique *bcl-2* fragment 3′ to the breakpoint region was developed from 20.5 kb germ line *bcl-2* (3′bcl-2 probe, Fig. 2) and it was shown that this probe detected a 4.4 kb rearranged fragment with *Bam*HI digestion and a 19 kb rearranged fragment with *Eco*RI digestion (Fig. 1a and 1b). A genomic library was constructed with size-selected DNA which was digested to completion with *Eco*RI, and

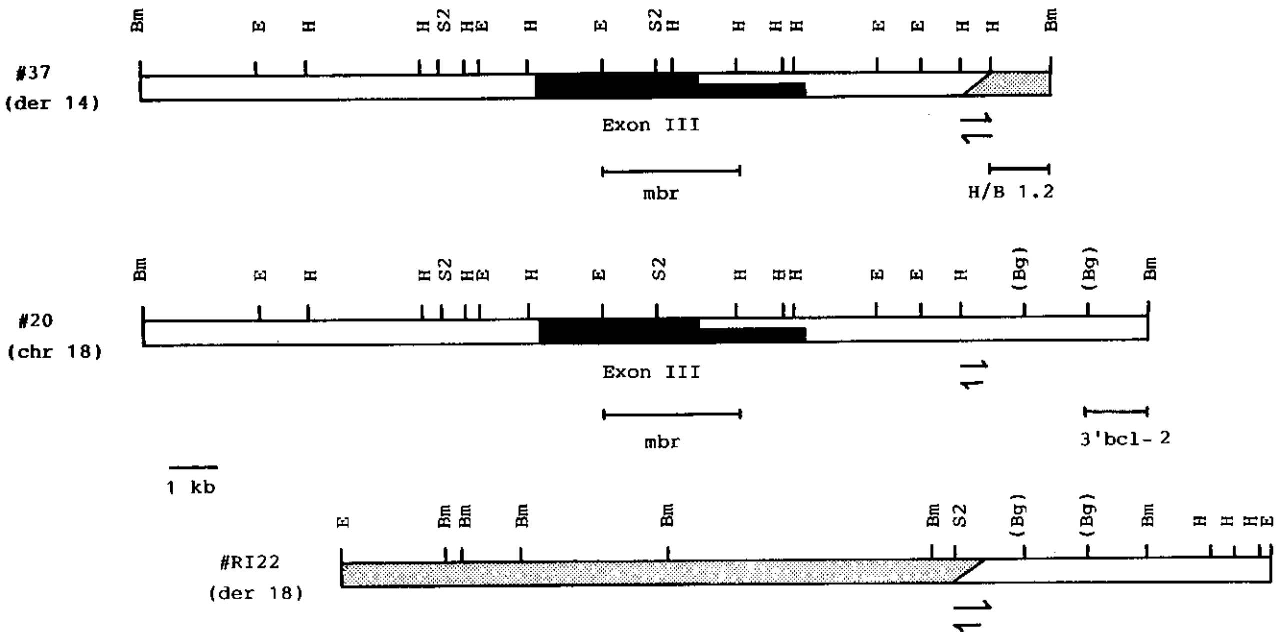

Fig. 2. Restriction enzyme map of rearranged *bcl-2* (#37, 18.5 kb *Bam*HI fragment; #RI22, 19 kb *Eco*RI fragment) and germline *bcl-2* (#20, 20.5 kb). Black boxes represent *bcl-2* exon III which has alternative poly A signals. White-and-black boxes represent *bcl-2* locus. Dotted boxes represent DNA fragments introduced into the *bcl-2* locus. Arrows indicate sequencing strategy to characterize breakpoint regions. Probes for the present study are indicated. *Bgl*II sites in parenthesis were determined only in short regions for finer comparison. *Bam*HI (Bm), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Sac*II (S2).

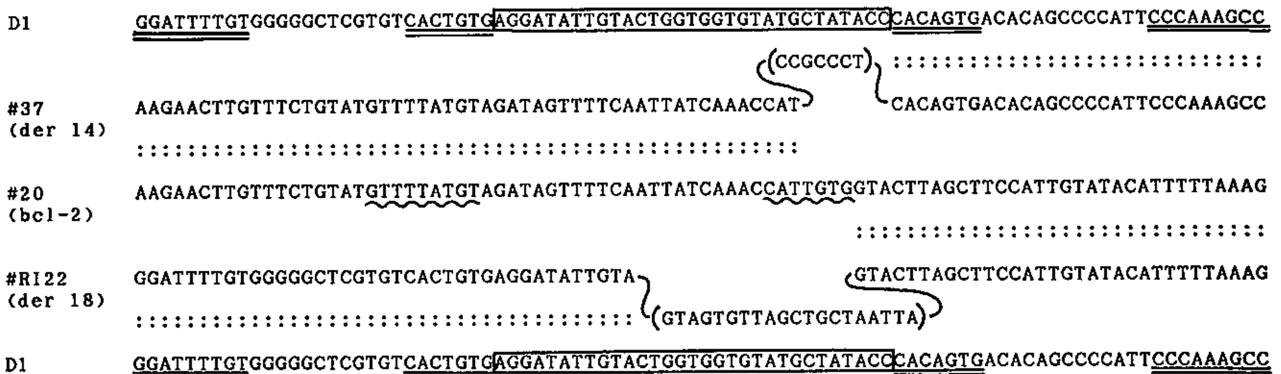

Fig. 3. Nucleotide sequences of breakpoint regions of #37, #20 and #RI22. Ig heavy chain diversity segment, D1 sequence, is aligned on the top and bottom lines for comparison. The D1 coding segment is boxed and the heptamer-nonamer recombination signal sequence (RSS) is double-underlined. Wavy lines indicate an RSS-like sequence in *bcl-2* which is compatible with the 12/23 rule for recombination.[27] An N-like sequence of unknown origin is parenthesized. Homologous regions are depicted by colons (:).

screened with 3′bcl-2 probe; two phages were obtained out of 4.5×10^5 recombinant clones. Both gave an identical restriction map and one of them, #RI22, was further analyzed.

Restriction mapping and sequence analysis Each phage insert was cloned in pBluescript and the restriction enzyme site was determined. As shown in Fig. 2, #37 demonstrated a different restriction map from #20 of

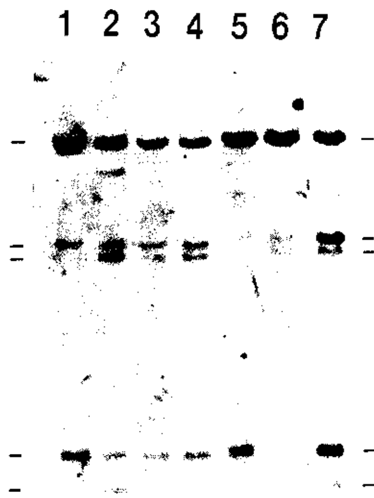

Fig. 4. Southern blot of various B cell lymphomas with H/B 1.2 kb probe (see Fig. 2) after *Bam*HI digestion. Lanes 1 to 6, B cell lymphomas; liver DNA in lane 7 revealed germline fragments of 18 kb, 7.4 kb, 6.6 kb, 1.8 kb and 1.6 kb (indicated by dashes), similar to the pattern demonstrated with a D region probe by Siebenlist *et al.*[20] All B cell lymphomas from lanes 1 to 6 showed either rearrangement, deletion or both.

germ line *bcl-2* beyond the most 3′ *Hin*dIII site, suggesting that the breakpoint is located close to this site. *Bgl*II, 3′ to the breakpoint region, to *Bam*HI of #RI22 showed an identical restriction map with #20 at this region, and 5′ to this region showed a different restriction map, suggesting that 5′ to this region contains the rearranged fragment juxtaposed to the 3′ *bcl-2* gene. *Hin*dIII/*Hin*dIII 350 bp fragment of #37 across the breakpoint, 0.9 kb *Hin*dIII/*Bgl*II fragment from #20 of germ line *bcl-2*, and *Sac*II/*Bgl*II 0.9 kb across the breakpoint from #RI22 were subcloned and sequenced.

Comparison of the nucleotide (nt) sequences of #37 and #20 revealed an identical sequence at the 5′ site and the sequence after the breakpoint of #37 demonstrated an identical sequence to that 3′ to the coding region of D1 or D4[20] after a stretch of 7 nt of CCGCCCT (Fig. 3). A heptamer-spacer-nonamer-like sequence compatible with Ig recombination existed near the breakpoint in #20 of germ line *bcl-2*. #RI22 breakpoint region was shown to possess the 5′ coding region of D1 (11 nt), which is followed by 20 nt of unknown origin and by a sequence identical to *bcl-2* which is broken precisely at 3′ to the heptamer-like signal of *bcl-2*. Four nt from the *bcl-2* breakpoint site and 20 nt of D1 coding segment were deleted (Fig. 3). The 3′ side beyond the breakpoint region of #37 was suggested to possess the D_H region by

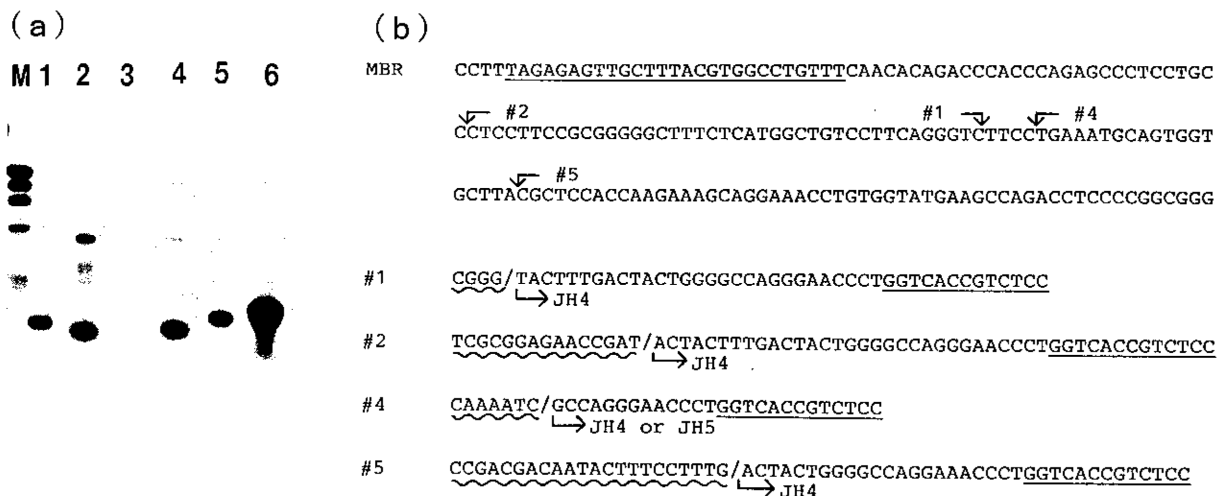

Fig. 5. Southern blots of PCR-amplified fragments probed with mbr probe (see Fig. 2) and the sequence of breakpoint regions of each patient. a) Cases with *bcl-2* rearrangement at the mbr which showed comigration with J_H fragment (lanes 1 to 5) were amplified with 5′ bcl-2 oligomer and 3′ anti-sense oligomer. Amplification was found in four out of five patients (1 and 5, follicular mixed; 2, follicular small cleaved; 3, diffuse small cleaved; 4, diffuse large). Lane 6 shows SUDHL-6 as a positive control which was previously demonstrated to be amplified with the same sets of oligomers.[18] M; ϕX174DNA/*Hae* III marker, 1.36 kb, 1.08 kb, 0.87 kb, 0.6 kb from top. b) Amplified fragments shown in a) were sequenced and the breakpoints of the patients are indicated by arrows in the mbr clustering region sequence (MBR line). The sequence following the *bcl-2* breakpoint of each patient is shown (#1–#5 line). The sequence of the PCR oligomer is underlined. The N-like segment between *bcl-2* and J_H is depicted by wavy lines and the J_H region of each breakpoint region is separated by (/) from the N-like segment and indicated below by an arrow.

Southern blotting of various B cell lymphomas using H/B 1.2 kb as a probe (Fig. 2). As shown in Fig. 4, all B cell lymphomas demonstrated either deletion, rearrangement or both when compared with the germ line pattern of liver DNA. This germline pattern of *Bam*HI digestion is identical to that published by Siebenlist *et al.*[20] The size of 1.2 kb *Hin*dIII/*Bam*HI suggested that this region is D1 since there is no such fragment at 3′ of the D4 region.[20]

Clustering of breakpoints in a narrow region of the mbr The present study, combined with the previous report, raised the possibility that *bcl-2* rearrangement in Japanese B cell lymphomas may occur at a more mature stage of B cell development, as compared with most American cases, in which *bcl-2* rearrangement takes place at D_H-J_H recombination. Thus, it is probable that even the cases showing comigration with J_H probe may have developed from the B cells at stages later than that of D_H-J_H recombination. Therefore, attempts were made to determine the breakpoints of five Japanese comigrated cases by PCR amplification with a combination of 5′ bcl-2 oligomer and 3′ anti-sense J_H consensus oligomer. Southern blot analysis (shown in Fig. 5a) demonstrated that the breakpoints of four out of five cases were clustered within a region of about 150 bp and that the breakpoint of the remaining case is outside this region. Thus, the ratio of clustering is broadly in accord with that of American cases.[15, 18] Sequence analysis revealed that none of these breakpoints showed an identical site and N-like segments of four to 22 nt were demonstrated (Fig. 5b).

DISCUSSION

Lower frequency of *bcl-2* gene rearrangement in Japanese B cell lymphoma, particularly in follicular lymphoma has been reported by us[12] and also by Amakawa *et al.*[11] we further demonstrated a unique case with *Igκ-bcl-2* rearrangement in which translocation took place at a more mature stage of B cell development than in American cases.[12] Case JA (diffuse lymphoma) reported in the present study showed no comigration of the *bcl-2* rearranged fragment with any of J_H, J_κ or C_λ fragment, suggesting a novel pattern of *bcl-2* translocation. Cloning and sequence analysis demonstrated that *bcl-2* was broken at 3′ to the mbr and 5′ *bcl-2* was translocated to the 3′ flanking region of D1, while 3′ *bcl-2* was translocated to the coding segment of D1. The question arose, what stage of B cell development was likely to be involved in translocation in this case? Since the 3′ end of D1 is involved in translocation, this resembles D_H-J_H joining where the sequence between 3′ to the D_H segment and 5′ to the J_H segment is deleted. If this translocation had taken place at the step of D_H-J_H joining, recombination machinery would have recombined and processed 3′ recombination signal sequence (RSS) to D_H and RSS-like sequence at *bcl-2* instead of 5′ RSS of J_H. In such a case, one of the J_H alleles should remain in the germ line configuration. Case JA, however, showed both J_H alleles rearranged, suggesting that the translocation had taken place at D_H-D_H J_H or V_H-D_H J_H joining, at least one step later than that of D_H-J_H joining when most *bcl-2* translocations take place in American cases.[15, 16]

Endemic and sporadic types of Burkitt's lymphoma were shown to involve different regions of Ig heavy-chain gene,[21] endemic Burkitt's being at J_H, while sporadic Burkitt's is at the switch region. It is speculated that the difference in the stage of B cell development at which *myc* translocation takes place is responsible for clinical, epidemiological, and phenotypic differences of Burkitt's lymphoma between these areas.[22] The fact that two out of 10 cases with *bcl-2* rearrangement were shown to have translocated at later steps than D_H-J_H joining may suggest that *bcl-2* translocations of Japanese cases have a tendency to take place at a later stage of B cell development, like sporadic type of Burkitt's lymphoma, although the remaining eight cases showed the common pattern of *bcl-2* translocation . If the susceptibility to *bcl-2* translocation shifts slightly to a more mature stage of B cell development, the possible number of B cells exposed to the translocation might decrease, leading to a lower frequency of *bcl-2* involvement for Japanese B cell lymphoma. Alternatively, the higher frequency of B cell lymphoma, particularly follicular lymphoma, and of *bcl-2* involvement in the USA might be caused by some unknown agent(s) which increases the susceptibility of B cells to *bcl-2* translocation. Recently, Adachi *et al.* reported three cases of *bcl-2* translocation with Ig light chain locus in B cell chronic lymphocytic leukemia (CLL),[23] which is much more frequent in the USA than in Japan (2.5 per 100,000 in the USA and 0.1 in Japan).[24] In the USA, *bcl-2* translocation at a more mature stage of B cell development may cause a distinct type of hematopoietic malignancy such as CLL.

A heptamer-spacer-nonamer-like sequence compatible with Ig recombination existed at the *bcl-2* breakpoint region and the location just 3′ to this sequence was cut and juxtaposed to the coding segment of D1 with extra nucleotides (Fig. 3). This suggested the involvement of recombinase in *bcl-2* breakage in Case JA. Breakpoints in the mbr in four out of five patients were demonstrated by PCR to be located in the narrow clustering region, suggesting that the same mechanism of clustering as has been reported in American cases plays a role in Japanese cases as well.[5, 15, 17, 18] Sequence analysis of PCR-amplified fragments demonstrated that the breakpoints in the mbr are not identical to each other, and that no heptamer-like sequence exists near these breakpoints, suggesting that

breakage at *bcl-2* is not necessarily brought about by recombinase in these cases, although involvement of recombinase for the Ig gene is suggested.

Extra nucleotides at the fusion site of *bcl-2* and the Ig gene in the present study vary in number from four to 22 nt. Lieber *et al.* demonstrated by using a transfection system that the N segment is at most 9 nt.[25] The present study showed that three out of six N-like segments are longer than those reported by Lieber *et al.*[25] Since *bcl-2* translocation was demonstrated to occur at a later stage than D_H-J_H recombination, it is possible that translocation occurs at V_H-D_H joining, too. In such a case, the sequence of *bcl-2*-N-D_H-N-J_H could exist, with more extra nucleotides. The present study, however, failed to demonstrate the sequence compatible with D_H coding segment (Fig. 5b), although this possibility still remains because characterization of human D_H segment is not completed yet.[26] The long extra-nucleotide insert in #RI22 of Case JA is, however, not explainable by the insertion of a D_H segment, since the heptamer-nonamer sequence of the non-coding segment of D1 follows after the extra nucleotides.

Recently, we have expanded our study on *bcl-2* involvement in B cell lymphoma and a total of 15 cases showing *bcl-2* rearrangement was accumulated. Interestingly, we found a new case of *bcl-2* translocation at the 5′ *bcl-2* region which demonstrated comigration with J_H fragment (Case MS, follicular lymphoma; unpublished data). This pattern of translocation is also not common, since most cases possessing comigration of J_H fragment have breakpoints at the 3′ *bcl-2* region, such as the mbr. It is, therefore, important to examine what stage of B cell development is involved in this *bcl-2* translocation, since the result might provide a clue to understanding the difference of *bcl-2* involvement between Japan and the USA.

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