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bcl-2 Gene Rearrangement Analysis of Japanese Follicular Lymphomas by Polymerase Chain Reaction in Formalin-fixed, Paraffin-embedded Tissue Specimens

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The prevalence of chromosomal translocation t(14;18) was examined in 41 cases of follicular lymphoma covering all histopathological subtypes, using the polymerase chain reaction and hybridization method with non-radioactive oligonucleotide probes. DNAs were extracted from unfixed fresh-frozen and/or formalin-fixed paraffin-embedded biopsy specimens. The t(14;18) chromosomal translocation, resulting in *bcl*-2-J_H (immunoglobulin heavy chain gene joining segment) fusion gene, was detected in 7 (36.8%) of 19 follicular small cleaved cell lymphomas and 6 (54.5%) of 11 follicular mixed, small cleaved cell and large cell lymphomas. Meanwhile, 11 cases of follicular large cell lymphoma revealed no *bcl*-2-J_H fusion gene. There was a statistically significant difference in the frequency of *bcl*-2-J_H rearrangement between follicular, small cleaved cell and large cell lymphoma (two-sided Fisher exact test, $P=0.049$). The difference of *bcl*-2 rearrangement frequency between follicular, mixed and large cell lymphoma was also statistically significant ($P=0.012$).

Key words: *bcl*-2 gene — Rearrangement — Lymphoma — PCR — Nonradioactive hybridization

The specific chromosomal translocations found in distinct types of neoplasms suggest that these gene rearrangements may play an important role in carcinogenesis.[1,2] In non-Hodgkin's lymphoma, the t(14;18) translocation is the most common chromosome abnormality following t(14;8) translocation. This karyotypic abnormality t(14;18) was observed in up to 85% of follicular lymphoma and in 28% of diffuse large cell lymphoma[3–7] in Europe and the U.S. The translocation t(14;18) joins *bcl*-2 gene on chromosome 18 with one of the six J segments of the immunoglobulin heavy chain locus on chromosome 14. This generates deregulated *bcl*-2-immunoglobulin fusion gene, resulting in an overproduction of *bcl*-2 mRNA and protein.[8–15] This deregulated *bcl*-2 gene expression delays the onset of cell death[16–18] and prolongs the survival of cells. On chromosome 18, approximately 60% of t(14;18) breakpoints occur within the 150 bp major break point region (mbr), which is located at the untranslated region within the third exon of *bcl*-2. In comparison, 25% of t(14;18) breakpoints occur at the minor cluster region (mcr), a second site located approximately 30 kb downstream from *bcl*-2 gene.[11,19] The clustering of breakpoints at mbr and mcr enabled the use of polymerase chain reaction (PCR) to detect *bcl*-2-immunoglobulin fusion gene by using a single chromosome 18 specific primer with a consensus immunoglobulin heavy chain primer.[20,21] No detailed analyses of histological subtypes of follicular lymphoma have been reported so far concerning this type of gene rearrangement. In this study, the authors observed the prevalence of translocation t(14;18) covering all histopathological subtypes of Japanese follicular lymphoma by PCR and hybridization with sequence-specific oligonucleotide, using formalin-fixed paraffin-embedded tissue specimens and/or unfixed fresh-frozen tissue specimens.

MATERIALS AND METHODS

Specimens and DNAs Eighty-two cases of non-Hodgkin's lymphoma were studied, including 41 cases of follicular lymphoma, 21 of diffuse B-cell lymphoma and 20 of diffuse T-cell lymphoma. The 41 cases of follicullar lymphoma consisted of 19 cases of follicular, small cleaved cell subtype, 11 of follicular, mixed subtype and 11 of follicular, large cell subtype as classified by the

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Working Formulation.[22] DNAs of 14 follicular lymphomas (6 cases of follicular, small cleaved cell subtype, 6 of follicular, mixed subtype and 2 of follicular, large cell subtype) and 41 diffuse lymphomas (21 B-cell lymphomas and 20 T-cell lymphomas) were extracted from unfixed fresh-frozen lymph node specimens.[23] DNAs of 19 follicular lymphomas (7 cases of follicular, small cleaved cell subtype, 3 of follicular, mixed subtype and 9 of follicular, large cell subtype) were prepared from formalin-fixed paraffin-embedded lymph node specimens in accordance with the method of Goelz *et al.*[24, 25] with minor modifications. In the remaining 8 follicular lymphoma cases (6 cases of follicular, small cleaved cell subtype and 2 of follicular, mixed subtype), DNAs were extracted from both unfixed fresh-frozen and formalin-fixed paraffin-embedded specimens.

Oligonucleotide primers and probes To detect chromosomal translocation t(14;18) at the mbr and J_H locus by the PCR technique and hybridization with sequence-specific oligonucleotide, the following oligonucleotides were synthesized[20] using a Cyclon Plus DNA Synthesizer (Milligen/Bioresearch, Tokyo): the 5′*bcl*-2 oligonucleotide primer, 5′-TTAGAGAGTTGCTTTACGTG-3′, the 3′anti-sense J_H consensus oligonucleotide primer, 5′-ACCTGAGGAGACGGTGACCAGGGT-3′ and the internal hybridization oligonucleotide probe, 5′-GCCTGTTTCAACACAGACCC-3′. To detect t(14;18) translocation at the mcr and J_H locus, we synthesized the following in a similar manner; the 5′ mcr-specific chromosome 18 oligonucleotide 5′-GACTCCTTTACGTGCTGGTACC-3′, the 3′ anti-sense J_H consensus oligonucleotide 5′-ACCTGAGGAGACGGTGACC-3′ and the mcr-specific oligonucleotide probe 5′-GATGGCTTTGCTGAGAGGTAT-3′.[21] The nonradioactive labeling of oligonucleotides was carried out by tailing them with digoxigenin-11-dUTP, using the DNA Tailing Kit (Boehringer Mannheim GmbH, Germany).

PCR and hybridization One microgram of DNA of each specimen was subjected to 50 cycles of PCR using a GeneAmp kit (Cetus, Norwalk, CT). Cycle conditions included denaturation at 95°C (2 min, first cycle: 5 min), annealing at 55°C (3 min), and extension at 72°C (3 min). After the final cycle, tubes were placed at the annealing temperature for 3 min, followed by incubation for 5 min at 72°C. One-tenth of the 100 μl reaction mixture was electrophoresed through a 3% Nusieve GTG agarose (FMC USA) gel. It was then transferred to nylon filters, Hybond-N^+ (Amersham Japan Inc., Tokyo), and hybridized with a digoxigenin-labeled oligomer probe overnight at 42°C, using a Dig-ELISA Kit (Boehringer Mannheim).

Statistical analysis A two-sided Fisher exact test[26] was used to examine the difference of the frequency of *bcl*-2-J_H rearrangement between two histological subtypes.

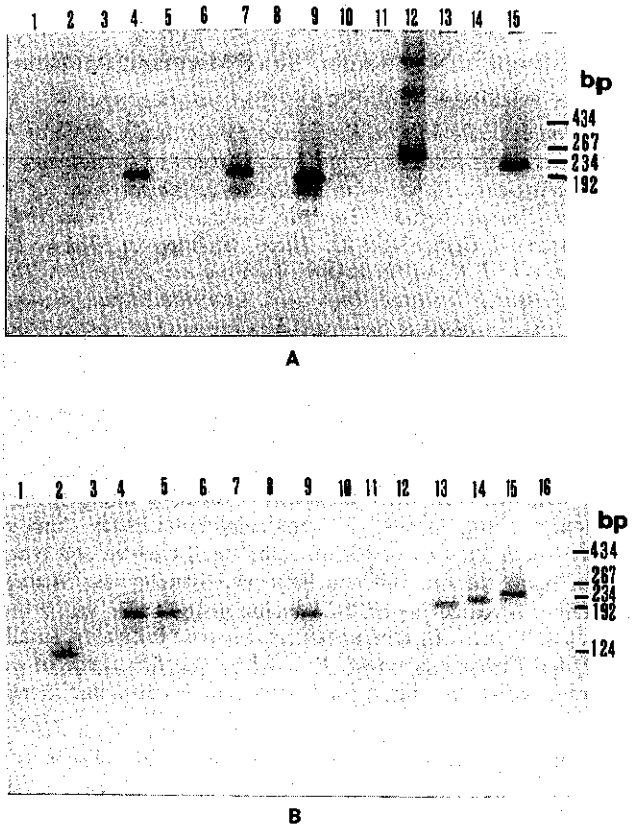

Fig. 1. A. Southern blot analysis of PCR product using fresh-frozen lymph node specimens. The sample in each lane was 10 μl of PCR mixture using DNA of SU-DHL-6 (lane 15), human placenta (lane 14), follicular lymphoma (lanes 4–12) and diffuse B cell lymphoma (lanes 1–3) specimens. Filters were hybridized with a digoxigenin-labeled mbr internal oligomer probe. Approximately 230 bp hybridized bands were detected in lanes 4, 7, 9, 12 and 15. B. Southern blot analysis of PCR product using formalin-fixed paraffin-embedded follicular lymphoma specimens. The samples were 10 μl of PCR mixture using DNA of human placenta (lane 16), SU-DHL-6 (lane 15) and follicular lymphomas (lanes 1–14). The filter was hybridized with a digoxigenin-labeled mbr oligomer probe. Hybridized bands were detected in lanes 2, 4, 5, 9 and 13–15.

RESULTS AND DISCUSSION

Sensitivity and specificity of the detection system To determine the sensitivity and specificity of the present detection system by PCR and hybridization with non-radiolabeled oligomer probe, the PCR was carried out using DNA of a cell line, SU-DHL-6,[12, 22] carrying the *bcl*-2(mbr)-J_H rearrangement which was diluted serially with human normal lymph node DNA. After electrophoresis of reaction mixtures and blotting, the filters were hybridized with digoxigenin-labeled mbr-specific oligo-

nucleotide probe. The results showed that 0.5 ng of translocated cell DNA was the minimal requirement for detection (data not shown). Consequently, the minimum number of rearranged copies per cell that could be detected was about 5.18×10^{-4} copies/cell.

Detection of *bcl*-2(mbr)-J_H rearranged cases As a result of hybridization with mbr-specific oligonucleotide probe following PCR using DNAs extracted from fresh-frozen tissue specimens of non-Hodgkin's lymphoma, approximately 230 bp rearranged bands were revealed in some cases of follicular lymphoma (Fig. 1A). Such rearranged bands were detected in 9 cases of 22 follicular lymphomas, but not in 21 diffuse B cell lymphomas and 20 diffuse T cell lymphomas (Table I). DNAs extracted from formalin-fixed paraffin-embedded tissue specimens revealed rearranged bands in 6 cases of 27 follicular lymphomas (Fig. 1B, Table I). It seems that the use of DNAs from formalin-fixed, paraffin-embedded tissue specimens did not substantially affect the results. In eight cases corresponding to lanes 2, 7, 8 and 10–14 in Fig. 1B, DNAs extracted from formalin-fixed paraffin-embedded tissue and unfixed fresh-frozen specimens were studied. Three of these 8 cases showed rearranged bands (lanes 2, 13, 14 of Fig. 1B). The cases in lanes 8, 10 and 14 of Fig. 1B, correspond to the cases in lanes 8, 11 and 7 of Fig. 1A respectively. Of these three, one case (in lane 14 of Fig. 1B and in lane 7 of Fig. 1A) showed a *bcl*-2 rearranged band, but two cases (in lanes 8 and 10 of Fig. 1B, in lanes 8 and 11 of Fig. 1A) did not. In the remaining five cases (lanes 2, 7 and 11–13 of Fig. 1B) similar results were obtained with DNA of unfixed fresh-frozen specimens (data not shown). Results of these 8 cases, based on formalin-fixed paraffin-embedded tissue specimens and unfixed fresh-frozen tissue specimens, were identical, suggesting that the studies on formalin-fixed paraffin-embedded tissues specimens were equally

Table I. Detection of *bcl*-2-J_H Rearrangement in Non-Hodgkin's Lymphomas by PCR and Hybridization with Non-radiolabeled Sequence-specific Oligonucleotide Probe

No. of cases	No. of analyzed specimens		No. of rearranged cases	
			mbr-J_H	mcr-J_H
Follicular lymphoma 41	Unfixed fresh-frozen tissue specimens	22 (8)[a]	9 (3)[a]	1
	Formalin-fixed paraffin-embedded tissue specimens	27 (8)[a]	6 (3)[a]	0
Diffuse B lymphoma 21	Unfixed fresh-frozen tissue specimens only	21	0	ND
Diffuse T lymphoma 20	Unfixed fresh-frozen tissue specimens only	20	0	ND

a) Numbers in parentheses indicate the numbers of specimens for which DNAs were extracted from both fresh-frozen specimens and formalin-fixed paraffin-embedded specimens.
ND: not done.

Table II. *bcl*-2-J_H Rearrangement and Histopathological Subtype of Follicular Lymphoma

	No. of cases analyzed	No. of *bcl*-2-J_H rearranged cases		
		mbr-J_H	mcr-J_H	Total
Follicular, small cleaved cell	19 (13)[a]	6 (4)[a]	1	7 (36.8%)[b]
Follicular, mixed, small cleaved cell and large cell	11 (5)[a]	6 (2)[a]	0	6 (54.5%)[b]
Follicular, large cell	11 (9)[a]	0	0	0
Total no. of cases	41 (27)[a]	12 (6)[a]	1	13 (31.7%)[b]

a) Numbers in parentheses indicate the numbers of formalin-fixed, paraffin-embedded specimens.
b) Numbers in parentheses indicate the positivity of the rearranged cases in each subtype.

valid. In a few cases, the size of rearranged bands was slightly different, as shown in Fig. 1A, B, reflecting the difference of the break point within the mbr. For example, in the case of lane 2 in Fig. 1B, the smallest rearranged band (approximately 124 bp) was detected. This seems to suggest that the break point within the mbr of chromosome 18 occurred at the position nearest to 5′ *bcl*-2 primer.

Detection of *bcl*-2(mcr)-J_H rearranged cases In only one case out of all 41 follicular lymphomas could we detect a rearranged band (approximately 500 bp), *bcl*-2-J_H rearranged at the mcr (data not shown). The case was follicular, small cleaved cell lymphoma (Table II). The analysis of *bcl*-2(mcr)-J_H rearrangement was not made in 41 diffuse B and T cell lymphomas.

Frequency of *bcl*-2-J_H rearrangement in each histopathological subtype of follicular lymphoma The results of *bcl*-2-J_H rearrangement in each histopathological subtype of 41 follicular lymphomas are summarized in Table II. The rearrangement was detected in 7 cases (36.8%) of 19 follicular, small cleaved cell lymphomas and 6 cases (54.5%) of 11 follicular, mixed, small cleaved cell and large cell lymphomas. In the combined group of follicular, small cleaved cell lymphomas and follicular mixed, small cleaved cell and large cell lymphomas, the frequency of *bcl*-2-J_H rearrangement was 43.3%. However, none of the 11 follicular, large cell lymphomas revealed *bcl*-2-J_H rearrangement. The difference in the frequency of *bcl*-2-J_H rearrangement was statistically significant between follicular, small cleaved cell and large cell lymphoma (two-sided Fisher exact test $P=0.049$). The difference of *bcl*-2-J_H rearrangement frequency was also significant between follicular, mixed lymphoma and follicular, large cell lymphoma ($P=0.012$). Moreover, a statistically significant difference was observed between the group of follicular large cell lymphoma and the combined group of follicular, small cleaved cell lymphoma and follicular, mixed lymphoma ($P=0.013$). The difference was not statistically significant between the follicular, small cleaved cell lymphoma and follicular, mixed lymphoma. It seems unlikely that the rarity of the rearrangement in follicular, large cell lymphoma in Table II can be attributed to the decreased reactivity of DNAs extracted from the formalin-fixed paraffin-embedded tissues. The reasons are as follows: (1) the frequency of the rearrangements in formalin-fixed paraffin-embedded tissues (4/13 in follicular, small cleaved cell lymphoma; 2/5 in follicular, mixed lymphoma) parallels the overall frequency (6/19 in follicular, small cleaved cell lymphoma; 6/11 in follicular, mixed lymphoma); (2) in 8 cases, DNAs which were extracted from the formalin-fixed paraffin-embedded tissue and fresh-frozen tissue in parallel did not show any discrepancy of the detected rearrangements depending on the source of the DNA in each case.

Our data showed that *bcl*-2 rearrangement occurred rarely in follicular, large cell lymphoma, or at least, it occurred significantly less frequently than in follicular, small cleaved cell lymphoma and follicular, mixed lymphoma. The data suggest two possibilities: first, in tumorigenesis of follicular, large cell lymphoma, the participation of *bcl*-2 rearrangement was minor, and some other oncogenes may have played important roles; and second, the *bcl*-2 rearrangements in these follicular, large cell lymphomas may have taken forms that were not detected by our analysis system.

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