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Detection of oncogene rearrangements in human non-Hodgkin's lymphomas.

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Abstract

Southern blot hybridization was used to detect the rearrangement and amplification of five proto-oncogenes (bcl-2, bcl-1, c-myc, c-myb and c-Ha-ras) and one tumor suppressor gene (RB-1) in 55 Japanese patients with non-Hodgkin's lymphoma; 16 with T-cell lymphomas and 39 with B-cell lymphomas (7 follicular and 32 diffuse lymphomas). Genetic abnormalities of the proto-oncogenes were detected in 7 of the 55 (13%). Genetic abnormalities of bcl-2 plus other genes were detected in 5 of 7 cases of follicular lymphoma (71%), rearrangements of bcl-2 and c-myc, rearrangement of bcl-2 and amplification of c-myb. Genetic abnormalities were observed in only three cases of diffuse lymphoma. In each of 3 cases of B-cell lymphoma, one of the genes, blc-2 mbr, bcl-2 mcr and c-myc, was rearranged respectively. The incidence of genetic abnormalities in diffuse lymphomas (6.3%) was lower than that in follicular lymphomas. None of diffuse lymphomas had double oncogene abnormality. No abnormalities were found in RB-1, bcl-1, and Ha-ras. These findings suggest that follicular lymphomas are associated with some abnormalities of oncogenes not restricted to bcl-2 that facilitate growth which may be associated with their clinical features.

KEYWORDS: malignant lymphoma, cellular oncogenes

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Detection of Oncogene Rearrangements in Human Non-Hodgkin's Lymphomas

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Southern blot hybridization was used to detect the rearrangement and amplification of five proto-oncogenes (*bcl-2, bcl-1,* c-*myc,* c-*myb* and c-Ha-ras) and one tumor suppressor gene (*RB-1*) in 55 Japanese patients with non-Hodgkin's lymphoma; 16 with T-cell lymphomas and 39 with B-cell lymphomas (7 follicular and 32 diffuse lymphomas). Genetic abnormalities of the proto-oncogenes were detected in 7 of the 55 (13%). Genetic abnormalities of *bcl-2* plus other genes were detected in 5 of 7 cases of follicular lymphoma (71%), rearrangements of *bcl-2* and c-*myc*, rearrangement of *bcl-2* and amplification of c-*myb*. Genetic abnormalities were observed in only three cases of diffuse lymphoma. In each of 3 cases of B-cell lymphoma, one of the genes, *blc-2* mbr, *bcl-2* mcr and c-*myc*, was rearranged respectively. The incidence of genetic abnormalities in diffuse lymphomas (6.3%) was lower than that in follicular lymphomas. None of diffuse lymphomas had double oncogene abnormality. No abnormalities were found in *RB-1*, *bcl-1*, and Ha-*ras*. These findings suggest that follicular lymphomas are associated with some abnormalities of oncogenes not restricted to *bcl-2* that facilitate growth which may be associated with their clinical features.

Key words : **malignant lymphoma, cellular oncogenes**

Recently it has been reported that non-Hodgkin's lymphomas exhibit characteristic, non-random chromosomal abnormalities; t(14;18) (q32;q21) in follicular lymphomas, t(8;14) (q24; q32) in Burkitt's lymphomas, t(11;14) (q13; q32) in diffuse, small lymphocytic lymphomas, del (6) (q16-q25) in B-cell lymphomas, and del (13) (q14) in acute lymphocytic leukemias, (ALLs) and lymphomas (1, 2). Many cellular oncogenes have been located within or near these abnormal chromosomal sites (3-6). Because comprehensive studies on the oncogene abnormalities in malignant lymphomas remain limited, we studied genetic abnormalities such as rearrangement and amplification of cellular oncogenes to confirm whether there is a correlation between genetic abnormalities and the histologic types or immunophenotypes of Japanese non-Hodgkin's lymphomas. We found that follicular lymphomas had a tendency to involve plural oncogene abnormalities which reflect their clinical futures.

In this study we examined five proto-oncogenes, *bcl-2, bcl-1,* c-*myc,* c-*myb* and c-Ha-*ras* and one recessive tumor suppressor gene, *RB-1.* The *bcl-2* proto-oncogene is encoded on chromosome 18q21 locus and might be activated by translocations t(14;18) causing the juxtaposition of the *bcl-2* gene with an IgH gene locus on

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chromosome 14q32 (3, 7-9). *bcl-1* gene is located on chromosome 11q13, which is related to the breakpoint of t(11;14) (q13;q32) chromosomal translocation (5, 6). The c-*myc*, c-*myb* belong to a group whose products bind to DNA. The c-*myc* gene on chromosome 8q24 locus appears to be activated in Burkitt's lymphomas carrying t (8; 14), t(8;22) or t(2;8) chromosomal translocations (2). The c-*myb* proto-oncogene could be involved in the 6q-aberrations which are frequently found in hematopoietic malignancies (1, 10, 11). The c-Ha-*ras* gene was reported to be amplified or rearranged in some non-Hodgkin's lymphomas (12, 13). The *RB* gene is located on chromosome 13q14 locus, and its homozygous deletion has been implicated in the pathogenesis of retinoblastomas (14). Inactivaion of the *RB* gene has been also reported in some ALLs and malignant lymphomas (15).

Materials and Methods

Patients. The present study was based on fresh, frozen samples taken from 55 patients with non-Hodgkin's lymphomas. As a control, two cases of reactive lymph node hyperplasia and one case of necrotizing lymphadenitis were analysed. Immunophenotypic analyses were performed on lymphoma specimens by the immunoperoxidase method using the following antibodies: Leucocyte common antigen (CD45RB + CD45), UCHL-1 (CD45RO), OPD-4 (anti-helper/inducer T-cell), lysozyme, α_1-antichymotrypsin, α_1-antitrypsin (Dacopatts), MT-1 (CD43), MB-1 (CD45R) (Bio-Science Product AG), Mx-PanB (Kyowa Medics Co.), and LN-1 (CDW75) (Techniclone International) for paraffin-embedded sections and anti-Leu-4 (CD3), anti-Leu-3a (CD4), anti-Leu-2a (CD8), anti-Leu-12 (CD19), anti-Leu-16 (CD20), and anti-Leu-14 (CD22) (Becton Dickinson) for frozen sections. Most of the neoplastic specimens were examined for the rearrangement of the T-cell receptor β chain gene and the immunoglobulin heavy chain gene by Southern blot hybridization using human T-cell receptor, $J\beta_1$ and $J\beta_2$ probes and the human immunoglobulin heavy chain joining region (J_H) to confirm their immunophenotypes and monoclonalities (data not shown). B-cell lymphomas were subclassified according to the Working Formulation Classification (16), and T-cell lymphomas were subclassified according to the updated Kiel classification (17, 18).

Southern blot hybridization. High molecular weight DNA was extracted from the fresh surgical materials by a modification of the method of Maniatis *et al.* (19) involving lysis of the cells in 0.5 % sodium dodecyl sulfate (SDS) with 100 ug/ml proteinase K and 5 μg/ml RNase followed by phenol-chloroform extraction. 10 μg of purified DNA were digested with the restriction enzymes: *Hin*dIII and *Bam*HI (Life Technologies, Inc.); *Eco*RI and *Bgl*II (Toyobo Co., Ltd.). The digests were fractionated by electrophoresis at the appropriate voltage on horizontal 0.8 % agarose gels overnight in 1 × Tris-borate EDTA buffer (19), and then transferred to nylon filters (Hybond-N^+, Amersham Int. Co.) using a Vacu Gene (Pharmacia 2016-010). Nylon filters were prehybridized with salmon sperm DNA in hybridization buffer (6 × SSC, 5 × Denhardt's solution, 0.1 % SDS and 50 % Dextran sulfate) at 42 ℃ for over 4 h and hybridized with ^{32}P-labelled probe DNA (approximately 10^6 cpm/ml) overnight at 65 ℃. Filters were washed under stringent conditions using 2 × SSC with 0.1 % SDS at room temperature followed by 2 × SSC with 0.1 % SDS at 65 ℃, then autoradiographed with Fuji AIFRX X-ray film and intensifying screens.

The c-DNA probes used in this study were heat-denatured and labelled with ^{32}P by random hexanucleotide priming utilizing Multiprime™ DNA labelling system kit (Amersham Int. Co.). λ/*Hin*dIII digest was used as a molecular size marker and was also labelled in the same way as described in c-DNA probes. In order to confirm the juxtaposition of a rearranged *bcl-2* gene or *myc* gene with a J_H gene locus on chromosome 14, or a Cκ gene locus on chromosome 2 and the amplification of *bcl-2* gene or *myb* gene, the same filters were rehybridized after complete removal of probe DNA by denaturation in 0.5 % SDS solution at a temperature above 95 ℃. Gene amplification was quantitatively analyzed by densitometry of the autoradiographed films.

The possibilities of artifacts were excluded from the evaluation of the rearranged bands in Southern blot hybridization. The reappearance of the rearranged bands on the same filter was confirmed by repeating the hybridization with the same probe after stripping the bands. The rearranged bands were confirmed by digesting the same genomic DNA with various restriction enzymes. About ten different DNA samples digested completely with the same restriction enzyme were simultaneously blotted on the same filter, and all the fragments obtained were of

different lengths from the original sample. The possibility of partial digestion was excluded by comparing the results of Southern blot hybridization with those using several other kinds of DNA probes, and Star activity in some restriction enzymes was scrupulously avoided by controlling the components of the reaction solutions.

DNA probes. Two DNA probes, pFL-1 and pFL-2, were used for detecting rearrangements of the *bcl-2* gene. pFL-1 is a 1.5kb *Eco*RI-*Hin*dIII fragment of the *bcl-2* gene involving the major breakpoint cluster region (mbr) (3). pFL-2 is a 4.0kb *Eco*RI fragment of the *bcl-2* gene involving the minor breakpoint cluster region (mcr) (8), both of which were provided by M.L. Cleary. The other cDNA probes of cellular oncogenes used in this study included a 2.1kb *Sst* I fragment of the *bcl-1* gene (6, 20) provided by Y. Tsujimoto, a 1.5kb *Sst* I fragment of the human *myc* proto-oncogene carrying the second *myc* exon (Amersham International plc.) (21, 22), a 2.0kb *Eco*RI fragment of the c-*myb* gene (23) provided by Japanese Cancer Research Resources Bank (JCRB), a 6.0kb *Eco*RI fragment of the c-Ha-*ras* gene (24) provided by JCRB, a 3.8kb *Eco*RI fragment of the *RB-1* locus named p2R3.8 (14) provided by T. Sekiya and Y. Murakami. The other probes used were a 0.55kb *Xba* I fragment of $J\beta_1$ gene, a 4.3kb *Eco*RI fragment of $J\beta_2$ gene (25) provided by J. Sklar, a 3.5kb *Eco*RI-*Hin* dIII fragment of J_H provided by Dr. T. Honjo through JCRB (26), a *Eco*RI fragment of the human immunoglobulin κ constant region ($C\kappa$) gene (27), a 2.5kb *Eco* RI fragment of human immunoglobulin μ constant region ($C\mu$) gene (28) provided by JCRB and a 0.77kb *Nco* 1-*Taq* I fragment of β-actin gene (ONCOR).

Statistical analysis. The chi-squared test was used to verify the significane of differences in the frequency of oncogene abnormalities between follicular and diffuse lymphomas, as well as between T-cell and B-cell lymphomas.

Results

The results of oncogenes analysis are summarized in Tables 1 and 2. Four of 7 follicular lymphomas showed rearrangements of *bcl-2* mbr detected with the pFL-1 probe (Fig. 1a, lanes 3, 5, 7 and 9). Rearranged fragments of *bcl-2* mbr of follicular lymphomas were identified in *Hin* dIII DNA digests in 3 cases and in *Bam* HI DNA digest in one case. In diffuse lymphomas, 2 of 48 cases showed rearrangements of the *bcl-2* gene, both of which were phenotipically B-cell lymphomas. One, which was histologically a large cell type with a vague nodularity, had a rearranged fragment of *bcl-2* mbr detected with pFL-1 in *Hin* dIII DNA digest (Fig. 1b, lane 3). The other, a small cleaved cell type, had a rearranged fragment involving *bcl-2* mcr detected with pFL-2 in *Hin* dIII DNA digest (Fig. 1b, lane 7). All the rearranged fragments of *bcl-2* mbr and mcr comigrated with rearranqed J_H region fragments, indicating t(14;18) (q32;q21) translocations (Figs. 1a and 1b).

Rearranged c-*myc* genes were detected in one patient with follicular lymphoma (Case 7), and in one patient with Burkitt's lymphoma of the small intestine (Case 37) (Fig. 2, lanes 1 and 4). However, Southern hybridization using the J_H probe did not disclose comigration of the re-

Table 1 Results of gene analysis of follicular lymphomas

Case No.	Histological type	*bcl-2*		*bcl-1*	*myc*	*myb*	*Ha-ras*	*RB*
		mbr	mcr					
1	Follicular, small cleaved	R	G	G	G	G(Amp)	G	G
2	Follicular, small cleaved	R	G	G	G	G	G	G
3	Follicular, small cleaved	G	G	G	G	G(Amp)	G	G
4	Follicular, small cleaved	G	G	G	G	G	G	G
5	Follicular, small cleaved	G	G	G	G	G	G	G
6	Follicular, mixed	R	G	G	G	G	G	G
7	Follicular, mixed	R	G	G	R	G	G	G
Total number of abnormalities		4	0	0	1	2	0	0

Abbreviation: R, gene rearraged configuration; G, germline configuration; Amp, gene amplification

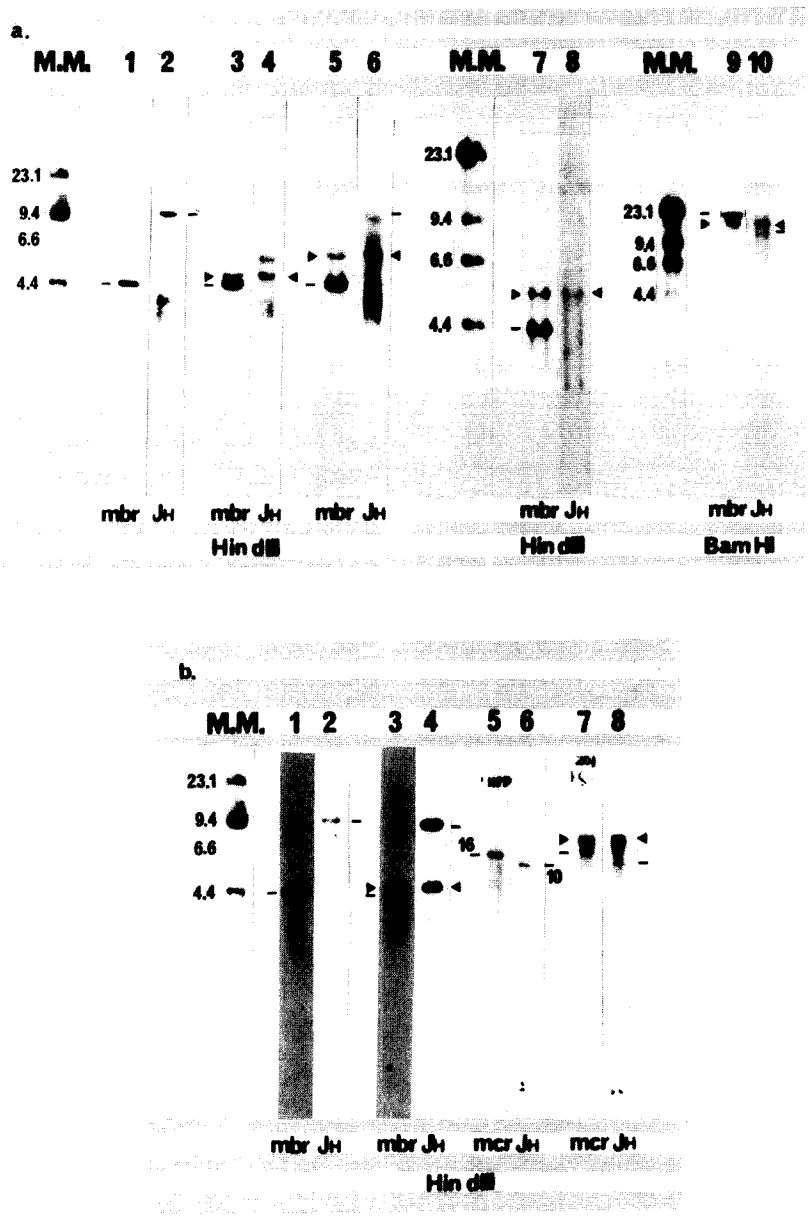

Fig. 1 Rearrangement of the *bcl-2* gene in non-Hodgkin's lymphomas. **a**: Southern blot hybridization of follicular lymphoma DNA. Lanes 1 and 2, control human placental DNA digested with *Hin* dIII ; lanes 3–10, DNA from 4 cases of follicular lymphomas digested with *Hin* dIII (lanes 3–8) or *Bam* HI (lanes 9, 10). **b**: Southern blot hybridization of DNA from patients with diffuse B-cell lymphomas. Genomic DNA was digested mith *Hin* dIII. Lanes 1, 2, 5 and 6, control DNA; lanes 3, 4, 7 and 8, DNA from lymphoma patients. Short bars indicate germline bands; the *Hin* dIII germline bands are 4.2 kb (*bcl-2* mbr), 16.0 kb (*bcl-2* mcr) and 10.0 kb (J_H), and the *Bam* HI germline bands are 23.0 kb (*bcl-2* mbr) and 18.5 kb (J_H). Closed arrow heads indicate rearranged fragments showing comigration.

Table 2 Rearrangement of cellular oncogenes in 48 non-Hodgkin's diffuse lymphomas

Case No.	Lymphoma subtype	No.	*bcl-2*		*bcl-1*	*myc*	*myb*	*Ha-ras*	*RB*
			mbr	mcr					
	B-cell lymphomas								
8–11	Small lymphocytic	4	0	0	0	0	0	0	0
12, 13	Small cleaved	2	0	1	0	0	0	0	0
14–16	Mixed	3	0	0	0	0	0	0	0
17–36	Large	20	1	0	0	0	0	0	0
37, 38	Burkitt's	2	0	0	0	1	0	0	0
39	Plasmacytoma	1	0	0	0	0	0	0	0
	T-cell lymphomas								
40, 41	Lymphoepithelioid	2	0	0	0	0	0	0	0
42–44	Angioimmunoblastic	3	0	0	0	0	0	0	0
45	Pleomorphic, small cell	1	0	0	0	0	0	0	0
46, 47	Pleomorphic, medium sized	2	0	0	0	0	0	0	0
48	Pleomorphic, medium and large	1	0	0	0	0	0	0	0
49–52	Pleomorphic, large cell	4	0	0	0	0	0	0	0
53	Immunoblastic	1	0	0	0	0	0	0	0
54	Unclassifiable, low grade	1	0	0	0	0	0	0	0
55	Unclassifiable, high grade	1	0	0	0	0	0	0	0
	Total No.	48	1	1	0	1	0	0	0

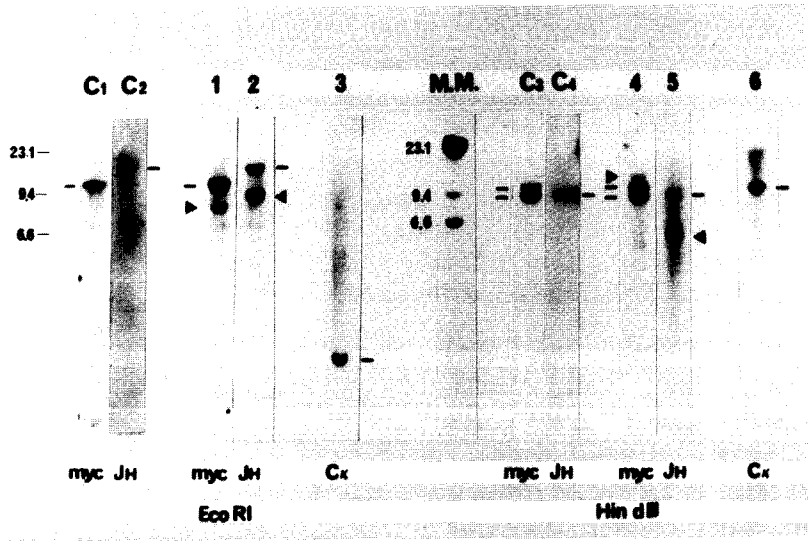

Fig. 2 Rearrangement of the c-*myc* gene in non-Hodgkin's lymphomas. Southern blot hybridization of *Eco* RI digest of DNA from a follicular lymphoma, Case 7 (lanes 1–3) and *Hin* dIII digest of DNA from a Burkitt's lymphoma, Case 37 (lanes 4–6). Lanes C_1 and C_2 are control human placental DNA digested with *Eco* RI. Lanes C_3 and C_4 are human placental DNA digested with *Hin* dIII. Short bars indicate germline bands; the *Eco* RI germline bands are 13.5 kb (c-*myc*) and 17.0 kb (J_H), and the *Hin* dIII germline bands are 11.1 kb and 9 kb (c-*myc*) and 10.0 kb (J_H). Closed arrow heads indicate the rearranged bands. M.M. shows molecular size markers.

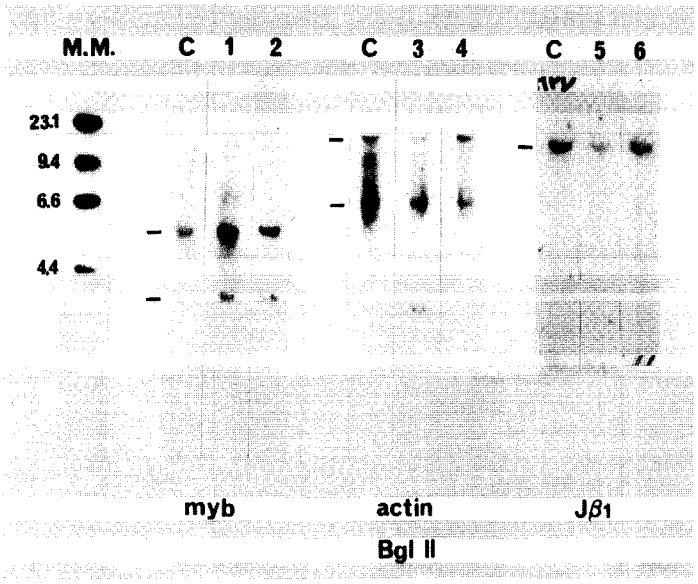

Fig. 3 Amplificationion of the c-*myb* gene in non-Hodgkin's lymphomas. The amplification of c-*myb* gene in *Bgl* II digests of follicular lymphoma DNA. Lanes 1, 3 and 5, DNA from Case 1; lanes 2, 4 and 6, DNA from Case 3; lane C, control DNA. Filters rehybridized with β-actin and Jβ_1 are used for densitometric analysis to compare with the *myb*-hybridized filter. Germline c-*myb* bands in *Bgl* II digests correspond to 6.2 kb and 4.0 kb. All fragments of c-*myb*, and Jβ_1 genes showed germline configurations. M.M. shows molecular size markers.

arranged c-*myc* band fragment with a rearranged J_H gene in either case. In addition, germline configurations were found in both cases by Southern blotting using the Cκ probe. Amplification of the c-*myb* gene was found in two patients with small cleaved cell follicular lymphoma, (Cases 1 and 3) (Fig. 3, lanes 1 and 2). Densitometric analyses showed the amplified fragment in Case 1 was over 20-fold more intense and the amplified fragment of Case 3 showed over 9-fold more intense than the control DNA when corrected by the intensity of rehybridized fragments of β-actin and Jβ_1 gene. In the detailed comparison of c-*myb* bands with those of β-actin, Case 1 showed 18-fold, and Case 3 showed 21-fold amplification, respectively. Also comparing c-*myb* bands with those of Jβ_1, Case 1 showed 40-fold, and Case 3 showed 6-fold amplification, respectively (Fig. 3; lanes 3, 4 and lanes 5, 6, respectively). No rearranged fragments of c-*myb* gene were detected.

In this study, no rearrangement of *bcl-1* gene was detected (data not shown). The cases examined for the c-Ha-*ras* gene only showed polymorphisms due to variable tandem repeat regions (data not shown). Abnormalities such as the deletion or rearrangement of the retinoblastoma gene (*RB-1*) were not detected in any of cases examined (data not shown).

Discussion

There are several breakpoint regions within or near the *bcl-2* sequence, which include the 5′ region of the *bcl-2* sequence containing parts of exons I and II, mbr and mcr (3, 7, 9, 25). Using c-DNA probes for *bcl-2* mbr (pFL-1) and

mcr (pFL-2), we detected the rearrangement of *bcl-2* gene in 6 of the 55 lymphoma cases; in 5 by pFL-1 and in one by pFL-2. All cases which showed rearrangement of the *bcl-2* gene were B-cell lymphomas including 4 of follicular lymphoma. The incidence of *bcl-2* gene rearrangements was approximately 57 % in follicular lymphomas and 6.3 % in diffuse B-cell lymphomas. This incidence is lower than that of American B-cell lymphomas, suggesting that the oncogenesis of Japanese B-cell lymphoma cannot be fully explained by the result from t(14;18) chromosomal translocation (29–31). Although all cases with rearrangements of the *bcl-2* gene had a juxtaposition of *bcl-2* gene with the immunoglobulin heavy chain gene, the patterns of recombination differed somewhat. There was a rare breaking pattern in the *bcl-2* sequence as seen in lane 9 of Fig. 1a which may be due to a difference of the timing of V-D-J recommbination on 14q32 locus.

Burkitt's lymphoma frequently carries a chromosomal translocation t(8;14) causing rearrangement of the c-*myc* locus. According to previous reports, there have been some patients with non-Hodgkin's lymphoma which had rearrangement of the c-*myc* gene without t(8;14) (32). The percentage of cases which showed a rearrangement of the c-*myc* locus was approximately 25 % in diffuse large cell lymphomas (1). In our series, two cases showed rearrangements of the c-*myc* gene without J_H locus comigration. One of the two specimens came from the gastrointestinal tract and the character of the specimen may differ from that of nodal lymphoma (33).

Amplification of c-*myb* gene was observed in two cases of follicular lymphomas. Barletta *et al.* (10) reported that DNA amplification of the c-*myb* gene was found in AML and that high level expressions of m-RNA which were over 50-fold higher than in normal lymphocytes were seen in not only AML but also in T-cell and B-cell malignancies carrying only a single copy of the c-*myb* gene. We could detect c-*myb* gene amplification with germline configuration in some follicular lymphomas.

No rearrangement of the *bcl-1* gene was detected. A recent report demonstrated that the rearrangement of *bcl-1* gene was only found in low-grade small cleaved or small lymphocytic lymphomas at a low frequency (34). So the absence of *bcl-1* rearrangement in our cases might reflect the low number of examined cases of those histological types, or the *bcl-1* gene may have little relationship with the oncogenesis of other types of non-Hodgkin's lymphomas with respect to genetic abnormalities.

Although the overexpression of c-Ha-*ras* gene has been demonstrated in malignant lymphomas by the *in situ* hybridization method (13), neither rearrangement nor amplification in the DNA level was detected in our study. From this, we infer that the *ras* gene is related to the oncogenesis of non-Hodgkin's lymphoma not by gene mutation at the DNA level, but by marked amplification at the RNA level.

Ginsberg *et al.* (15) reported that lymphoma or leukemia cells from patients with clinically poor prognoses had *RB* locus abnormalities detected by Southern or Northern analysis. That we could not detect abnormalities at the DNA level using Southern blot hybridization may be due to the fact that lymphoma tissues are often intermingled with much more reactive non-neoplastic cells and that DNA from these admixed non-neoplastic cells may conceal the deletion of the *RB* locus.

In this study, oncogene abnormalities were more frequently in follicular lymphomas than in diffuse lymphomas (P value = 0.0018). Even if abnormality of the *bcl-2* gene is excluded, there was a significant difference between the 2 types of lymphomas. There was no significant difference between T-cell and B-cell lymphomas (P = 0.28). The follow-up data was not sufficient to evaluate the association between oncogene abnormalities and prognosis, although it has been reported that cases with *bcl-2* gene rearrangements have relatively poor prognoses (35). Diffuse lymphomas usually follow a more aggressive clinical course than follicular lymphomas, and are known to have

some consistent chromosomal abnormalities and/or genetic abnormalities (2). In, the current study, the reverse result was observed not only for *bcl-2*, but also for the other cellular oncogenes. Follicular lymphomas grow slowly and are easily led to complete remission with chemotherapy, and then to show a late relapse and stead progress to a fatal outcome (36). It seems possible that follicular lymphomas involve some oncogenetic abnormalities in addition to the *bcl-2* gene to gain a growth advantage during a long, but steady progression as suggested in the acute B-cell leukemia cell line 380 in which there are t(14;18) and t(8;14) translocations (20, 37).

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