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Loss of Heterozygosity at the *bcl*-2 Gene Locus and Expression of *bcl*-2 in Human Gastric and Colorectal Carcinomas

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The loss of heterozygosity (LOH) at the *bcl*-2 gene locus and the expression of the *bcl*-2 gene were examined in gastric and colorectal carcinoma cell lines and carcinoma tissues. LOH at the *bcl*-2 locus was detected in 24% (4/17) of gastric and 60% (6/10) of colonic carcinomas, all of which were well differentiated adenocarcinomas, whereas LOH was not seen in poorly differentiated ones. On the other hand, 24% (5/21) of poorly differentiated stomach cancers overexpressed *bcl*-2 gene, whereas no overexpression was detected in well differentiated stomach cancer. Three gastric and three colorectal carcinoma cell lines, all of which were derived from poorly differentiated adenocarcinomas, expressed considerable levels of *bcl*-2 mRNA and protein. These results suggest that LOH at the *bcl*-2 locus is frequently associated with well differentiated adenocarcinomas of the stomach and colon, and *bcl*-2 overexpression has implications for the development of poorly differentiated adenocarcinomas of the gastrointestinal tract.

Key words: *bcl*-2 — Loss of heterozygosity — Expression — Gastrointestinal carcinoma — Programmed cell death

The *bcl*-2 gene is a membrane-associated protein that extends the survival of certain cells by blocking programmed cell death independently of promoting cell division.[1–3] The *bcl*-2 gene was first identified at the t(14;18) translocation present in leukemias and B-cell lymphomas. In these malignancies, due to t(14;18) translocation, *bcl*-2 gene is placed adjacent to Ig heavy chain gene, causing overexpression of the *bcl*-2 gene.[4] Although the translocation exists, hybrid transcripts continue to encode structurally normal *bcl*-2 protein of 26 kDa.[5] *bcl*-2 plays a role not only in hemopoietic cells, but also in a variety of tissues characterized by apoptotic cell death, including neuronal cells.[6,7] Although there are many reports concerning gene alterations and expression of *bcl*-2 in hematopoietic malignancies,[8,9] little is known about the gene alterations and expression of *bcl*-2 in epithelial tumors. An association of *bcl*-2 expression with androgen-independent tumors has been demonstrated in prostate carcinomas.[10] It has also been reported that *bcl*-2 protein is expressed by Epstein-Barr virus-associated nasopharyngeal carcinomas.[11] However, there is no other report in the literature dealing with the expression and genetic alterations of *bcl*-2 gene in human carcinomas.

In the present study, we examined loss of heterozygosity (LOH) at the *bcl*-2 locus and the expression of *bcl*-2 in human gastric and colonic carcinoma cell lines and carcinoma tissues, as well as corresponding non-neoplastic mucosa, to elucidate the possible role of *bcl*-2 in the development and progression of gastrointestinal carcinomas.

MATERIALS AND METHODS

Cell lines Six human gastric carcinoma cell lines (KATOIII, HSC-39, TMK-1, MKN-74, MKN-45 and MKN-28) and seven human colorectal carcinoma cell lines (Colo201, Colo320DM, DLD-1, LoVo, SW837, WiDr and TCO) were used. TMK-1, a poorly differentiated gastric adenocarcinoma cell line and TCO, a poorly differentiated colonic adenocarcinoma cell line were established in our laboratory.[12,13] KATOIII and HSC-39, gastric carcinoma cell lines derived from signet ring cell carcinoma were kindly provided by Dr. M. Sekiguchi (University of Tokyo, Tokyo) and Dr. K. Yanagihara (Hiroshima University, Hiroshima).[14] The three gastric carcinoma cell lines of the MKN series (MKN-28, well differentiated adenocarcinoma; MKN-45, poorly differentiated adenocarcinoma and MKN-74, well differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima). Colorectal carcinoma cell lines (CoLo 201, CoLo320DM, DLD-1, LoVo, SW837 and WiDr) and a promyelocytic leukemia cell line (HL60) were provided by the Japanese Cancer Research Resources Bank (JCRB). All cell lines

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were routinely maintained in RPMI-1640 (Nissui Co., Tokyo) supplemented with 10% fetal bovine serum (Whittaker, Maryland), penicillin (0.1 mg/ml) and streptomycin (0.1 mg/ml) under conditions of 5% CO_2 in air at 37°C.

Tissue samples A total of 45 gastric and 23 colonic carcinomas and corresponding non-neoplastic mucosa obtained by surgery were examined. A representative tumor specimen and non-neoplastic mucosa from the surgical margin were frozen in liquid nitrogen immediately after surgical resection and stored at −80°C for molecular analysis. Samples for immunohistochemistry were fixed by the AMeX method.[15] We confirmed histopathologically that the tumor specimen consisted mainly of carcinoma tissue and that the non-neoplastic mucosa did not exhibit tumor cell invasion. Lymphocytic infiltration in tumor stroma and non-neoplastic mucosa adjacent to those taken for molecular analysis, was evaluated by microscopical examination of hematoxylin and eosin sections by three independent observers (A.A., W.Y., H.Y.) and was arbitrarily subdivided into four groups, (−) to (+++), depending on the number of lymphocytes. The definition of stage grouping and histological classification were made according to the criteria of the Japanese Research Society for Gastric Cancer[16] and the Japanese Research Society for Cancer of Colon and Rectum.[17]

Southern blot analysis High-molecular-weight DNAs were prepared from tissue samples, digested with *Eco*RI restriction enzyme and subjected to Southern blot analysis as described previously.[18] *bcl*-2 cDNA probe used was #58, which contains the entire coding region.[15] The allele loss was determined by densitometric tracing if the intensity of a band in the tumor was less than 50% of that in the corresponding non-neoplastic mucosa.[18]

Northern blot analysis Poly A^+-selected RNAs from the cell lines or 29 gastric and 8 colorectal carcinomas as well as corresponding non-neoplastic mucosal samples were prepared by the standard guanidium isothiocyanate/cesium chloride method and subjected to Northern blot analysis with ^{32}P-labeled *bcl*-2 probe (the same probe as used for Southern blot hybridization) as described previously.[19] β-Actin probe was used as an internal control. The comparative intensity of RNA signals between tumor and normal mucosa was determined by one-dimensional densitometric tracing after normalizing with respect to the internal control.

Western blot analysis Whole cell lysates from the cell lines were prepared as described previously.[20] The lysates were subjected to Western blot analysis using 15% SDS-polyacrylamide gel. Monoclonal anti-*bcl*-2 antibody (DAKO-bcl-2; 124, Denmark) and peroxidase-conjugated anti-mouse IgG antibody (TAGO Inc., Burlingame, California) were used in the primary and secondary reactions, respectively. The immune complex was visualized using the ECL Western blot detection system (Amersham, Aylesbury, UK).

Immunostaining A modification of the immunoglobulin enzyme bridge technique (ABC method) was employed on AMeX-fixed, paraffin-embedded sections.[21,22] Deparaffinized sections were incubated with anti-*bcl*-2 antibody (DAKO-bcl-2), biotinylated anti-mouse IgG horse antibody, and avidin dehydrogenase biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector, Burlingame, California) according to the instructions of the supplier. A negative control monoclonal antibody (TrpE, Oncogene Science, Manhasset, New York) was used at the same concentration as in the primary reaction to confirm the specificity of the immunoreactivity.

Immunofluorescent staining was performed by the indirect method.[23] Cells on chamber slides (Nunc Inc., Naperville, Illinois) were fixed with acetone and subjected to immunostaining. Anti-*bcl*-2 antibody and FITC-conjugated anti-mouse IgG antibody (DAKO) were used in the primary and secondary reactions, respectively.

RESULTS

The results of LOH at the *bcl*-2 gene locus in gastric and colorectal carcinomas are summarized in Table I. Four (36%) of the 11 well differentiated stomach cancers had LOH at the *bcl*-2 locus, whereas none of the six poorly differentiated stomach cancer cases did so. In colorectal carcinoma cases, 6 (60%) of the 10 well differentiated adenocarcinomas showed LOH. Representative cases with LOH are shown in Fig. 1. The 23.1

Table I. Loss of Heterozygosity at *bcl*-2 Gene Locus in Human Gastric and Colorectal Carcinoma

Histology[a]	Number of cases	Informative cases	Cases with LOH
Gastric carcinoma			
Well	25	11	4 (36%)
Poor	20	6	−
Total	45	17	4 (24%)
Colorectal carcinoma			
Well	22	10	6 (60%)
Poor	1	−	−
Total	23	10	6 (60%)

a) According to the criteria of the Japanese Research Society for Gastric Cancer and the Japanese Research Society for Cancer of Colon and Rectum.[16,17] Well includes well and moderately differentiated carcinomas and poor includes poorly differentiated medullary type and scirrhous type carcinomas.

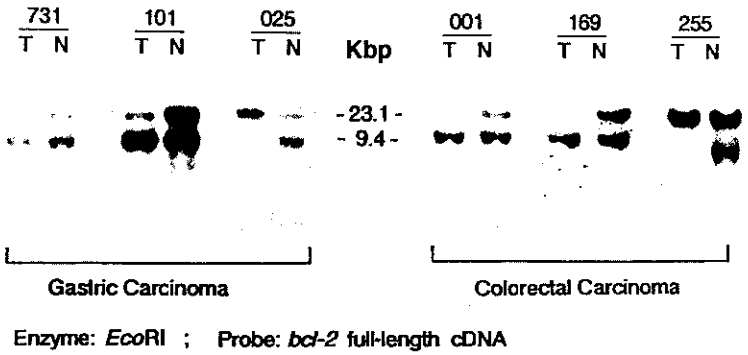

Fig. 1. LOH at the *bcl*-2 gene locus in human gastric and colorectal carcinomas. *Eco*RI digested DNA (10 μg) was subjected to Southern blot analysis with *bcl*-2 full-length cDNA probe. Numbers above the lanes indicate case numbers. T, tumor tissue; N, non-neoplastic mucosa.

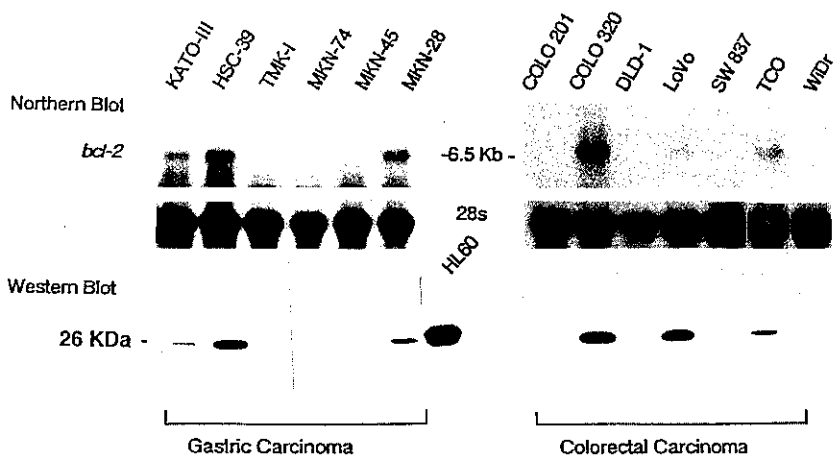

Fig. 2. Expression of *bcl*-2 mRNA and protein in human gastric and colorectal carcinoma cell lines. poly $(A)^+$-selected RNA (5 μg) was subjected to Northern blot analysis. For Western blotting, whole cell lysates (50 μg) were applied to 15% SDS-polyacrylamide gels.

kbp band of Cases 731, 101, 001 and 169, as well as the 9.4 kbp band of Cases 025 and 255 revealed LOH. The intensity of the 9.4 kbp band of Case 731 was lower in the tumor sample (T) than in the normal mucosa (N) because we accidentally applied a smaller amount of the tumor sample in this lane; this was confirmed by hybridizing the filter with β-actin probe (data not shown). Although the same amounts of DNA from Case 025 were applied, the 23.1 kbp band of the T sample from Case 025 was more intense than that of the N sample, suggesting duplication of the remaining allele. Amplification or rearrangement of *bcl*-2 gene was not detected.

We next examined the expression of *bcl*-2 mRNA in gastric and colorectal carcinoma cell lines by Northern blot analysis (Fig. 2). Out of the six gastric carcinoma cell lines, KATOIII, HSC-39 and MKN-28 expressed *bcl*-2 mRNA at high levels. Both HSC-39 and KATOIII are derived from signet ring cell carcinoma of the stomach, while MKN-28 is a well differentiated gastric carcinoma cell line. MKN-45 showed a faint band of *bcl*-2 mRNA. Among the 7 colorectal carcinoma cell lines, CoLo320DM, LoVo and TCO, derived from poorly differentiated carcinomas (LoVo contains signet ring cells), expressed *bcl*-2 mRNA at various levels. The expression of *bcl*-2 protein detected by Western blotting was relatively well correlated with the steady-state transcripts of *bcl*-2 for both gastric and colorectal cell lines, although there were some exceptions. MKN-45 expressed *bcl*-2 mRNA without a detectable level of the protein, while SW837 expressed a very low level of *bcl*-2 protein with undetectable mRNA expression, which might be due to the different sensitivity of the two methods. Colo320 and LoVo cell lines expressed *bcl*-2 protein at nearly the same level, while the level of *bcl*-2 mRNA was very much higher in Colo320 than in LoVo. This may raise the possibility of a small structural alteration in *bcl*-2 mRNA affecting the epitope of the antibody, or different status of translational efficiency or

stability of the protein. HL60 was used as a positive control. The expression of *bcl*-2 protein in the *bcl*-2 mRNA-positive cell lines (KATOIII and HSC-39) was confirmed by immunofluorescence staining (Fig. 3). *bcl*-2 was detected in the cytoplasm of some KATOIII cells and most HSC-39 cells.

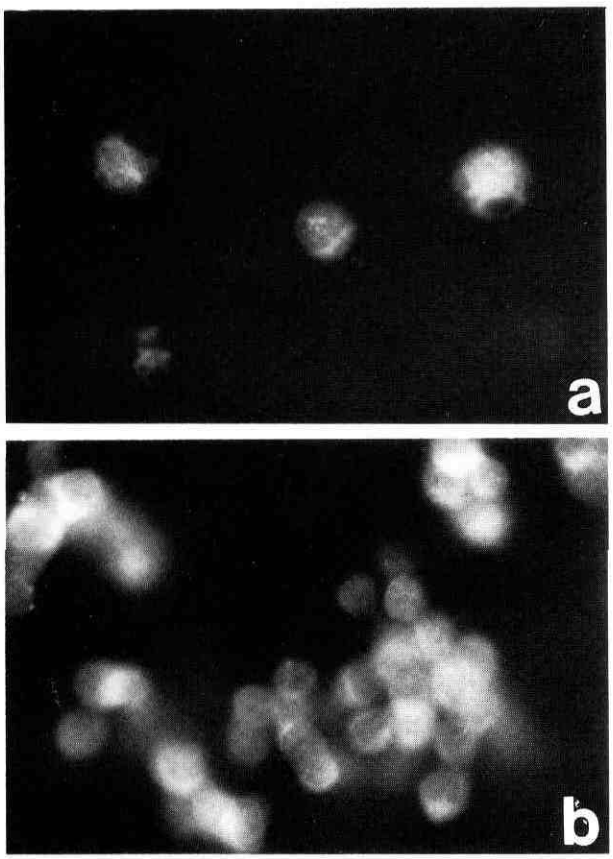

Fig. 3. Immunofluorescence staining of *bcl*-2 protein on human gastric carcinoma cell lines. Indirect immunofluorescence staining was performed as described in "Materials and Methods." KATOIII (a) and HSC-39 (b).

Since some of the gastric and colorectal carcinoma cell lines, especially of poorly differentiated type, expressed *bcl*-2, we next examined the *in vivo* situation. *bcl*-2 mRNA expression in representative cases of gastric and colorectal carcinoma are shown in Fig. 4. Cases 501, 506 and 510 (poorly differentiated adenocarcinomas) showed higher levels of *bcl*-2 mRNA expression, while Case 504, a moderately differentiated adenocarcinoma, had a lower level of *bcl*-2 mRNA than its non-neoplastic mucosa. The intensity of the 6.5 kb band of *bcl*-2 mRNA was evaluated by densitometric scanning and the results for the tumor tissues and non-neoplastic mucosa are compared in Table II. Twenty-four percent (5/21) of the poorly differentiated adenocarcinomas of the stomach expressed *bcl*-2 gene at a higher level than non-neoplastic mucosa. On the other hand, there was no example which showed a high level of *bcl*-2 gene expression in tumor tissue among well differentiated gastric adenocarcinomas. The colorectal carcinoma tissues also expressed *bcl*-2 at various levels. However, because the distribution of histological differentiation of the colorectal carcinomas examined was not even, most being well differentiated carcinomas, a relation between *bcl*-2 expression and histological type could not be detected.

It is well known that normal lymphocytes express *bcl*-2 gene to some degree. To distinguish *bcl*-2 expression by tumor cells from that by normal lymphocytes, we compared the *bcl*-2 mRNA expression with the degree of lymphocytic infiltration in the stroma of tumor tissues (Table III). The degree of lymphocytic infiltration in tumor stroma was not correlated with the level of *bcl*-2 expression as shown by T/N ratio. For example, although Case S5 had a lesser amount of lymphocytes in tumor stroma than in its non-neoplastic mucosa, *bcl*-2 mRNA expression in tumor tissue was 6.3 times more

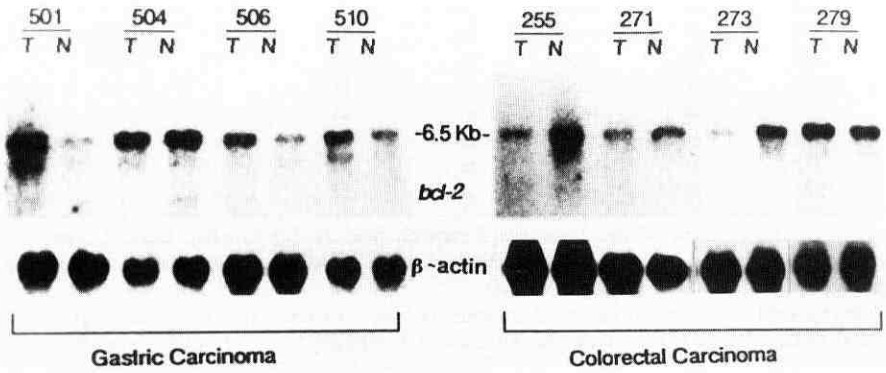

Fig. 4. Expression of *bcl*-2 mRNA in surgically resected samples of human gastric and colorectal carcinomas. Northern blot analysis was performed as described in Fig. 2.

Table II. Expression of *bcl*-2 Gene in Human Gastric and Colorectal Carcinomas

Histology[a]	Number of cases	Level of expression[b]		
		T>N	T=N	T<N
Gastric carcinoma				
Well	8	0	5 (62%)	3 (37%)
Poor	21	5 (24%)	10 (48%)	6 (28%)
Total	29	5 (17%)	15 (22%)	9 (31%)
Colorectal carcinoma				
Well	7	1 (14%)	2 (29%)	4 (57%)
Poor	1	—	1	—
Total	8	1 (13%)	3 (37%)	4 (50%)

a) According to the criteria of the Japanese Research Society for Gastric Cancer and the Japanese Research Society for Cancer of Colon and Rectum.[16, 17] Well includes well and moderately differentiated type carcinomas, poor includes poorly differentiated medullary type and scirrhous type carcinomas.
b) The intensities of *bcl*-2 specific bands of Northern blots were analyzed by one-dimensional densitometric scanning after normalizing with respect to the internal control and presented as a comparison of tumor and normal tissues. T, tumor tissue; N, non-neoplastic mucosa.

Table III. *bcl*-2 Gene Expression and Clinicopathological Parameters in Gastric and Colorectal Carcinomas

Gastric carcinoma[a]

No.	Case	Mac.	Hist.	Inv.	Stage	Lymphocyte inf[b]		*bcl*-2 mRNA T/N[c]
						T	N	
S1	244	2	well	ss α	2	+	++	1.0
S2	245	3	well	ss β	2	+	++	1.0
S3	246	2	well	ss β	2	−	+	0.6
S4	247	4	poor	ss γ	3	++	+++	1.2
S5	501	2	poor	pm	2	+	++	6.3
S6	504	3	mod	se	3	−	+	0.9
S7	507	4	poor	sei	4	−	+++	0.9
S8	509	5	poor	sm	2	+	++	0.8
S9	510	4	poor	ss γ	3	+	++	1.7
S10	514	4	poor	sei	4	+	++	2.3

Colorectal carcinoma[a]

No.	Case	Dukes	Hist.	Inv.	Stage	Lymphocyte inf[b]		*bcl*-2 mRNA T/N[c]
						T	N	
C1	255	A	well	pm	I	+	+	0.4
C2	271	B	well	ss	II	+	++	1.0
C3	272	C	mod	ss	III	+++	++	0.2
C4	273	B	well	ss	II	+	+	0.6
C5	279	B	well	a1	II	+	+	2.1
C6	281	B	well	ss	II	+	++	0.8
C7	282	B	mod	ss	II	+++	+	1.0
C8	288	B	poor	ss	II	+	++	1.0

a) According to the criteria of the Japanese Research Society for Gastric Cancer, and the Japanese Research Society for Cancer of Colon and Rectum.[16, 17] Mac., macroscopic classification; Hist., histological type; Inv., depth of tumor invasion.
b) Lymphocytic infiltration was evaluated by microscopical examination of hematoxylin-eosin sections of tumor and normal tissues, based on the number of lymphocytes. T, tumor tissue; N, non-neoplastic mucosa.
c) The intensity of the *bcl*-2 specific band in Northern blots was analyzed by one-dimensional densitometric scanning, normalized with respect to the internal control and presented as T/N ratio.

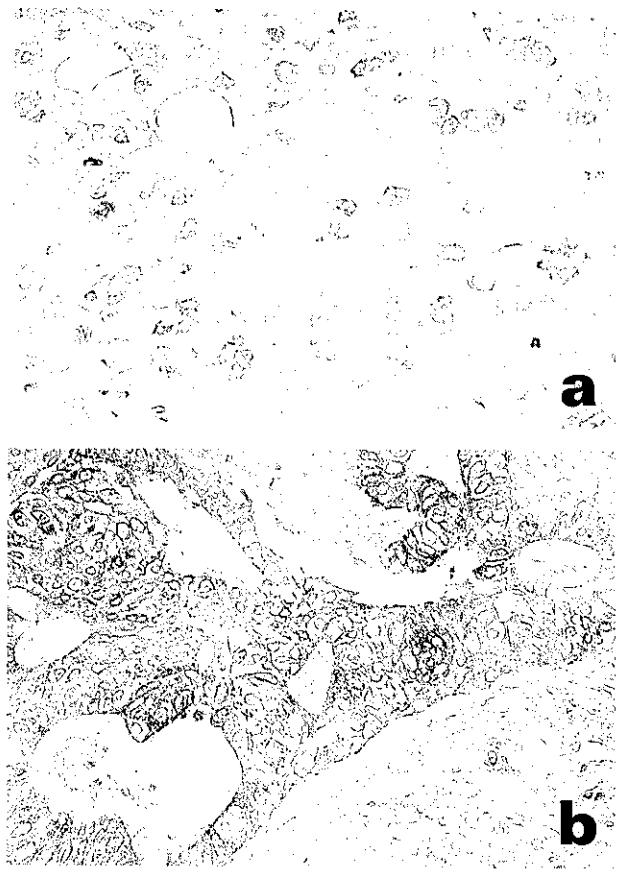

Fig. 5. Immunohistochemical staining of *bcl*-2 in gastric and colonic carcinoma cases. AMeX-fixed, paraffin-embedded sections were subjected to immunohistochemistry by the ABC method using anti-*bcl*-2 antibody. A poorly differentiated gastric carcinoma (a) and a well differentiated colonic carcinoma (b).

than in non-neoplastic mucosa. On the other hand, Case C7 contained more lymphocytes in tumor stroma than its non-neoplastic mucosa, but *bcl*-2 mRNA expression was almost equal. Furthermore, the expression of *bcl*-2 protein was immunohistochemically detected within the tumor cells (Fig. 5). Therefore, we concluded that the expression of *bcl*-2 mRNA and protein is not solely by the infiltrating lymphocytes, but tumor cells are also responsible for the *bcl*-2 expression detected in tumor tissues.

DISCUSSION

We detected LOH at the *bcl*-2 gene locus in 24% and 60% of gastric and colorectal carcinomas, respectively. All the tumors showing LOH at the *bcl*-2 locus were well differentiated adenocarcinomas, although the difference in incidence of LOH between well differentiated type and poorly differentiated type was not significant because of the small number of cases examined. If *bcl*-2 is a tumor suppressor gene, LOH of one allele, possibly associated with a point mutation of the remaining allele, would cause loss of function. Another possible explanation for this phenomenon may be the proximity of *bcl*-2 locus (18q21.3) to a tumor suppressor gene, *DCC* (deleted in colorectal carcinoma) locus (18q21.3). *DCC* is frequently deleted in the colorectal tumors and simultaneous loss of the two during carcinogenesis might be expected.[24] In fact, we have demonstrated that well differentiated gastric cancers develop through the same genetic pathway as colorectal cancers, and loss at the *DCC* locus is a common event, occurring in 50% of well differentiated type gastric adenocarcinomas.[25] We should examine the alteration of the *DCC* gene in the same cases to clarify the relation between LOH at the *bcl*-2 and *DCC* loci.

The possibility exists that LOH might be underestimated in poorly differentiated gastric carcinomas, because considerable amounts of normal stromal and inflammatory cells are usually present as contaminants, although we confirmed that the tumor specimens consisted mainly of carcinoma tissue histologically. Tamura *et al.*[26] recently reported that LOH at the specific locus in poorly differentiated gastric carcinomas could be frequently detected after tumor cell enrichment by cell sorting. Confirmation is needed using samples rich in tumor cells.

Twenty-four percent of the poorly differentiated gastric carcinoma cases expressed *bcl*-2 mRNA at a higher level in tumor tissues than in non-neoplastic mucosa, while such a high expression in tumor tissues was not detected in well differentiated adenocarcinomas. Most of the gastric and colorectal carcinoma cell lines which expressed *bcl*-2 mRNA were also poorly differentiated carcinomas. The expression of *bcl*-2 protein by tumor cells was verified by immunostaining. Accordingly, this is the first report to demonstrate *bcl*-2 expression in human gastrointestinal carcinomas. Our findings suggest that the expression of *bcl*-2 may participate in the development of poorly differentiated adenocarcinomas of the stomach and colon, or at least a certain fraction of them. These findings are consistent with the different pathogenesis of the two types of stomach cancer.[25]

However, the absolute level of *bcl*-2 mRNA among samples taken from non-neoplastic mucosa seemed to be highly variable, and we do not know the reason for this. The degree of lymphocytic infiltration in the stroma of non-neoplastic mucosa was not correlated with the level of *bcl*-2 mRNA expression. *bcl*-2 is not always expressed by all infiltrating lymphocytes.[27] *bcl*-2 protein can be detected in small B lymphocytes in the mantle zone and many cells within T cell areas. Very few cells in germinal

centers express *bcl*-2. We need to look carefully at the kinds of lymphocytes infiltrating the mucosal stroma. Even if cases containing highly variable *bcl*-2 expression in normal mucosa such as Cases 501 and 255 (Fig. 4) were neglected, a similar tendency in T/N ratio between well differentiated adenocarcinomas and poorly differentiated adenocarcinomas was detected.

Although the coexistence of a high percentage of LOH at the *bcl*-2 locus and the expression of *bcl*-2 gene in gastric and colorectal carcinomas seems to be rather confusing, our data indicated that these events are independent in individual cases. We did not observe LOH in poorly differentiated tumors, while higher expression in well differentiated carcinomas was rare, suggesting that LOH and overexpression may be confined to well differentiated and poorly differentiated adenocarcinomas, respectively. Furthermore, although the number of cases with LOH for which mRNA analysis could be performed was small, the level of *bcl*-2 mRNA was lower than in the non-neoplastic mucosa, suggesting that strong expression of *bcl*-2 is unlikely due to increased message from the remaining allele.

bcl-2 is known to be a gene that blocks programmed cell death, although its biochemical mode of action has not been clarified. Gene introduction experiments showed that *bcl*-2 promotes the short-term survival of cells after growth factor deprivation.[2] Therefore, it is possible that *bcl*-2 provides a distinct survival signal to the cell and may contribute to neoplasia by allowing the cell to persist until other synergistic oncogenes such as c-*myc* become activated,[28,29] and/or by abrogating apoptosis induced by those oncogenes.[30,31] Regulation of apoptosis by *bcl*-2 gene may be significant for physiologic events such as embryonic development and antigenic selection, as well as tumorigenesis.[32,33]

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