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Microsatellite Instability and Frameshift Mutations in the *Bax* Gene in Hereditary Nonpolyposis Colorectal Carcinoma

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We studied microsatellite instability (MI) and *bax* gene abnormalities in colorectal carcinomas from 36 patients diagnosed as having hereditary nonpolyposis colorectal cancers (HNPCC) according to the clinical criteria (12 with confirmed HNPCC in group A and 24 at high risk of HNPCC in group B) and from 20 randomly selected patients with other colorectal cancers. MI was examined at 4 dinucleotide microsatellite loci and one mononucleotide locus. Frameshift mutations in the *bax* gene were detected by polymerase chain reaction-single strand conformation polymorphism analysis. MI was detected in 7 of the 12 patients in group A and 12 of the 24 in group B. Three MI patterns were identified: type 1, MI in both mono- and dinucleotide repeats; type 2, MI only in mononucleotide repeats and type 3, MI only in dinucleotide repeats. Most MI-positive patients in group A showed type 1 MI, whereas in group B, 5 showed type 1, 3 showed type 2 and 4 showed type 3. Frameshift mutations in the *bax* gene correlated strongly with type 1 and type 2 MI. These results indicate that mutations of different DNA mismatch repair genes may cause several types of MI and result in several different clinical phenotypes of HNPCC. The *bax* gene may be one of the target genes which play a role in the tumorigenesis of HNPCC.

Key words: Hereditary nonpolyposis colorectal cancer — Microsatellite instability — *Bax* gene — Frameshift mutation

Microsatellite instability (MI) or replication errors in simple repeated sequences have been reported in hereditary nonpolyposis colorectal cancers (HNPCC).[1, 2] These are principally due to germ-line mutations of the mismatch repair (MMR) genes, which include *hMSH2*,[3] *hMLH1*,[4, 5] *hPMS1* and *hPMS2*.[6] Recently, somatic or germline mutations in *hMSH3*[7, 8] and *hMSH6* (*GTBP*)[9–11] have been reported to be associated with HNPCC or HNPCC-like disease in patients.[12] Mutations of these six MMR genes produce genetic instability, and are thought to reflect MI in mononucleotide, dinucleotide, and tri- or multinucleotide repetitive sequences, with different patterns occurring according to the particular gene mutation involved.[8, 11]

Frameshift mutations in the small repeat sequences of the transforming growth factor-β type II receptor (TGF-β RII) have been reported to be closely associated with MI-positive colorectal cancers.[6, 13, 14] These frameshift mutations have frequently been detected in both the carcinoma and adenoma types of HNPCC, and are suspected to be an early event in HNPCC carcinogenesis.[15] Recently, frameshift mutations in the *bcl-2* associated X protein (*bax*) gene, which is involved in the *p53* apoptotic pathway and has a (G)8 tract in exon 3,[16, 17] have been reported in the mutator phenotype of colorectal cancers[18, 19] or HNPCC.[20, 21] *Bax* was initially identified as a *bcl-2*-associated 21-kDa protein, with extensive amino acid homology to the bcl-2 protein.[16] It is believed to play a role in the activation of *bcl-2* by forming *bcl-2*/*bax* complex.[22, 23] *Bax* is also believed to be closely associated with drug resistance, oncogenic transformation and tumorigenesis due to the attenuation of *p53*-dependent apoptosis.[24, 25] In neuroendocrine lung carcinomas, immunohistochemical analysis indicated that *p53* abnormalities were positively correlated with high *bcl-2*/*bax* ratios.[26]

We have previously reported the relationship between MI and clinicopathological features in HNPCC patients diagnosed according to clinical criteria (Table I).[2] In this study, we analyzed MI using mono- and dinucleotide microsatellite loci, and found several different patterns of MI in HNPCC patients. We also analyzed frameshift mutations in the *bax* gene including the (G)8 tract, in addition to bax and p53 protein expression, in order to clarify the role of the *bax* gene in HNPCC carcinogenesis.

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Table I. Criteria for Hereditary Nonpolyposis Colorectal Cancer

Minimum Criteria:
1. At least three relatives with histologically verified colorectal cancers; one of the relatives should be a first-degree relative of the other two
(familial adenomatous polyposis should be excluded).
2. At least two successive generations should be affected.
3. In one of the relatives, colorectal cancers should have been diagnosed at below 50 years of age.

Clinical Criteria:
A. A patient with three or more first-degree relatives having colorectal cancers.
B. A patient with two first-degree relatives having colorectal cancers and with any of the following criteria:
   a) Age at onset of colorectal cancer(s) younger than 50 years
   b) Right colon involvement
   c) Synchronous or metachronous multiple colorectal cancers
   d) Association with extracolorectal malignancy (familial polyposis coli should be excluded)

MATERIALS AND METHODS

Patients We studied 36 patients with colorectal cancer who underwent surgical resection between 1978 and 1995 at the National Cancer Center Hospital (Tokyo) or the University Hospital of Tsukuba (Ibaraki) and were diagnosed as having HNPCC according to clinical criteria (Table I). These included 12 patients with confirmed HNPCC (group A) (7 patients were within the Amsterdam criteria) and 24 patients at high risk for HNPCC (group B).[2] A further 20 randomly selected colorectal cancer patients (group C) who underwent surgical resection during the same period were also examined. Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue as previously described.[27] About 20 to 60 ng of DNA was used for each polymerase chain reaction (PCR) amplification.

Analysis of microsatellite instability Four dinucleotide ($(CA)^n$ or $(CT)^n$) microsatellite loci, *D1S158*, *D5S107*, *D8S199* and *D18S39*, were analyzed as previously reported.[2] One poly(A) microsatellite locus, *BAT 26*, was also analyzed in all 56 patients as described previously.[28] Briefly, PCR of *BAT 26* was performed with the primers 5′-TGACTACTTTTGACTTCAGCC-3′ and 5′-AACCATTCAACATTTTTAACCC-3′. PCR was carried out over 30 cycles, each comprising 30 s at 95°C, 60 s at 45°C and 60 s at 70°C, in the presence of 0.2 mCi of [γ-^{32}P]dCTP. The PCR products were separated in 6% denaturing polyacrylamide gel. Electrophoresis was performed at 55 W for 1.5 h at room temperature. MI positivity was defined as the presence of extra bands in the PCR products amplified from tumor DNA that were not visible in the products from corresponding noncancerous tissue.

PCR-single strand conformation polymorphism (SSCP) analysis of the *bax* gene PCR of the *bax* gene (including the (G)8 tract) was performed as described previously.[29] Briefly, a 94-base pair region including the (G)8 tract was amplified by PCR with the primers 5′-ATCCAGGATCGAGCAGGGCG-3′ and 5′-ACTCGCTCAGCTTCTTGGTG-3′. PCR was carried out over one cycle of 4 min at 94°C, followed by 30–35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C. Electrophoresis was performed using precast homogenous 12% polyacrylamide gel (TEFCO Corporation, Tokyo) with 1× TBE (Tris-borate EDTA). After electrophoresis, the gel was stained with silver (Daiichi Co., Ltd., Tokyo). Abnormal bands observed during SSCP analysis were cut out and the DNA was extracted from the gel. The extracted DNA (20–50 ng) was reamplified by PCR under the conditions described above, and the products (200–250 ng) were sequenced directly using a Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham International plc, Bucks, UK).

Histological and immunohistochemical analyses The tumors were histologically classified according to the criteria set by the World Health Organization.[30] *Bax*, *p53* and *bcl-2* immunostaining was performed on formalin-fixed, paraffin-embedded tissues using the ABC method.[27] After heat treatment by microwaving at 90°C for 10 min, non-specific IgG binding and endogenous peroxidase activity were suppressed with 5% normal swine serum and 0.8% hydrogen peroxide, respectively. The primary antibodies were applied to the slides and incubated at 4°C overnight. Bax protein was detected with the N-20 rabbit polyclonal antibody, which was raised against a peptide corresponding to amino acids 11–30 mapping at the amino terminus of human *bax* and does not cross-react with *bcl-2* or *bcl-x* (1/200; Santa Cruz Biotechnology, Santa Cruz, CA). *p53* was detected using the rabbit polyclonal antibody, RSP-53 (Nichirei Co., Tokyo).[2] Bcl-2 protein was detected with a mouse monoclonal antibody (1/100; Dako Japan Co., Ltd., Kyoto). For *bax*, positive staining was defined as an intensity equal to or greater than that of the apical portions of normal colonic epithelial cells present on the same slide. The immunostaining score for *bax* was graded as 1+: less than 10% of the cancer cells were stained positively; 2+: 10 to 50% of the cancer cells were stained positively; 3+: over 50% of the cancer cells were stained positively. *P53* immunostaining was considered positive when stained tumor cells were distributed zonally.[31] For *bcl-2*, specimens containing more than 10% stained cells were defined as positive.

Statistical analysis The two-tailed Fisher's exact test was used to analyze the significance of differences between the patient groups for each analysis. The Scheffe

Table II. Microsatellite Instability in Colorectal Cancers

Microsatellite markers		Microsatellite instability				
		type 1	type 2	type 3		
Dinucleotide		+	−	+	−	
Mononucleotide		+	+	−	−	MI(+)/total
Group	A (12)	6	0	1	5	7/12[a)]
	B (24)	5	4	3	12	12/24[a)]
	C (20)	1	0	0	19	1/20

MI, microsatellite instability; dinucleotide, *D1S158*, *D5S107*, *D8S199*, *D18S39*; mononucleotide, *BAT 26*.
+, −: positive or negative for microsatellite instability.
Group A, B: HNPCC patients diagnosed according to the clinical criteria, group C: randomly selected patients.
a) $P<0.01$, compared with group C determined by two-tailed Fisher's exact test.

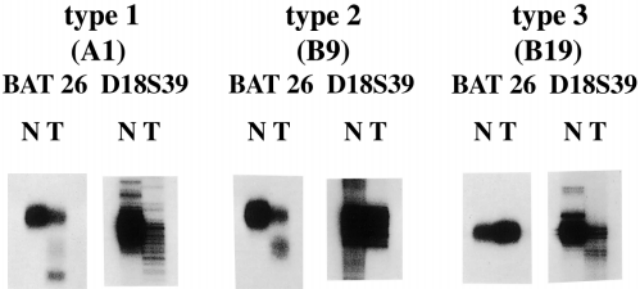

Fig. 1. Three types of microsatellite instability. Type 1: microsatellite instability in both mono- and dinucleotide repeats (case A1). Type 2: microsatellite instability only in mononucleotide repeats (case B9). Type 3: microsatellite instability only in dinucleotide repeats (case B19). N: non-cancer. T: cancer.

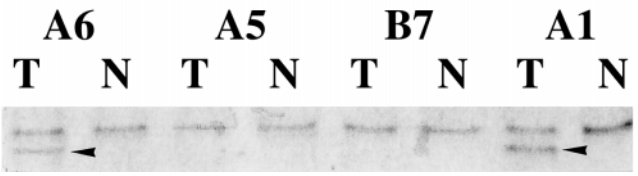

Fig. 2. PCR-SSCP analysis of the *bax* gene. T: cancer. N: non-cancerous tissue. Arrowheads: extra bands.

F test was used in the analysis of the association between MI and frameshift mutations in the *bax* gene.

RESULTS

Microsatellite instability in colorectal cancers The result of the MI analysis is summarized in Table II. The proportion of patients with MI was higher ($P<0.01$) in both HNPCC groups (group A: 58.3%, B: 50.0%) than in group C (5.0%). Three MI patterns were identified: type 1, MI in both mono- and dinucleotide repeats; type 2, MI only in mononucleotide repeats and type 3, MI only in dinucleotide repeats (Fig. 1). In group A, 6 of the 7 patients with MI met the Amsterdam minimum criteria and all 6 showed MI in both mono- and dinucleotide repeats (type 1). In group B, 7 of the 12 patients positive for MI were classified as having either type 2 or 3 MI. Patients with type 2 MI were found only in group lB. In group C, one patient showed type 1 MI.

PCR-SSCP and sequencing analysis of the *bax* gene Representative results from the SSCP analysis of the *bax* gene, including the (G)8 tract, are shown in Fig. 2. Extra bands were observed in 5 of the 12 specimens from group A, 6 of the 24 specimens from group B and 1 of the 20 from group C, and all these extra bands showed the same mobility shift pattern. To confirm the results of the SSCP analysis, the extra bands from 3 randomly selected specimens were cut out of the gel and sequenced. All three were revealed to have one base deletion in the (G)8 tract (Fig. 3).

The incidence of frameshift mutations was significantly higher in group A ($P<0.05$) or in group A+B ($P<0.05$) than in group C. The relationship between MI and frameshift mutations in the *bax* gene is summarized in Table III. In the MI-positive (MI(+)) patients (type 1 or 2), the incidence of frameshift mutations in the *bax* gene was significantly higher than that in MI-negative (MI(−)) patients with HNPCC ($P<0.05$ and 0.01, respectively). There were no patients with frameshift mutations in the *bax* gene among the MI(+) (type 3) and MI(−) patients with HNPCC. In group C, one MI(+) patient (type 1) had a frameshift mutation in the *bax* gene, while the other MI(−) patients had no frameshift mutations. The correlation between MI and the clinical characteristics of HNPCC in patients diagnosed according to clinical criteria was also

examined. The incidence of multiple colorectal cancers was higher in type 1 patients (9/11, 81.8%) than in MI(−) patients (6/17, 35.3%).

Immunostaining of bax, p53 and bcl-2 proteins The results are summarized in Table IV and Fig. 4. All 12 patients who had frameshift mutations in the *bax* gene were graded 1+ for the immunostaining of bax protein. Two of the three diagnosed as having moderately differentiated adenocarcinoma showed heterogenous staining for bax protein in their tumor specimens. Histologically, in moderately differentiated adenocarcinoma, the tumors contained poorly differentiated areas, and no *bax*-positive cells were detected in these areas. Positive cells were however, detected in the main, moderately differentiated parts of the tumor (Fig. 5). Forty-four tumors were negative for frameshift mutations in the *bax* gene and 15 of these showed weak immunoreactivity for bax protein. Eleven of these 15 tumors were positive for p53 protein (Table IV). A decrease in bax protein expression was closely associated with *bax* gene or *p53* abnormalities ($P<0.0001$). The proportion of *p53*-positive tumors was significantly higher in group C (10/20, 50%) than in group A (1/12, 8.3%) or B (4/24, 16.7%) ($P<0.05$). Overexpression of *bcl-2* was observed in three tumors (two in group B and one in group C), but we could find no relationship between the expressions of bax protein and *bcl-2*, or *p53* and *bcl-2*.

Histological differentiation and frameshift mutations in the *bax* gene in HNPCC patients Thirty-six tumors diagnosed as HNPCC according to clinical criteria were histologically classified according to the criteria of the World Health Organizaton,[30] and were analyzed with

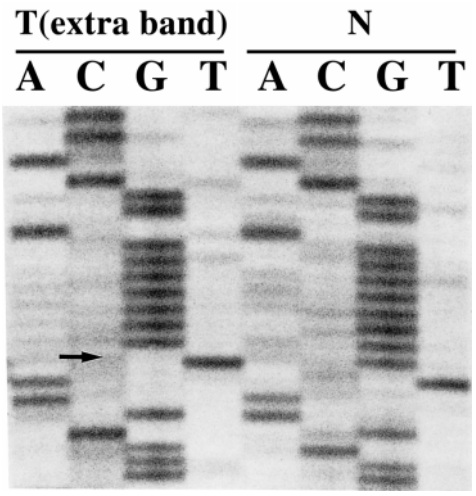

Fig. 3. Sequence of the extra band (case A6) detected by PCR-SSCP analysis of the *bax* gene. T: cancer. N: non-cancer. Arrow: one G deletion in the (G)8 tract.

Table IV. Frameshift Mutations and Immunohistochemical Staining of Bax Protein in Colorectal Cancers

Bax immunoreactivity	Frameshift mutation		
	positive	negative	total
1+	12(0)	15(11)	27
2+ or 3+	0	29(4)	29

Bax immunoreactivity 1+: the proportion of positively stained cells is less than 10% in a minimum of five high-power fields (HPFs) (40× objective lens), 2+: positively stained cells are 10% to 50%, 3+: positively stained cells are over 50%.
Parenthesis: number of cases positive for p53 protein immunostaining.

Table III. Microsatellite Instabilities and Frameshift Mutations in the *Bax* Gene in HNPCC

Microsatellite markers		Microsatellite instability			
		type 1	type 2	type 3	
Dinucleotide		+	−	+	−
Mononucleotide		+	+	−	−
FM positive	A (12)	5/6[a)]	0	0/1	0/5
	B (24)	3/5[a)]	3/4[b)]	0/3	0/12
	Total	8/11[a)]	3/4[b)]	0/4	0/17

Dinucleotide, *D1S158*, *D5S107*, *D8S199*, *D18S39*; mononucleotide, *BAT 26*.
+, −: positive or negative for microsatellite instability.
FM: frameshift mutations in the *bax* gene.
A, B: HNPCC patients diagnosed according to the clinical criteria.
a), *b*) $P<0.05$ and 0.01, compared with patients showing negative for microsatellite instability in the same group determined by two-tailed Fisher's exact test.

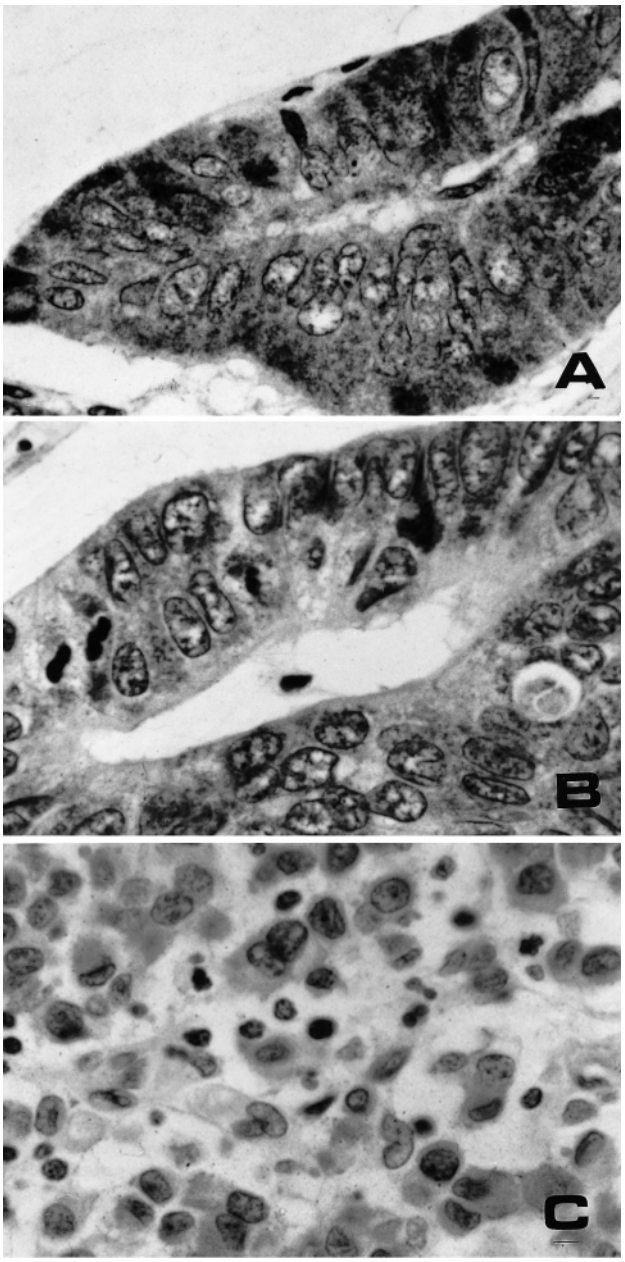

Fig. 4. Immunostaining of bax protein (×400). A: most of the tumor cells show strong immunoreactivity (grade 3+). B: 10 to 50% of the tumor cells show strong immunoreactivity (grade 2+). C: less than 10% of the tumor cells show strong immunoreactivity (grade 1+).

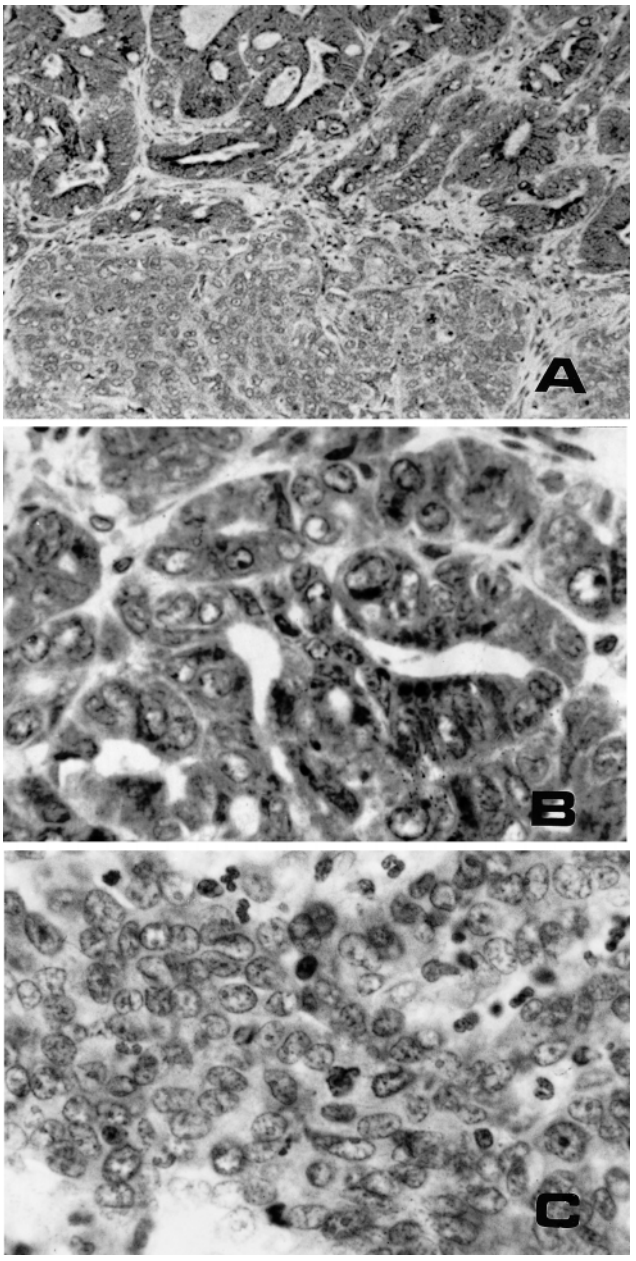

Fig. 5. Immunostaining of bax protein (case B8). A: low magnification of the tumor tissue. Bax protein was stained heterogenously (×100). B: more differentiated tumor cells showed strong stainability for bax protein (×400). C: less differentiated tumor cells were weakly stained (×400).

respect to the relationship between their histological differentiation and the incidence of frameshift mutations in the *bax* gene (Table V). There were no tumors with frameshift mutations among 7 well differentiated adenocarcinomas, and no marked correlations between histological differentiation and MI.

DISCUSSION

Mutations of *hMSH2*, *hMLH1* and *hPMS2* induce genetic instability in both mono- and dinucleotide repeats.[3–6, 32, 33] In the present study, three types of MI(+) patients were grouped as types 1, 2 and 3 (see “Results”).

Table V. Histological Differentiation and Frameshift Mutations in the *Bax* Gene in HNPCC

Differentiation	Frameshift mutation	
	positive	negative
W/D	0	7[a)]
M/D	7	15
P/D	3	2
MUC	1	1
total	11	25

HNPCC: diagnosed according to the clinical criteria.
W/D: well differentiated, M/D: moderately differentiated, P/D: poorly differentiated, MUC: mucinous.
a) $P=0.078$, compared with positive for frameshift mutations determined by two-tailed Fisher's exact test.

Most of the patients with MI in group A had alterations in both mono- and dinucleotide repeats (type 1). In particular, seven patients diagnosed according to the minimum criteria showed a high incidence (6/7, 86%) of MI and all had alterations in both mono- and dinucleotide repeats. These data are consistent with previous reports that HNPCC is principally due to germ-line mutations of MMR genes, such as *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2*.

On the other hand, in this study, 4 of the MI(+) patients in group B showed MI only within mononucleotide repeats (type 2), while the other 3 patients showed MI only within dinucleotide repeats (type 3) (Table II). The abnormalities in these patients may have been related to some specific MMR gene, such as *GTBP* (*hMSH6*). Tumors with *GTBP* mutations primarily showed alterations in mononucleotide repeats.[9)] Reportedly,[12)] the *hMSH2-hMSH3* complex recognizes certain single or multiple nucleotide insertion/deletion loops, while the *hMSH2-hMSH6* (*GTBP*) complex recognizes G/T mismatches and some +1 nucleotide insertion/deletion loops. On the basis of these findings, we hypothesized that (a) the 4 patients in group B with type 2 MI may have had (germ-line) mutations of one of the MMR genes, such as *GTBP*, and (b) the 3 patients in group B and one patient in group A with type 3 MI may have had (germ-line) mutations of one of the MMR genes, such as *hMSH3*. Recently, somatic mutation of *GTBP* has been reported in a patient with hereditary colorectal cancer-like disease.[12)] The patient developed two colonic adenomas and one rectal cancer at 62 years of age. Somatic mutations of *GTBP* were detected in all three tumors and somatic mutation of *hMSH3* was also detected within the rectal cancer. MI was detected in mononucleotide repeats in all three tumors, and MI in dinucleotide or other repeats were detected within the rectal cancer, but not in the adenomas. These results are consistent with our hypothesis.

Recently, it was reported that about 50% of colorectal cancers showing MI had frameshift mutations in the *bax* gene.[18, 19–21)] In the present study, the incidences of frameshift mutations were 65% in MI(+) colorectal cancers and 31% in HNPCC patients diagnosed according to clinical criteria. In particular, frameshift mutations were observed in more than 71% of patients diagnosed according to minimum criteria. The nucleotides involved in frameshift mutations in the *bax* gene are almost the same as those involved in TGF-β RII alterations.[13, 14, 19–21)] These frameshift mutations in the *bax* gene were strongly associated with MI, especially MI in both mono- and dinucleotide repeats (type 1) or within mononucleotide repeats (type 2).

MI only in mononucleotide repeats (type 2) is closely associated with single base deletions in the (G)8 tract of the *bax* gene as well as MI in both mono- and dinucleotide repeats (type 1) (Table III). These data suggest that the tumorigenesis of colorectal carcinomas with type 1 and 2 MI is closely associated with mutations in certain critical target genes with mononucleotide repetitive sequences, such as the *bax* gene. We could find no significant differences in clinical characteristics between the three types of MI in HNPCC patients, except for a higher incidence of multiple colorectal cancers in patients showing type 1 MI when compared with MI(−) patients.

Decreases in bax protein, as detected by immunostaining were strongly correlated with frameshift mutations in the *bax* gene or abnormalities in *p53* ($P<0.0001$) (Table V). This is compatible with the fact that the *bax* gene is a transcriptional target of *p53* and is involved in the *p53* apoptotic pathway.[17)] All 12 patients with frameshift mutations in the *bax* gene showed decreased bax protein expression. Two tumors with frameshift mutations showed heterogenous decreases in *bax* immunoreactivity, together with loss of histological differentiation. These heterogeneic alterations in bax protein expression may depend upon whether the abnormality of the *bax* gene is heterozygous or homozygous. Our genomic and immunohistochemical results indicated that *bax* gene alterations are associated with histological differentiation.

The negative correlation between abnormalities in *p53* immunostaining and MI is in agreement with previous observations.[2)] Furthermore, none of the patients with *p53* gene abnormalities as detected by immunostaining ($n=12$) showed frameshift mutations in the *bax* gene. These results suggest differences in the tumorigenic pathway between MI(+) and MI(−) patients.

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