**ORIGINAL ARTICLE**

**Bcl-2 expression and frequency of apoptosis correlate with morphogenesis of colorectal neoplasia**

Y Suzuki, T Honma, S Hayashi, Y Ajioka, H Asakura

**Aim:** To investigate whether the expression of apoptosis and cell proliferation related proteins is related to the macroscopic form of colorectal neoplasia.

**Methods:** The extent of apoptosis, using the 3′ end DNA labelling method, and the immunohistochemical expression of cell proliferation (Ki-67) and apoptosis related proteins (Bcl-2, Bak, and p53) were investigated in 64 colorectal adenomas and 22 early carcinomas extending no further than the upper submucosal region. The specimens were classified into three types of macroscopic form (polypoid, flat, and depressed).

**Results:** The Ki-67 labelling index and the Bak score did not differ significantly among each macroscopic form. In contrast, the apoptotic index and the Bcl-2 score changed significantly according to the macroscopic forms. Compared with polypoid and flat tumours, depressed tumours had a significantly lower apoptotic index (2.84, 2.28, and 1.44, respectively) and a significantly lower Bcl-2 score (3.18, 2.70, and 1.64, respectively). The proliferation/apoptosis ratio was significantly lower in polypoid tumours than in the other two macroscopic forms. The Bcl-2 score became significantly lower as the tumours flattened or took on a depressed form. Immunohistochemical p53 overexpression did not correlate with the macroscopic forms.

**Conclusions:** These results suggest that differences in both Bcl-2 expression and apoptosis may play an important role in the morphogenesis of colorectal neoplasia.

**Assessment of macroscopic form**

The extirpated specimens were stretched on to styrofoam and immersed in 10% buffered formalin. The macroscopic form was determined according to the following three types: polypoid, over 3 mm in height; flat, 3 mm in height or less; and depressed, lower than the surrounding normal mucosa. The specimens were then embedded in paraffin wax, and histological diagnoses were made on routine haematoxylin and eosin stained sections.

**Immunohistochemistry**

We applied the streptavidin–biotin, technique using a Histofine SAB-PO kit (Nichirei Corporation, Tokyo, Japan) for immunostaining. Serial sections heated in a microwave oven in 0.01M sodium citrate buffer (pH 5.9) for five cycles of three minutes were incubated with the anti-Ki-67 antibody (MIB-1; Zymed Laboratories, San Francisco, USA), anti-Bcl-2 antibody (clone 124; Dako, Copenhagen, Denmark), anti-Bak antibody (TC102; Oncogene Research Products, Cambridge, USA), or anti-p53 antibody (PAb1801; Oncogene Research Products). The colour was developed with 3,3′-diaminobenzidine (DAB) solution. The sections were counterstained with haematoxylin and mounted. Negative controls were established by replacing

**METHODS**

**Patients**

Eighty-six colorectal tumours (64 tubular or tubulovillous adenomas and 22 carcinomas extending no further than the upper submucosal region) were resected surgically or endoscopically from 59 patients (44 men and 15 women; age range, 40–83) at Niigata University Hospital. Histological classification was determined according to the criteria of the Japanese Research Society for Cancer of the Colon and Rectum.**

**Abbreviations:** AI, apoptotic index; DAB, 3,3′-diaminobenzidine; KI, Ki-67 index; TUNEL, 3′ end DNA labelling
The classified macroscopic form were compared using the Mann-Whitney U test. The AIs, KIs, and the Bcl-2 and Bak scores in groups were defined as apoptosis positive. Positive controls were obtained by DNase treatment of the sections.

**Detection of apoptosis**

DNA fragmentation associated with apoptosis was identified by means of the 3' end DNA labelling (TUNEL) method using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, USA), according to the method described by Gavrieli et al. The steps were performed according to the manufacturer's instructions, with minor modifications. The DAB reaction, counterstaining, and setting up of negative controls were performed using the same procedures as those used for immunohistochemistry. Positive controls were found for Ki-67, lymphocytes within the lamina propria for Bcl-2, and epithelial cells of normal crypts for Bak.

**Evaluation**

Cytoplasmic staining was considered positive for Bcl-2 and Bak. A semiquantitative evaluation was performed, the reproducibility of which was confirmed by two of the authors. Because various degrees of staining were found, the proportion of positive to whole tumour cells on each section was scored as follows: 0, < 1%; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, > 75%. For p53, only nuclear staining was considered positive; it was evaluated by a slightly modified method (previously described by Kobayashi and colleagues) as follows: –, negative; 1+, sporadic positive; and 2+, diffuse positive.

**Statistical analysis**

The unpaired *t* test was used to compare tumour sizes in each group. The AIs, KIs, and the Bcl-2 and Bak scores in groups classified for macroscopic form were compared using the Mann-Whitney U test. The *χ*² test was used to compare the results of p53 staining. *p* values < 0.05 were regarded as significant.

**RESULTS**

Table 1 details the clinicopathological features of each macroscopic form of the tumours investigated. The mean size of polypoid tumours was significantly greater than that of flat and depressed ones (*p* < 0.01 and *p* < 0.05, respectively). The ratio of carcinoma in depressed tumours was higher than that in polypoid and flat ones.

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Table 1 shows the p53 immunoreactivity in each macroscopic form of the 86 colorectal tumours investigated.

**Relation between macroscopic form and apoptosis**

The KIs and the Bak scores for each macroscopic form were similar (table 3 and fig 3). In contrast, the mean AI of depressed tumours was significantly lower than that of polypoid (*p* < 0.01) and flat (*p* < 0.05) type tumours. The ratio of Ki-67 positivity was significantly larger (*p* < 0.01) than those with a superficial pattern (data not shown).
DISCUSSION
In our present study, we focused on the possible role of cell proliferation and loss in early colorectal tumorigenesis, particularly in terms of macroscopic form. There are several proteins in the Bcl-2 family, and their expression differs according to the histological type of the tumour. Among them, antiapoptotic Bcl-2 and proapoptotic Bak are major proteins that have been reported to show characteristic expression patterns in colorectal neoplasia. To clarify how they are involved in the morphogenesis of colorectal adenomas and early carcinomas, we investigated the immunohistochemical expression of these two proteins, together with Ki-67 and apoptosis.

“Antiapoptotic Bcl-2 and proapoptotic Bak are major proteins that have been reported to show characteristic expression patterns in colorectal neoplasia”

We found that both the AI and the Bcl-2 score were significantly higher in polypoid tumours than in the other two macroscopic forms. Although a direct inverse relation between

Figure 1  [A] Histology of a polypoid intramucosal carcinoma in adenoma. [B] The increased Ki-67 positive cells are seen mainly in the upper region of the crypts. [C] The apoptotic cells (arrow) occur in the neoplastic epithelium. Diffuse staining for [D] Bcl-2 and [E] Bak is seen in both the epithelial cells and the intramucosal lymphocytes.
Bcl-2 expression and apoptosis (which would be expected in view of the fact that the Bcl-2 protein can block apoptosis) has been found in some organs/tissues, this may not hold true during the morphogenesis of colorectal tumours, as has been reported in the lung and ovary. The results of our present study showing that the KI/AI ratio is lowest in polypoid tumours suggests that polypoid adenomas and carcinomas do not grow simply because of increased proliferation and reduced apoptosis. As hypothesised for gastric neoplasia, the diffuse expression of Bcl-2 and a superficial distribution of proliferating cells may contribute to the formation of a polypoid configuration in colorectal tumours. This is supported by our results of a higher Bcl-2 score in polypoid tumours, 60.7% of which showed a superficial distribution of Ki-67 positive cells, than in the other types of tumour.

Although flat and/or depressed tumours of the large intestine have become apparent in recent years, the mechanism by which the non-polypoid configuration is formed is unknown. Unlike previous reports, the proliferative index did not vary significantly with the different macroscopic forms or histopathological scores in our study. This may be because the tumours investigated in our study were smaller and earlier than those in previous reports. Consequently, our results might suggest that, in the extremely early phase of colorectal tumorigenesis, the relative increase of the KI/AI ratio following a significant reduction of AI contributes to the growth of flat and, especially, depressed tumours with high malignant potential, perhaps through some unknown mechanisms unrelated to Bcl-2, or through the accumulation of genetic changes.

We used the TUNEL technique in our present study to identify apoptotic cells. One known shortcoming of this method is that any type of DNA fragmentation, including that of necrotic cells, might be detected. However, because extensive necrosis caused by severe inflammation or ischaemia rarely occurs in

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<th>Table 2</th>
<th>p53 staining for each macroscopic form of the 86 colorectal tumours investigated</th>
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<tbody>
<tr>
<td>p53 staining</td>
<td>Macroscopic form</td>
</tr>
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<td></td>
<td>Polypoid</td>
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Values in parentheses are the numbers of cancers.

<table>
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<th>Table 3</th>
<th>KIs and AIs for each macroscopic form of the 86 colorectal tumours investigated</th>
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</thead>
<tbody>
<tr>
<td>Macroscopic form</td>
<td>KI</td>
</tr>
<tr>
<td>Polypoid [n=28]</td>
<td>22.7 (7.45)</td>
</tr>
<tr>
<td>Flat [n=47]</td>
<td>25.6 (8.24)</td>
</tr>
<tr>
<td>Depressed [n=11]</td>
<td>24.5 (8.09)</td>
</tr>
</tbody>
</table>

Each pair of values is a mean (SD).
*p<0.05; **p<0.01.
KI, Ki-67 index; AI, apoptotic index.
of colorectal neoplasia, we demonstrated that the frequency of proliferation and death are related to the early morphogenesis in some cells, damage from entering the cell cycle, which results in apoptosis and those proteins involved in the apoptotic pathway in early colorectal tumorigenesis may be mediated directly by wild-type p53. However, more detailed molecular analysis would be necessary to determine the involvement of p53.

In conclusion, to investigate the possibility that cell proliferation and death are related to the early morphogenesis of colorectal neoplasia, we demonstrated that the frequency of apoptosis and Bcl-2 expression differed significantly according to the macroscopic form. Further investigations may be required once more detailed mechanisms of apoptosis are elucidated.

ACKNOWLEDGEMENTS
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REFERENCES
**Keratan sulphate MAB 5-D-4 and βig-h3 protein accumulation in corneas**

Build up of keratan sulphate and βig-h3 protein in the cornea causes opacity in Maroteaux-Lamy syndrome (MLS) type B. Recently, mutations in the βig-h3 gene, which codes for βig-h3 protein of the extracellular matrix, have been shown in four autosomal dominant corneal dystrophies. Ahktar et al looked at how proteoglycans, keratan—a byproduct of incomplete breakdown of proteoglycan of the cell envelope and extracellular matrix—and βig-h3 were distributed in diseased corneal tissue from a 16 year old girl with MLSVI type B undergoing a corneal transplant and a normal cornea obtained from necropsy.

In MLS epithelial cells, intercellular spaces, and cell junctions contained proteoglycans and the stroma was heavily loaded. Keratocytes were vacuolated and their lysosomes packed with proteoglycan. The normal cornea βig-h3 protein appeared evenly throughout, save in keratocytes and endothelial cells, and keratan sulphate uniformly within the stroma.

Aryl sulphatase deficiency leads to build up of keratan sulphate within cells, causing keratocytes to degenerate and abnormal deposits of proteoglycan to form. This, the authors conclude, increases light scattering and the opacity of the cornea.

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