Detection of cancer clones in human colorectal adenoma as revealed by increased DNA instability and other bio-markers

Y. Jin,[1] A. Sun,[2] S. Noriki,[1] Y. Imamura,[3] M. Fukuda[4]

[1]Department of Oncological Pathology, Faculty of Medicine, University of Fukui, Matsuoka, Eiheiji-cho, Yoshida-Gun, Fukui, Japan; [2]Department of Pathology, Shaoxing People's Hospital, the First Affiliated Hospital, Shaoxing University Shaoxing, China; [3]Division of Surgical Pathology, Fukui University Hospital, Matsuoka, Eiheiji-cho Yoshida-Gun, Japan; [4]Secretary's office, University of Fukui, Bunkyo Fukui, Japan

An immunohistochemical differential staining of cancerous cells with anti-cytidine antibody after denaturation of nuclear DNA by acid hydrolysis with 2N HCl at 30°C for 20 min (DNA-instability test) has been used as a marker for malignancy. The test was applied to bioptic tissues of human colorectal polyps assessed histopathologically as hyperplastic polyp (11 cases), tubular adenoma of mild (68 cases), moderate (102 cases), and severe (46 cases) dysplasia, and adenocarcinoma (30 cases). The serial sections of the same tissues were also subjected to immunohistochemical staining for Ki67, p53, DNA-fragmentation factor 45 (DFF45) and vascular endothelial growth factor (VEGF). The DNA-instability test was positive in 30 (100%) adenocarcinoma cases, 46 (100%) severe dysplasia adenoma cases, 36 (35.29%) moderate dysplasia adenoma cases, and 8 (11.76%) mild dysplasia adenoma cases, indicating malignancy. All hyperplastic polyps were negative to the DNA-instability test. Furthermore, the percentage of glands positive in the DNA-instability test steadily increased in going from mild (10%), to moderate (35%), to severe (100%) dysplasia, and adenocarcinoma (100%). All other biological markers tested in the present study showed significantly higher values in those adenoma glands that werepositive to the DNA-instability test, irrespective of the dysplasia grade, as compared to the markers in the adenoma glands that were negative to DNA instability testing. Furthermore, the former values were comparable to those in adenocarcinoma. The results indicate that cancer cell clones are already present at the adenoma stages showing positivity to DNA instability testing, enhanced proliferative activity, p53 mutation and induction of DFF45 and VEGF, at a time when the degree of morphological atypia are not yet large enough for them to be identified as cancer. These factors promote cancer cell proliferation, produce heterogeneous subclones due to DNA instability, enhance their survival by escaping apoptosis, and provide abundant nutrients by neovascularization during the early-stage progression of colorectal cancer.

Key words: human colon adenoma, procancer clone, DNA-instability test, Ki67, p53, DFF45, VEGF.

Correspondence: prof. Masaru Fukuda, vice president, University of Fukui,
Bunkyo-3-9-1, 910-8507, Fukui, Japan
E-mail: mitamu_m@sec.icpc.fukui-u.ac.jp

Paper accepted on November 16, 2006

European Journal of Histochemistry
2007; vol. 51 issue 1 (Jan-Mar): 1-10

Human colorectal adenoma is defined by the World Health Organization (WHO) as a circumscribed benign neoplasm composed of tubular and/or villous structures lined by dysplastic epithelium (Jass and Sobin, 1989). WHO classified adenomas according to the grade of epithelial dysplasia into mild, moderate or severe dysplasia or low grade (mild and moderate) and high grade (severe) dysplasia. Hamilton *et al.* (2000) defined adenoma as a precursor lesion from the presence of intraepithelial neoplasia, characterized by hypercellularity with enlarged, hyperchromatic nuclei, varying degrees of nuclear stratification, and loss of polarity.

It is well accepted that the majority of invasive overt cancer of the human colon and rectum arise through the *adenoma-carcinoma sequence* (Jackman and Mayo, 1951; Muto, 1975; Sinya and Wolff, 1979; Lotfi, 1986; Winawer, 1987; Atkin, *et al.*, 1992; Hamilton, l992; Winawer *et al.*, 1993; Leslie *et al.*, 2002). The concept of an adenoma-carcinoma sequence in colorectal carcinoma is supported by the following evidence: (1) the distribution of adenomas of the large bowel parallels the distribution of colorectal carcinomas (Jackman and Mayo, 1951; Sinya and Wolff, 1979; Hamilton, 1992); (2) the mean age of patients with adenomas is approximately five years younger than patients with invasive carcinomas (Winawer, 1987; Hamilton, 1992); (3) the risk of colorectal carcinoma in the patients with neoplastic polyps is higherthan that in the general population (Lotfi, 1986); (4) most colorectal cancers develops in patients whose adenomas had been inadequately removed, and complete endoscopic removal of adenomatous polyps reduces the long-term risk of colorectal cancer (Atkin *et al.*, 1992; Winawer *et al.*, 1993; Leslie *et al.*, 2002); and (5) histopathologic examination of adenomas sometimes reveals small areas of infiltrating adenocarcinoma within it (Hamilton, 1992).

On the other hand, the importance of *de novo can-*

cer (Kuramoto and Oohara, 1987; Shimoda *et al.*, 1989; Mueller *et al.*, 2002) has been repeatedly stressed. The development of endoscopical techniques has made it possible for us to detect small human colorectal carcinoma at an early stage. Since these tumors are very small in the absence of adenomatous elements in their vicinity, it appears that they do not originate from any precursor lesion; hence the name *de novo* carcinoma has been given to these tumors, although they probably account for only a small percentage of colorectal carcinomas (Mueller *et al.*, 2002).

Of the various colorectal polyp types, only the neoplastic polyps, including tubular, villous and villotubular adenomas, are regarded as having malignant potential in the *adenoma-carcinoma sequence* (Shinya and Wolff, 1979). Evaluation of the malignant potential of adenomatous polyps has been extensively studied by surgical pathologists, and adenoma size, the degree of dysplasia, and the participation of villous components have been used to predict the malignant potential of colorectal adenomas (Shinya and Wolff, 1979; Sugai *et al.*, 1999). On the other hand, it has been repeatedly stressed that only a small proportion of adenomas progress to carcinoma, and there are no reliable criteria available for the precise prediction of adenoma progression or recurrence (Hamilton, 1992; Gazelle *et al.*, 2000; Leslie *et al.*, 2002).

The morphological criteria for evaluating the potential malignancy or the probability of transition to overt cancer of colorectal adenoma are quite ambiguous and controversial, and the validity of them is confusing in everyday diagnosis (Nitta *et al.*, 1993). In order to solve this problem, many histochemical and molecular biological methods have been applied in colorectal adenoma and cancer in the search for reliable markers of malignancy and potential malignancy. For example, Ki67 (Hoang *et al.*, 1989; Johnston *et al.*, 1989; Sugai *et al.*, 1999; Saleh *et al.*, 2000), p53 (Baker *et al.*, 1990; Ohue *et al.*, 1994; Saleh *et al.*, 1998; Sugai *et al.*, 1999; Saleh *et al.*, 2000), DNA-ploidy analysis (Suzuki *et al.*, 1995; Sugai *et al.*, 1999; Sugai *et al.*, 2003; Torres *et al.*, 2005), vascular endothelial growth factor (VEGF) (Wong *et al.*, 1999; Kondo *et al.*, 2000; Hanrahan *et al.*, 2003), and genetic instability (Lengauer *et al.*, 1998; Ishiguro *et al.*, 2006) have been used for this purpose.

All of the above diagnostic tools have demonstrated the presence of abnormal gene expression and protein synthesis, disturbed cytodifferentiation, stimulated cell proliferation activity and DNA abnormalities during the course of overt malignant progression in colorectal adenoma. However, none of them can be used as a specific marker for malignancy diagnosis although some of the abnormalities are statistically significant.

In this regard, Fukuda *et al.,* (1986, 1993, 2005) found that nuclear DNA of cancerous cells was always, and without exception, much more unstable than that of comparable benign tumor cells and normal cells, irrespective of epithelial or mesenchymal origin.They also developed the method of differential fluorescent or immunohistochemical staining of cancerous cells after denaturation of DNA by acid hydrolysis. Using this method (the DNA-instability test), cancerous clones can be identified at early stages of carcinogenesis in the so-called borderline or precancerous lesions such as gastric adenoma (Otaki *et al.*, 1994; Sun *et al.*, 2003), otorhinolaryngeal borderline lesions (Tsuzuki *et al.*, 1994), bone giant cell tumor (Azuchi *et al.*, 1998), uterine cervical dysplasia (Khaled *et al.*, 2000), oral leukoplakia (Iwasa *et al.*, 2001), 20-methylcholanthrene-induced squamous cell carcinoma of mouse epidermis (Hirai *et al.*, 2001), and dysplasia and non-papillary low grade cancer of the urinary bladder (Hirose *et al.*, 2005).

In the present study, we used the DNA-instability test as a malignancy marker to identify cancer clones in human colorectal adenoma in 68, 102, and 46 cases of mild, moderate and severe dysplasias, respectively. Cancer clones were identified by their increased DNA instability in colorectal adenoma and their significantly higher Ki67 index values, immunoreactivity for p53, DNA fragmentation factor 45 (DFF45), and vascular endothelial growth factor (VEGF) as compared with the DNA-instability test-negative lesions.

Materials and Methods

A total of 257 biopsy specimens from human colorectal polyps assessed histopathologically as hyperplastic polyp (11 cases), tubular adenoma of mild (68 cases), moderate (102 cases), and severe (46 cases) dysplasia and adenocarcinoma (30 cases) were used in the present study. The histopathological classification of dysplasia grade of tubular adenoma was made based on the degree of structural and cellular atypia of the glands (Jass and Sobin, 1989).

DNA-instability test

Paraffin-embedded 4 μm thick sections were prepared after fixation with 10% buffered formalin for 24 hours at room temperature (RT). They were deparaffinized with xylene, then replaced with ethanol.

Preblocking

After washing in water, the intrinsic peroxidase was blocked with 0.3% H_2O_2 in absolute methanol at RT for 15 min and rinsed with PBS (pH 7.4). The sections were mounted with 2% skim-milk (Yukijirushi, Sapporo, Japan) dissolved in PBS (pH7.4) at 37°C for 20 min to block the background adsorption of antiserum. They were then reacted with blocking solution [Histofine, Nichirei, PO (R) Japan] at RT for 5 min.

HCl-hydrolysis and immunohistochemical staining of single-stranded DNA

The sections were then washed in water and treated with 2N HCl at 30°C for 20 min for acid hydrolysis followed by re-washing in water. They were reacted with polyclonal antibody to cytidine [Biogenesis, UK, 1:1500 dilution in PBS (pH 7.4)] at 4°C overnight and rinsed with PBS (pH 7.4). The sections were reacted with biotinylated goat anti-rabbit IgG [Histofine] at 37°C for 40 min, and rinsed with PBS (pH 7.4), followed by reaction with the avidin-biotin peroxidase complex [Histofine] at 37°C for 30 min, and further rinsed with PBS (pH 7.4).

Co-DAB reaction

In order to visualize the peroxidase colour reaction, the sections were incubated with DAB (3,3′-diaminobenzidine tetrahydrochloride) cobalt solution [DAB, Dojin, Kumamoto, Japan, 5 mg dissolved in 100 mL of 0.05 M Tris-HCl buffer (pH 7.4), added with 2 mL of 1% $CoCl_2$ and 10 μL of 30% H_2O_2 solution] at 20°C for 10 min and washed in water. Nuclear counterstaining was carried out with Kernechtrot (Kernechtrot, Chroma, Stuttgart, Germany, 0.1 g and aluminium sulphate 5 g dissolved in 100 mL distilled water).

Detection of abnormal mitosis in DNA-instability test-positive adenoma glands

The number of normal mitosis and abnormal mitosis were counted in the DNA-instability test-positive and -negative glands, respectively.

Immunohistochemical staining of Ki67, p53, DFF45 and VEGF

Serial 4 μm-thick sections were prepared from the same specimens used for the DNA-instability test as described above. After washing with water, the intrinsic peroxidase activity was blocked as described above and washed with PBS (PH 7.4). The sections were irradiated in a polypropylene slide holder with a cap filled with 10 mM Na-citrate buffer (pH 6.0) over a period of 5 min using an autoclave (Hirayama, 1.5 KW, Japan) for all immunohistochemical stainings except for VEGF. For VEGF, the sections were treated with proteinase K (DAKO, S-3020) for 8 min at RT. They were then reacted with the blocking solution [Histofine] at 20°C for 5 min. This was followed by reactions at 4°C overnight with the following primary antibodies: anti-Ki67 antigen monoclonal antibody [Immunotech, MIB-1, 1:50 dilution in PBS (PH 7.4)], anti-human p53 tumor suppressor protein monoclonal antibody [Dako, Clone DO-7, 1:100 dilution in PBS (PH 7.4)]. The sections were then washed with PBS (7.4), and further reacted with a biotinylated rabbit anti-mouse IgG secondary antibody [Histofine] at 37°C for 30 min, followed by rinsing with PBS (PH 7.4). They were allowed to react with the avidine-biotin- peroxidase complex at 37°C for 30 min and rinsed with PBS (PH 7.4). In order to visualize the peroxidase colour reaction, the sections were incubated with 0.02% DAB [20 mg DAB dissolved in 100 mL of PBS (PH 7.4)] at RT for 10 min and washed in water. Nuclear counterstaining was performed with hematoxylin.

Immunohistochemical staining for DFF45 and VEGF were performed in a similar fashion, except for using polyclonal anti-DFF45 antibody [NCL-DFFp, Novocastra, Newcastle, UK, 1:400 dilution in PBS (PH 7.4)] and polyclonal anti-human VEGF antibody [Santa-cruz A-20; 1:200 dilution in PBS (PH 7.4)] as the primary antibody, respectively. Biotinylated goat anti-rabbit IgG was used in this case as the secondary antiserum with the blocking solution for rabbit.

Evaluations of immunohistochemical staining for the DNA-instability test, Ki67, p53, DFF45 and VEGF

For the DNA-instability test, a gland with more than five positively stained cells was regarded as a positive gland (although a gland containing even a single positive cell should be regarded as a positive

gland); artificially damaged cell nuclei can also be positively stained showing pseudo-positivity to the DNA-instability test and anadenoma and adenocarcinoma with more than one positive gland was counted as a positive lesion. The percentage of positive glands, normal mitosis and abnormal mitosis in the DNA-instability test-positive and -negative glands were also determined in all lesions. In serial sections of the same specimens, the percentages of cells positive for Ki67 (Ki67 index, KI) was determined by counting the positively-stained nuclei among more than 500 cells in each entire lesion, and was also determined in both DNA-instability test-positive and -negative glands, respectively. As for P53, DFF45 and VEGF, a case with more than 5% positively-stained cells, as determined in the same way used for counting KI, was regarded as positive.

Statistical analysis

The Ki67 index (KI) is given as the mean±S.D. Data were analysed using Student's t-test, with a p value <0.05 considered significant. Positive staining for p53, DFF45, VEGF and DNA-instability test are shown as percentages. Data were compared in different cases and between DNA-instability test-positive and -negative adenomas by the Chi-square test. A p value<0.05 denoted a significant statistical difference.

Results

The results of the DNA-instability test, immunohistochemical staining of Ki67, p53, DFF45 and VEGF are summarized in Table 1. The data on Ki67, p53, DFF45 and VEGF in relation to the results of the DNA-instability test are also shown in Table 2. Comparison of various biomarkers in the DNA-instability test-positive adenoma and carcinoma is shown in Figure 3.

DNA-instability test

After hydrolysis with 2N HCl at 30°C for 20min, all cancer cells (Figure 1d) and all cells of severely dysplastic adenoma (Figure 1c) were positively stained with anti-cytidine antibody without exception, indicating increased DNA-instability in both lesions (positive DNA-instability test). On the other hand, normal epithelial cells, stromal fibroblasts, vascular endothelial cells and inflammatory cells in the same slide were negative. About 11.76% (8/68) of mildly dysplastic adenomas were positive to DNA-instability testing, in which about 10% of dysplastic glands were positively stained (Figure 1a). About 35.29% (36/102) of moderately dysplastic adenomas were positive to the DNA-instability test, in which about 35% of dysplastic glands were positively stained (Figure 1b). These positively-stained glands showed increased DNA instability comparable to that of cancer cells. In the remaining cases with negative DNA instability, no positive glands were found. Thus, the extent of immunostaining with anti-cytidine antibody increased with histological grade (Table 1), which was statistically significant (p<0.01). No apparent morphological differences were, however, identified between DNA-instability test-positive and -negative glands in the adenoma of the same dysplastic grade.

Presence of abnormal mitosis in DNA-instability test positive glands

The numbers of normal mitosis and abnormal mitosis were counted in the DNA-instability test-positive and -negative glands, respectively (Figure 2). Abnormal mitosis was only observed in DNA-instability test-positive glands, and the average numbers were 0.15%, 0.25%, 0.95%, and 1.15%, in mild, moderate, severe adenoma and adenocarcinoma, respectively. Although the numbers of normal mitosis tended to be slightly larger in the DNA-instability test-positive glands, no statistical difference was found as compared to those in DNA-instability test-negative glands. Averages were 0.23%, 0.57%, 1.9%, and 2.2%, in mild, moderate, and severe adenoma and adenocarcinoma, respectively.

Ki67 immunohistochemistry

Ki67 immunoreactivity was observed exclusively in the nuclei of cells (Figure 1e-h). In hyperplastic polyp and normal mucosal epithelium surrounding adenoma or carcinoma, ki67-positive cells were observed only sporadically. In adenoma and carcinoma, Ki67 positively stained cells were present diffusely, indicating a loss of polarity of proliferating cells. KI was significantly higher in adenocarcinoma, severe, moderate and mild dysplasia than in hyperplastic polyp (Table 1). Furthermore, KI of the positive DNA-instability test cases was statistically higher than that of -negative cases (p<0.01), both in mild and moderate dysplasia (Table 2). Moreover, the KI of the DNA-instability test-positive adenoma glands were comparable to that of adenocarcinomas, irrespective of adenoma grade (Figure 3).

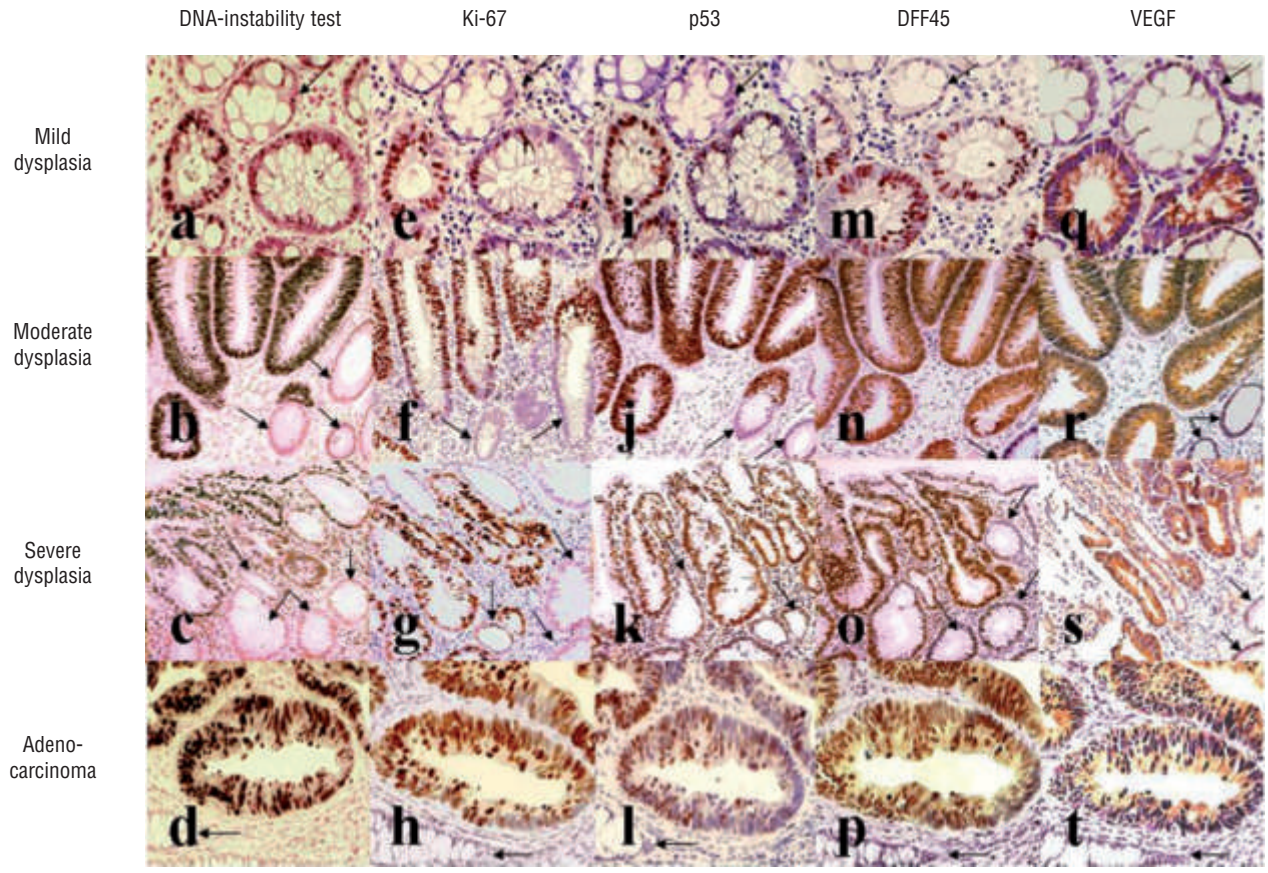

Figure 1. The results of Immunohistochemical staining. Mild dysplasia (a, e, i, m, q), moderate dysplasia (b, f, j, n, r), and severe dysplasia (c, g, k, o, s), magnification ×250; adenocarcinoma (d, h, l, p, t), magnification, ×500. DNA-instability testing (a-d), Ki67 (e-h), p53 (i-l), DFF45 (m-p), VEGF (q-t). Normal glands were indicated by arrows.

P53 immunohistochemistry

P53 immunoreactivity was limited to the nuclei of cells (Figure 1i-l). Foveolar hyperplastic polyps and normal mucosal epithelium surrounding adenoma or carcinoma were negative for p53. On the other hand, as shown in Table 1, the percentages of p53-positive cases in mild, moderate, and severe dysplasia, whole dysplastic lesion and adenocarcinoma were 17.65%, 31.37%, 65.21% 34,26% and 70%, respectively. There was a significantly higher p53 positivity in carcinoma, severe and moderate dysplasia than in mild dysplasia ($p<0.01$) (Table 1). Furthermore, among the 8 mild and 36 moderate dysplasia with a positive DNA-instability test, 6 (75%) and 24 (66.67%) cases were also positive for p53, whereas among 60 mild and 66 moderate dysplasia with a negative DNA-instability test, only 6 (10%) and 8 (12.12%) cases expressed p53 positivity, respectively. The proportions of p53-positive cases with a positive DNA-instability test were statistically higher than those with a negative DNA-instability test ($p<0.01$), both in mild and moderate dysplasia (Table 2). Moreover, in all dysplasia and adenocarcinoma lesions positive to DNA instability testing, the distribution of p53-positive cells coincided well with those of DNA-instability test-positive cells, and the percentages of p53-positive cells in the DNA-instability test-positive adenoma glands were comparable to those of adenocarcinomas, irrespective of adenoma grade (Figure 3).

DFF45 immunohistochemistry

The proportions of DFF45-positive cases in hyperplastic polyp, adenoma of mild, moderate and severe dysplasia, adenoma as a whole and adenocarcinoma were 9.09%, 35.29%, 58.82%, 65.22% and 86.67%, respectively. The proportions of DFF45-positive cases in adenoma of moderate and severe dysplasia and adenocarcinoma were sig-

nificantly different from those in hyperplastic polyp and adenoma of mild dysplasia (Table 1).

As shown in Table 2, the proportion of DFF45-positive cases with positive DNA-instability test scores were significantly higher than that with negative DNA-instability test scores both in mild and moderate dysplasia ($p<0.05$). In the DNA-instability test-positive cases, the distribution of DFF45-positive cells correlated well with that of DNA-instability test-positive cells in all cases (Figure 1m-p).

VFGF immunohistochemistry

The proportions of VEGF-positive cases in adenoma of mild, moderate, and severe dysplasia, adenoma as a whole and adenocarcinoma were 14.71%, 35.29%, 73.91%, 37,04% and 80%, respectively. Adenoma of moderate and severe dysplasia and adenocarcinoma had a significantly higher VEGF expression rate than that of adenoma of mild dysplasia (Table 1). In addition, the distribution of VEGF positive cells correlated well with that of

Table 1. The results of DNA-instability testing, and the immunohistochemical staining of Ki67, p53, DFF45, and VEGF. The statistical differences of KI values were examined by the Student's t-test using the value of hyperplastic polyp lesion as the standard. The statistical differences of DNA-instability testing, p53, DFF45, and VEGF positive test were examined by the chi-square test using the values of mildly dysplastic adenoma as the standard. The values with meaningful differences are shown by asterisks (*$p<0.05$, **$p<0.01$).

	DNA-instability test (%)	*KI (mean±S.D.)*	*p53 (%)*	*DNA-Fragmentation Factor 45 (DFF45) (%)*	*VEGF (%)*
Hyperplastic polyp (n=11)	0.00 (0/11)	25.10±9.43	0.00 (0/11)	9.09 (1/11)	0.00 (0/11)
Mild dysplasia (n=68)	11.76 (8/68)	31.33±10.75*	17.65 (12/68)	35.29 (24/68)	14.71 (10/68)
Moderate dysplasia (n=102)	35.29** (36/102)	45.30±11.55**	31.37 (32/102)	58.82* (60/102)	35.29* (36/102)
Severe dysplasia (n=46)	100** (46/46)	53.90±13.77**	65.21** (30/46)	65.22* (30/46)	73.91** (34/46)
Dysplasia total (n=216)	41.67 (90/216)	42.64±14.49**	34.26 (74/216)	52.78 (114/216)	37.04 (80/216)
Carcinoma (n=30)	100** (30/30)	54.65±12.51**	70** (21/30)	86.67** (26/30)	80** (24/30)

Table 2. Comparison of different biological markers in DNA-instability testing-positive and -negative adenomas. The statistical differences of KI values were examined by Student's t-test. The statistical differences of p53, DFF45, and VEGF positivity were examined by the chi-square test. Significant differences are indicated by asterisks (*$p<0.05$, **$p<0.01$).

	DNA-instability test	*(%)*	*KI (mean±S.D.)*	*p53 (%)*	*DNA-Fragmentation Factor 45 (DFF45) (%)*	*VEGF (%)*
Mild dysplasia (n=68)	positive (n=8)	11.76	54.13±1.32**	75** (6/8)	75* (6/8)	75** (6/8)
	negative (n=60)	88.24	28.30±7.08	10 (6/60)	30 (18/60)	6.67 (4/60)
Moderate dysplasia (n=102)	positive (n=36)	35.29	52.16±9.32**	66.67** (24/36)	83.33* (30/36)	83.33** (30/36)
	negative (n=66)	64.71	41.55±11.02	12.12 (8/66)	45.45 (30/66)	9.09 (6/66)
Severe dysplasia (n=46)	positive (n=46)	100	53.90±13.77	65.21 (30/46)	65.21 (30/46)	73.91 (34/46)
Total dysplasia (n=216)	positive (n=90)	41.67	53.23±11.37**	66.67** (60/90)	73.33** (66/90)	77.78** (70/90)
	negative (n=126)	58.33	35.24±11.43	11.11 (14/126)	41.27 (52/126)	7.94 (10/126)

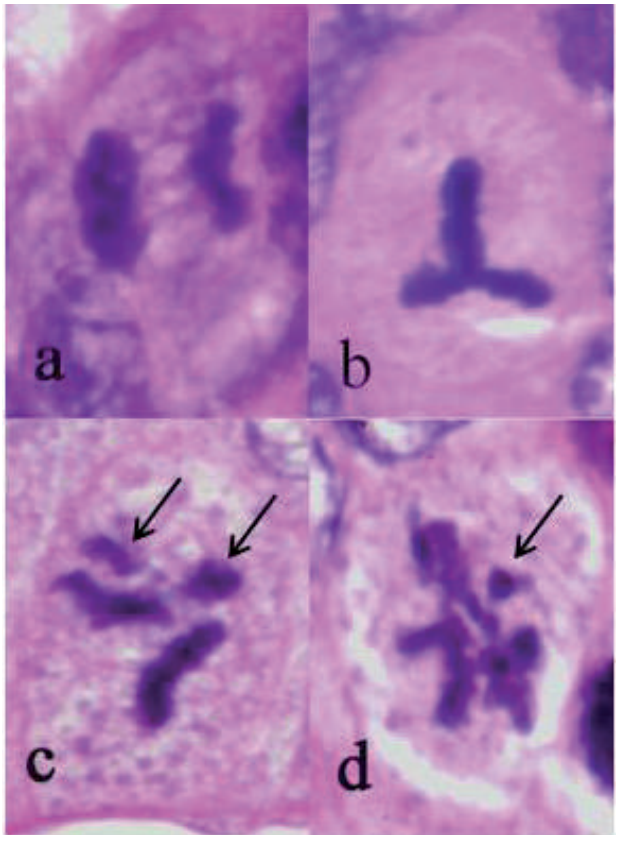

Figure 2. Abnormalities in mitosis figures seen exclusively in the DNA-instability testing-positive glands in adenomas. Asymmetric mitosis (a), tripolar mitosis (b), small chromosomal fragment separated (indicated by the arrow), (c,d). Magnification × 1000.

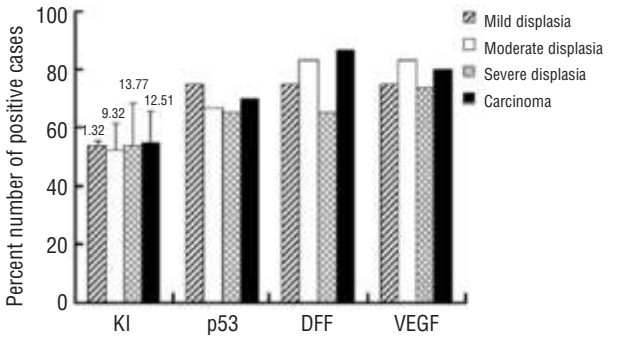

Figure 3. Comparison of various biomarkers in the DNA-instability testing-positive glands of adenoma and adenocarcinoma. The positive proportions of all biomakers in adenoma were comparable to those in carcinomas, irrespective of the dysplasia grade, without statistical significance.

DNA-instability test-positive cells (Figure 1q-t) in all cases. Furthermore, the proportions of VEGF-positive cases in cases with a positive DNA-instability test were significantly higher than that in cases with a negative DNA-instability test, both in mild and moderate dysplasia ($p<0.01$). In the DNA-instability test-positive adenomas, 75% (6/8) of mildly dysplastic adenoma, 83.33% (30/36) of moderately dysplastic adenomas, and 73.91% (34/46) of severely dysplastic adenomas were positive for VEGF, with these values being just comparable to those of adenocarcinoma, 80% (24/30) (Figure 3).

Discussion

The probability of malignant transformation of colorectal adenoma varies with different reports. Histopathological studies have demonstrated foci of malignancy within colorectal adenomas in 0.2-8.3% of cases (Gillespie *et al.*, 1979; Shinya and Wolff, 1979; Collacchio *et al.*, 1981; Cranley *et al.*, 1986; Leslie *et al.*, 2002). On the other hand, remnants of adenomatous tissue contiguous with invasive cancer have been identified in 14-23% of all colorectal cancers (Muto *et al.*, 1975; Eide, 1983; Leslie *et al.*, 2002). The discrepancies in the reported frequencies of malignant transformation of colorectal adenoma may be partly attributable to the significant differences between Japanese and western pathologists' criteria for the diagnosis of colorectal carcinoma (Shibata *et al.*, 1997; Mueller *et al.*, 2002). In western countries, colorectal carcinoma is diagnosed when evident invasive growth of neoplastic epithelium into the lamina propria of the mucosa or beyond is observed, while most Japanese pathologists make a diagnosis of carcinoma on the basis of nuclear and structural atypia irrespective of whether or not there is invasion into the lamina propria (Tada *et al.*, 1995; Mueller *et al.*, 2002). In order to detect the cancer clones at very early stages of progression without invasion, where cellular and structural atypia are insufficient as the basis of cancer diagnosis, certain breakthroughs are necessary.

Based on the finding that all cancerous cells produce much more single-stranded DNA than normal cells after acid hydrolysis, Fukuda *et al.* (1986, 1993, 2005) developed a method (the DNA-instability test) of staining cancerous cells specifically with acridine orange or anti-cytidine antibody after acid hydrolysis as a specific marker for malignancy, irrespective of epithelial or mesenchymal origin. It is known that DNA in cancer cells is much more unstable than in normal cells and is more prone to denaturing by HCl hydrolysis, reflecting its accumu-

lated genome-wide alterations (Lengauer *et al.*, 1998). Other possible mechanisms of increased DNA-instability to acid hydrolysis in malignancy have been described (Hatchoh *et al.*, 1992; Fukuda *et al.*, 1993; Nitta *et al.*, l993; Otaki *et al.*, 1994; Sun *et al.*, 2003; Fukuda and Sun, 2005). Since the first report by Fukuda *et al.* (l986), many malignancy cases and so-called borderline malignancy cases have been tested by this method (Nitta *et al.*, 1993; Otaki *et al.*, 1994; Tsuzuki *et al.*, 1994; Azuchi *et al.*, 1998; Khaled *et al.*, 2000; Hirai *et al.*, 2001; Ishida *et al.*, 2001; Iwasa *et al.*, 2001; Sun *et al.*, 2003; Hirose *et al.*, 2005), and all cancerous cells were found to be positively stained in the DNA-instability test, while comparable normal cells, vascular endothelial cells, fibroblasts and inflammatory cells present in the same slides were completely negative.

In the present study, all adenocarcinoma cells showed, without exception, diffusely-stained positivity to the DNA-instability test (Figure 1d, Table 1), indicating their malignancy. The percentage of DNA-instability test-positive cases in adenomas of mild, moderate, and severe dysplasia were 11.76%, 35.29%, and 100%, respectively, indicating that they also already contain cancer clones, although the degree of morphological atypia is not sufficient yet in these lesions to make histopathological diagnosis of carcinoma possible (Table 1). Furthermore, the percentage of glands positive to the DNA-instability test steadily increased when going from mild (10%), to moderate (35%), to severe (100%) dysplasia adenoma, and adenocarcinoma (100%), reflecting the expansion of cancer clones along the course of cancer progression.

In the above described studies and also in the present study, The DNA-instability test-positive lesions showed significantly higher values of positivity of other biomarkers. These results indicate that cancer clones exhibit enhanced proliferative activity as revealed by Ki67 (an antigen expressed in all phases of cell cycle except G0) (Hoang *et al.*, 1989; Johnston *et al.*, 1989; Sugai *et al.*, 1999; Saleh *et al.*, 2000;) immunohistochemistry, mutation of p53 (a gene producing a protein which inhibits the entrance of abnormal cells through the Gl-S checkpoint and push these to apoptosis even after slipping into the S phase) (Finlay *et al.*, 1988; Hollstein *et al.*, 1991), induction of DFF45 (an inhibitory protein which inhibits DNA-fragmentation at the final step of apoptosis) (Liu *et al.*, 1997; Enari *et al.*, 1998; Sabol *et al.*, 1998; Sasaki *et al.*, 1999) and paracrine secretion of VEGF (a protein which induces vascular endothelial cell proliferation) (Leung *et al.*, 1989) with the induction of neovascularization. All these changes should favor an increase in the number of descendants of the cancer clones to accept abundant nutrients, and have a higher chance to survive by escaping the apoptotic machinery (Hirai *et al.*, 2001; Iwasa *et al.*, 2001, Sun *et al.*, 2003) in the process of cancer progression and clonal expansion from the early stage of carcinogenesis in colorectal adenoma to full-blown adenocarcinoma. Interestingly, the degree of positivity of these biomarkers were statistically not different in the DNA-instability test positive glands in all mild, moderate and severe dysplasia of adenoma and adenocarcinoma (Table 2, Figure 3), thereby indicating that these glands in both adenoma and carcinoma are essentially identical.

In accordance with the malignant character of the DNA-instability test-positive glands, many abnormal mitosis were found predominantly in such glands (Figure 2), as seen also in other reports (Tsuzuki *et al.*, 1994; Azuchi *et al.*, 1998).

The concept of *procancer* (not precancer) to designate the cancer clones as an early stage in the course of carcinogenesis and cancer progression has been proposed (Hirai *et al.*, 2001; Iwasa *et al.*, 2001, Sun *et al.*, 2003, Fukuda and Sun, 2005). *Procancer* lesions represent the cancer clones at a very early stage of malignancy without distinguishable morphological atypia but with a positive DNA-instability test and other biomarkers such as Ki67, p53 DFF45 and VEGF. They also define the abnormal positivities of these biomakers including the DNA-instability test as *functional atypia*, compared with the commonly used *morphological atypia*. While morphological criteria and existence of invasion seem to be the most reliable markers for the diagnosis of malignancy, it appears that a certain period is required before the degree of morphological atypia is manifested histopathological to allow the diagnosis of malignancy, even after the formation of cancer clones. During the extremely long, incipient (1-4 year) and early (14-21 year) phases of tumor growth of gastric cancer (Fujita, 1978, for example), the DNA alterations induced in cancer cells, which should be minute initially, will become intensified and exaggerated by repeated cell divisions (Hatchoh *et al.*, 1992; Sun *et al.*,

2003; Fukua and Sun, 2005) during these long phases so as to make the diagnosis of cancer by pathologists possible based on the degree of morphological atypia. According to this concept, the cancer clones present in the adenoma lesions found in the present study could be regarded as procancer clones. Further progression of cancer clones will produce the subclones with the potency to invade.

Thus, all colorectal adenoma lesions should be completely removed by EMR or polypectomy irrespective of the histological grades, although their progression to full blown carcinoma with sufficient morphological atypia and invasive potency may need more than several years. As already described, the DNA-instability test was positive in 11.76, 35.29, and 100% of mild, moderate and severe dysplasia adenoma cases, and the percentage of the glands positive in the DNA-instability test steadily increased when going from mild (10%), to moderate (35%), and to severe (100%) dysplasia adenoma, showing the heterogeneity of DNA-instability in adenomas. Therefore, all adenoma lesions should be completely removed by endoscopical resection, and the existence of DNA-instability should be examined thereafter. Even if the totally resected adenoma specimens show no DNA-instability, it would be impossible for us to know this already in only partly resected bioptic specimens because of the existence of heterogenous distribution of DNA-instability in adenomas.

References

Atkin WS, Morson BC, Cuzick J. Long-term risk of colorectal cancer after excision of rectosigmoid adenomas. N Engl J Med 1992; 326: 658-62.

Azuchi M, Baba H, Imura S, Noriki S, Imamura Y, Fukuda M. Demonstration of biological aggressiveness of bone giant cell by the comparative study of immunohistochemical detection of DNA-instability and cortical bone destruction by CT. Acta Histochem Cytochem 1998; 31: 401-10.

Baker SJ, Preisinger AC, Jessup JM, Paraskeva C, Markowitz S, Willson JK, et al. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Res 1990; 50: 7717-22.

Colacchio TA, Forde KA, Scantlebury VP. Endoscopic polypectomy: inadequate treatment for invasive colorectal carcinoma. Ann Surg 1981; 194: 704-7.

Cranley JP, Petras RE, Carey WD, Paradis K, Sivak MV. When is endoscopic polypectomy adequate therapy for colonic polyps containing invasive carcinoma? Gastroenterology 1986; 91: 419-27.

Eide TJ. Remnants of adenomas in colorectal carcinomas. Cancer 1983; 51: 1866-72.

Enari M. Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated Dnase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 1998; 391: 43-50.

Finlay CA, Hinds PW, Tan TH, Eliyahu D, Oren M, levine AJ. Activating mutations for transformation by p53 produce a gene product that forms an hac 70-p53 complex with an altered half-life. Mol cell Biol 1988; 8: 531-9.

Fujita S. Biology of early gastric carcinoma. Pathol Res Pract 1978; 163: 297-309.

Fukuda M, Miyoshi N, Hattori T, Sugihara H, Hosokawa Y, Nakanishi K. Different instability of nulear DNA at acid hydrolysis in cancerous and noncancerous cells as revealed by fluorescent staining with acridine orange. Histochemistry 1986; 84: 556-60.

Fukuda M, Noriki S, Imamura S, Miyoshi N, Kimura S, Koizumi K, et al. Differential immunohistochemical staining of cancerous cells with anti-single-stranded DNA antiserum in ordinary pathological paraffin section after DNA-denaturation by acid hydrolysis. Eur J Histochem 1993; 37: 309-19.

Fukuda M, Sun A. The DNA-instability test as a specific marker of malignancy and its application to detect cancer clones in borderline malignancy. Eur J Histochem 2005; 49: 11-26.

Gazelle GS, McMahon PM, Scholz FJ. Screening for colorectal cancer. Radiology 2000; 215: 327-35.

Gillespie PE, Chambers TJ, Chan KW, Doronzo F, Morson BC, Williams CB. Colonic adenomas- a colonoscopy survey. Gut1979; 20: 240-5.

Hamliton SR. The Adenoma-Adenocarcinoma sequence in the large bowel: Variations on a theme. J Cell Biochem 1992; 16: 41-6.

Hamliton SR, Vogelstein B, Kudo S, Riboli E, Nakamura S, Hainaut P, et al. Carcinoma of the Colon and Rectum. In: Hamilton SR. and Aaltonen LA, eds. World Health Organization Classification of Tumours Pathology and Genetics Tumours of the Digestive System. IARC Press, Lyon, 2000, pp. 104-19.

Hanrahan V, Currie MJ, Gunningham SP, Morrin HR, Scott PA, Robinson BA, et al. The angiogenic switch for vascular endothelial growth factor (VEGF)-A, VEGF-B, VEGF-C, and VEGF-D in the adenoma-carcinoma sequence during colorectal cancer progression. J Pathol 2003; 200: 183-94.

Hatchoh M, Ueda K, Imamura Y, Noriki S, Fukuda M. Qualitative and quantitative changes in nuclear DNA and phenotypic gene expression in human malignant skin tumors during their progression. Eur J Histochem 1992; 36: 289-302.

Hirai K, Kumakiri M, Ueda K, Imamura Y, Noriki S, Nishi Y, et al. Clonal evolution and progression of 20-methylcholanthrene-induced squamous cell carcinoma of mouse epidermis as revealed by DNA instability and other malignancy markers. Eur J Histochem 2001; 45: 319-32.

Hirose M, Sun A, Okubo T, Noriki S, Imamura Y, Fukuda M. Detection of non-papillary, non-invasive transitional cell G1 carcinoma as revealed by increased DNA instability and other cancer markers. Eur J Histochem 2005; 49: 199-209.

Hoang C, Polivka M, Valleur P, Hautefeuille P, Nemeth J, Galian A. Immunohistochemical detection of proliferating cells in colorectal carcinomas and adenomas with the monoclonal antibody Ki-67. Preliminary data. Virchows Arch A Pathol Anat Histopathol 1989; 414: 423-8.

Hollstein M, Sidransky D, Vogelstein B, Haris CC. P53 mutations in human cancers. Science 1991; 253: 49-53.

Ishida H, Kumakiri M, Ueda K, Lao LM, Yanagihara M, Asamoto K, et al. Comparative histochemical study of Bowen's disease and actinic keratosis: preserved normal basal cells in Bowen's disease. Eur J histochem 2001; 45: 1777-90.

Ishiguro K, Yoshida T, Yagishita H, Numata Y, Okayasu I. Epithelial and stromal genetic instability contributes to genesis of colorectal adenomas. Gut 2006; 55: 695-702.

Iwasa M, Imamura Y, Noriki S, Nishi Y, Kato H, Fukuda M. Immunohistochemical detection of early-stage carcinogenesis of oral leukoplakia by increased DNA-instability and various malignancy markers. Eur J Histochem 2001; 45: 333-46.

Jackman RJ, Mayo CW. The adenoma-carcinoma sequence in cancer of the colon. Surg Gynecol Obstet 1951; 93: 327-30.

Jass JR, Sobin LH. Histological typing of intestinal tumours. In: World Health Organization. International histological classification of tumours. Second edition. Springel-Verlag, Berlin 1989, pp.29-31.

Johnston PG, O'Brien MJ, Dervan PA, Carney DN. Immunohistochemical analysis of cell kinetic parameters in colonic adenocarcinomas, adenomas, and normal mucosa. Hum Pathol 1989; 20: 696-700.

Khaled A, Imamura Y, Noriki S, Fukuda M. Early progression stage of malignancy of uterine cervical dysplasia as revealed by immunohis-

tochemical demonstration of increased DNA instability. Eur J Histochem 2000; 44: 143-56.

Kondo Y, Arii S, Furutani M, Isigami S, Mori A, Onodera H, et al. Implication of vascular endothelial growth factor and p53 status for angiogenesis in noninvasive colorectal carcinoma. Cancer 2000; 88: 1820-7.

Kuramoto S, Oohara T. Minute cancers arising *de novo* in the human large intestine. Cancer 1987; 61: 829-34.

Lengauer C, Kinzler KW, Vogelstein B.œ Genetic instabilities in human cancers. Nuture 1998; 396: 643-49.

Leslie A, Carey FA, Pratt NR, Steele RJ. The colorectal adenoma-carcinoma sequence. Br J Surg 2002; 89: 845-60.

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989; 246:1306-9.

Liu X, Zou H, Slaughter C, Wang X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 1997; 89: 175-84.

Lotfi AM, Spencer RJ, Ilstrup DM, Melton LJ. Colorectal polyps and the risk of subsequent carcinoma. Mayo Clin Proc 1986; 61: 337-43.

McLeod HL, Murray GI. Tumour markers of prognosis in colorectal cancer. Br J Cancer 1999; 79: 191-203.

Mueller JD, Bethke B, Stolte M. Colorectal *de novo* carcinoma: a review of its diagnosis, histopathology, molecular biology, and clinical relevance. Virchows Arch 2002; 440: 453-60.

Muto T, Bussey HJ, Morson BC. The evolution of cancer of the colon and rectum. Cancer 1975; 36: 2251-70.

Nitta Y, Suzuki K, Kohli Y, Fujiki N, Imamura Y, Noriki S, et al. Early progression stage of malignancy of human colon border-line adenoma as revealed by immunohistochemical demonstration of increased DNA-instability. Eur J Histochem 1993; 37: 207-18.

Ohue M, Tomita N, Monden T, Fujita M, Fukunaga M, Takami K, et al. A frequent alteration of p53 gene in carcinoma in adenoma of colon. Cancer Res 1994; 54: 4798-804.

Otaki T, Kohli Y, Fujiki N, Imamura Y, Fukuda M. Early progression stage of malignancy as revealed by immunohistochemical demonstration of DNA instability; I, human gastric adenomas. Eur J Histochem 1994; 38: 281-90.

Sabol SL,Li R, Lee TY, Abdul-khalek R. Inhibition of apoptosis-associated DNA fragmentation activity in nonapoptotic cells:the role of DNA fragmentation factor-45 (DFF45/ICAD). Biochem Biophys Res Commun 1998; 253: 151-8.

Saleh HA, Aburashed A, Bober P, Tabaczka P. P53 protein immunohistochemical expression in colonic adenomas with and without associated carcinoma. Am J Gastroenterol 1998; 93: 980-4.

Saleh HA, Jackson H, Banerjee M. Immunohistochemical expression of bcl-2 and p53 oncoproteins: correlation with Ki67 proliferation index and prognostic histopathologic parameters in colorectal neoplasia. Appl Immunohistochem Mol Morphol 2000; 8: 175-82.

Sasaki H, Suzuki T, Funaki N, Hoshi T, Iwabuchi M, Ohi R, Sasano H. Immunohistochemistry of DNA fragmentation factor in human stomach and colon: its correlation to apoptosis. Anticancer Res 1999; 19: 5277-82.

Shibata H, Toyama K, Shioya H, Ito M, Hirota M, Hasegawa S, et al. Rapid colorectal adenoma formation initiated by conditional targeting of the APC gene. Science 1997; 278: 120-3.

Shimoda T, Ikegami M, Fujisaki J, Matsui T, Aizawa S, Ishikawa E. Early colorectal carcinoma with special reference to its development de novo. Cancer 1989; 64: 1138-46.

Shinya H, Wolff WI. Morphology, anatomic distribution and cancer potential of colonic polyps. Ann Surg 1979; 190: 679-83.

Sugai T, Nakamura S, Habano W, Uesugi N, Sato H, Yoshida T, et al. Usefulness of proliferative activity, DNA ploidy pattern and p53 products as diagnostic adjuncts in colorectal adenomas and intramucosal carcinomas. Pathol Int 1999; 49: 617-25.

Sugai T, Takahashi H, Habano W, Nakamura S, Sato K, Orii S, et al. Analysis of genetic alterations, classified according to their DNA ploidy pattern, in the progression of colorectal adenomas and early colorectal carcinomas. J Pathol 2003; 200: 168-76.

Sun A, Noriki S, Imamura Y, Fukuda M. Detection of cancer clones in human gastric adenoma by increased DNA-instability and other biomarkers. Eur J Histochem 2003; 47: 111-22.

Suzuki S, Mizuno M, Tomoda J, Ohmori M, Tsuji T. Flow cytometric analysis of the DNA content in colorectal adenomas with focal cancers. Gastroenterology 1995; 109: 1098-104.

Tada T, Iida M, Matsumoto T, Yao T, Aoyagi K, Koga H, et al. Small flat cancer of the rectum: clinicopathologic and endoscopic features. Gastrointest Endosc 1995; 42: 109-13.

Tsuzuki H, Saito H, Imamura Y, Noriki S, Fukuda M. Early progression stage of malignancy as revealed by immunohistochemical demonstration of DNA instability; II, otorhinolaryngeal borderline neoplastic lesions. Eur J Histochem 1994; 38: 291-302.

Torres MA, Merlo RR, Ortega AR, Veguillas ADL, Munoz JV, Simon SR, et al. DNA aneuploidy in colorectal adenomas. Role in the adenoma-carcinoma sequence. Rev Esp Enferm Dig 2005;97:7-15.

Winawer SJ. Screening for colorectal cancer. In: Devita VTJr, Hellman S, Rosenberg SA, eds. Updates for cancer: principles and practice of oncology. 2nd ed. 1987; No6, Page 4.

Winawer SJ, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS,et al. Prevention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup. N Engl J Med 1993; 329: 1977-81.

Wong MP, Cheung N, Yuen ST, Leung SY, Chung LP. Vascular endothelial growth factor is up-regulated in the early pre-malignant stage of colorectal tumour progression. Int J Cancer 1999; 81: 845-50.