

Detection of cancer clones in human gastric adenoma by increased DNA-instability and other biomarkers

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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 of malignancy. The test was applied to bioptic tissues of human gastric polyp assessed histopathologically as foveolar hyperplastic polyp (13 cases), mild (58 cases), moderate (86 cases), and severe (20 cases) dysplasia, and adenocarcinomas (14 cases). The serial sections of the same tissues were also subjected to immunohistochemical staining for Ki67, p53, DNA-fragmentation factor (DFF45), and basic fibroblast growth factor (bFGF). The DNA-instability test was positive in 14 (100%) adenocarcinoma cases, 20 (100%) severe dysplasia cases, 52 (60.5%) moderate dysplasia cases, and 12 (20.7%) mild dysplasia cases, indicating malignancy. All foveolar hyperplastic polyps were negative to the DNA-instability testing. Furthermore, the percentage of glands positive in the DNA-instability test steadily increased in going from mild (10%), to moderate (40%), to severe (100%) dysplasia, and adenocarcinoma (100%). All other biological markers tested in the present study showed significantly higher values in the adenoma glands, being positive to DNA-instability testing, irrespective of the dysplasia grade, as compared to those in the adenoma glands that were negative to DNA-instability testing. Furthermore, the former values were comparable to those in adenocarcinoma. These results indicate that cancer cell clones are already present at the adenoma stages showing a positive DNA-instability test, enhanced proliferative activity, p53 mutation, induction of DFF45 and bFGF. These factors allow cancer cell proliferation, producing heterogeneous subclones due to DNA-instability, enhancing their survival by escaping apoptosis, and providing abundant nutrients during the early-stage progression of gastric cancer. Based on these findings, we herein propose the concept of "procancer" (as opposed to "pre-cancer") as being a unique stage during the course of carcinogenesis and cancer progression. We designate the term to cancer clones at the very early stages of malignant progression that do not show distinguishable morphological atypia but do show positive DNA-instability testing and positive staining for various biomarkers such as Ki67, p53, DFF45, and bFGF. We also define the abnormal positive staining of these biomarkers, including the DNA-instability test as "functional atypia", compared to the ordinary morphological atypia.

Key words: human gastric adenoma, DNA instability, Ki67, p53, DFF45, bFGF

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Gastric adenoma is defined by the World Health Organization (WHO) as *circumscribed benign lesions, composed of tubular and/or villous structures showing intraepithelial neoplasia* (Fenoglio-Preiser et al., 2000), and is widely considered to be a premalignant lesion (Correa et al., 1992). According to the degree of cellular and structural dysplasia, gastric adenoma is divided into two qualitative grades (low-grade and high-grade dysplasia) or three qualitative grades (mild, moderate and severe dysplasia) (Ming et al., 1984; Tosi et al., 1989; Goldstein and Lewin, 1997). Many authors have investigated the relationship between gastric adenoma and cancer, and showed that the frequency of malignant transformation from adenoma to carcinoma depends on size and histological grade of adenoma (Nakamura et al., 1988; Xuan et al., 1991; Tsujitani et al., 1992). Most investigators agree that severe, or high grade dysplasia is the most important precursor of gastric cancer and strongly recommend gastrectomy, endoscopic mucosal resection (EMR) or polypectomy, particularly in light of the high percentage of early gastric cancer (Lansdown et al., 1990; Farinati et al., 1991; Di Gregorio et al., 1993). However, there remains disagreement regarding of the risk of malignant potential for adenomas with mild and/or moderate dysplasia. Some authors believe that adenomas with mild and moderate dysplasia progress slowly, remain stable or regress (Saraga et al.,

1987; Farinati et al., 1989), while others have reported that the progression to severe dysplasia or evolution into gastric carcinoma ranged between 19~21% and 33~40% in mild and moderate dysplasia, respectively (Rugge et al., 1991; Fertitta et al., 1993; Rugge et al., 1994).

The morphological criteria for evaluating the potential malignancy, or the probability of transition to overt cancer of gastric adenoma, however, are quite ambiguous and controversial, and the validity of them is confusing in everyday diagnosis. In order to solve this problem, many histochemical and molecular biological methods have been applied in gastric adenoma and cancer in the search for reliable marker(s) of malignancy and potential malignancy. For example, brain type glycogen phosphorylase (Shimada et al., 1999), sulphomucins (Jass and Filipe, 1980), p53 (Matozaki et al., 1992; Shiao et al., 1994), c-erbB-2 (Sasano et al., 1993), bcl-2 (Ayhan et al., 1994), PCNA (Miracco et al., 1995; Ozercan et al., 2000), DNA topoisomerase (Yabuki et al., 1996), Ki67 (D'Errico et al., 1991; Hoshi et al., 1998), AgNOR (Ozercan et al., 2000), APC LOH and mutation (Nakatsuru et al., 1993; Sanz-ortega et al., 1996), genetic instability (Semba et al., 1996; Kim et al., 1999; Kim et al., 2000; Kashiwagi et al., 2000), DNA-ploidy analysis (Bearzi et al., 1992) and *H. pylori* infection (Wu et al., 1997; Wang et al., 2002) have been used for this purpose.

All 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 gastric adenoma. However, none of them could be used as a specific marker for malignancy diagnosis, although some of the abnormalities were statistically significant.

In this regard, Fukuda et al (1986,1993) 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 could be identified during early stages of carcinogenesis in so-called borderline or precancerous lesions such as colorectal ade-

noma (Nitta et al., 1993), otorhinolaryngeal borderline lesions (Tsuzuki et al., 1994), bone giant cell tumors (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). Otaki et al. (1994) also demonstrated that all adenoma lesions with severe dysplasia can be regarded as malignant in nature existing at an early stage of cancer progression, using the DNA-instability test compared with PCNA immunohistochemistry and AgNORs parameter analysis. However, no data were obtained for mild dysplasia and the number of moderate dysplasia examined was only limited in the study.

In the present study, we used this DNA-instability test as a malignancy marker to identify cancer clones in human gastric adenoma including 58, 86 and 20 cases of mild, moderate and severe dysplasias, respectively. Cancer clones were identified by increased DNA-instability in gastric adenoma and they exhibited significantly higher values of Ki67 index and enhanced immunoreactivity for p53, DNA fragmentation factor 45(DFF45) and bFGF, compared with DNA-instability test-negative lesions.

Materials and Methods

A total of 191 biopsy specimens from human stomach, including 13 foveolar hyperplastic polyps, 164 adenomas and 14 adenocarcinomas were examined. The gastric adenoma lesions were divided into mild (58 cases), moderate (86 cases) and severe dysplasia (20 cases), based on the degree of structural and cellular atypia of glands (Rubio and Kato, 1988).

DNA-instability test. Paraffin-embedded 4 μ m thick sections were prepared after fixation with 10% buffered formalin for 24 hours at room temperature. They were deparaffinized with xylene and replaced by ethanol.

(a) preblocking

After washing in water, the intrinsic peroxidase was blocked with 0.03% H₂O₂ solution dissolved in absolute methanol at room temperature for 15 min and rinsed with PBS (pH 7.4). The sections were mounted with 2% skim-milk (Yukijirushi, Sapporo, Japan) dissolved in PBS (pH 7.4) at 37°C for 20 min to block the background adsorption of anti-serum. Then they were reacted with the blocking

solution [Histofine, Nichirei, PO(R) Japan] at room temperature, for 5 min.

(b) 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, Nichirei, PO(R), Japan] at 37°C for 40 min, and rinsed with PBS (pH 7.4), followed by reaction with the avidin-biotin peroxidase complex [Histofine, Nichirei, PO(R), Japan] at 37°C for 30 min, and further rinsed with PBS (pH 7.4).

(c) 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% CoCl₂ and 10 µL of 30% H₂O₂ 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).

Immunohistochemical staining of Ki67, p53, bFGF and DNA- fragmentation factor (DFF45).

Serial 4 µm-thick sections were prepared from the same specimens used for DNA-instability testing 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 poly-propylene 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), and then reacted with the blocking solution [Histofine, Nichirei, PO (M), Japan] at 20°C for 5 min. This was followed by reactions at 4°C overnight with following primary antibodies: anti-Ki67 antigen monoclonal antibody [Immuotech, MIB-1, 1:50 dilution in PBS (pH7.4)], anti-human p53 tumor suppressor protein monoclonal antibody [Dako, Clone DO-7, 1:100 dilution in PBS (pH 7.4)], and anti human

bFGF monoclonal antibody [Wako, clone78, 1:100 dilution in PBS (pH 7.4)]. Then sections were washed with PBS (pH7.4), and further reacted with biotinylated rabbit anti-mouse IgG (Histofine, Nichirei, PO (M), Japan) 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 (Histofine, Nichirei, PO (M), Japan) 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 room temperature for 10 min and washed in water. Nuclear counterstaining was performed with hematoxylin.

Immunohistochemical staining for DNA-fragmentation factor was performed in a similar fashion except for using polyclonal anti-DNA-fragmentation factor antibody [NCL-DFFp, Novocastra, Newcastle, UK, 1:400 dilution in PBS (pH 7.4)] as the primary antibody. Biotinylated goat anti-rabbit IgG was used this time as the secondary antiserum with the blocking solution for rabbit [Histofine, Nichirei, PO(R), Japan].

Evaluations of immunohistochemical staining for DNA-instability, Ki67, p53, DFF45 and bFGF

As for the DNA-instability test, the gland with more than several positively-stained cells was regarded as positive and the adenoma and adenocarcinoma with more than one positive gland was counted as positive lesion. The percent numbers of positive glands, mitosis and abnormal mitosis in the DNA-instability test-positive and -negative glands was also determined in all lesions. In serial sections of the same specimens, the percent number 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 bFGF, 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 presented as mean±S.D. Data were analysed using Student's t-test with a p value < 0.05 being considered significant. Staining for p53, DFF45, bFGF, and DNA-instability test are shown as positive percentage. Data were compared

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

	DNA-instability test (%)	KI (mean±S.D.)	p53 (%)	DNA-fragmentation factor (%)	bFGF (%)
Hyperplastic poly (n=13)	0.0E+01 (n=0)	17.76±7.93	0.0E+01 (n=0)	7.7 (n=1)	0.0E+01 (n=0)
Mild dysplasia (n=58)	20.7 (n=12)	33.68±7.20*	34.5 (n=20)	58.6 (n=34)	32.8 (n=19)
Moderate dysplasia (n=86)	60.5 (n=52)	48.64±8.58*	65.1 (n=56)	69.8 (n=60)	47.7 (n=41)
Severe dysplasia (n=20)	100 (n=20)	75.19±12.58*	90.0 (n=18)	90 (n=18)	75.0 (n=15)
Dysplasia total (n=164)	51.2 (n=84)	46.59±15.74*	57.3 (n=94)	68.3 (n=112)	45.7 (n=75)
Carcinoma (n=14)	100 (n=14)	77.13±14.98*	85.7 (n=12)	100 (n=14)	57.1 (n=8)

in different cases and between DNA-instability test positive and negative adenomas by Chi-square test. A p value < 0.05 denoted the presence of a significant statistical difference.

Results

The results of the DNA-instability test, immunohistochemical staining of Ki67, p53, DFF45 and bFGF are summarized in Table 1. The data of Ki67, p53, DFF45 and bFGF in relation to the results of the DNA-instability test are listed in Table 2. Comparison of various biomarkers in 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 without exception with anti-cytidine antibody, 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. A quote of 20.7% (12/58) of mildly dysplastic adenoma were positive in the DNA-instability test, in which about 10% of dysplastic glands were positively stained (Figure

Table 2. Comparison of different biological markers in DNA-instability test-positive and -negative adenomas. The statistical differences of KI values were examined by Student's t-test. The statistical differences of p53, DFF45, and bFGF positivity were examined by the Chi-square test. Significant differences are indicated by asterisks.

	DNA-instability test	%	KI (mean±S.D.)	p53 (%)	DNA-fragmentation factor (%)	bFGF (%)
Mild dysplasia (n=58)	positive (n=12)	20.7	49.89±11.21**	83.3** (10/12)	100** (12/12)	75.0** (9/12)
	negative (n=46)	79.3	20.69±9.96	21.7 (10/46)	47.8 (22/46)	21.7 (10/46)
Moderate dysplasia (n=86)	positive (n=52)	60.5	68.25±10.11**	80.8** (42/52)	94.2** (49/52)	65.4** (34/52)
	negative (n=34)	39.5	43.33±15.54	41.2 (14/34)	32.4 (11/34)	20.6 (7/34)
Severe dysplasia (n=20)	all-positive (n=20)	100	75.19±12.58	90.0 (18/20)	90.0 (18/20)	75.0? (15/20)
Total dysplasia (n=164)	positive (n=84)	51.2	67.78±18.74**	83.3** (n=70)	94.1** (n=79)	69.1** (n=58)
	negative (n=80)	48.8	36.45±10.45	30.0 (n=24)	41.3 (n=33)	21.3 (n=17)

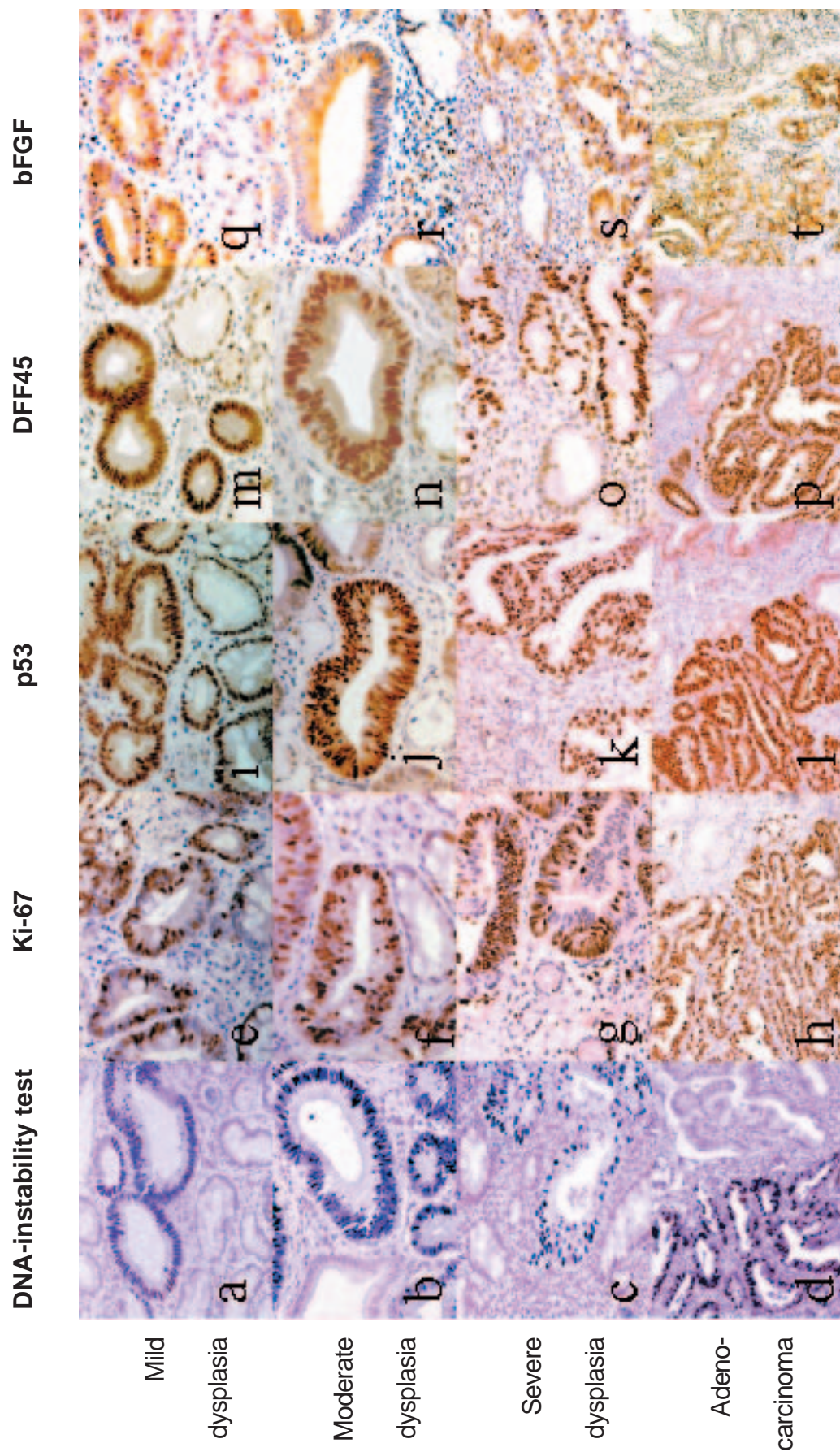


Figure 1. The results of immunohistochemical staining. Mild dysplasia (a, e, i, m, q, r), Magnification $\times 250$; moderate dysplasia (b, f, j, n, r), Magnification, $\times 500$; severe dysplasia (c, g, k, o, s), and carcinoma (d, h, l, p, t), Magnification, $\times 250$. DNA-instability-test (a-d), Ki67 (e-h), p53 (i-l), DFF45 (m-p), bFGF (q-t).

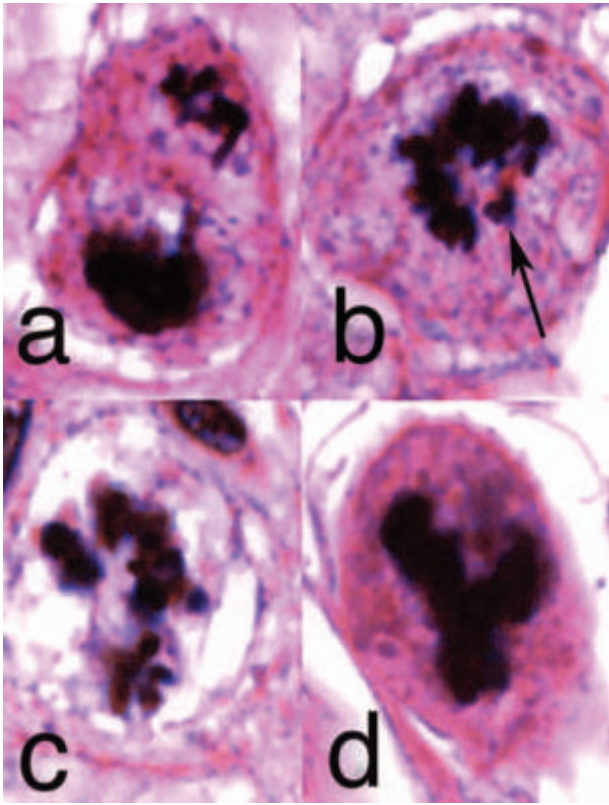


Figure 2. Abnormalities in mitosis figures seen exclusively in the DNA-instability test-positive glands in adenomas. Asymmetric mitosis (a), small chromosomal fragment separated (indicated by the arrow), (b), tripolar mitosis (c, d). Magnification $\times 1000$.

1a). About 60.5% (52/86) of moderately dysplastic adenoma were positive in the DNA-instability test, in which about 40% 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 ($p < 0.05$). No apparent morphological differences were, however, identified between DNA-instability test-positive and -negative glands.

Presence of abnormal mitosis in DNA-instability test-positive glands

We also counted the percent numbers of normal mitosis and abnormal mitosis in the DNA-instability test-positive and -negative glands, respectively

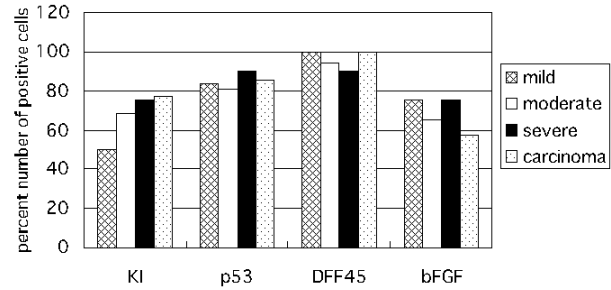


Figure 3. Comparison of various biomarkers in the DNA-instability test-positive glands of adenoma and carcinoma. The positive proportions of all biomarkers in adenoma were comparable to those in carcinomas, irrespective of the dysplasia grade, without statistical significance.

(Figure 2). Abnormal mitosis was only observed in DNA-instability test-positive glands, and the average numbers were 0.3%, 0.5%, 1.9%, and 2% in mild, moderate, severe adenoma and adenocarcinoma, respectively. Whereas the numbers of normal mitosis tended to be slightly larger in the DNA-instability test-positive glands, no statistical difference was found compared to that in DNA-instability test-negative glands. The average numbers were 0.5%, 1.1%, 4.1%, and 3.9%, 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 foveolar 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 the loss of polarity of proliferating cells. KI was significantly higher in adenocarcinoma, severe and moderate dysplasia than those in foveolar hyperplastic polyp and mild dysplasia (Table 1). Furthermore, KI of the DNA-instability test-positive cases was statistically higher than that of -negative cases ($p < 0.01$), both in mild and moderate dysplasia (Table 2). Moreover, KI of DNA-instability test-positive adenoma glands were comparable to that of adenocarcinomas, irrespective of adenoma grade (Figure 3).

P53 immunohistochemistry

P53 immunoreactivity was limited to the nuclei of cells (Figure 1i-l). Foveolar hyperplastic polyp 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 34.5, 65.1, 90, 57.3 and 85.7, respectively. There was a significantly higher p53 positivity in carcinoma, severe and moderate dysplasia than that in mild dysplasia ($p < 0.05$) (Table 1). Furthermore, among the 12 mild and 52 moderate dysplasias with a positive DNA-instability test, 10 (83.3%) and 42 (80.8%) cases were also positive for p53, whereas among 46 mild and 34 moderate dysplasias with a negative DNA-instability test, only 10 (21.7%) and 14 (41.2%) 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 with a positive DNA-instability test, the distribution of p53-positive cells coincided well with those of DNA-instability test-positive cells, and the percent numbers of p53-positive cells in the DNA-instability test-positive adenoma glands were comparable to that of adenocarcinomas, irrespective of adenoma grade (Figure 3).

DNA-fragmentation factor 45 immunohistochemistry

The proportions of DFF45-positive cases among foveolar hyperplastic polyp, mild, moderate and severe dysplasia, whole dysplastic lesions and adenocarcinoma were 7.7, 58.6, 69.8, 90, 68.3, and 100%, respectively. The proportion of DFF45-positive cases in severe dysplasia and adenocarcinoma were significantly different from that in mild dysplasia, but were not different between moderate dysplasia and mild dysplasia (Table 1).

As shown in Table 2, the proportion of DFF45-positive cases with a positive DNA-instability test were significantly higher than that with a negative DNA-instability test. The distribution of DFF45-positive cells in all cases correlated well with that of DNA-instability test-positive cells (Figure 1 m-p).

bFGF immunohistochemistry

bFGF immunoreactivity was detected in cyto-

plasm. The proportions of bFGF-positive cases among mild, moderate, and severe dysplasia, whole dysplastic lesion and adenocarcinoma were 32.8, 47.7, 75, 45.7 and 57.1%, respectively. Severe dysplasia had a significantly higher bFGF expression rate than that of mild and moderate dysplasia (Table 1). In addition, the distribution of bFGF-positive cells correlated well with that of DNA-instability test-positive cells (Figure 1q-t). Furthermore, the proportion of bFGF-positive cases with a positive DNA-instability test were significantly higher than that with a negative DNA-instability test, both in mild and moderate dysplasia (Table 2). In the DNA-instability test-positive adenomas, 75% (9/12) of mildly dysplastic adenoma, 65.4% (34/52) of moderately dysplastic adenoma, and 75% (15/20) of severely dysplastic adenoma were positive for bFGF, and these values were comparable to those of adenocarcinoma, 57.1% (8/14) (Figure 3).

Discussion

As for the degree of malignant potential of gastric adenoma, a great number of controversial reports have been so far proposed. Some authors believe that the malignant transformation of gastric adenoma is rare (Carey and Hay, 1948; Hay, 1953; Plachta and Speer, 1957; Monaco et al., 1962) but others reported the high incidence of malignant transformation of gastric adenoma, regarding it as precancerous (Nakamura et al., 1966; Sugano et al., 1971; Tomasulo, 1971; Morson et al., 1980; Kamiya et al., 1982; Nakamura et al., 1988; Ruge et al., 1991; Farinati et al., 1993). Apparent cancer lesion has, in fact, been discovered in gastric adenoma (Sugano et al., 1971; Kamiya et al., 1982; Coma-del Corral et al., 1991).

The incidence of gastric carcinoma is reported to be very high in Japan in comparison with Western countries, and 30-50% of all gastric carcinomas in Japan are diagnosed as early gastric cancer without invasion beyond the submucosal layer (Kaneko et al., 1977; Hirota and Ming, 1992; Sano et al., 1992).

In Western countries, gastric adenoma is diagnosed when evident invasive growth of neoplastic epithelium into the lamina propria of the mucosa or beyond is observed (Lansdown et al., 1990; Lewin et al., 1992), but most Japanese pathologists, on the other hand, make diagnosis of carcinoma on the basis of nuclear and structural atypia irrespective of

whether or not there is invasion into the lamina propria. This will partly explain the discrepancies in the reported frequencies of malignant transformation of gastric adenoma (Schlemper et al., 1997).

In order to detect the cancer clones in the very early stage of progression, 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) developed the method of staining cancerous cells specifically with acridine orange or anti-cytidine antibody after acid hydrolysis and called it as the *DNA-instability test* as a specific marker for malignancy irrespective of epithelial or mesenchymal origin. Since the first report (Fukuda et al., 1986), more than 20,000 malignancy cases and so-called borderline malignancy cases were 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), 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 these studies, and also in the present study, the DNA-instability-positive lesions showed significantly higher values of positivity of other biomarkers. Furthermore, many abnormal mitoses were found predominantly in the DNA-instability test-positive lesions as shown in the present study (Figure 2) and other reports (Tsuzuki et al., 1994; Azuchi et al., 1998); and the frequency of chromosome 17 polysomies and the percentage of hyperdiploid cells measured by means of the chromosome index using interphase cytogenetics increased significantly and exclusively in the DNA-instability test-positive lesions (Khaled et al., 2000; Ishida et al., 2001). This is also supported by the report that numerical chromosomal aberration is associated with cancer progression (Bulten et al., 1998). These findings clearly showed the existence of DNA or chromosomal abnormalities in these DNA-instability test-positive lesions, indicating their malignant character, although no drastic progression showing marked morphological atypia is taking place.

The mechanism of increased DNA-instability against acid hydrolysis in malignancy is not fully understood, but possible mechanisms have been proposed (Hatchoh et al., 1992; Fukuda et al., 1993;

Nitta et al., 1993; Otaki et al., 1994). Nuclear pleomorphism with altered chromatin structures and contents, used as the most reliable morphological criteria for daily pathological diagnosis of malignancy, will be a direct reflection of a changed chemical nature and spatial configuration of nuclear DNA. Various carcinogens will induce genomic damage with single and double DNA-strand breaks, and erroneous repair and successive cell proliferation with DNA synthesis will exaggerate the damage, resulting in increased DNA-instability. In order to accomplish the effective and complete packing of nuclear DNA, cells will need sufficient amounts of histones to form nucleosomes in proportion to the amount of nuclear DNA. Disturbed functions of histone production by certain gene damages will disturb the DNA packing function and it may result in abnormal chromatin configuration that will also induce increased DNA-instability upon acid hydrolysis.

Another possible mechanism of increased DNA-instability of malignancy may be attributable to the production of active oxygen by cancerous cells (Noriki et al., 1989; Fukuda et al., 1993). In the cell membrane of cancerous cells, the amount of unsaturated fatty acids is known to be increased (Wood et al., 1985) with increased lipid peroxide (Noriki et al., 1989; Otamiri and Sjothol, 1989), because unsaturated fatty acids are susceptible to peroxidation to produce lipid peroxide. The latter is included in the group of active oxygen, and may be the source of radical chain reactions producing further active oxygen. Active oxygen modifies enzymatic and structural proteins, rendering them susceptible to proteolytic attack (Levine et al., 1981; Dean, 1987), and gives rise to membrane damage through lipid peroxidation. This harmful chain reaction induces the inactivation of the enzymes needed as scavengers. These finally induce serious DNA damage by modification, strand-break, and oxidative degeneration (Van Hemmen and Meuling, 1975; Peskin and Shlyanova, 1986; Kasai and Nishimura, 1986; Noriki et al., 1989).

Recently, a very large number of reports concerning DNA-instability have been presented using methods for detecting chromosomal fragile sites (Ohta et al., 1996; Pennisi E, 1996), loss of heterozygosity (White et al., 1985; Yokota et al., 1989a; Yokota et al., 1989b; Kallioniemi A., 1992), and microsatellite instability (Hearne et al., 1992; Jago et al., 1993; Thibodeau et al., 1993; Thompson

et al., 1992). It was shown that the microsatellite instability was particularly closely related to the progression of gastric adenoma to adenocarcinoma, reflecting the abnormalities of the DNA mismatch repair system (Semba et al., 1996; Kim et al., 1999; Kashiwagi et al., 2000). These methods, however, only demonstrated the presence of qualitative or quantitative changes in certain specific genomic loci. In contrast to these commonly used methods to reveal the DNA-instability at certain specific DNA regions, the DNA-instability test used in the present study revealed the presence of physical DNA-instability in the entire DNA molecule as revealed by increased liability to denature upon HCl hydrolysis.

The DNA-instability test used in the present study was also previously applied to the analysis of chemical carcinogenesis of mouse skin cancer by 20-methylcholanthrene and succeeded in detecting cancer clones in the very early stage of its progression only 2 weeks after starting the topical applications (Hirai et al., 2001). These cancer clones demonstrated enhanced proliferative activity as revealed by PCNA (Bravo et al., 1987; Wong et al., 1987) immunohistochemistry, mutation of p53 (a gene producing a protein which inhibits the entrance of abnormal cells through the G1-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 (vascular endothelial growth factor, 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).

Similar results were obtained in the present study. The proliferative activation, this time, expressed as KI (Schlüter et al., 1993), p53 mutation, induction of the DFF45, and bFGF (a protein which has a similar effect as VEGF) (Folkman and Klagsbrum, 1987; Russell, 1989), were all predominantly seen in the DNA-instability test-positive glands (Table 2).

But the degrees of positivity of these biomarkers were statistically not different in the DNA-instabil-

ity test-positive glands in all mild, moderate, and severe dysplasia of adenoma and cancer (Figure 3). Judging from these results, the DNA-instability test-positive glands in mildly dysplastic adenoma should be already regarded as cancer clones at the early stage of progression. 20.7% of mildly dysplastic adenoma were positive in the DNA-instability test in which only 10% of dysplastic glands were positively stained. Therefore, a large number of glands involved in mildly dysplastic adenoma should be reactive in nature showing simple hyperplasia, although small cancer buds co-exist in some of them.

We previously proposed the concept of *procancer* (not pre-cancer) to designate the cancer clones as a stage in the course of carcinogenesis and cancer progression (Hirai et al., 2001; Iwasa et al., 2001). *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 PCNA, Ki67, p53, VEGF, bFGF, and DFF45. We also defined the abnormal positivities of these biomarkers 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 histopathologically to allow the diagnosis of malignancy, even after the formation of cancer clones. During the extremely long, incipient (1-4 years) and early (14-21 years) phases of tumor growth of gastric cancer (Fujita, 1978), 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) during these long phases so as to make the diagnosis of cancer by pathologists possible based on the degree of morphological atypia. Further progression of cancer clones will produce the subclones with the potency to invade.

The cancer clones present in the adenoma lesions could be regarded as *procancer clones*. Reflecting the malignant character of the *procancer clones* in adenomas, abnormal mitoses are exclusively encountered in the DNA-instability test-positive glands, reflecting the chromosomal abnormalities evoked by DNA- or genomic instability (Steinbeck, 2001).

Thus, all gastric adenoma lesions should be completely removed by EMR or polypectomy irrespective of the histological grades, although their progression to full blown carcinomas with sufficient morphological atypia and invasive potency may need more than several years.

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A. Sun et al.

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