DNA Ploidy Patterns in Gastric Adenocarcinoma

To assess the value of DNA ploidy, flow cytometric analysis was performed on unfixed fresh materials obtained from 86 patients with gastric cancer who underwent stomach resection. We evaluated the DNA content of gastric carcinoma cells from four different sites and compared it with Ki-67 proliferating activity, and other pathologic parameters. The incidence of aneuploid and diploid was similar (48.8% vs. 51.1%). Early gastric carcinoma showed a higher rate of the diploid pattern (75%) compared to that of advanced gastric carcinoma (47.3%). DNA diploidy was noted increasingly in diffuse-type tumors according to Lauren, in signet ring cell type tumor according to WHO classification and in poorly differentiated tumors ($p<0.05$). Well and moderately differentiated carcinomas revealed the aneuploid pattern more frequently than poorly differentiated tumors. The aneuploidy was associated with high S phase fraction and high proliferative index. Aneuploidy was noted in the mucosa adjacent to the tumor (26%), in the close normal-looking mucosa (7%) and in the remote normal-looking mucosa (3%). This result suggest the possible role of field cancerization in the development of gastric adenocarcinoma.

Key Words : *Ploidies; Ki-67 Antigen; Adenocarcinoma; Stomach Neoplasms*

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INTRODUCTION

In spite of improved endoscopic diagnosis and surgical treatment, gastric carcinoma is still an important cause of death. The most important factor determining the prognosis of stomach cancer is the tumor stage with 5-year survival rates for patients in treatable groups of 30-40% and for early cancer (tumor infiltration limited to mucosa or submucosa) of up to 90% (1). Of possible additional prognostic values are tumor grade, growth pattern, histological type (2), and the DNA content of tumor cells (3). Abnormalities in DNA content in human cancers may have an important association with the clinical course (4-7).

Dysplasia, intestinal metaplasia and atrophy of the normal mucosa of the stomach are thought to provide the morphological background for gastric adenocarcinoma (8). Some transitional dysplastic changes are noted in the normal-looking gastric mucosa adjacent to the neoplastic glands. Alterations in chromosomal numbers in normal epithelial glands, in the intestinal metaplasia and in the gastric carcinoma (9) and c-erbB-2 protooncogene amplification in the normal tissue adjacent to tumor

tissue could be a premalignant changes in a small proportion of gastric carcinoma patients (9). Similar phenomenon was observed in the head and neck cancer and Slaughter et al. (10) proposed the concept of "field cancerization". Now it is generally accepted that most sporadic solid tumor result from a multistep process of accumulated genetic alterations (11).

Some tumors show variable morphologic features, site by site, suggesting subpopulations with different properties (12). Different cell clones from the primary tumor could be responsible for lymph node and distant metastasis (13, 14). Multiploidy, defined as more than one aneuploid cell clone, was noted in the gastric cancer (15). Multiple biopsy specimen also showed different ploidy patterns (16). Because of cellular heterogeneity and regional differences in DNA ploidy within the same tumor, multiple sampling of sites may be appropriate for accurate DNA measurement (14).

Another method to measure cell proliferation is by immunohistochemical staining with proliferation markers, such as Ki-67 antigen. This recognizes a nuclear antigen in the proliferating cell (17). The level of Ki-67 immunoreactivity correlates with the degree of tumor prolifer-

ation (18). However, it is still controversial that Ki-67 could be a prognostic factor in gastric carcinoma (19, 20).

The purpose of this study was to assess the value of the DNA content of gastric carcinoma cells from four different sites and to compare it with Ki-67 proliferating activity, and other pathologic parameters.

MATERIALS AND METHODS

Specimen collection

Formalin-fixed, paraffin-embedded tissue sections from 86 patients with gastric adenocarcinoma diagnosed between 1995 and 1998 were obtained from the files of the Department of Diagnostic Pathology at the Sanggye Paik Hospital. The material consisted of total or subtotal gastrectomy specimens. We examined the pathology reports and the hematoxylin & eosin (H&E) slides from each case to evaluate the clinicopathologic findings. This information included age, sex, size of the tumor, differentiation of the tumor, depth of invasion, perineurial invasion, angioinvasion and metastasis to lymph nodes. Gastric adenocarcinoma was divided into 2 groups according to the depth of invasion (21). Early gastric carcinoma (EGC) was defined as a lesion confined to the mucosa and submucosa, regardless of the presence or absence of perigastric lymph node metastasis. Advanced gastric carcinoma (AGC) is a neoplasm that has extended below the submucosa into the muscular wall.

Flow cytometric analysis for DNA ploidy

The samples were harvested from unfixed gross gastrectomy resections at the four different sites, that is, at the tumor and at the normal-looking gastric mucosa adjacent to the tumor, at the normal-looking mucosa 3 cm apart from the tumor, and at the normal-looking mucosa more than 5 cm apart from the tumor. DNA flow cytometric studies were performed using a method modified from that of Hedley et al. (22). The collected tissue specimens were minced with a scalpel and lysed cells were filtered through a nylon mesh (50 μm). Routine H&E stain of cytospin were prepared to confirm the presence of cancer cells using a small amount of the sample. The remaining solution was treated with trypsin (0.03 g/L, Sigma Chemical Co., St. Louis, U.S.A.) in 0.1% spermine and 5% Triton, followed by 0.5% trypsin inhibitor (Sigma), in 0.1% RNase (Sigma) for 10 min at 4℃. After staining with 50 μg/mL propidium iodide (Aldrich Chemical Co., U.S.A.) in 0.1% spermine and 5% Triton for 10 min at dark field, the samples were analyzed. DNA analysis was made using a FACScan cell

analyzer model (Becton Dickinson). Cell cycle distribution was analyzed by a Cell Fit program. To construct each histogram, more than 10,000 cells were analyzed. A tumor with a single G0/1 was considered diploid and the evidence of an additional G1 peak indicated the presence of aneuploidy. The DNA index (DI) was calculated as a ratio by dividing the channel number of the abnormal peak by the channel number of the diploid peak. Samples showing a single peak with a coefficient of variation (CV) greater than 10 were reanalyzed with the formalin-fixed paraffin-embedded tissue. Five separate pieces of 10 μm-thick paraffin sections including the tumor portion and normal mucosa were dewaxed with xylene, and then progressively rehydrated in decreasing concentrations of alcohol. After the specimens were washed with distilled water, they were incubated in a 0.25% trypsin solution (Sigma) at 37℃. After overnight incubation, the specimen was minced and filtered through a 50 μm filter. The following procedures were the same as the previous method using fresh specimens. The mean CV of all measured DNA-diploid peaks was 4.48± 0.85%. Proliferation index was determined by evaluating the percentage of cells in the S and G2-M phases (23). After the flow cytometric analysis, the remaining specimens were smeared to confirm their cytologic adequacy.

Immunohistochemical analysis

Ki-67 antigen expression was determined by the immunohistochemical analysis of paraffin-embedded specimens from 86 patients with gastric adenocarcinoma with Ki-67 monoclonal antibody (DAKO, Denmark). Unstained 4 μm sections were deparaffinized and rehydrated. Hydrogen peroxide was first applied to quench endogenous peroxidase activity, followed by a protein block to reduce nonspecific binding of the primary or secondary antibodies. The primary antibody was applied at a 1:100 dilution for 1 hr at room temperature. Slides were sequentially incubated with the universal biotinylated secondary immunoglobulin antibody, avidin-horseradish peroxidase conjugate and 3,3'-diaminobenzidine substrate. Then the slides were washed to stop the reaction and remove any unbound reagent. The slides were counterstained with hematoxylin and coverslipped.

Nuclear staining was regarded as positive. The number of stained nuclei out of 1,000 nuclei at the maximum staining area was recorded as the Ki-67 labeling rate.

Statistical analysis

Statistical comparisons were made with the student's t-test, χ^2 test and Pearson correlation test. We considered a p-value of less than 0.05 to be significant.

RESULTS

Clinical and pathologic findings

Of the total 86 tumors, 74 cases were AGC and the remaining 12 cases were EGC. The mean ages were 59.5 ±13.2 years in the AGC group and 57.0±13.1 years in the EGC group. Fifty-seven patients were men and 29 patients were women. The mean size of the tumor was 5.43 cm (±2.46 cm) in the AGC group and 2.62 cm (±1.30 cm) in the EGC group.

DNA ploidy

Forty-two cases (48.8%) were aneuploidy and the remaining 44 cases (51.1%) were diploidy. Number of aneuploidy was 39 in the AGC group (52.7%) and only 3 (25%) in the EGC group. The ploidy patterns according to the sampling sites were shown in Table 1. Of the

Table 1. DNA ploidy patterns in 86 cases of gastric adeno-carcinoma

| Site	Aneuploidy (%)	Diploidy (%)
Tumor	39 (45)	47 (54)
Adjacent mucosa	22 (26)	64 (72)
Close mucosa	6 (7)	80 (93)
Remote mucosa	3 (3.5)	83 (96.5)

Adjacent mucosa: normal-looking mucosa adjacent to tumor
Close mucosa: normal-looking mucosa, 3 cm apart from the adenocarcinoma
Remote mucosa: normal-looking mucosa, more than 5 cm apart from the adenocarcinoma

samples from the tumor and from the adjacent normal-looking mucosa, 39 cases (45%) and 22 cases (26%) were aneuploidy, respectively (Fig. 1). The aneuploidy pattern was also noted in the close normal-looking mucosa (6 cases, 7%) and in the remote normal-looking mucosa (3 cases, 3.5%), (Fig. 2), respectively. Cases of aneuploidy
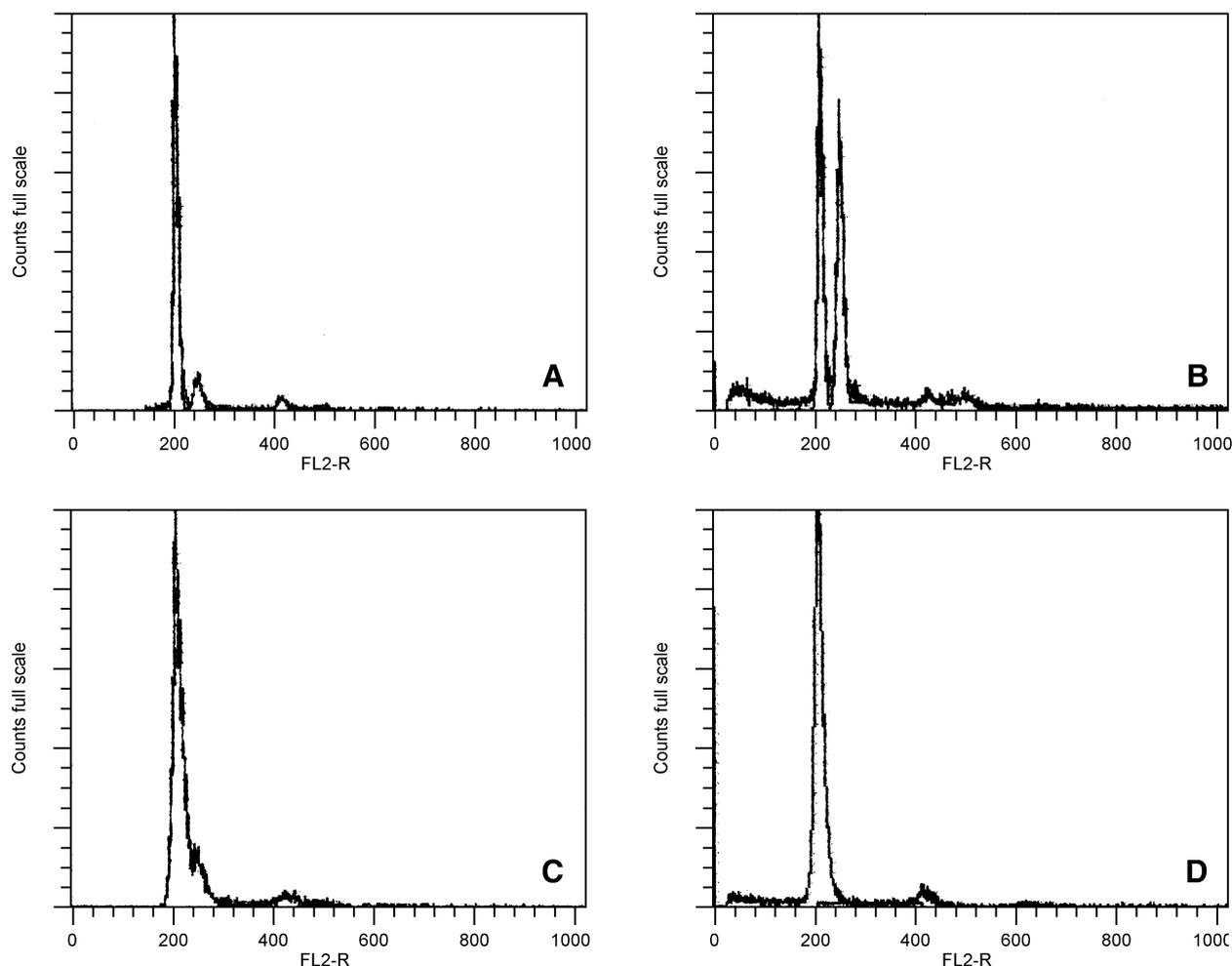
Fig. 1. Flow cytometric analysis reveals an aneuploidy pattern in the normal-looking mucosa adjacent to tumor (B). A: tumor, B: normal-looking mucosa adjacent to tumor, C: close normal-looking mucosa, 3 cm apart from the adenocarcinoma, D: remote normal-looking mucosa, more than 5 cm apart from the adenocarcinoma.
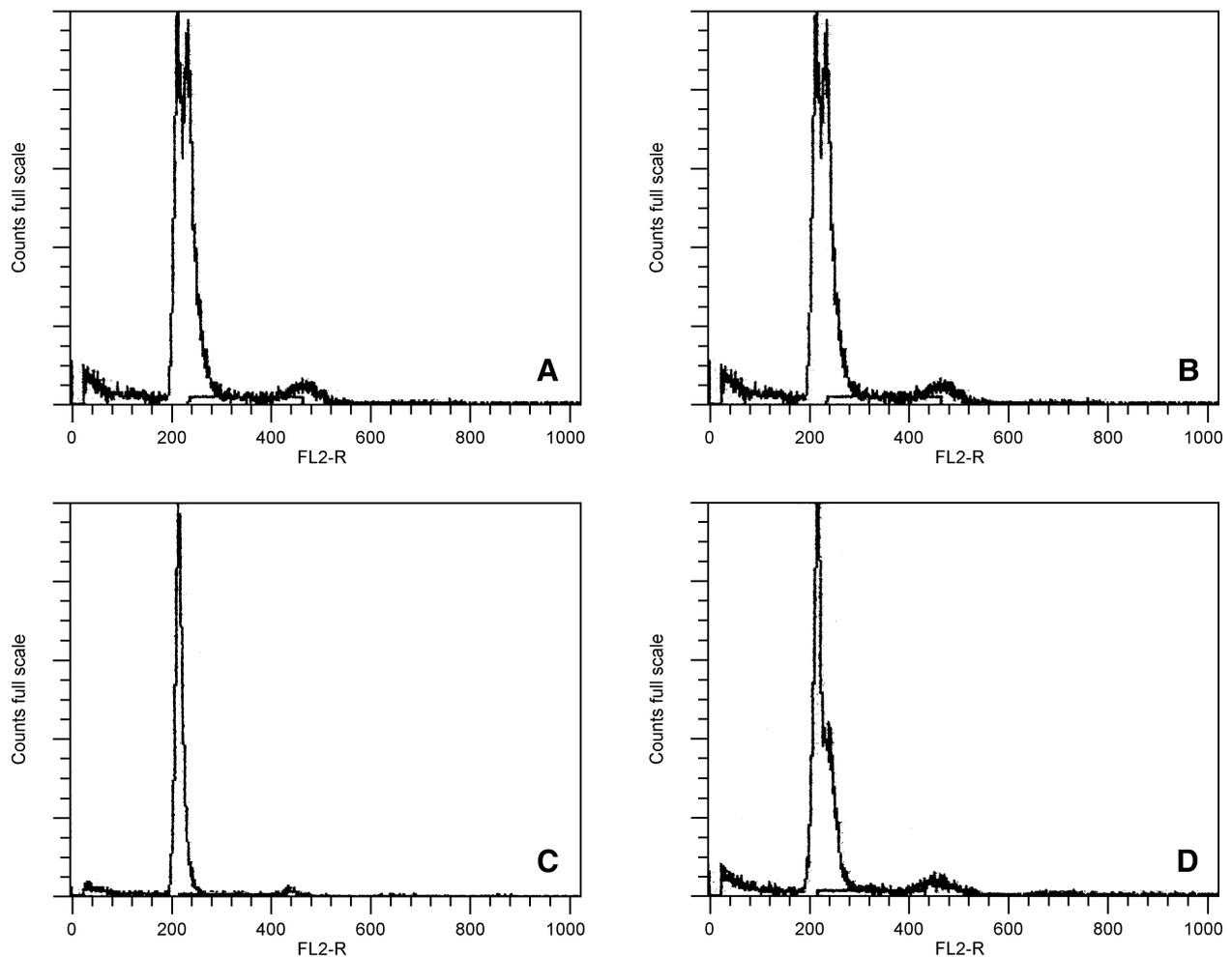
Fig. 2. The aneuploidy pattern is noted in the remote normal-looking mucosa (D). **A:** tumor, **B:** normal-looking mucosa adjacent to tumor, **C:** close normal-looking mucosa, 3 cm apart from the adenocarcinoma, **D:** remote normal-looking mucosa, more than 5 cm apart from the adenocarcinoma.

in the normal-looking mucosa are presented in Table 2. Among them, two were EGC and six were AGC. Seven out of eight cases showed aneuploidy pattern in the main tumor.

Correlation between DNA ploidy, Ki-67 and pathologic parameters

The correlation between DNA ploidy, Ki-67 labeling rate and pathologic features are presented in Table 3, 4. The aneuploidy group revealed higher S phase fraction (20.1 ± 9.2 vs. 11.9 ± 7.1, $p < 0.05$), higher proliferation index (28.8 ± 10.6 vs. 18.5 ± 9.4, $p < 0.05$) and higher Ki-67 labeling rate (39.1 ± 15.1 vs. 30.7 ± 24.5) compared to the diploidy group. Metastasis to lymph nodes was higher in the aneuploidy group (73.8%) than in the diploidy group (54.5%). According to the differentiation of the tumor, well and moderately differentiated adenocarcinomas showed a higher aneuploidy pattern (22/35,

62.9%), but poorly differentiated tumors showed a higher diploidy pattern (23/37, 62.2%). That was statistically significant ($p < 0.05$). Signet ring cell carcinoma also revealed a higher diploidy pattern (8/14, 57.1%). According to Lauren's classification, intestinal type tumor revealed high incidence of aneuploidy pattern (24/39, 61.5%) compared to diffuse type tumor (18/47, 38.3%), ($p < 0.05$).

The Ki-67 labeling rate was higher in the well and moderately differentiated groups than in the poorly differentiated carcinoma and signet ring cell carcinoma groups (39.8 ± 20.9 vs. 31.7 ± 20.3, $p < 0.05$), and higher in the intestinal type tumor than in the diffuse type tumor (40.1 ± 21.6 vs. 31.1 ± 19.5, $p < 0.05$). There were no differences according to the size of the tumor, lymphatic invasion, perineurial invasion and angioinvasion.

Because of the short follow-up period (mean 13.4 months), the survival time was not significantly different between aneuploidy and diploidy groups.

Table 2. Cases of aneuploidy in the normal-looking gastric mucosa of gastric adenocarcinoma

No.	Age (years)	Sex	Type	Tumor	Adjacent mucosa	Close mucosa	Remote mucosa
1	53	M	EGC	A	A	A	D
2	58	F	EGC	A	A	D	A
3	67	F	AGC	A	A	A	D
4	57	M	AGC	A	A	A	D
5	80	M	AGC	A	A	A	D
6	48	F	AGC	A	A	D	A
7	81	M	AGC	A	D	A	A
8	42	F	AGC	D	A	A	D

Adjacent mucosa: normal-looking mucosa adjacent to tumor
Close mucosa: normal-looking mucosa, 3 cm apart from the adenocarcinoma
Remote mucosa: normal-looking mucosa, more than 5 cm apart from the adenocarcinoma
M, male; F, female; EGC, early gastric adenocarcinoma; AGC, advanced gastric adenocarcinoma; A, aneuploidy; D, diploidy

Table 3. Correlation between DNA ploidy and pathologic parameters

	Aneuploidy (42)	Diploidy (44)
S phase fraction*	20.1±9.2	11.9±7.1
Proliferation index*	28.8±10.6	18.5±9.4
Ki-67 rate (%)	39.1±15.1	30.7±24.5
No. of LN (+) cases	31 (73.8%)	24 (54.5%)
AGC (74, pm:ss:se:si)	39, 7:8:20:4	35, 6:9:17:3
EGC (12, m:sm)	3, 2:1	9, 2:7
Differentiation[†]		
(W:M:P:S)	1:21:14:6	0:13:23:8
Differentiation (I:D)[†]	24:18	15:29

$*p<0.05$
[†]W: well-differentiated adenocarcinoma, M: moderately differentiated adenocarcinoma, P: poorly differentiated adenocarcinoma, S: signet ring cell carcinoma, [†]I: intestinal type by Lauren's classification, D: diffuse type by Lauren's classification
LN, lymph node; AGC, advanced gastric adenocarcinoma; EGC, early gastric adenocarcinoma; pm, proper muscle; ss, subserosa; se, serosa exposed; si, serosa infiltrative; m, mucosa; sm, submucosa

Table 4. Correlation between Ki-67 labeling rate and pathologic parameters

	Ki-67 labeling rate	
Differentiation*(W+M:P+S)	39.8±20.9	31.7±20.3
Differentiation*(I:D)	40.1±21.6	31.3±19.5
Depth (AGC:EGC)	36.6±20.7	24.7±18.9
LN (+:−)	37.4±19.4	30.6±22.7
Size (<5 cm:≥5 cm)	33.9±19.2	36.2±22.1
Lymphatic invasion (+:−)	35.9±20.4	31.8±22.5
Nerve invasion (+:−)	33.4±19.9	36.9±21.9
Angioinvasion (+:−)	34.8±21.8	35.3±20.6

$*p<0.05$
W+M: well differentiated adenocarcinoma and moderately differentiated adenocarcinoma
P+S: poorly differentiated adenocarcinoma and signet ring cell carcinoma
AGC, advanced gastric carcinoma; EGC, early gastric carcinoma; LN, lymph node

DISCUSSION

DNA ploidy pattern has been shown to be a prognostic indicator in gastric cancer by some authors (16, 24), whereas others have found no such correlation (25). Conflicting results concerning the correlation of DNA ploidy with clinicopathologic findings have also been reported. Meanwhile, there is general agreement that ploidy abnormalities become more frequent in advanced gastric cancer compared with earlier stage (26). This study showed a higher aneuploidy pattern in the AGC than in the EGC. However, it seems that the number of EGC included in the study was too small to compare the ploidy patterns and it requires further evaluation. A correlation was noted between aneuploidy and lymph

node metastasis (25, 27, 28). But we only observed a tendency between lymph node metastasis and aneuploidy patterns.

The relationship between DNA ploidy and differentiation of the tumor is also controversial (15, 25, 26, 29-36). Some authors (32, 33) found no significant differences in DNA ploidy and histologic type. Another insisted poorly differentiated adenocarcinoma and signet ring cell carcinoma were predominantly of the diploidy pattern and well and moderately differentiated adenocarcinoma had a dominant DNA distribution in the aneuploidy range (15, 25, 26, 29, 30, 34). However, undifferentiated tumors showed a remarkable increase in frequency of aneuploidy (31). A correlation between the DNA ploidy and Lauren's classification was also noted (28, 32). In this study, we observed well and moderately differentiated tumor groups showed a high rate of aneuploidy, but poorly differentiated adenocarcinoma and signet ring cell carcinoma showed a high incidence of diploidy. Small

cells with homogeneous nuclei might explain the low aneuploidy rate in the poorly differentiated carcinoma (15). Because some poorly differentiated tumors or diffuse type tumors were accompanied by large infiltration of inflammatory cells, careful attention must be paid to interpret the results.

Some tumors show variable morphologic features (12), multiploidy in one tumor (16) and different ploidy patterns in multiple biopsy specimens (16, 37) suggesting subpopulations with different properties. DNA ploidy was concordant among the three samples in 63.3% and the DNA histograms of two of the three multiple samples were similar in 36.6% (4). However, the ploidy of the third sample was discordant. A single sample detected aneuploid in 60% of cases, but multiple samples increased the detection rate to 80% (38). Therefore one sample from each tumor could lead to a biased result. Intratumoral heterogeneity has been used to explain differences in tumor chemosensitivity, radiosensitivity and response to hormonal manipulation (39). The heterogeneity is also observed when samples from primary tumors and metastasis were compared (12-14, 40). One of the most practical recommendations resulting from the study of heterogeneity or regional tumor variation is that multiple tissue samples should be submitted for accurate DNA measurement (14).

Although the morphologic changes were not prominent, the detection of the abnormalities at the cellular level was possible from the multiple different site samplings. The ploidy patterns from the tumor and adjacent normal-looking mucosa were different in 26.7% (23/86 cases) of the study. Some aneuploidy peaks were noted in the close and remote normal-looking mucosa. In most of these cases, the main tumor showed an aneuploidy peaks. This suggests the presence of the so-called 'field cancerization', the biology of which has not yet been extensively analyzed. The 'field cancerization' was introduced by Slaughter et al. (10). They examined the resection specimens of invasive squamous cell carcinoma of the oral cavity and found histopathologic abnormalities in the epithelium surrounding the invasive cancer. They (10) and other investigators (41) have reported an increased incidence of second primary head and neck, pulmonary, and esophageal cancers in patients with head and neck cancer.

Califano et al. (42) observed the spectrum of chromosomal loss progressively increased at each histopathological step from benign hyperplasia to carcinoma in situ to invasive cancer. They proposed adjacent areas of tissue with different pathological appearance shared common genetic changes, but the more histopathologically advanced areas exhibited additional genetic alterations. The c-erbB-2 protooncogene was more frequently amplified in

the advanced or metastatic adenocarcinomas compared to early gastric adenocarcinoma and non-metastatic gastric carcinoma (43, 44).

Although the remaining specimens were smeared to confirm their cytologic adequacy after the flow cytometric analysis, all specimens were harvested from unfixed gross gastrectomy and did not have histologic correlation. It is often difficult to separate the early gastric carcinoma from the adjacent nonneoplastic epithelium. Also we would not be able to rule out satellite foci of neoplasm or angiolymphatic tumor emboli. The significance of aneuploidy in normal-looking mucosa requires further investigation.

The survival rate was not significantly different from aneuploidy and diploidy groups because of short followup period. However, there was a tendency that diploid group showed a longer survival period but more followup study is needed to confirm the prognosis.

Ki-67 is a nuclear antigen present in proliferating cells in the late G1, S, G2 and M phase (45) and Ki-67 labeling rate is related to the probability of metastatic dissemination and prognosis of patient (46). This study showed a rough correlation between Ki-67 labeling rate, tumor differentiation, depth of invasion, and lymph node metastasis.

In summary, aneuploidy, correlated with high proliferative activity, was higher in advanced gastric carcinoma, in well and moderately differentiated tumor, and in intestinal type tumor. Aneuploidy was noted even in the normal-looking gastric mucosa suggesting field cancerization.

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