DNA Diploidy of Gastric Cancer from the Aspects of DNA Heterogeneity and Chromosomal Numerical Aberrations

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DNA heterogeneity, which is a problem we encounter during DNA ploidy analysis, was studied on the basis of analyzing numerical aberrations (that is, in the direction of gain) of chromosomes 7, 11 and 17 in 33 cases of gastric cancer, using fluorescence in situ hybridization (FISH). Emphasis was placed on clarification of the DNA aneuploidy formation process by comparing and characterizing three types of gastric tumor: homogeneously DNA diploid tumors (DD), the diploid portion of tumors showing DNA heterogeneity (DD-H) and the aneuploid portion of tumors showing DNA heterogeneity (DA-H).

DNA heterogeneity and chromosomal heterogeneity increased markedly as gastric tumor advanced from 'm' (mucosal) cancer to 'sm' (submucosal) cancer in early cancer. Thus, 'sm' cancer exhibited some features of advanced cancer. When DD and DD-H, both of which are DNA diploid, were compared in terms of chromosomal numerical aberrations, the number of chromosomes 11 and 17 was significantly higher in DD-H. When DD-H and DA-H were compared, the number of chromosomes 7 and 11 was significantly higer in DD-H, while the number of chromosome 17 was approximately the same in both. Based on these results of DNA ploidy analysis of gastric cancer, the numerical aberrations of chromosomes 11 and 17 strongly suggest the presence of DNA heterogeneity even in DNA diploid cases, and DNA diploid tumors with abnormal numbers of chromosomes 11 and 17 have features similar to those of DNA aneuploid tumors. When the DNA aneuploidy formation process was studied on the basis of chromosomal numerical aberrations, it appeard that the number of chromosomes 11 and 17 increases first, and that chromosomes 7 and 11 are closely involved in the change of a tumor into a DNA aneuploid tumor.

Introduction

As a result of the recent spresd in the use of flow cytometry (FCM), simple and rapid measurement of the nuclear DNA content of various solid tumors is now possible. Numerous studies have been conducted concerning the relationship between DNA ploidy and clinicopathological factors. These studies revealed that the incidence of lymph node metastasis or hematogenous metastasis is higer for DNA aneuploid tumors than for DNA diploid tumors. Based on this finding, DNA ploidy has begun to be adopted clinically as a prognostic indicator [1-4] .

However, it is known that the prognosis is sometimes poor even in DNA diploid cases, and that some tumors show DNA heterogeneity. Therefore, it has been pointed out that ploidy assessment on the basis of measurement at only one site of a tumor can lead to erroneous judgments, and that two or more sites need to be measured.

According to recent reports, the incidence of DNA heterogeneity was high for cancer of many organs, e. g., 44 % for esophageal cancer [5] , 67 % for gastric cancer [6] , 78 % for colorectal cancer [7] , 80 % for breast cancer [8] and 90 % for pulmonary cancer [9] . Therefore, DNA heterogeneity requires particular attention when DNA ploidy is to be assessed. Some investigators noted that the prognosis was poorer for tumors with DNA heterogeneity than for tumors without it, suggesting the usefulness of DNA heterogeneity as an indicator of tumor malignancy level [10] .

DNA heterogeneity has two forms : (1) tumors showing an aneuploid pattern alone, and (2) tumors composed of a mixture of diploid areas (DD-H) and aneuploid areas (DA-H). The DD-H in the latter case differs from the homogeneously DNA diploid tumor (DD) which shows no DNA heterogeneity. The DD-H appears to occupy an intermediate position between DNA diploidy and DNA aneuploidy, although this view has not been confirmed. When the DNA ploidy and chromosomal numerical aberrations were studied for several types of solid cancer [11] , there were some DNA diploid cases in which chromosomal numerical aberrations were detected, and the prognosis in these cases was poor. this observation indicates the necessity of checking for chromosomal numerical aberrations in cases of DNA diploid tumors.

In the past, chromosome analysis of solid cancers was quite difficult because cells in the mitotic phase were difficult to obtain. In 1986, however, Plinkel et al. developed fluorescence in situ hybridization (FISH). The use of FISH made it possible to detect chromosomal numerical aberrations even in interphase nuclei, resulting in remarkable advances in the study of chromosomal numerical aberrations of solid tumors [12-15] . Using this

technique, we recently analyzed chromosomal numerical aberrations of gastric cancer in relation to DNA heterogeneity, and examined DNA diploid tumors with respect to chromosomal numerical aberrations, for the goal of determining whether or not chromosomal numerical aberrations provide additional information to assess DNA ploidy of gastric cancer. During this study, emphasis was placed on comparing DD (homogeneously DNA diploid tumors) with DD-H (DNA diploid portions of DNA heterogeneous tumors), and on clarifying the DNA aneuploidy formation process.

Materials and Methods

1. Subjects

The subjects were 33 patients with primary gastric cancer who underwent surgical resection at the First Department of Surgery, Nagasaki University Hospital or at its related facilities between February 1993 and November 1993. The diagnosis was based on the 12th Edition of the General Rules for the Study of Gastric Cancer prepared by the Japanese Society of Gastric Cancer [16] . There were 21 males and 12 females, with ages ranging from 33 to 81 years old (mean : 62 ± 11.7 years). The cancer was histologically classified as papillary adenocarcinoma (pap) in 2 cases, tubular adenocarcinoma (tub) in 12, poorly differentiated adenocarcinoma (por) in 17, signet-ring cell carcinoma (sig) in 1 and mucinous adenocarcinoma (muc) in 1. The depth of tumor invasion was 'm' (mucosal) in 8 cases, 'sm' (submucosal) in 8, 'mp' (invading the musculus propria) in 3, 'ss' (invading beyond the tunica externa but not reaching the serosa) in 5, ' se' (invading the serosa) in 7 and 'si' (invading beyond the serosa) in 2. thus, 16 patients had early gastric cancer and 17 had advanced gastric cancer. The tumor stage was Ia in 13 cases, I b in 5, II in 7, IIIa in 3, IIIb in 4, IVa in 1 and IVb in none (Table 1).

2. Sample preparation

From each fresh sample of gastric cancer, two tissue specimens were collected from two neighboring regions at each of the following four sites of the sample, using forceps designed for endoscopic biopsy : (A) the oral side, (B) the anal side, (C) the side facing the anterior wall, and (D) the side facing the posterior wall. One specimen was subjected to FISH and flow cytometry (FCM). The other specimen was fixed in 10 % neutral formalin buffer, embedded in paraffin and stained with HE for histological examination to confirm that the collected tissue was cancer (Fig. 1).

3. Fluorescence in situ hybridization (FISH)

Touch smears of fresh tissue were prepared using an APS-coated slide glass. The smears were fixed in Carnoy's solution (ethanol : acetic acid = 3 :1, v/v) and stored frozen at $-80\,°C$. Immediately before use, the slide glass was washed in PBS and incubated at 37 °C for 15 minutes in 0.01 % pepsin/0.2N HCl. The sample was then dehydrated at 4 °C in ethanol series (80 %, 95 % and 100 %), followed by incubation at room temperature for 10 minutes in 0.25 % acetic anhydrate/0.1M Tris-HCl (pH 8.0). After being washed in 2 x SSC (0.3M NaCl, 30 mM sodium citrate, pH 8.0), target DNA was denatured at 70 °C for 2 minutes in 70 % formamide/2 x SSC. The sample was then dehydrated again in cold ethanol series (70, 80, 95 and 100 %).

Alpha-satellite DNA probes specific to chromosomes 7, 11 and 17 (biotinylated D7Zl, D11Zl, Dl7Zl ; Oncor, USA) were used. A hybridization mixture composed of a DNA probe (0.5 μg/ml), 50 % formamide, herring sperm DNA (500 μg/ml), 2x SSC and 10 % dextran sulfate was prepared. DNA denaturation was induced at 70 °C for 10 minutes. After 20 μl of the hybridization mixture was added to each slide, the slide was incubated overnight at 37 °C in a moist chamber. After hybridization, the sample was washed in 2 x SSC at 42 °C for 10 minutes, in 60 % formanide/2 x SSC at 42 °C for 10 minutes and in 4 x SSC (0.05 % Tween-20/4 x SSC) at 42 °C for 5 minutes. The sample was then incubated at 37 °C for 45 minutes in FITC conjugated avidin DCS (5 μl/ml ; Vector Labs, USA).

Table. 1 Clinicopathological features of the subjects.

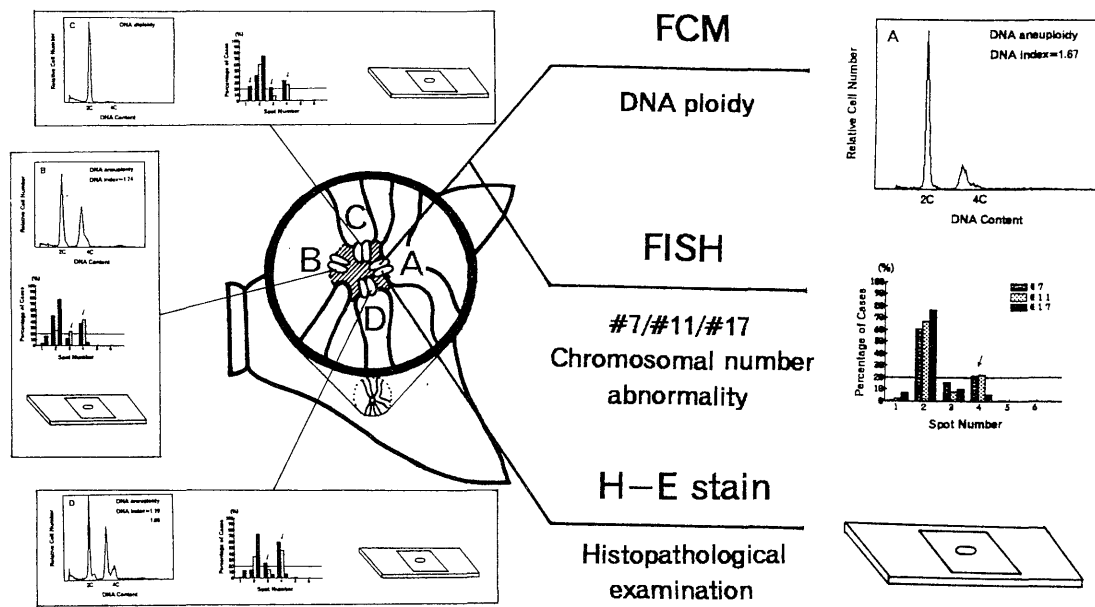|	N	33
Sex Male : Female		21 : 12
Age (mean) years old		33~81 (62)
Histological	pap	2
type	tub	12
	por	17
	sig	1
	muc	1
Histological	m	8
depth	sm	8
	mp	3
	ss	5
	se	7
	si	2
n	n (−)	20
	n (+)	13
Conclusive	I a	13
stage	I b	5
	II	7
	IIIa	3
	IIIb	4
	IVa	1
	IVb	0

Fig. 1 Sample preparation

From each fresh sample of gastric cancer, two tissue specimens were collected from two neighboring regions at each of the following four sites of the sample. (A) the oral side, (B) the anal side, (C) the side facing the anterior wall, and (D) the side facing the posterior wall. One specimen was subjected to FISH and flow cytometry. The other specimen was subjected for histological examination.

After being washed in 4 x SSC at 42 °C for 5 minutes and in 0.05 % Tween-20/PBS at 42 °C for 5 minutes, the sample was counter stained with propidium iodide (1 μ g/ml ; Sigma, USA). Under a fluorescence microscope (BH-2, Olympus Japan), the number of signal spots per 200 nuclei of each sample was counted, with special attention to those samples having large nuclei and high numbers of spots.

4. *Flow cytometry (FCM)*

The specimen after touch smears were cut into small pieces using scissors and suspended in 0.1 % Triton-X 100/ PBS. The cell suspension thus obtained was filtered through a 50 μ m Nylon mesh and stained RNase-added propidium iodide at a final concentration of 50 μ g/ml. DNA contents in 10000 or more nuclei were measured by FACS can (Becton Dickinson, USA) and DNA histograms were obtained. DNA ploidy was assessed on the basis of the DNA index (DI) calculated by dividing the cancer cell G_0/G_1 peak channel number by the normal cell's G_0/G_1 peak channel number. A DI equal to 1.0 was regarded as indicating diploidy, and a DI not equal to 1.0 was regarded as indicating aneuploidy. Only cases where the coefficient of variation of the cancer cell G_0/G_1 peak was 8.0 or less were included into the evaluation.

5. *Analysis of heterogeneity*

DNA heterogeneity was determined on the basis of the DI. Cases in which the difference in the D I among different peaks of the same tumor was 0.1 and over (i.e., a 10 % or greater difference in diploid DNA contents) were regarded as showing DNA heterogeneity.

The chromosome number was regarded as being abnormal in cases where the cells with spot numbers other than 2 occupied 20 % or more of the total cells examined [17] . In cases where at least one of the 4 sites of the tumor showed a different tendency in spot numbers, as compared to other sites, a judgment of chromosomal heterogeneity was made. In this study, the term "chromosome heterogeneous" was used to indicate those cases where at least one of the chromosomes 7, 11 and 17 showed heterogeneity.

6. *Statistical analysis*

Significance of differences was tested using chi-square test. $p < 0.05$ (both-sided) was regarded as significant.

Results

Figures 2 and 3 present typical cases.

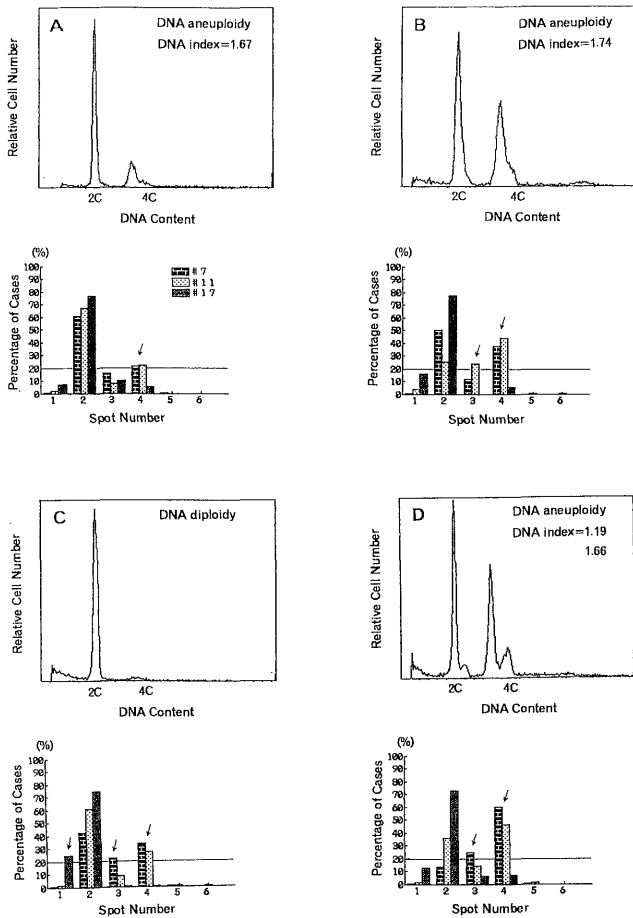Case 1 was a 63-year-old male with advanced gastric cancer of type 0 (IIc), depth 'mp' and dimensions of 20 x

Fig. 2   Case 1

Case 1 was 63-year-old male with advanced gastric cancer of type 0 (II c), depth 'mp', and dimensions of 20 x 20 mm, histologically classified as tub 2, ly2, vl and n(-). This case was DNA heterogeneous and showing chromosomal heterogeneity of all of chromosomes 7, 11 and 17, although each pattern of chromosomal numerical aberrations was not same.

Fig. 3   Case 2

Case 2 was 66-year-old male with early gastric cancer of type 0 (II c), depth 'm', and dimensions of 25 x 15 mm, histologically classified as por, ly0 , v0 and n(-). This case showed DNA homogeneous diploidy. And this case showed chromosome homogeneous as to chromosomes 11 and 17, but showed chromosome 7 heterogeneity. Then this case was classified as chromosome heterogeneous.

20 mm. This case was classified as tub2, ly2, vl and n (−). Of the four sites of the tumor, A, B and D were DNA aneuploid, while C was DNA diploid. Therefore, this case was DNA heterogeneous. Of the aneuploid sites, D contained a clone with a D I = 1.19, which was absent in sites A and B. This also supports the judgment of DNA heterogeneity. When chromosomal numerical aberrations were examined, cells with four spots of chromosome 7 showed significant (20 % or greater) increase in all of the 4 examined sites of the tumor, without showing heterogeneity. However, a significant increase of cells with 3 spots of chromosome 7 was only seen in sites C and D. Heterogeneity was observed in this chromosome. Cells with 4 spots of chromosome 11 showed a similar degree of increase in all 4 of the examined sites, while cells with 3 spots of chromosome 11 increased in only in site B, thus
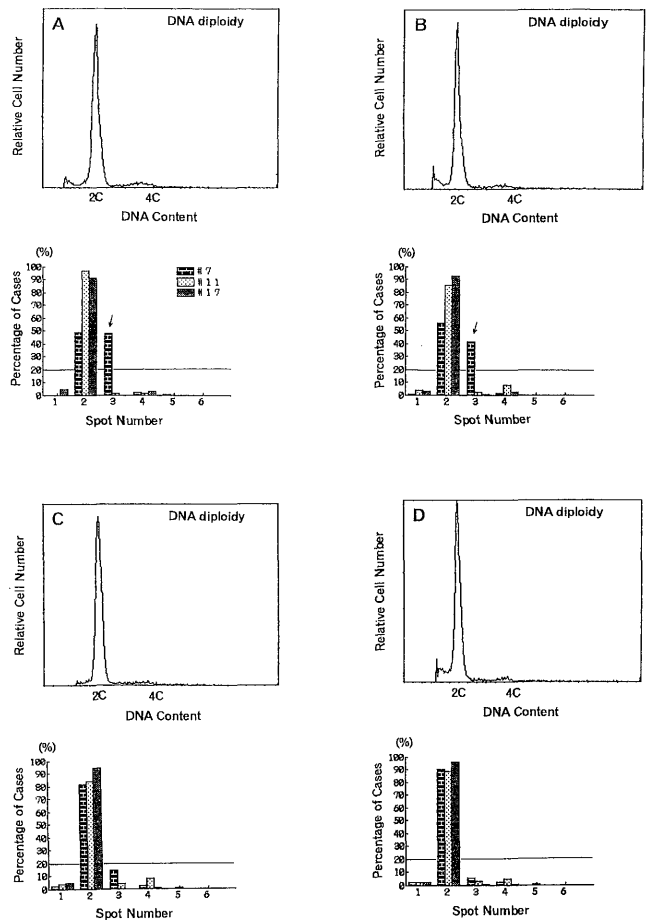
showing heterogeneity. This case, showing heterogeneity of all of chromosomes 7, 11 and 17, was therefore classified as chromosome heterogeneous (Fig.2).

Case 2 was a 66-year-old male with early gastric cancer of type 0 II c, depth 'm' and dimensions of 25 x 15 mm. This case was histologically classified as por, ly0, v0 and n (−). All 4 of the examined sites were diploid, thus showing DNA homogeneity. When chromosomal numerical aberrations were examined, chromosomes 11 and 17 showed no numerical aberrations in any of the four sites, while cells with 3 spots of chromosome 7 increased significantly in sites A and B although no such abnormalities were seen in sites C and D. This case, showing chromosome 7 heterogeneity, was classified as chromosome heterogeneous (Fig.3).

When the incidence of DNA heterogeneity was analyzed

Table. 2　The incidence of DNA heterogeneity and chromosomal heterogeneity in relation to the depth of tumor invasion.

	early		advance		total (%)
	m	sm	mp-ss	se-si	
DNA heterogeneity	10/16 (62.5)		12/17 (70.6)		22/33 (66.7)
	3/8 (37.5)	7/8 (87.5)	8/8 (100)	4/9 (44.4)	
Chromosomal heterogeneity	9/16 (56.3)		11/17 (64.7)		20/33 (60.6)
	3/8 (37.5)	6/8 (75)	7/8 (87.5)	4/9 (44.4)	

Table. 3　The incidence of chromosomal heterogeneity in relation to the depth of tumor invasion for each of chromosomes 7, 11, and 17.

	early		advance		total (%)
	m	sm	mp-ss	se-si	
#7	9/15 (60)		11/17 (64.7)		20/32 (62.5)
	3/8 (37.5)	6/7 (85.7)	7/8 (87.5)	4/9 (44.4)	
#11	5/14 (35.7)		10/17 (58.8)		15/31 (48.4)
	1/7 (14.3)	4/7 (57.1)	7/8 (87.5)	3/9 (33.3)	
#17	7/16 (43.8)		9/17 (52.9)		16/33 (48.5)
	2/8 (25)	5/8 (62.5)	6/8 (75)	3/9 (33.3)	

on the basis of nuclear DNA contents as measured using FCM, the incidence did not differ significantly between the early cancer group (62.5 %, 10/16) and the advanced cancer group (70.6 %, 12/17). However, when the incidence was analyzed by the depth of tumor invasion, it was much higer for 'sm' cases (87.5 %, 7/8) than for 'm' cases (37.5 %, 3/8), although this difference was not significant (P = 0.1213). As the tumor depth increased from 'm' to 'sm' and to 'mp-ss', the incidence of DNA heterogeneity increased, but the incidence decreased when the tumor depth reached the 'se-si' level (Table 2).

When chromosome numbers were measured using FISH, the incidence of chromosomal heterogeneity, as assessed based on a general evaluation of chromosomes 7, 11 and 17, did not differ significantly between the early cancer group (56.3 %, 9/16) and the advanced cancer group (64.7 %, 11/17). When this incidence was compared among cases of early cancer, the incidence was higher for 'sm' cases (75 %, 6/8) than for 'm' cases (37.5 %, 3/8), as shown in Table 2. When chromosomal heterogeneity was examined separately for each of chromosomes 7, 11 and 17, a tendency similar to that observed for DNA heterogeneity and overall chromosomal heterogeneity was noted (Table 3).

The relationship between DNA heterogeneity and

chromosomal numerical aberrations was then analyzed by dividing tumors into three types (Table 4) : (1) homogeneously DNA diploid tumors (DD ; tumors which showed no DNA heterogeneity, e. g., Case 2), (2) the diploid portions of DNA heterogeneous tumors (DD-H ; e. g., part C of Case 1) and (3) the aneuploid portions of DNA heterogeneous tumors (DA-H ; e. g., parts A, B and D of Case 1).

For each of the three chromosomes examined (chromosomes 7, 11 and 17), the incidence of numerical aberrations was significantly higher for DA-H than for DD (p < 0.0001). When DD and DD-H, both of which are diploid, were compared, the incidence of numerical aberrations of chromosome 7 did not differ between them, while the incidence of numerical aberrations of chromosomes 11 and 17 was significantly higer for DD-H than for DD (chromosome 11 ; 40 % or 12/30 vs. 0 % or 0/33, p < 0.0005; chromosome 17 ; 41 % or 13/32 vs. 3 % or 1/40, p < 0.0005). Furthermore, when chromosomal numerical aberrations were compared between DD-H and DA-H, the incidence of numerical aberrations of chromosomes 7 and 11 was significantly higher for DA-H than for DD-H (p < 0.0001 for chromosome 7 and p < 0.01 for chromosome 11), while the incidence for chromosome 17 did not differ significantly. These results indicate that the number of

Table. 4 The incidence of chromosomal numerical aberrations in relatin to the three DNA ploidy types.

	DNA diploid tumor (DD)	DNA heterogeneouus tumor	
		diploidy part (DD-H)	aneuploidy part (DA-H)
# 7 Total	8/40 (20 %) 1*	11/30 (37 %) 6*	50/57 (88 %) 1* 6*
#11 Total	0/33 (0 %) 2* 4*	12/30 (40 %) 4* 7*	42/58 (72 %) 2* 7*
#17 Total	1/40 (3 %) 3* 5*	13/32 (41 %) 5*	35/60 (58 %) 3*

$p < 0.0001 : 1*, 2*, 3*, 6*$ $p < 0.0005 : 4*, 5*$ $p < 0.01 : 7*$

Table. 5 The incidence of chromosomal numerical aberrations in relation to the three DNA ploidy types and the depth of tuumor invasion (early and advanced gastric cancer).

		DNA diploid tumor (DD)	DNA heterogeneous tumor	
			diploidy part (DD-H)	aneuploidy part (DA-H)
# 7	early	4/20 (20 %) 1*	3/13 (23 %) 9*	20/27 (74 %) 1* 9* 12*
	advance	4/20 (20 %) 2*	8/17 (47 %) 10*	30/30 (100%) 2* 10* 12*
#11	early	0/16 (0 %) 3*	3/13 (23 %) ·	15/27 (56 %) 3* 13*
	advance	0/17 (0 %) 4* 7*	9/17 (53 %) 7* 11*	27/31 (87 %) 4* 11* 13*
#17	early	1/20 (5 %) 5*	5/15 (33 %)	12/29 (41 %) 5* 14*
	advance	0/20 (0 %) 6* 8*	8/17 (47 %) 8*	23/31 (74 %) 6* 14*

$p < 0.0001 : 2*, 4*, 6*, 10*$ $p < 0.001 : 1*, 3*$ $p < 0.005 : 7*, 8*$ $p < 0.01 : 9*$
$p < 0.05 : 5*, 11*, 12*, 14*$

chromosome 7 is abnormal in about 20 % of DNA diploid tumors, but that abnormal numbers of chromosomes 11 and 17 are only seen in DD-H and DA-H. In other words, these results indicate that the sites where chromosomal numerical aberrations appear vary depending on individual chromosomes. I also noted that the incidence of numerical aberrations of chromosomes 7 and 11 was significantly higher for DA-H than for DD-H, while the incidence for chromosome 17 did not differ significantly between DD-H and DA-H.

I then compared the incidence of numerical aberrations of chromosomes 7, 11 and 17 between early and advanced cancers. When this comparison was limited to DNA diploid tumors (DD and DD-H), there was no significant difference in the incidence for any chromosome between early and advanced cancers. When the comparison was limited to DNA aneuploid tumors (DA-H), the incidence of numerical aberrations of each of the three chromosomes was significantly higher in advanced cancer than in early cancer (p < 0.05), as shown in Table 5.

Discussion

As a result of the recent spread in the use of flow cytometry, measurement of nuclear DNA contents of cells is simpler even in the case of solid cancers. For this reason, numerous studies have been carried out concerning the relationship between DNA ploidy and clinicopathological factors. Those studies revealed that the incidence of lymph node metastasis and hematogenous metastasis is higher for DNA aneuploid tumors than for DNA diploid tumors. As a result, DNA ploidy has begun to be clinically adopted as a new indicator of tumor malignancy level. In addition, it has been reported that some tumors show DNA heterogeneity, that is, are composed of a mixture of regions with different DNA ploidy patterns. The percentage of such tumors is high even in the case of gastrointestinal cancers. Based on these findings, it has been pointed out that ploidy judgment based on nuclear DNA measurement of only one site of a tumor can be erroneous. It is therefore considered necessary to measure nuclear DNA content in two or more

sites of a tumor before making an DNA ploidy judgment. To date, however, there is no widely accepted view about the optimal number of sites where nuclear DNA content should be measured in a given case of tumor.

Tumor DNA heterogeneity has two forms : (1) tumors showing an aneuploid pattern alone, and (2) tumors composed of a mixture of diploid areas (DD-H) and aneuploid areas (DA-H). DD-H causes a problem when we make a judgment as to ploidy. Unlike DD, which is a homogeneously diploid tumor, DD-H seems to occupy an intermediate position between DNA diploidy and DNA aneuploidy, although this view has not been confirmed. In the present study, I compared DD with DD-H from the viewpoint of chromosomal numerical aberrations to determine whether or not chromosomal numerical aberrations provide information useful for the judgment of DNA ploidy of gastric cancer. At the same time, the DNA aneuploidy formation process, as viewed from chromosomal numerical aberrations, was studied.

In the past, this kind of chromosome analysis was quite difficult because cells in the mitotic phase were difficult to obtain. However, the use of FISH, which was developed in 1986 by Pinkel et al., has made it possible to detect chromosomal numerical aberrations even in inter-phase unclei. As a result, studies of chromosomal numberical aberrations of solid cancers have advanced remarkably. In the present study, this method was used to check for numerical aberrations of chromosomes 7, 11 and 17 of gastric cancer cells. An advantage of this method lies in that only small amounts of tisue are needed, if the tissue is made into a touch smear sample, thus allowing examination of two or more sites even in the case of a small tumor. This feature of FISH seems to be highly useful in the study of heterogeneity within a tumor.

Chromosomes 7, 11 and 17, which were examined in the present study, carry various genes associated with the onset, growth and progression of gastric cancer. The long arm of chromosome 7 is known to contain a locus for c-met and a locus for its ligand hepatocyte growth factor (HGF). The incidence of amplification of loci for c-met and HGF is particularly high in the case of scirrhous cancer of the stomach. The short arm of chromosome 7 is known to contain a locus for epidermal growth factor receptor (EGFR) which is thought to be involved in the proliferation of cancer cells and the tumor malignancy level through forming an autocrine-paracrine system with EGF and TGF-α. The long arm of chromosome 11 has loci for hst-1 and int-2 which are associated with fibroblast growth factor (FGF), but these loci are relatively nonspecific to particular organs and are thought to be associated with esophageal, mammary and hepatic cancers in addition to gastric cancer. The short arm of chromosome 11 has a locus for H-ras which is often seen in human cancers and is thought to be involved in carcinogenesis through binding to GTP and GDP and exerting GTPase activity.

The long arm of chromosome 17 has c-erbB2 gone locus which has a structure similar to EGFR, and is known to be marked amplified in the case of breast cancer. This gene is considered to be associated with differentiated gastric cancer. The short arm of chromosome 17 has an suppressor-oncogene p53, which appears to be involved in the onset of many types of tumor including gastric cancer [18, 19]. Thus, the chromosomes examined in the present study carry many cancer-related genes. Numerical aberrations of these chromosomes may be associated with abnormalities of the cancer-related genes on these chromosomes. [20-24]

Previous studies of the relationship between DNA heterogeneity and the depth of gastric cancer invasion suggested that the incidence of DNA heterogeneity increases with the depth of tumor invasion from 'm' to 'sm' [6]. Also in the present study, the incidence of DNA heterogeneity was higher for 'sm' tumors (87.5%) than for 'm' tumors (37.5%). The incidence of DNA heterogeneity was 70.6% for advanced cancers ('mp' through 'si'). The incidence differed significantly between 'm' cases and 'sm-mp-ss' cases. Thus, 'sm' cancer appeared to have assumed some features of advanced cancer. The incidence of chromosomal heterogeneity for 'sm' cases (75%) was also higher than that for 'm' cases (37.5%) and close to the incidence for advanced cancer (64.7%). These results indicate that although 'sm' cancer is regarded as an early cancer, as is the case with 'm' cancer, according to the current General Rules for the Study of Gastric Cancer, the cytobiological characteristics of 'sm' cancer seem to resemble those of advanced cancer. When 'm' and 'sm' cancers were compared in terms of clinicopathological factors, the incidence of lymph node metastasis was higher for 'sm' cancer (15-20%) than for 'm' cancer (about 3%), and hematogenous metastasis was noted among 'sm' cases [25, 26]. These clinicopathological differences between 'm' and 'sm' cancers were clarified in the present study from the aspects of nuclear DNA content and chromosomal numerical aberrations.

As the depth of tumor invasion increased from 'm' to 'sm' 'mp' and to 'ss', the incidence of DNA heterogeneity increased. However, when the depth tumor invasion reached the 'se' or 'si' level, the incidence decreased. Considering that four sites of each tumor were examined in the present study irrespective of the tumor size, the decreased incidence of DNA heterogeneity for 'se-si' is probably because the spresd and progression of cancer resulted in competition of several clones and eventually in the appearance of a predominant clone. In this connection, Fujimaki et al., who collected one sample per 1 cm² cancer tissue and at least 4 samples in the case of a small lesion, reported that the incidence of DNA heterogeneity was highest for 'sm', 'mp' and 'ss' cases, but that the incidence was slightly lower for 'se' and 'si' cases [6]. Cancer that has invaded the serosa is usually large and a large number

of sample can be collected from this cancer. Despite these facts, the incidence of DNA heterogeneity for serosa-invading cancer was low. This can be attributed only to an unbalanced distribution due to competition among different clones, or to a decrease in the number of clones. In other words, it is likely that multiple clones are present in the relatively early stages of carcinogenesis ('m' and 'sm' cancer), and that during repetition of clonal evolution, several clones become predominant at some stages ('mp' and 'ss' cancer) and the most predominant clone occupy the greatest area in the last stages of cancer progression ('se' and 'si' cancer). According to Ooiwa et al. and Tadaoka et al. who studied serosa-invading gastric cancer in the direction perpendicular to the gastric wall, the incidence of DNA heterogeneity was about 30 %, which is much lower than the incidence often reported for gastric cancer in general [10, 24]. The evaluation of chromosomal heterogeneity on the basis of the assessment of chromosomes 7, 11 and 17 also revealed a tendency similar to that observed for the incidence of DNA heterogeneity (a decreased incidence in 'se' and 'si' cancer), as shown in Tables 2 and 3.

Although DNA ploidy is promising as a prognostic indicator of gastric cancer, the presence of DNA heterogeneity within a tumor can lead to erroneous judgments as to DNA ploidy. That is, there is a possibility that observation of the diploid portion of a tumor with DNA heterogeneity leads to an erroneous judgment that this tumor is a homogeneously diploid tumor. For this reason, it has been pointed out that multiple sites of a tumor need to be examined to make an accurate judgment as to ploidy. However, there is no widely accepted view as to the optimal number of sites of a tumor to be examined. In the present study, I paid attention to heterogeneous tumors composed of a mixture of areas with different DNA ploidy patterns, and compared the incidence of chromosomal numerical aberrations among the diploid portions of heterogeneous tumors (DD-H), the aneuploid portions of heterogeneous tumors (DA-H) and homogeneously diploid tumors (DD). When DNA diploid tumor was compared with DNA aneuploid tumor in past studies, the comparison was always made between DD and DA-H. In the present study, a comparison between DD and DA-H revealed that the incidence of numerical aberrations for each of the three chromosomes was significantly higher for DA-H than for DD. When a comparison was made between DD and DD-H (both of which are diploid tumors) and between DD-H and DA-H (two different regions of a heterogeneous tumor), the incidence of chromosomal numerical aberrations differed (Table 4).

The incidence of numerical aberrations of chromosome 7 was relatively high (20 %) even in DD, but it did not differ significantly between DD and DD-H. However, the incidence differed significantly between DD-H and DA-H, suggesting that chromosomal numerical aberrations

increase as DNA ploidy changes. In other words, it is likely that chromosome 7 can show two types of numerical aberration : (1) aberration which accompanies a diploid pattern, and (2) aberration which occurs when an aneuploid pattern appear. In DD, the incidence of numerical aberrations of chromosomes 11 and 17 was close to zero. In DD-H, however, it was significantly higher. The incidence of numerical aberrations of chromosome 11 was even higher in DA-H than in DD-H, although the incidence for chromosome 17 was similar between DA-H and DD-H. This tendency was significant in advanced cancer, and was noted even in early cancer although not significant. Thus, during DNA ploidy analysis of gastric cancer, the presence of numerical aberrations (in the direction of gain) of chromosomes 11 and 17 strongly suggests DNA heterogeneity even when the tumor appears to be diploid, and that such a tumor has characteristics similar to those of aneuploid tumor.

I then compared numerical aberrations of each chromosome between early and advanced cancers. In DD, the incidence of numerical aberrations of any of the three chromosomes did not differ significantly between early and advanced gastric cancer. However, the incidence in DD-H tended to be higher for advanced cancer, and the incidence of aberrations of each chromosome in DA-H was significantly higher for advanced cancer than for early cancer. These results suggest that numerical aberrations of chromosomes 7, 11 and 17 can serve as an indicator of the progression of DNA heterogeneous tumors. This finding is identical to the observation by Yamaguchi concerning chromosomal numerical aberrations of DNA aneuploid gastric cancer [21]. In homogeneously diploid tumor, on the other hand, it is likely that none of these three chromosomes is involved in cancer progression, or that the progression of this tumor occurs in a completely different manner.

The DNA aneuploidy formation process was compared among DD, DD-H and DA-H by analyzing the incidence of chromosomal numerical aberrations. The total incidence of chromosomal numerical aberrations was high in the order of DD < DD-H < DA-H. When analyzed for individual chromosomes, the incidence of numerical aberrations of chromosomes 11 and 17 was higher in DD-H than in DD, and the incidence for chromosomes 7 and 11 was higher in DA-H than DD-H. These findings suggest that an increase in chromosomes 11 and 17 occurs first to yield DD-H, and that a subsequent increase in chromosome 7 and a further increase in chromosome 11 makes the tumor DNA aneuploid. Since the incidence of numerical aberrations of chromosome 7 was high (20 %) even in diploid tumors, this chromosome seems to be involved even in early stages of carcinogenesis.

A clonal evolution theory has been proposed to explain how a tumor becomes malignant [28, 29]. According to this theory, a tumor acquires new genetic characters to

become more malignant, eventually leading to chromosomal numerical aberrations and abnormal nuclear DNA contents. Briefly, the increase in the malignancy level of a tumor is understood as an increase in genetic instability, according to this theory. When viewed from nuclear DNA contents, genetic instability seems to be higher for DNA heterogeneous tumor than for homogeneously diploid tumor. It is also likely that genetic instability is high in the order of DD < DD-H < DA-H, if the incidence of chromosomal numerical aberrations is taken into consideration. Therefore, if DNA ploidy analysis is combined with the analysis of chromosomal numerical aberrations, it will be possible to identify those types of DNA diploid tumor which have high genetic instability and are more likely to become malignant. In conclusion, the combination of DNA ploidy analysis and chromosomal numerical aberration analysis provides information useful for the diagnosis of hematological tumor which is often considered to be DNA diploid and the diagnosis of borderline tumor for which a judgment of benign or malignant nature is difficult.

Conclusion

1. Even in the case of gastric cancer which is classified as DNA diploid on the basis of DNA ploidy analysis of one site of the tumor, the presence of numerical aberrations (in the direction of gain) of chromosome 11 and 17 suggests a high probability that this tumor is DNA heterogeneous. Such tumors need to be regarded as DNA aneuploid tumors.

2. When the DNA aneuploidy formation process for gastric cancer is viewed from the aspect of chromosomal numerical aberrations, it seems that an initial increase in chromosomes 11 and 17 yields DD-H, and that a subsequent increase in chromosome 7 and a further increase in chromosome 11 makes the tumor DNA aneuploid.

3. If DNA ploidy analysis is combined with the analysis of chromosomal numerical aberrations using FISH, it will be possible to identify those particular types of DNA diploid tumor which have higher genetic instability and a higher likelihood to become malignant when compared the other types of DNA diploid tumor. Therefore, the combination of these two analyses will provide information valuable to determining the malignant level of a given DNA diploid tumor.

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