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Amplification of growth factor receptor genes and DNA ploidy pattern in the progression of gastric cancer

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Abstract To study the background of oncogene amplification in gastric cancers, we examined the correlation between occurrence of oncogene amplification and DNA ploidy pattern. In 57 primary gastric cancers, amplifications of *c-erbB*, *c-erbB-2*, *c-met* and *K-sam* genes were investigated by Southern blot analysis, and the DNA ploidy pattern was determined by static cytofluorometry and by flow cytometry. Oncogene amplification was detected in 11 cancers, 10 of which were advanced gastric cancers and 1 was an early differentiated type. The amplification of *c-erbB-2* and *K-sam* genes was found exclusively in differentiated- and undifferentiated-type cancers, respectively. Of the 11 cancers, 5 were DNA-diploid and 6 were DNA-aneuploid. All the 11 tumours with oncogene amplification contained polyploid cell populations (polyploidy), whereas none of the tumours without polyploidy showed oncogene amplification. In differentiated-type cancers the incidence of polyploidy was high in both early and advanced stages, while in undifferentiated-type cancers it was low in early stages but significantly higher in advanced stages. It was thus shown that amplification of growth factor receptor genes is closely related to the presence of polyploidy, irrespective of any different stemline DNA-ploidy mode. The time-course of oncogene amplification and kinds of genes amplified may differ between differentiated- and undifferentiated-type gastric cancers.

Key words Gastric cancer · Growth factor receptor gene · DNA-ploidy pattern · Cytofluorometry · Gene amplification

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Introduction

Malignant tumours often involve amplifications of various oncogenes. In gastric cancers, it has been shown that growth factor receptor genes such as *c-erbB*, *c-erbB-2*, *c-met* and *K-sam* are often amplied [18, 20, 35, 36]. These are cellular proto-oncogenes that encode epidermal growth factor receptor (EGFR), EGFR homologue, hepatocellular growth factor receptor (HGFR), and keratinocyte growth factor receptor (KGFR), respectively [5, 10, 15, 26]. These oncogenes have transforming activity, and their products have tyrosine kinase activity [1, 3, 15, 17, 22, 32, 33]. It has been reported that amplification of such oncogenes is closely related to histological type in gastric cancers; amplifications of *c-erbB-2* and *K-sam* occur in differentiated and in undifferentiated types, respectively [20, 35]. However, *c-met* is amplified in both histological types, especially in scirrhous carcinoma [18]. These results were based on observations in advanced gastric cancers, and it remains unclear how those gene amplifications occur during development and in the earlier stages of gastric cancers. To clarify this point, we studied the incidence of amplification of four growth factor receptor genes in early and advanced gastric cancers and compared the difference between two major histological types.

Alterations of the DNA-ploidy pattern are often detected in various types of human malignant tumours and apparently to correlate with malignant biological behaviours. The abnormality in DNA ploidy patterns generally reflects large-scale changes in chromosomal number and structure, as demonstrated in several studies on the correlation between DNA ploidy pattern and karyotypes [23, 24]. Cytogenetic studies have revealed that the tumours with gene amplifications frequently also have numerical and structural chromosome aberrations [25]. Therefore, alterations of DNA ploidy pattern and gene amplifications might occur simultaneously against a common background. We investigated the relationship between the occurrence of oncogene amplification and DNA-ploidy pattern, paying particular attention to the occur-

rence of polyploidy as well as DNA-aneuploidy. For a detection of small polyploid and DNA-aneuploid cells in the cancer, we adopted static cytofluorometry as the basic method of ploidy analysis.

Materials and methods

We examined 57 surgically resected primary gastric cancers, including 20 early cancers (without invasion to/beyond the proper muscle layer) and 37 advanced cancers (with invasion deeper than the proper muscle layer). Histologically, 27 were of a differentiated type and 30 of an undifferentiated type. The former included 19 well-differentiated adenocarcinomas (10 early and 9 advanced cancers) and 8 moderately differentiated adenocarciomas (8 advanced cancers). The latter contained 9 solid-type, poorly differentiated adenocarciomas (1 early and 8 advanced cancers), 9 non-solid-type, poorly differentiated adenocarcinomas (1 early and 8 advanced cancers), 9 signet ring cell carcinomas (8 early and 1 advanced cancers) and 3 mucinous adenocarcinomas (3 advanced cancers). Histological classification, staging and other pathological analyses were based on the general rules of the Japanese Research Society for Gastric Cancer (GRSGC) [16]. A small part of the cancer and the normal gastric mucosa were sampled fresh from each case and stored at –80°C. The rest of the tissue was fixed in formalin.

For Southern blot analysis the probes used were 2.4 kbp cDNA of EGFR, 0.44 kbp genomic DNA of *c-erbB-2* and 1.6 kbp genomic DNA of *c-met*, which were provided by the JCRB (Japanese Cancer Research Resource Bank) [8, 26, 31]. The 0.46-kbp *K-sam* cDNA probe was synthesized from the total RNA of Kato III cells by reverse transcription and polymerase chain reaction using two synthetic oligonucleotide primers: 5'-TGTTGAAAGATGCCGCCGTGAT-3' and 5'-GTGTGATTGATGGACCCGTATT-3' [14]. The DNA sequence of the synthesized probe was confirmed by the dideoxy method. As internal control, a 1.2 kb β-actin cDNA probe was used. These probes were labelled with ^{32}P by the random priming method.

From the tumour and the normal tissue stored in –80°C, high-molecular-weight DNA fragments were isolated by phenol chloroform after digestion with proteinase K. The DNAs were digested completely with two types of restriction enzymes, HindIII and EcoRI (Takarashuzo, Ohtsu, Japan), after which 10 μg of the DNAs was electrophoresed in 1% agarose gel, denatured, neutralized, and transferred to nylon membrane (Hybond N+, Amersham International, Amersham, UK). The membranes were hybridized with ^{32}P labelled *c-erbB*, *c-erbB-2*, *c-met*, *K-sam* and β-actin probes. After washing, autoradiography and densitometric quantification were performed. Significant gene amplification was defined as 3-fold or greater increase of the signal intensity in comparison with the corresponding normal tissue.

Cytofluorometric analysis was carried out on formalin-fixed, paraffin-embedded tumour tissue taken from the portion adjacent to the sample used for frozen specimen: serial 4-μm and 100-μm thick sections were taken. Sections 4-μm thick were stained with haematoxylin and eosin for histological examination, and 100-μm-thick sections were used for cell isolation. After deparaffinization of 100-μm sections, we isolated tumour tissues by removing the normal tissue under a stereoscopic microscope. The isolated tissue fragments were digested with 100 μg/ml of proteinase K (Sigma Chemical, St. Louis, Mo.) and were homogenized mildly to facilitate mechanical cell isolation. The nuclear suspensions obtained were smeared on glass slides with an automatic smear maker (Auto Smear CF12C, Sakura Seiki, Tokyo, Japan).

The smear preparations were stained with DAPI (4',6-diamidino- 2-phenylindole dihydrochloride) solution (50 ng/ml of DAPI in antifade solution: 10 mM Tris/10 mM EDTA/100 mM NaCl/10 mM 2-mercaptoethylamine, pH 7.4) for 30 min at room temperature and mounted with staining solution [11]. The nuclear DNA content was measured on each smear with a fluorescence cytophotometer (Nikon P1, Nikon, Tokyo, Japan). The DNA contents of 40 lymphocytes that were concomitant with cancer cells on the slide were measured first, and the mean value was determined as the internal diploid (2C) standard. We then measured 300 tumor nuclei randomly and the nuclear DNA content was expressed in frequency histograms.

The DNA histograms with the modal values between 1.8 C and 2.2 C were considered to be DNA-diploid stemline. The histograms with well-defined peaks comprising over 5% of all the counts outside the DNA-diploid or DNA-tetraploid (3.7 C–4.3 C) ranges and those with the peak comprising over 20% of the counts in the DNA-tetraploid range were considered to be DNA-aneuploid. In addition, we paid particular attention to the presence of a polyploid cell population as polyploidy, where the DNA content was greater than 5 C in DNA-diploid tumours or 2.5 times the stemline DNA content in DNA-aneuploid tumours.

For cases with oncogene amplification, we determined the DNA-ploidy pattern by flow cytofluorometry in addition to the static cytometry. After treatment with 0.1% RNase in 0.1 M phosphate buffer (pH 7.0), the nuclear suspension was stained in propidium iodide (20 μg in 1 ml of 0.034 M sodium citrate for 2 h at 37°C) according to Vindelov's method [34]. The nuclear DNA contents of 10,000 cells in suspension were measured with FACScan flow cytometer (Becton Dickinson Immunocytometry System, Mountain View, USA). The DNA index (DI) was calculated, with the major peak of the internal reference cells taken as diploid. The DNA histograms of the single stemline with their DI ranging from 0.95 to 1.05 were defined as DNA-diploid. The histograms with well-defined peaks comprising over 5% of all the counts outside the DNA-diploid and DNA-tetraploid (1.90–2.10 of DI) ranges and those with the peak comprising over 20% of the counts in the tetraploid range were interpreted as DNA-aneuploid. The coefficient of variation of the diploid peak varied from 5.5% to 9.2%.

Results

Among 57 gastric cancers, oncogene amplification was detected in 11 tumours. Amplification of *c-erbB*, *c-erbB-2*, *c-met* and *K-sam* genes was detected in 1 (2%), 4 (7%), 5 (9%) and 3 (5%) gastric cancer(s), respectively (Table 1). By densitometric comparison of signal intensity between the tumours and the normal DNA, *c-erbB*, *c-erbB-2*, *c-met* and *K-sam* were amplified 11-fold, 3- to 8-fold, 3- to 6-fold, and 3- to 13-fold, respectively. There were 2 gastric cancers with co-amplification of *c-erbB* and *c-erbB-2*, and *c-erbB-2* and *c-met* (Fig. 1, Table 1). Of all the 11 cancers with oncogene amplification, 10 were advanced cancers (27% of the advanced cancers) and the remaining case was an early cancer of differentiated type (5% of the early cancers). There was a significant difference in incidence of the gene amplification between the early and the advanced gastric cancers (Chi-square test, $P < 0.05$). Amplification of the *c-erbB-2* gene occurred only in differentiated-type cancers (18% of the differentiated-type advanced cancers), whereas the *K-sam* gene was amplified only in undifferentiated-type (15% of the undifferentiated-type advanced cancers). Amplification of the *c-met* gene was found in both differentiated- and undifferentiated-type cancers.

Static cytofluorometry showed that 33 gastric cancers (58%) were DNA-diploid and 24 tumours (42%) were DNA-aneuploid (Table 2). The incidence of DNA-aneuploidy tended to be correlated with tumour stage in both histological types: the incidence of DNA-aneuploidy was

Table 1 Incidence of amplification of growth factor receptor genes

	Number of patients	Oncogene amplification (%)				Total
		c-erbB	*c-erbB-2*	*c-met*	*K-sam*	(%)
Differentiated type						
Early	10	0 (0)	1 (10)	0 (0)	0 (0)	1 (10)[b]
Advanced	17	1 (6)	3 (18)	2 (12)	0 (0)	4[a] (24)[c]
Undifferentiated type						
Early	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)[d]
Advanced	20	0 (0)	0 (0)	3 (15)	3 (15)	6 (30)[e]
Total	57	1 (2)	4 (7)	5 (9)	3 (5)	11 (19)

[a] Including two co-amplification cases
[b–e] For b+d versus c+e, Chi-square = 4.1 ($P<0.05$)

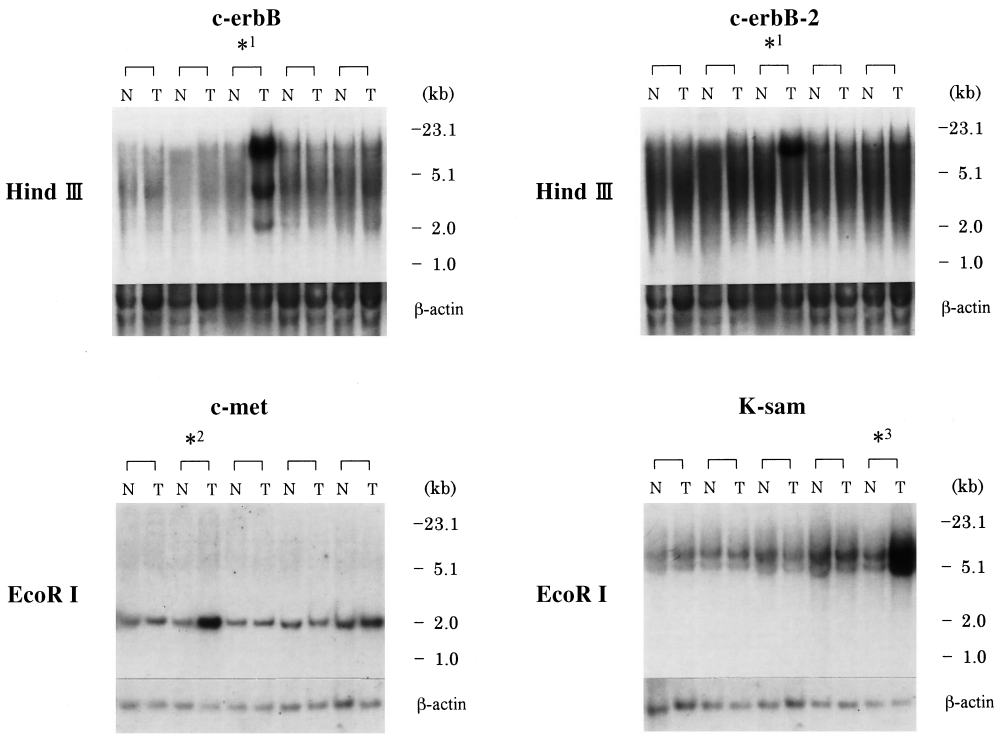

Fig. 1 Southern blot analysis with *c-erbB*, *c-erbB-2*, *c-met*, and *K-sam* probes. **1* A case with co-amplification of *c-erbB* and *c-erbB-2* genes. **2* A case with amplification of the *c-met* gene. **3* A case with amplification of the *K-sam* gene (*T* tumour tissue, *N* corresponding normal mucosa)

lower in the early cancers, especially in undifferentiated type, than in the advanced cancers, although the difference was not statistically significant.

Polyploidy was detected in 20 out of 33 DNA-diploid tumours (61%) and in 21 of 24 DNA-aneuploid tumours (88%) (Table 2). The incidence of polyploidy in the DNA-diploid tumours was lower in the early than in the advanced cancers in undifferentiated-type tumours, but was similar in early and advanced tumours in differentiated-type cancers. In the DNA-aneuploid tumours polyploidy seemed to occur more frequently in differentiated-type cancers, irrespective of tumour stage. Statistically, there was a significant correlation between tumour stage and incidence of polyploidy in undifferentiated-type cancers (Chi-square test, $P < 0.01$).

The DNA ploidy pattern and clinicopathological features of the tumours with oncogene(s) amplification are shown in Table 3. Among the 10 tumours tested both by static cytofluorometry and by flow cytometry, 9 showed good concordance in the ploidy mode. In the remaining case, which was a scirrhous carcinoma (non-solid undifferentiated-type cancer) with an abundance of stromal components, detection of the DNA-aneuploid peak was difficult by flow cytometry alone. The flow cytometric analysis could not distinguish a polyploid peak from the background noise in any tumours in which polyploidy was detected in static cytofluorometry (Fig. 2).

Table 4 showed the relationship between the oncogene amplification and the DNA ploidy pattern. The oncogene amplification was found in 5 of the 33 DNA-diploid cancers (15%) and in 6 of the 24 DNA-aneuploid cancers (25%). There was no statistically significant correlation between oncogene amplification and DNA-ploidy mode (Chi-square test, $P > 0.05$). However, all the

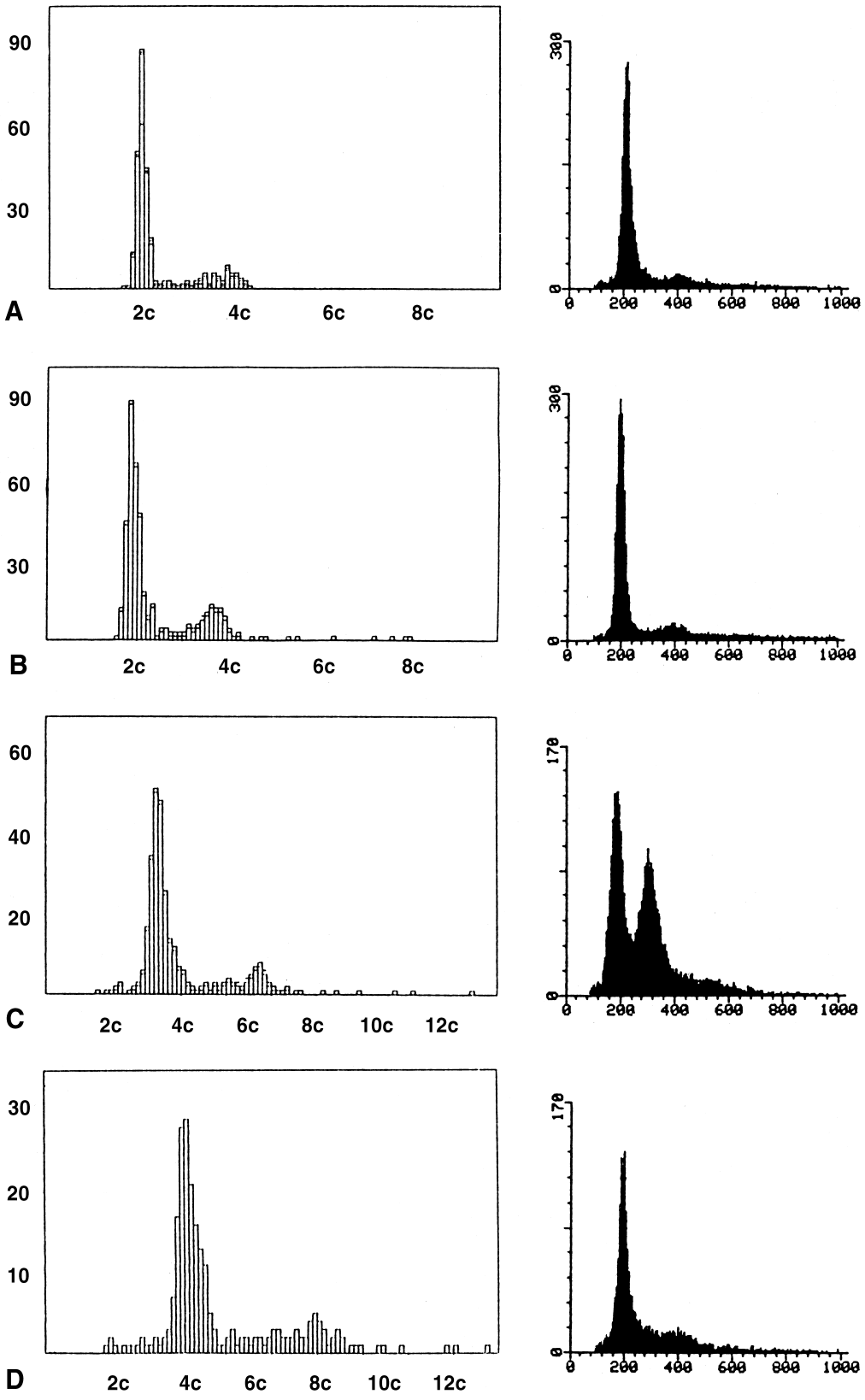

Fig. 2 A–D Comparison of DNA ploidy patterns determined by static and flow cytometries. Corresponding sets of DNA histograms of static cytofluorometry of tumour cells (*left side*) and flow cytometry of tumour and normal cells (*right side*) in representative cases. **A** A case showing the DNA-diploid pattern without polyploid component. **B** A case showing the DNA-diploid pattern with polyploid component. No polyploid peak is discernible on flow cytometry. **C** A case showing the DNA-aneuploid pattern. The G_0G_1 peak of static cytofluorometry corresponds to the second G_0G_1 peak of the flow cytometry with their mode values concordant to each other. Polyploid component is revealed only on static cytofluorometry. **D** A case showing the DNA-aneuploid pattern with polyploidy on static cytofluorometry and the DNA-diploid pattern on flow cytometry

Table 2 DNA ploidy pattern (with polyploidy) of gastric cancers studied

	Differentiated type		Undifferentiated type		Total
	Early	Advanced	Early	Advanced	
Number of patients	10	17	10	20	57
Diploidy (%)[a]	7 (70)	6 (35)	9 (90)	11 (55)	33(58)
Polyploidy(+) (%)[b]	5 (71)	4 (67)	2 (22)[a1]	9 (82)[b1]	20(61)
Aneuploidy (%)[a]	3 (30)	11 (65)	1 (10)	9 (45)	24(42)
Polyploidy(+) (%)[b]	3 (100)	11 (100)	0 (-)[a2]	7 (78)[b2]	21(88)

[a] Percentage of DNA-diploid or DNA-aneuploid tumours in each number of patients
[b] Percentage of tumour with polyploidy in DNA-diploid or DNA-aneuploid tumour in each column
[a1–b2] For a1+ a2 versus b1+ b2, Chi-square = 10.0 (P<0.01)

Table 3 Clinicopathological features and DNA ploidy pattern of gastric cancers with oncogene amplifications (*Un* undifferentiated-type of gastric cancer, *Di* Differentiated-type of gastric cancer, *tub1* well-differentiated adenocarcinoma, *tub2* moderately differentiated adenocarcinoma, *por1* poorly differentiated adenocarcinoma, solid type, *por2* poorly differentiated adenocarcinoma, non-solid type, *se* exposed on serosal surface, *ss* subserosa sm submucosa, *P* peritoneal dissemination, *H* liver metastasis, *A* aneuploidy, *D* diploidy, *N.T.* not tested)

Patient no.	Age sex	Histology	Depth of invasion	Lymph node metastasis	Distant metastasis	Oncogene amplification	Static cytofluorometry		Flow cytometry	
							Ploidy	DNA content	Ploidy	DNA Index
Advanced cancer										
1	60 F	Un (por2)	se	+	–	c-met	D	2	D	1
2	60 M	Di (tub2)	ss	–	-	c-erbB-2	A	2.6, 3.3	A	1.4, 1.6
3	72 M	Di (tub1)	se	+	–	c-erbB, c-erbB-2	A	3.6	A	1.8
4	59 M	Un (por1)	se	+	–	K-sam	A	2.3	A	1.2
5	49 M	Un (por1)	ss	–	-	c-met	D	2	D	1
6	47 F	Di (tub2)	se	+	P	c-erbB-2, c-met	A	3.6	N.T.	N.T.
7	62 M	Un (por2)	se	+	P	c-met	A	4.2	D	1
8	83 M	Un (por2)	se	+	P,H	K-sam	D	2	D	1
9	57 F	Di (tub1)	se	+	–	c-met	A	3	A	1.6
10	78 M	Un (por2)	se	–	-	K-sam	D	2	D	1
Early cancer										
11	53 M	Di (tub1)	sm	–	-	c-erbB-2	D	2	D	1

tumours with oncogene amplification had polyploidy irrespective of DNA-ploidy mode, while none of the tumors without polyploidy showed oncogene amplification. There was a significant difference in the incidence of oncogene amplification between the tumours with and without polyploidy (Fisher's exact test, $P < 0.05$).

Discussion

Our present study shows that several growth factor receptor genes are amplified in gastric cancer. Several gene abnormalities were common to the two main histological types, and others differed, with *c-erbB-2* in differentiated-type and *K-sam* in undifferentiated-type gastric cancers, confirming earlier descriptions [18, 20, 35, 36]. Compared with breast and colon cancers, gastric cancers have a wider range of histological variation and biological behaviour. The amplification of these various growth factor receptor genes may delineate the different characteristics of gastric cancers.

The aetiology of oncogene amplification is not fully elucidated, and it is not clear how oncogene amplification is related to carcinogenesis and tumour progression [25]. In the present study, most of the tumours with oncogene amplification were advanced. There are at least two possible explanations for this. Oncogene amplification may occur as a late event in the progression of gastric cancer. It is also possible that the oncogene amplification may occur in earlier stages but that such tumours progress so rapidly that they present as advanced cancers.

For further analysis on the time-course of gene amplification, we have to search for useful factors that are closely correlated with oncogene amplification and tumour progression. One candidate we have examined is the DNA-ploidy pattern, because it seemed very important to examine quantitative change of DNA to study a mechanism of oncogene amplification at an earlier stage. In addition, the occurrence of both DNA-aneuploidy and DNA-polyploidy has been reported by us to correlate with the progression of gastric cancer [12, 13, 28]. The

Table 4 Correlation between oncogene amplification and DNA ploidy pattern with and without polyploidy

	Number of patients	Amplification (+)	Amplification (–)
Diploidy (%)[a]	33	5 (45)[d]	28 (62)[d]
Polyploidy(–) (%)[b]	13	0 (0)[f]	13 (46)[f]
Polyploidy(+) (%)[b]	20	5 (100)[g]	15 (54)[g]
Aneuploidy (%)[a]	24	6 (55)[e]	18 (38)[e]
Polyploidy(–) (%)[c]	3	0 (0)[h]	3 (17)[h]
Polyploidy(+) (%)[c]	21	6 (100)[i]	15 (83)[i]
Total	57	11	46

[a] Percentage of DNA-diploidy or DNA-aneuploidy in each tumors with or without gene amplification
[b] Percentage of tumor with or without polyploidy in each DNA-diploid tumors with or without gene amplification
[c] Percentage of tumor with or without polyploidy in each DNA-aneuploid tumors with or without gene amplification
[d–i] For d vs e difference is not significant (Chi-square test, $P>0.05$) For f+h versus g+i, $P<0.05$ (Fisher's exact test)

occurrence of DNA-aneuploidy, however, did not show any relationship to oncogene amplification (Table 4). There are several reports stating that in breast cancer [2, 29] and soft tissue sarcoma [4] DNA-aneuploidy is not correlated with amplification of *c-erbB-2* and *c-myc* genes, respectively, and that in neuroblastomas amplification of *N-myc* gene occurs in DNA-diploid tumours [7].

Extending our attention to the occurrence of polyploidy in this study, we found that all the tumours with oncogene amplification were accompanied by polyploidy, whereas none of the tumours without polyploidy had oncogene amplification. We consider that it is important to detect polyploidy to know about chromosome instability in the tumour, because polyploidy is detected quite commonly in DNA-aneuploid tumours but not in the normal gastric mucosa [12, 28]. Furthermore, there is experimental and clinical evidence that polyploidization precedes the occurrence of DNA-aneuploidy and further evolution of new stemlines from the DNA-aneuploid cell population during tumour progression [6, 9, 21, 27]. In this respect, the occurrence of polyploidy in the tumour is considered to reflect enhanced chromosome instability, and the oncogene amplification may result from this enhanced genetic instability.

Cytometrically determined DNA-diploidy does not always indicate true diploidy, but it also includes "near-diploid" aneuploidy, which has some numerical chromosomal changes within the range of DNA-diploidy. This cannot be studied by conventional cytometric methods. In this point, our present study did not rule out the possibility that "DNA-diploid" cancers with polyploidy have minor numerical changes of chromosome. A close linkage between near-diploid aneuploidy and the occurrence of polyploidy must be further studied by another method, such as a cytogenetic study in combination with fluorescence in situ hybridization study on centromere numbers [19]. It is thus possible that oncogene amplification may occur only in such cytogenetically aneuploid cells, although they seem to be DNA-diploidy on conventional cytometric studies.

The close linkage between oncogene amplification and polyploidy suggests that the occurrence of polyploidy may predispose to oncogene amplification. From this point of view, we can speculate how oncogene amplification occurs in gastric cancers. As shown in Table 2, differentiated-type cancers showed a higher incidence of polyploidy in both early and advanced stages, whereas in undifferentiated-type cancers the incidence of polyploidy was low in the early stages. It increased significantly in advanced stages. It seems that oncogene amplification is unlikely to occur in earlier stages of undifferentiated-type gastric cancers. However, gene amplification may occur in earlier stages in differentiated-type gastric cancers. In this study, the early cancer with the oncogene amplification was of the differentiated-type. Since the incidence of the oncogene amplification was much lower in earlier than in advanced stages in differentiated-type cancers, this type of cancer appears to progress very rapidly to an advanced stage. This hypothesis is partially supported by the report that *c-erbB* and *c-erbB-2* are amplified more frequently in metastatic tumours than in primary (differentiated-type) tumours of the stomach [30].

From a technical point of view, it was difficult to detect a small polyploid component by flow cytometry. In addition, in a tumour with abundant stroma, such as scirrhous carcinoma, isolated DNA-aneuploid cells can be so small in number relative to the stromal cells that they are readily masked in flow cytometry, as demonstrated in one of the tumours in our study (Fig. 2, Table 4) [4, 28]. This limitation could be overcome by static cytofluorometry, which can readily discriminate a small number of tumour cells from normal stromal cells. Although static cytofluorometry may be thought to be less objective than flow cytometry, the mode values of the stemline ploidy attained good concordance between flow and static cytometries [28].

In conclusion, the amplification of growth-factor-receptor genes was found to occur in close relationship to the presence of polyploidy, irrespective of the DNA-ploidy modes. It was suggested that the occurrence of polyploidy, which may reflect enhanced genetic instability, predisposes to oncogene amplification. Considering the incidence of polyploidy, both the time-course of amplification and the type of amplifying gene may differ between differentiated-type and undifferentiated-type gastric cancers.

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