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DNA-ploidy and survival in gastric carcinomas: a flow-cytometric study

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Summary. In 125 gastric carcinomas the nuclear DNA content was determined by flow cytometry from formalin-fixed and paraffin-embedded tissue of surgical specimens. The carcinomas were of intestinal or mixed type (85), and diffuse type (40). DNA-aneuploidy was found in 46% of the intestinal type and in 42% of the mixed type, but only in 15% of the diffuse-type carcinomas ($P<0.01$). The total rate of DNA-aneuploidy was 34%. Carcinomas localized in the cardia were more frequently DNA-aneuploid than tumours in other localizations ($P<0.01$). DNA-aneuploid carcinomas had metastasized more frequently to regional lymph nodes ($P<0.05$) whereas no correlations with tumour stage and cytological/histological grade were detected. In 94 patients follow-up data were available. DNA-aneuploidy was associated with a statistically significant poorer prognosis when compared to DNA-diploid tumours only in advanced gastric carcinomas with lymph node metastases ($P=0.0488$) and in the subgroup of advanced intestinal and mixed-type tumours ($P=0.0289$).

Key words: Flow cytometry – Gastric cancer – DNA-aneuploidy – Prognosis – Histological type

Introduction

In spite of the decrease in incidence and mortality of gastric carcinoma in many developed countries (Becker et al. 1984; Braun 1988), gastric cancer is still an important cause of death. In 1988 14500 patients with gastric carcinoma died in the Federal Republic of Germany (Statistisches Bundesamt, 1988) despite improved endoscopic diagnosis and surgical treatment. The 5-year survival rate for all stromach carcinomas is about 10–15% with broad variations depending on the cohort studied, the tumour histology and therapy (Bizer 1983; Braun 1988; Dupont et al. 1978; Korenaga et al. 1988; Pagnini and Rugge 1985). The most important factor determining prognosis of stomach cancer is the tumour stage (UICC 1989) with 5-year survival rates for patients in treatable groups of 30–40% and for early cancers (tumour infiltration limited to mucosa or submucosa) of up to 90%. Tumour grade, growth pattern (Ming 1977) and histological type (Lauren 1965) seem to be of additional prognostic value (Santini et al. 1987; Stemmermann and Brown 1974; Viste et al. 1986). Despite the widespread use of these determinants, the conventional tumour classifications are only of limited value in predicting the biological aggressiveness of a malignant neoplasm. In the literature the DNA content of tumour cells is suggested to be an additional variable of prognostic significance (Atkin and Kay 1979; Baisch 1984; Barlogie et al. 1983; Böhm and Sandritter 1975; Friedlander et al. 1984). The purpose of the present study was to assess the prognostic value of the DNA content of gastric carcinoma cells and to compare it with usual tumour classifications. Special attention was paid to the different histological types of gastric carcinomas according to Lauren's 1965 classification.

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Materials and methods

The study was done on formalin-fixed and paraffin-embedded tumour material obtained from resection specimens of 125 patients with primary gastric cancer: sixty-five (52%) were female and 60 (48%) were male. Ages ranged from 39 to 87 years (mean age 66.8 ± 11.3 years, Table 1). The patients underwent surgery between 1979 and 1986. Despite less intensive histological examination of the material (especially the lymph nodes) before 1985, these cases were chosen because of the longer observation time. From 110 cases an average of five lymph nodes had been prepared. Each lymph node was examined routinely in four sections. The localization of the lymph nodes in most cases (before 1985) had not been described exactly, and therefore only a classification of cases with or without metastatic infiltration in the lymph nodes was performed in this study. For comparative DNA study between primary tumours and lymph node metastases, only lymph nodes with a

Table 1. Gastric carcinomas ($n=125$): DNA ploidy, histological type (Lauren), and pT stage

	pT1	pT2	pT3	pT4	Total – diploid – aneuploid
Intestinal	9	51	0	1	61 (49%)
– diploid	8	24	0	1	33 (54%)
– aneuploid	1	27	0	0	28 (46%)
Mixed	7	15	2	0	24 (19%)
– diploid	4	9	1	0	14 (58%)
– aneuploid	3	6	1	0	10 (42%)
					$P<0.01$
Diffuse	8	23	3	6	40 (32%)
– diploid	8	18	3	6	35 (87.5%)
– aneuploid	0	5	0	0	5 (12.5%)
Total	24 (19%)	89 (71%)	5 (4%)	7 (6%)	125 (100%)
– diploid	20 (83%)	51 (57%)	4 (80%)	7	82 (66%)
– aneuploid	4 (17%)	38 (43%)	1 (20%)	0	43 (34%)

tumour infiltration of at least 0.5 cm extent were analysed (56 cases). The tumours were classified according to pT stages (UICC 1989) and to Lauren (1965), and graded according to the WHO classification (Watanabe et al. 1990). Those carcinomas with a blend of both intestinal and diffuse components (at least about 25%) were classified as mixed type. Of the 125 patients, 13 died within 30 days after operation and in 18 cases only incomplete follow-up data were available. The remaining 94 patients were followed up to 108 months (mean time of observation 27.9 months).

After excising necrotic areas and excess of normal tissue according to the H&E slides, nuclear preparation was performed using a modification of the method of Hedley et al. (1983, 1985). One to four (on average, two) paraffin blocks were available from each primary tumour, depending on tumour size. Five to 15 sections (30 µm) were cut from each paraffin block. The sections were dewaxed in xylol on a shaker (30 min) and rehydrated by passing through a series of alcohols (100%, 98%, 70% and 40% ethanol). The sections were washed twice in distilled water and minced by scissors. The next step was an enzymatic digestion with 5 ml 0.5% pepsin (Sigma, St. Louis, Mo., P-6887) in 0.9% NaCl adjusted to pH 1.1 with 1 M HCl and incubated at 37° C for 75 min on a rocking table. After filtration (30 µm nylon mesh) the probes were centrifuged at 1000 U/min for 10 min and stained by suspending them in a solution of propidium iodide (0.13 mg/ml; Aldrich, Steinheim, FRG, no. 28,707-5) containing 0.1% RNase (Sigma, R-4875). The suspensions were adjusted to a concentration of $2-4\times10^6$ nuclei/ml. Nuclear DNA content was measured using an Ahrens FCM "Phoenix" (Ahrens, Bargteheide, FRG), a modification of the instrument described by Steen and Lindmo (1979) with a mercury arc lamp as light source. Excitation wavelength was 546 ± 14 nm (Leitz filter block M2). The signals were registered by a photomultiplier; from each probe 20000 impulses were measured and the data were analysed with the FDAS software package (Ahrens). In diploid tumours the resulting histograms showed only one peak demonstrating a coincidence of tumour cells and normal cells after excision of residual diploid cells from surrounding tissue, lymphocyts, and stromal cells. In aneuploid carcinomas one or more additional abnormal stem lines were present. For each tumour a DNA index (DI) was determined by the ratio of fluorescence intensity of the tumour cell peak (channel number) and fluorescence intensity of the normal/diploid peak (channel number). For DNA-aneuploidy a minimal DI of 1.1 was defined. The coefficient of variation (CV) determined by

$$CV=\frac{\text{number of channels at half of peak}\times e^{-1/2}}{\text{mean channel number of peak}}$$

served as a parameter for the quality of the measurements. No histograms exceeding CV 8.0% were accepted. The mean CV of all measured DNA-diploid peaks was $5.49\%\pm1.51$.

In a preliminary series of 5 gastric carcinomas the ploidy was determined from fresh material and from corresponding formalin-fixed and paraffin-embedded tissue. The results concerning DNA ploidy and DI showed no differences, whereas the rate of nuclear debris and the CV values were higher in the latter group (Baretton et al. 1987).

Correlations between DNA ploidy and clinical and pathological features were examined by using the chi-square test, Kaplan-Meier survival curves and the log rank test (SPSS statistical software).

Results

Using the Lauren classification 61 (43%) tumours were of the intestinal type, 40 (32%) were of the diffuse type and in 24 (19%) of the carcinomas a mixed histological pattern was present with tubular and diffuse portions within the same tumour. DNA-aneuploidy was observed in a statistically significant higher rate in carcinomas of the intestinal (46% of cases) and mixed types (42% of cases) as compared to the diffuse gastric carcinomas (12.5% of cases; $P<0.01$; Table 1, Figs. 1, 2). Without differentiation of the histological type 82 (66%) of the 125 cases studied were DNA-diploid, 43 (34%) were DNA-aneuploid (Table 1). In 4 cases (3%) a heterogeneous DNA ploidy was detected. No correlation was found between DNA ploidy and patients' sex and age. Diffuse-type carcinomas showed a trend to be in more advanced tumour stages, but no clear correlations were found between ploidy and either the pT stage (Table 1) or the histological/cytological grade according to the WHO classification (Watanabe et al. 1990; Table 2). The frequency of lymph node metastases, however, was significantly higher in DNA-aneuploid primary tumours (77% vs 55% in DNA-diploid carcinomas, $P<0.05$) in the 110 cases in which a complete lymph node status was available (Table 3). The comparison of DNA content in primary tumours and lymph node metastases ($n=56$) resulted in a difference of DNA ploidy in 46% (11/24) of DNA-aneuploid cancers, but only in 6% (2/32) of DNA-diploid tumours ($P<0.01$).

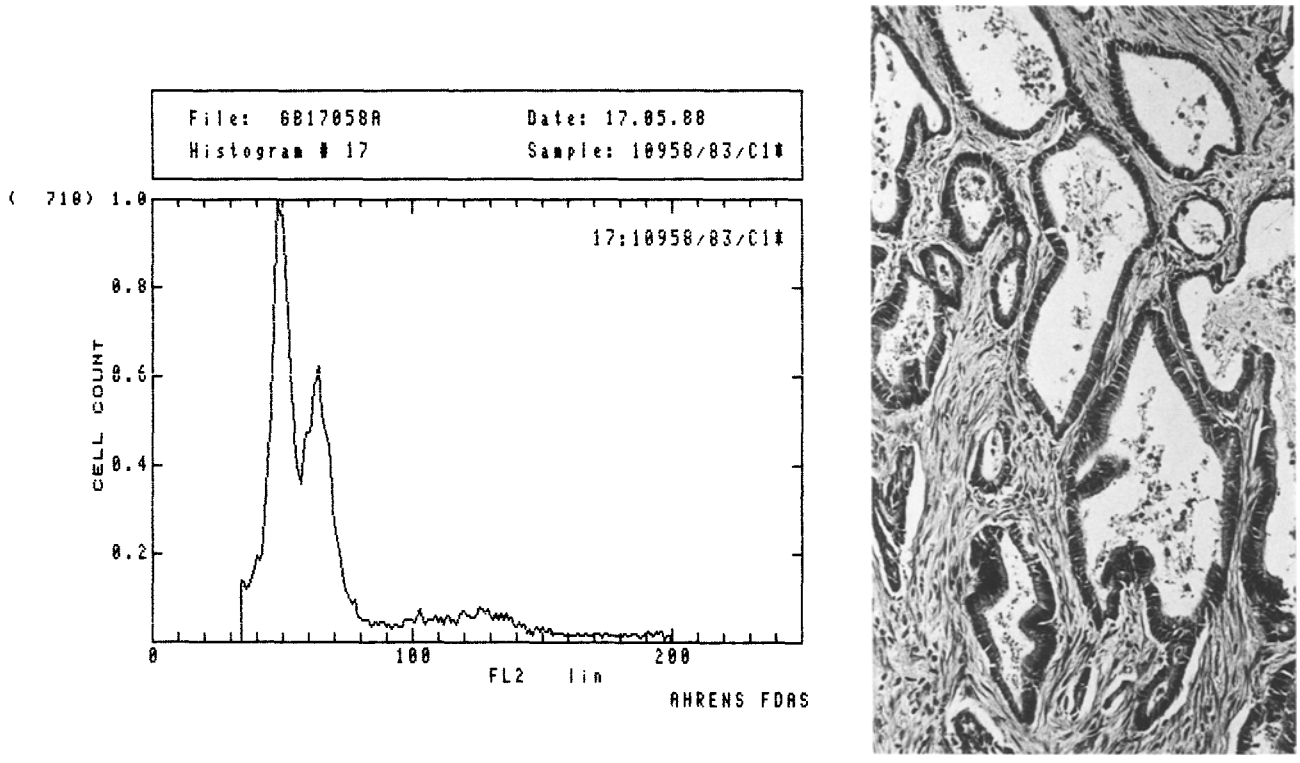

Fig. 1. Gastric carcinoma, intestinal type (pT2, pN pos, G1, H&E × 100) with an aneuploid DNA content (DI = 1.3, CVI = 5.5%, CV2 = 6.7%)

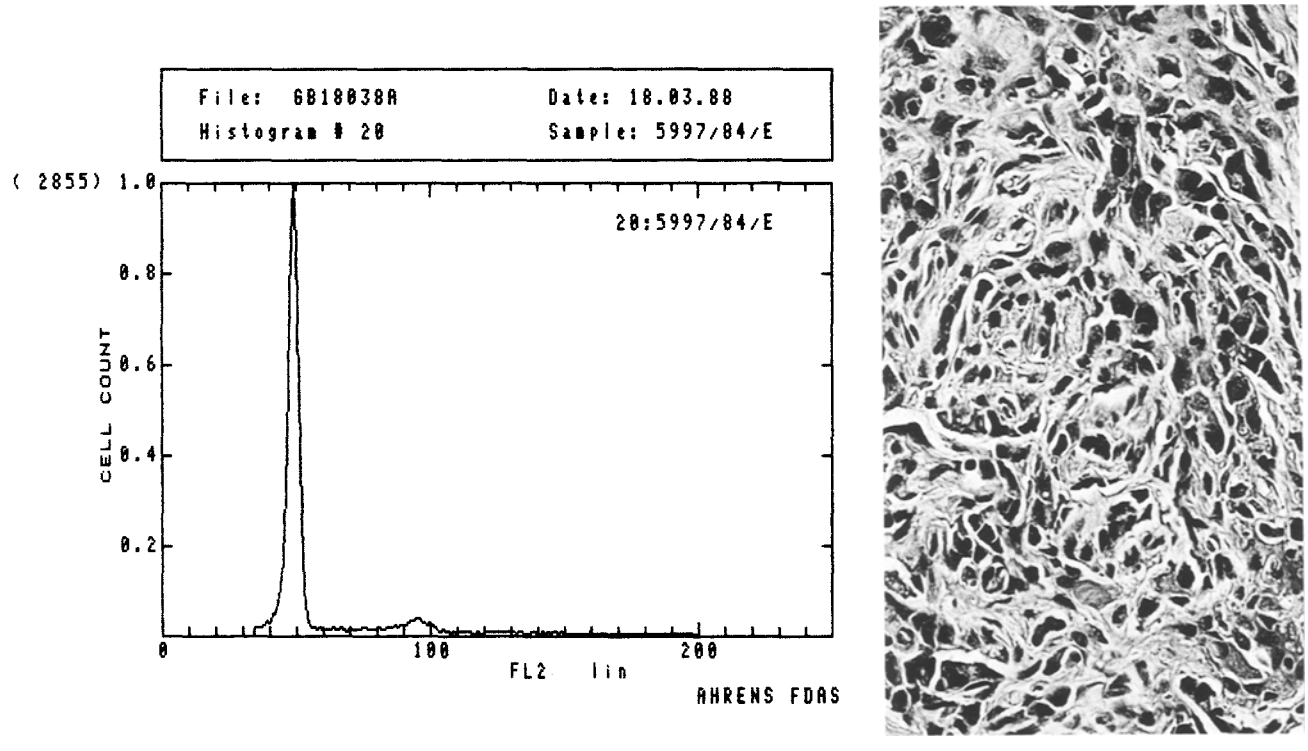

Fig. 2. Gastric carcinoma, diffuse type (pT2, pN pos, G3, H&E × 100) showing DNA diploidy (CV = 3.6%)

Tumours localized in the cardia were more frequently DNA-aneuploid (75%, $P < 0.01$, Table 4), whereas the majority of cancers arising in gastric stumps ($n = 13$) were DNA-diploid (85%, Table 4).

In 94 cases complete clinical follow-up data were analysed. In this group 65 (69%) patients had DNA-diploid tumours and 29 (31%) were found to have DNA-aneuploid carcinomas. The mean age and sex did not differ

Table 2. Gastric carcinomas ($n=125$): DNA ploidy, histological type (Lauren), and grade (WHO)

	G1	G2	G3	Total – diploid – aneuploid
Intestinal	2 (3%)	16 (26%)	43 (71%)	61
– diploid	1	10	22	33
– aneuploid	1	6	21	28
Mixed	0	1 (4%)	23 (96%)	24
– diploid	0	0	14	14
– aneuploid	0	1	9	10
Diffuse	0	0	40 (100%)	40
– diploid	0	0	35	35
– aneuploid	0	0	5	5
Total	2 (2%)	17 (13%)	106 (85%)	125
– diploid	1 (50%)	10 (59%)	71 (67%)	82
– aneuploid	1 (50%)	7 (41%)	35 (33%)	43

Table 3. Gastric carcinomas ($n=110$): DNA ploidy and frequency of lymph node metastases (except pNx; $n=15$)

	pN pos.	pN neg.	Total – diploid – aneuploid
Intestinal	34 (67%)	17 (33%)	51
– diploid	16	11	27
– aneuploid	18	6	24
Mixed	15 (63%)	9 (37%)	24
– diploid	7	7	14
– aneuploid	8	2	10
Diffuse	20 (57%)	15 (43%)	35
– diploid	16	14	30
– aneuploid	4	1	5
Total	69 (63%)	41 (37%)	110
– diploid	39 (55%)	32 (45%)	71
	$P<0.05$		
– aneuploid	30 (77%)	9 (23%)	39

between both groups. As expected, early cancers (pT1) proved to have a statistically significant better course (median survival 96 months) when compared with advanced tumour stages (pT2–pT4; median survival 18.24 months, $P=0.004$, Table 5), independent of histological type and ploidy.

Among the cases with advanced carcinomas a trend to longer median survival times for tumours of the intestinal and mixed types was registered, but the differences with diffuse-type carcinomas were not statistically significant (Table 5). DNA-aneuploidy was found to be a statistically significant indicator for worse prognosis only for advanced carcinomas with lymph node metastases (pT2–pT4/Ln pos.; $P=0.0415$; Table 6, Fig. 4) and for the subgroup of advanced carcinomas with tubular differentiated tumour areas (intestinal and mixed types, pT2–pT4; $p=0.0289$; Table 6, Fig. 5a). In all other tumour stages except early cancers, a tendency to a more unfavourable course of DNA-aneuploid tumours was detected (Table 5).

Table 4. Gastric carcinomas ($n=125$): DNA ploidy, histological type (Lauren), and localization

	Cardia	Corpus/ fundus	Antrum/ pylorus	Gastric stump	Total – diploid – aneuploid
Intestinal	6 (10%)	25 (41%)	25 (41%)	5 (8%)	61
– diploid	1	12	17	3	33
– aneuploid	5	13	8	2	28
Mixed	3 (12.5%)	5 (21%)	13 (54%)	3 (12.5%)	24
– diploid	0	2	9	3	14
– aneuploid	3	3	4	0	10
Diffuse	3 (7.5%)	14 (35%)	18 (45%)	5 (12.5%)	40
– diploid	2	13	15	5	35
– aneuploid	1	1	3	0	5
Total	12 (10%)	44 (35%)	56 (45%)	13 (10%)	125 (100%)
– diploid	3 (25%)	27 (61%)	41 (73%)	11 (85%)	82 (66%)
	$P<0.01$				
– aneuploid	9 (75%)	17 (39%)	15 (27%)	2 (15%)	43 (34%)

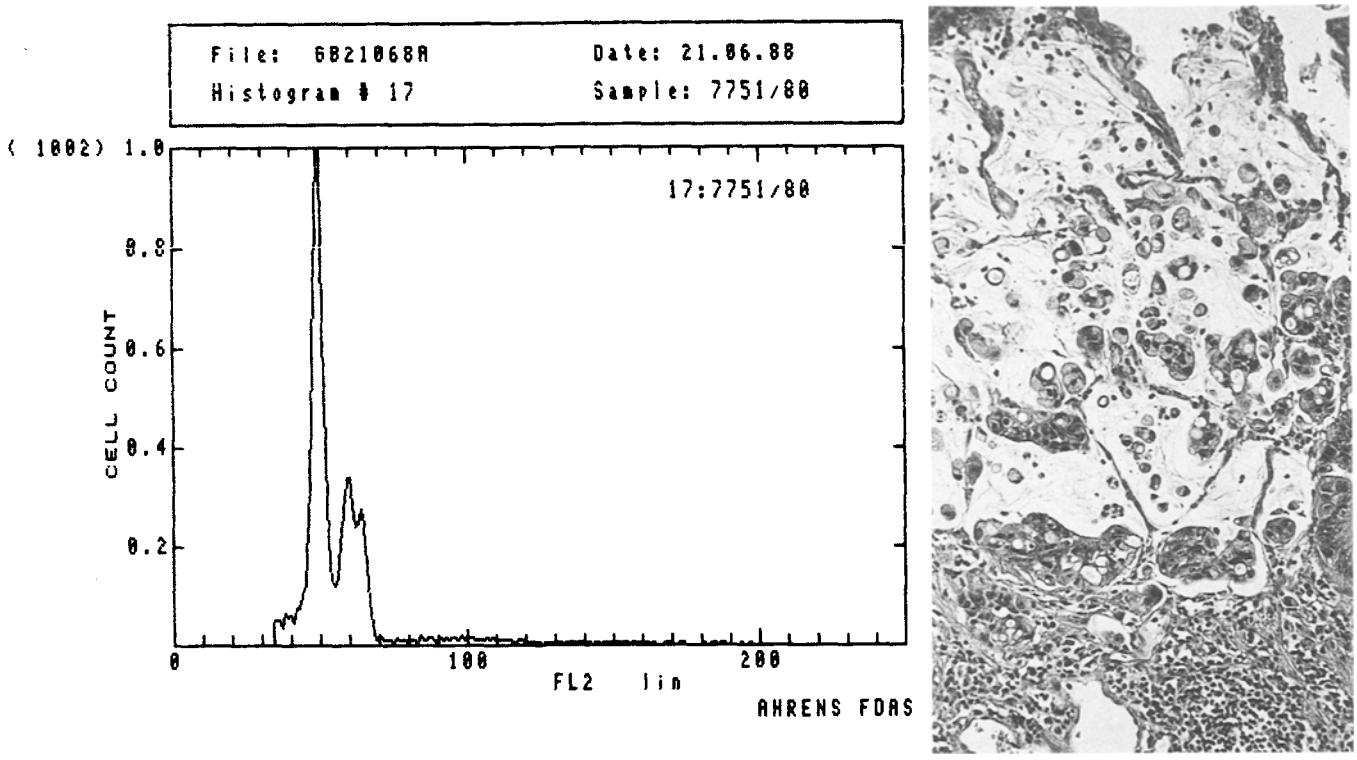

Fig. 3. Gastric carcinoma, mixed type (pT1, pN pos, G2, H&E × 100), here predominantly mucinous intestinal differentiation with two different abnormal stem lines (DI1 = 1.2, DI2 = 1.3, CV1 = 3.6%, CV2 = 3.9%, CV3 = 2.6%)

Table 5. Gastric carcinomas (n=94): DNA ploidy, tumour stage, histological differentiation (Lauren), and median survival (Kaplan-Meier)

Stage	Median survival (months)			
	Total	Diploid	Aneuploid	P value (log rank test)
pT1 (early ca.)	96.00 n=19	70.29 n=16	84.00 n=3	NS
pT2–pT4	18.24 n=75	25.49 n=49	14.00 n=26	NS
pT2–pT4 pN pos.	16.23 n=47	31.50 n=30	10.50 n=17	0.0415
Histological type and stage intestinal type				
pT1 (early ca.)	48.00 n=6	48.00 n=6	– n=0	–
pT2–pT4	19.58 n=36	42.46 n=17	13.50 n=19	NS (0.067)
Intestinal and mixed types				
pT2–pT4	25.02 n=47	33.96 n=25	16.00 n=22	0.0289
Diffuse type				
pT1 (early ca.)	96.00 n=7	96.00 n=7	– n=0	–
pT2–pT4	10.27 n=28	11.22 n=24	6.00 n=4	NS

NS, Not significant

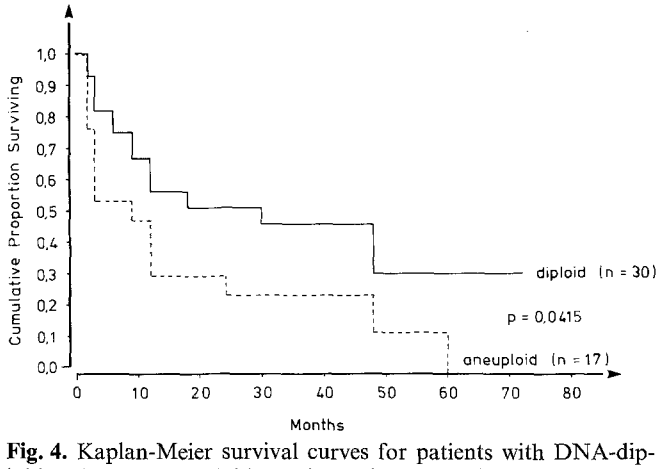

Fig. 4. Kaplan-Meier survival curves for patients with DNA-diploid and DNA-aneuploid gastric carcinomas with lymph node metastases (pT2–pT4, pN pos)

Discussion

The rate of DNA-aneuploidy in gastric carcinomas reported in the literature shows a broad variation from 39% (Odegaard et al. 1987) to 89% (Teodori et al. 1984, Table 6). Comparison of the data from different studies is problematical for several reasons. The differences might be explained by the relatively low number of cases in many investigations, patient selection and technical reasons. Considering case number, only Tosi et al. (1988) studied more than 100 tumours (Table 6). Patient selection is especially relevant in retrospective studies, in

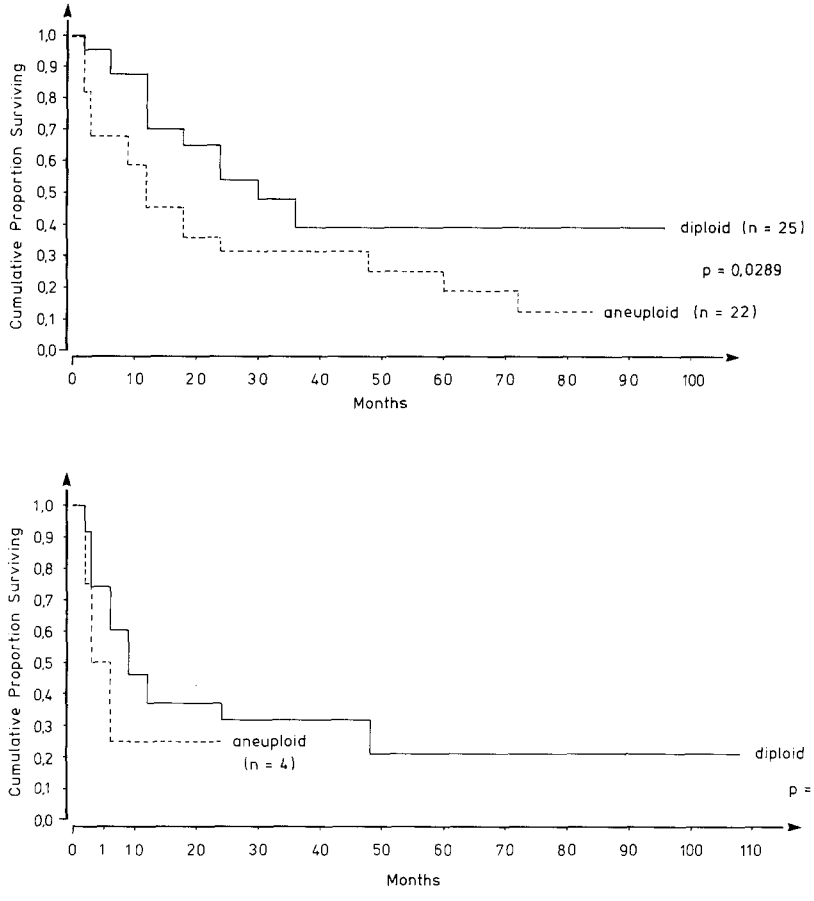

Fig. 5. Kaplan-Meier survival curves for patients with DNA-diploid and DNA-aneuploid gastric carcinomas (pT2–pT4) of **a** intestinal and mixed types (above) **b** diffuse type (below)

which there might be poor patient definition (site, grade, histological type, stage, and other standard tumour definitions). Different technical factors can also influence the results: kind of material used (resection specimens or biopsies, type of fixation and paraffin-embedding procedure, and in particular variations in the preparative techniques of the nuclei for DNA analysis. The mean coefficient of variation of 5.5% in the present study as an indicator of the quality of measurements is one of the most favourable results when compared with the published data in similar investigations (Table 6).

In our investigation, the DNA content of gastric carcinomas did not correlate with sex or mean age of the patients. The histological differentiation, however, showed a statistically significant higher rate of DNA-aneuploidy in stomach cancers with tubular differentiated areas; the intestinal (46% aneuploidy) and mixed types (42% aneuploidy), when compared with the diffuse-type tumours (12.5%). Similar, but not statistically significant data were reported by Macartney et al. (1986), Petrova et al. (1980) and Wyatt et al. (1989). Another investigation (Deinlein et al. 1983) came to the opposite conclusion, whereas Ballantyne et al. (1987), Mellin et al. (1986) and Tosi et al. (1988) did not find any correlation with histological type (Table 6). The statistically significant higher prevalence of DNA-aneuploid carcinomas in the cardia, recently described by Nanus et al. (1989), was confirmed. The pathogenetic reason for this finding is not yet clear. It might be suggested that the activity of mucosal enzymes metabolizing putative cancerogenic substances in the food (Pfeiffer 1976; Thies and Siegers 1989) could differ regionally in the stomach.

Early cancers and carcinomas arising in gastric stumps were mostly diploid. In agreement with most other investigations (Table 6), however, statistically significant correlations between DNA ploidy and the depth of infiltration (pT stages) or the histological/cytological grade were not detected (Table 2).

Independent of the histological type DNA-aneuploid carcinomas showed more frequent lymph node metastases ($P<0.05$, Table 3). An association of DNA-aneuploidy and a higher rate of lymphatic metastases was previously reported by Tosi et al. (1988). The comparison of DNA content in primary tumours and in lymph node metastases ($n=56$) resulted in a difference of ploidy in 46% of the aneuploid tumours, but only in 6% of the DNA-diploid carcinomas ($P<0.01$). Comparable results were obtained in colorectal carcinomas (Baretton et al. 1990; Rübe et al. 1988). Due to our technique of cutting out excess normal tissue from the paraffin blocks, a sampling error seems unlikely as an explanation for this finding. This phenomenon may indirectly indicate the presence of heterogeneous stem lines with possibly different metastatic or growth potential in the primary tumour, or the development of various stem lines from the primary tumour in the metastases (Rübe et al. 1988). A regression of aneuploid subpopulations in the lymph node metastases is also possible. Heterogeneity of DNA content is well known in human cancers and its detection

Table 6. Review of flow-cytometric analyses of gastric carcinomas in the literature, regarding the number of cases, the preparation [fresh (fr) or paraffin-embedded (pa) tissue from resected specimens (res) or biopsy material (bio)], the coefficient of variation (CV %), the percentage of aneuploidy and correlations between DNA ploidy and histological type, grade, depth of infiltration, lymph node metastases, and prognosis (significant positive, +; weak correlation, (+); negative, −; no investigation, *; no statement, 0)

Reference	Number of cases	Material		Median CV (%)	Percentage DNA-aneu	Correlation between DNA-ploidy and				
						Hist. type	Grade	Depth	LN state	Prog.
de Aretxabala et al. (1989)	37	res	fr/pa	5.8	59	*	−	(+)	*	
Ballantyne et al. (1987)	77	res	pa	6.8	62	−	−	−	*	−
Bronzo et al. (1989)	13	bio	fr	3.2	62	*	*	*	*	(+)
Danova et al. (1987)	9	res	fr/pa	0	67	*	(+)	*	*	*
Deinlein et al. (1983)	19	bio	fr	0	58	(+)	*	*	*	*
Macartney et al. (1986)	56	res	pa	5.3	73	(+)	*	−	−	*
Mellin et al. (1986)	55	0	0	0	80	−	−	−	*	*
Nanus et al. (1989)	50	res	fr	0	70	+	−	−	−	(+)/+
Odegaard et al. (1987)	18	bio	fr	7.2	39	*	*	*	*	*
Petrova et al. (1980a)	45	bio	fr	0	69	*	*	*	*	*
Petrova et al. (1980b)	23	res	fr	0	44	(+)	*	*	*	*
Sasaki et al. (1988)	15	res	fr	0	80	*	*	*	*	*
Teodori et al. (1984)	18	res/bio	fr	0	89	*	*	*	*	*
Tosi et al. (1988)	133	res	pa	2.5–7.0	62	−	−	+	+	+
Wyatt et al. (1989)	76	res	pa	8.5	42	(+)	−	−	−	(+)/+
Submitted investigation	125	res	pa	5.5	34	+	−	−	+	(+)/+

depends directly on the number of probes investigated (Baretton et al. 1989; Heppner 1984). The rate of 4 cases (3%) with heterogeneous ploidy in different tumour regions or different DNA-aneuploid stem lines in the same probe from one paraffin block (Fig. 3) is probably too low, because of the limited material available in this retrospective study, when compared with prospective and extensive investigations (de Aretxaballa et al. 1989; Sasaki et al. 1988).

Few reports dealing with the prognostic value of the flow-cytometric DNA content of gastric carcinomas have been published and the results are contradictory (Table 6). Mostly, a weak predictive value of DNA-aneuploidy for a more unfavourable postoperative course has been found (Bronzo et al. 1989; Nanus et al. 1989; Tosi et al. 1988; Wyatt et al. 1989). Studies from Japan on early gastric cancer using cytophotometric techniques have also shown an association between DNA content of tumour cells and biological behaviour (Inokuchi et al. 1983; Kamegawa et al. 1986), although such techniques are not directly comparable with flow cytometry.

Our results support the value of the usual tumour classifications, especially the importance of the depth of infiltration with the best prognosis for carcinomas limited to mucosa and submucosa (early cancers). Moreover, the limited value of the histological differentiation was shown by the tendency to a better outcome of tumours with tubular differentiated areas (intestinal and mixed types) in advanced stages. Within these subgroups a survival advantage for patients with DNA-diploid tumours was detected for all advanced tumours with statistically significant differences for carcinomas with lymphatic metastases (Table 5, Fig. 4) and for tumours with a tubular differentiated portion (intestinal and mixed-type tumours; Table 5, Fig. 5a). Our results on this point correspond with the findings of Wyatt et al. (1989), who also showed an association of DNA-aneuploidy and shorter survival only in patients with intestinal-type tumours. These authors, however, studied only patients undergoing potentially curative operations; the correlation of histological type together with tumour stage to DNA ploidy was not analysed, and mixed-type tumours resembled diffuse-type rather than intestinal-type cancers in DNA content and clinical behaviour. In the early cancers neither DNA ploidy nor histological differentiation seem to be of prognostic importance in our material.

A remarkable result is the high rate of DNA-diploidy in diffuse gastric cancers, which seemed to have a poorer prognosis than the intestinal types. This finding suggests that not only histogenesis and phenotype but other pathogenetic mechanisms such as tumour initiation and/or promotion might be also distinct in both types of stomach carcinomas. Nevertheless, in some diffuse gastric cancers DNA-aneuploidy is present and these cases seem to have the most unfavourable outcome (Table 5, Fig. 5b). In the present study the combination of usual tumour classifications and the flow-cytometric determination of DNA content in gastric carcinomas provided more detailed information about the further course of the patients than conventional tumour classifications alone. DNA-aneuploidy was a statistically significant indicator for a poorer prognosis in advanced gastric carcinomas with lymph node metastases and in advanced tumours with tubular differentiation (intestinal and mixed types). Those patients with the additional risk factor DNA-aneuploidy need closer surveillance and would perhaps profit from adjuvant forms of therapy.

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