DNA Aneuploidy and High Proliferative Activity but Not K-*ras*-2 Mutations as Independent Predictors of Clinical Outcome in Operable Gastric Carcinoma

Results of a 5-Year Gruppo Oncologico dell'Italia Meridionale (GOIM) Prospective Study

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BACKGROUND. The prognostic value of DNA ploidy, S-phase fraction (SPF) and K-*ras*-2 mutations in gastric carcinoma (GC) has not yet been clearly defined. The aim of this study was to clarify the association between biomolecular variables, tumor characteristics, and clinical outcome in GC patients.

METHODS. Resected specimens from a consecutive series of 69 patients with GC who underwent potentially curative surgery were studied prospectively. DNA ploidy and SPF were assessed by flow cytometry on multiple frozen tumor samples, whereas K-*ras*-2 mutations were detected by polymerase chain reaction followed by single-strand conformation polymorphism. All the patients involved in this study were followed up for a mean of 95 months.

RESULTS. DNA aneuploidy was present in 72% of the cases (50 of 69), whereas 10% of these (5 out of 50) showed multiclonality. Mutations of K-*ras*-2 were detected in 8% of the tumors (5 of 63). Both DNA ploidy and SPF were associated with TNM stage (American Joint Committee on Cancer [AJCC] staging system) and node status. Moreover, DNA aneuploidy was significantly related to high SPF. K-*ras*-2 mutations were not associated with clinicopathologic variables or flow cytometric indicators. At univariate analysis, advanced TNM stage, node involvement, diffuse histotype, depth of invasion, DNA aneuploidy, and high SPF proved to be significantly related to quicker tumor relapse and to shorter overall patient survival. With multivariate analysis, DNA aneuploidy, high SPF, and depth of invasion were related to risk of tumor relapse and patient death, whereas diffuse histotype was independently related to patient risk of tumor relapse.

CONCLUSIONS. DNA ploidy and SPF, when associated with clinicopathologic staging, might be useful for the identification of GC patients who have different risks for death or relapse of disease. ***Cancer* 2001;92:294–302.**

KEYWORDS: DNA ploidy, S-phase fraction, K-*ras*-2, gastric carcinoma, staging, prognosis.

Supported by grants from the Gruppo Oncologico dell'Italia Meridionale special project entitled Caratterizzazione Biomolecolare del Carcinoma Gastrico Resecato (Protocol 9602 bis); and by Consiglio Nazionale della Ricerca, PF Clinical Application of Cancer Research (ACRO), (Contract 9202199.PF39). Simona Corsale is supported by a fellowship from Federazione Italiana per la Ricerca sul Cancro (FIRC).

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Received September 6, 2000; accepted March 20, 2001.

Gastric carcinoma (GC) accounts for more than 95% of stomach tumors and still is one of the most common causes of death from cancer in developing countries.[1] Most of these tumors would seem to be of sporadic origin, although geographic variations have been observed. These variations are found mostly in men (two men to one woman) and involve dietary factors and the presence of *Helicobacter pylori* infection.[1] The biomolecular mechanisms behind gastric tumorigenesis are still unknown; its biologic, genetic, and clinical aspects are heterogeneous, and Lauren[2] has, in fact, described two histologic subtypes of GC, one intestinal and one diffuse, that present different biologic features and clinical behavior. The identification of prognostic factors that are more closely related to tumoral biology may help in the identification of those patients with more aggressive tumors and resulting higher relapse risk who would benefit from more efficient therapeutic choices.

Cell populations making up a tumor present morphologic and functional features that vary with time as a result of different selective stimuli.[3] A clear sign of destabilization is the presence of an abnormal cell DNA content (DNA aneuploidy), a high proliferative activity (high S-phase fraction [SPF]) and the resulting accumulation of mutations into oncogenes, tumor suppressor genes, and genes controlling DNA replication and/or repair mechanisms.[4] In recent years, DNA aneuploidy and high SPF have been recognized as malignancy markers with possible prognostic significance in many neoplasias, for example GC.[5,6,7,8] Although in most solid tumors, mutations of the oncogene K-*ras*-2 are associated with a poor clinical outcome,[9,10] its involvement in GC development and progression still is not understood clearly.[11] The aim of our prospective study was to assess SPF, DNA ploidy, and the mutational status of K-*ras*-2 in a consecutive series of 69 GCs to identify any possible links between these biologic variables and traditional clinicopathologic features and to evaluate the prognostic significance of these variables on the clinical outcome of the patients.

MATERIALS AND METHODS

Clinicopathologic Variables

Resected specimens from a consecutive series of 69 patients with GC who had undergone potentially curative surgery were studied prospectively. All patients were operated on between January 1992 and December 1996 at the same Institute (Department of Oncology, University of Palermo). None of them had received chemotherapy or radiotherapy before surgery. Curative resection included gastrectomy and extensive dissection of regional lymph nodes. The resected gastric carcinomas and lymph nodes were examined by pathology and staged according to the American Joint Committee on Cancer (AJCC) TNM classification and staging system.[12] Histologic sections from all the 69 surgical specimens were examined by two separate pathologists (R.M.T. and V.M.) who were methodologically blinded to previous and each other's findings. Tumors were classified according to Lauren criteria[2] into intestinal or diffuse. The grade of differentiation also was evaluated and ranked as G1, G2, or G3 (respectively, well, moderately, and poorly differentiated). A standard questionnaire of more than 100 clinicopathologic and research variables was available for each patient at the time of surgery and was maintained on a computerized database. A family history was obtained in every case; none of the patients included in the present study had a family history of GC. The group was comprised of 47 men (68%) and 22 women (32%), with a mean age of 64.4 $\pm$ 10 years (range, 39–79 yrs). The GC was located in the cardia or fundus in 23 (33%) patients, in the corpus in 29 (42%) patients, and in the antrum in 17 (25%) patients. Thirty-three (48%) of the tumors were $\leq$ 5 cm, and 36 (52%) were $>$ 5 cm. Invasion depth was PS (−) (into the muscularis propria or into the subserosa without infiltrative growth) in 42 (61%) cases and PS (+) (into the subserosa with infiltrative growth) in 27 (39%). Fourteen (20%) patients had Stage I disease, 27 (39%) had Stage II, 24 (35%) had Stage III, and 4 (6%) had Stage IV. Lymph-node metastases were present in 49 (71%) patients. Tumors were 5 (7%) in G1, 26 (38%) in G2, and 38 (55%) in G3. Fifty-five (80%) GCs were of the intestinal type, and 14 (20%) were of the diffuse type. Patients were followed up every 3 months for the first 3 years, at 6-month intervals for the next 2 years, and annually thereafter. All reasonable attempts were made to document disease relapse (local recurrence or distant metastases) cytologically or histologically. All patients with relapsed disease after surgery received a standard chemotherapeutic regimen for GC (5-fluorouracil, epirubicin, methotrexate, etoposide, doxorubicin, and cisplatin).

Tissue Handling

After patients provided their informed consent, multiple samples (3 to 10) of the primary GC tissue were taken from different representative areas and processed within 30 minutes of surgical resection. All tissues were carefully trimmed to remove as much nonneoplastic material as possible, avoiding the nonviable areas. In addition, from each patient multiple samples of normal-appearing mucosa were taken in a corresponding nontumoral area, as far as possible from the tumor site, to be used as a standard reference

for flow cytometric and biomolecular analyses. Tissues were bisected, and one-half of each sample was fixed in 70% ethyl alcohol and embedded in paraffin for pathologic examination. The remaining half of the sample pool was immediately frozen and stored at −80 °C until analysis. Histopathologic examination on cryostat sections stained with hematoxylin and eosin was then performed; only those samples containing > 80% of neoplastic cells were used for biomolecular studies. Where present, areas with a high content of nonneoplastic cells were removed from the frozen block with a scalpel. Evaluation of each biomolecular variable (DNA ploidy, SPF, and K-*ras*-2 mutations) was performed independently by researchers who had no knowledge of the clinical data for the samples.

Cellular DNA Content and S-Phase Fraction Flow Cytometric Analyses

DNA flow cytometry was performed on mechanically disaggregated samples of frozen tumor tissue as previously described.[13] A FACSort flow cytometer (Becton Dickinson, CA) was used to obtain data. DNA histogram analysis was performed by means of a Multicycle Software Program (Phoenix Flow Systems, San Diego, CA), including systematic background subtraction.[14,15] DNA ploidy, DNA index (DI), and SPF were determined as previously reported.[13] Briefly, healthy gastric mucosa was used as an internal DNA-diploid control for each sample. Tumors with DI = 1 were defined as DNA-diploid and those with lower or higher DI values were considered DNA-aneuploid if they contained >10% aneuploid cells. DNA-aneuploid results were further subdivided into monoploid (with only one aneuploid peak) and multiploid (with two or more aneuploid peaks).

Detection of K-*ras*-2 Mutations

High molecular-weight genomic DNA was extracted as previously described[16] from normal gastric mucosa (as internal control), and primary GC tissue samples that had been stored frozen at −80 °C and then pulverized in liquid nitrogen by MicroDismembrator U (B. Braun, Melsungen Ag, Braun Apparate, 3508 Melsungen, Germany). In 6 of the 69 patients, K-*ras*-2 analysis could not be performed because frozen material was not available. Polymerase chain reaction (PCR) amplification of the the first codifying exon of K-*ras*-2 gene was performed as described previously.[16] Briefly, 0.1 mg DNA aliquots were incubated in a final volume of 50 mL in the presence of 200 mM dNTPs, 30 pmol of each amplimer, 1.5 mM$MgCl_2$, 50 mM KC1, 10 mM Tris-HCl (pH 8.3). After denaturation at 94 °C for 5 minutes, 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Branchburg, NJ) were added to each sample, which was further incubated for 28 cycles as follows: 30 seconds at 93 °C, 90 seconds at 60 °C and then 10 minutes at 60 °C. In every instance, negative controls (DNA was replaced with water) were amplified by PCR and included in the experiment. In all PCR assays, aerosol-resistant pipette tips were used to avoid cross-contamination. The quality and the concentration of the amplification products were verified by 1.5% agarose-gel electrophoresis and ethidium-bromide staining. One hundred ng aliquots of the amplified DNA fragments, purified and concentrated by filtration through Microcon 50 columns (Amicon, Beverly, MA), were denatured and analyzed by electrophoresis at 21 °C on a 20% polyacrylamide gel (8 × 15 × 0.1 cm) in TBE (90 mM Tris-borate, 2 mM EDTA) buffer, at 400 V for 1.5–2 hours, essentially as described by Hongyo et al.[17] To keep the DNA temperature constant, we performed the electrophoretic run in a DGGE-2000 System (C.B.S. Scientific Company, Del Mar, CA), equipped with a KR-50A immersion chiller (Polyscience, Niles, IL). After the run, the gel was stained for 20 minutes with 0.5 mg/mL ethidium bromide in TBE 1X and destained for 5 minutes in TBE 1X. The DNA fragments were observed by illumination with long-wave (312 nm) ultraviolet light for the shortest possible time. PCR and single-strand conformation polymorphism (SSCP) analyses were repeated twice for each sample to minimize the possibility of artifact due to contamination or polymerase errors. DNA of normal gastric mucosa from each patient was also amplified and run in parallel with matched tumoral DNA samples on SSCP gels, to see if germline mutations or polymorphisms had occurred.

Statistical Analysis

Clinicopathologic factors included age and sex; tumor location (cardia or fundus, corpus and antrum); tumor size (≤ 5 cm and > 5 cm); depth of invasion (PS [−] and PS [+]); TNM stage (I–IV); node status (negative and positive); histologic grade (G1, G2, and G3); histotype (intestinal and diffuse). The association between DNA ploidy, SPF, K-*ras*-2 mutations and clinicopathologic variables was evaluated by means of the chi-square test and, where appropriate, Yates test. Disease-free survival (DFS) was measured from the day of primary surgery to the date of first relapse (locoregional or metastatic) and overall survival (OS) from the day of surgery to the day of death caused specifically by the tumor. If patients did not relapse or die, they were censored at the time of their last follow-up. Clinical and morphobiologic variables were examined by univariate analysis in accordance with the Kaplan–Meier method;[18] significance of differences for each prognostic factor was assessed by the log-

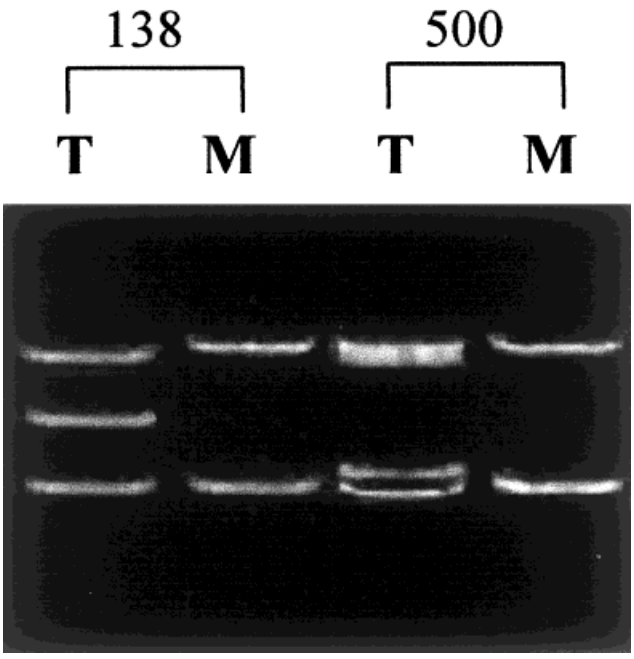

FIGURE 1. Representative ethidium bromide staining pattern obtained by cold SSCP analysis of the K-*ras*2 gene status in gastric mucosa (M) and gastric carcinoma (T) from 2 patients. The wild type ssDNA fragments can be seen in lanes 2 and 4, and the mutated ssDNA fragments appear in lanes 1 and 3.

TABLE 1
Relation of DNA-ploidy Status to Clinicopathologic Variables in Gastric Carcinoma

	DNA ploidy			
Variable	Diploid	An. monoclonal	An. multiclonal	*P*
TNM stage				
I	10	3	1	
II	7	19	1	
III–IV	2	23	3	< 0.01
Node status				
node negative	11	8	1	
node positive	8	37	4	< 0.05
SPF				
≤ 15.7	17	17	—	
> 15.7	2	28	5	< 0.01
Total patients	19	45	5	

An.: aneuploid.

rank and Wilcoxon tests or trend tests where appropriate.[19] Multivariate analysis was performed by means of Cox logistic regression model, using a backward procedure.[20] The null hypothesis $\beta = 0$ was tested by the Wald statistic. The relative contribution of clinicopathologic and biologic variables was assessed by means of the likelihood ratio test. For the prognostic variables contributing significantly to the model, the effect was calculated in terms of relative risk (RR) and the associated 95% confidence limits (CI). *P* values < 0.05 were considered statistically significant.

TABLE 2
Relation of SPF to Clinicopathologic Variables in Gastric Carcinoma

	SPF		
Variables	≤ 15.7%	< 15.7%	*P*
TNM stage			
I	10	4	
II	15	12	
III–IV	9	19	< 0.05
Node status			
node negative	15	5	
node positive	19	30	< 0.01
Total patients	34	35	

RESULTS

General Outcome

The mean follow-up of patients was of 95 months (range, 5–137 mos) duration. At the time of analysis (May, 2000), 41 patients had relapsed, of which 2 had locoregional recurrence, and 39 had distant metastases), whereas 40 had died from tumor-related causes. The overall 5-year survival rate was of 44.4 % (standard error [SE] ± 6.4) for the whole series.

DNA Ploidy and SPF Analysis

Adequate DNA histograms were obtained for all normal and tumoral gastric tissues by means of flow cytometry. The mean coefficient of variation of the diploid G0/G1 peaks of all the tumoral samples examined was 3.5% (range, 2.6–4.8). Seventy-two percent (50 out of 69) GCs were DNA aneuploid tumors, whereas 10% (5 of 50) of these showed multiclonality. The SPF ranged from 3.5–37.2% (mean, 15.7%, and interquartile range 12.1–20.1%). SPF mean value was used as the cutoff point between high (> 15.7%) and low (≤ 15.7%) SPF tumors.

Mutation Analysis of K-*ras-2* Gene

PCR-SSCP mutation analysis of exon 1 was performed on genomic DNA from normal and tumoral gastric tissues. Examples of K-*ras*-2 mutations detected by SSCP analysis are shown in Figure 1. Aberrantly migrating bands were found in 8% (5 of 63) of the cases. No germline mutations were found, indicating that the changes were somatic.

TABLE 3
Univariate and Multivariate Analyses of Clinicopathologic and Biologic Variables on Disease-Free Survival (DFS)

		Univariate				Multivariate			
Variables	Patients (*N*)	DFS (%) 2 yrs	DFS (%) 5 yrs	O/E	*P*	RR	CI (95%)	*β*	*P*
Depth of invasion									
PS(−)	42	69	52	0.74		1			
PS(+)	27	51	35	1.60	< 0.05	2.12	1.09–4.13	0.75	< 0.05
TNM stage									
I	14	86	79	0.34					
II	27	74	51	0.89					
III–IV	28	38	20	2	< 0.01				
Node status									
node negative	20	80	75	0.60					
node positive	49	55	32	1.27	< 0.05				
Histotype									
intestinal	55	70	54	0.83		1			
diffuse	14	28	9	2.32	< 0.01	2.93	1.34–6.40	1.07	< 0.01
DNA ploidy									
diploid	19	89	83	0.30		1			
an. monoclonal	45	56	34	1.32		3.18	1.10–9.19	1.16	< 0.05
an. multiclonal	5	20	—	4.39	< 0.01	12.7	2.94–55.2	2.54	< 0.01
SPF									
≤ 15.7	34	82	75	0.52		1			
> 15.7	35	43	12	1.90	< 0.01	2.83	1.25–6.38	1.04	< 0.05
Total patients	69	62	46						

O/E: observed/expected; RR: relative risk; CI: confidence interval; PS(−): invasion into the muscularis propria or into the subserosa without infiltrative growth; PS(+): invasion into the subserosa with infiltrative growth; an.: aneuploid.

Flow Cytometric Variables, K-*ras*-2 Mutations and Clinicopathologic Features

Both DNA aneuploidy and high SPF were associated with advanced TNM stage and node involvement (Tables 1 and 2). Further, DNA aneuploidy was significantly related to high SPF. No association was found between flow cytometric variables and any of the following factors: age, sex, tumor location and size, depth of invasion, histologic grade and type. Finally, K-*ras*-2 mutations were not associated with clinicopathologic variables and flow cytometric indicators (data not shown).

Univariate and Multivariate Analysis

Univariate analysis showed that advanced TNM stage, node involvement, diffuse histotype, invasion into the subserosa with infiltrative growth, DNA aneuploidy, and high SPF were significantly related to relapse risk (Table 3) and death (Table 4). Univariate analysis failed to reveal any significant association between relapse risk or survival and age, sex, histologic grade, tumor location and size, or K-*ras*-2 mutations. Figure 2 shows the probability of a) disease-free interval and b) OS in relation to DNA-ploidy status. Figure 3 shows the probability of a) disease-free interval and b) OS according to SPF. To identify independent factors that predict relapse or death, we performed a multivariate analysis of the 69 patients. In the Cox model for this, only the significant variables at univariate analysis were considered. The results of the multivariate analysis using the Cox proportional hazards model are summarized in Tables 3 and 4. Only DNA aneuploidy (and particularly DNA multiploidy, which appeared to be the most relevant indicator of relapse or death), high SPF, and invasion into the subserosa with infiltrative growth were found to be independent prognostic variables for relapse or death, whereas diffuse histotype was independently related to relapse risk.

DISCUSSION

All over the world, GC is one of the main causes of death due to cancer[1] and is the most common form of stomach neoplasia. Until now, traditional clinicopathologic factors such as the TNM system and histologic classification have been the most important variables for deciding whether or not a tumor was operable and have conditioned both the disease-free interval and OS. Nevertheless, patients showing exactly the same clinicopathologic aspects may present different clinical courses. New biomarkers must therefore be found

TABLE 4
Univariate and Multivariate Analyses of Clinicopathologic and Biologic Variables on Overall Survival (OS)

		Univariate				Multivariate			
Variables	Patients (*N*)	OS (%) 3 yrs	OS (%) 5 yrs	O/E	*P*	RR	CI (95%)	*β*	*P*
Depth of invasion									
PS(−)	42	73	52	0.69		1			
PS(+)	26	44	29	1.85	< 0.01	3.24	1.61–6.52	1.18	< 0.01
TNM stage									
I	14	86	78	0.33					
II	27	77	52	0.83					
III–IV	27	33	14	2.33	< 0.01				
Node status									
node negative	20	80	75	0.59					
node positive	48	56	30	1.29	< 0.05				
Histotype									
intestinal	55	71	51	0.87					
diffuse	13	33	17	1.82	< 0.05				
DNA ploidy									
diploid	18	94	89	0.23		1			
an. monoclonal	45	57	28	1.44		3.20	1.02–9.98	1.16	< 0.05
an. multiclonal	5	—	—	4.67	< 0.01	12.2	2.76–54.3	2.50	< 0.01
SPF									
≤ 15.7%	33	81	78	0.48		1			
> 15.7%	35	44	8	2.05	< 0.01	4.79	2.05–11.2	1.57	< 0.01
Total patients	68	63	44						

O/E: observed/expected; RR: relative risk; CI: confidence interval; PS(−): invasion into the muscularis propria or into the subserosa without infiltrative growth; PS(+): invasion into the subserosa with infiltrative growth; an.: aneuploid.

for the identification of a subgroup of patients at higher relapse and death risk who would benefit from particular therapeutic choices, more aggressive forms of treatment, and more specific follow-up modulations.

The main genetic changes occurring in GC include chromosome alterations (losses, duplications, translocations), genic alterations (small deletions and insertions, point mutations),[21,22] anomalies of cell DNA content (DNA ploidy), and/or anomalies of cell kinetics (SPF).[5] One of the most commonly used methods for quantifying cellular DNA and proliferative activity in various tumors, including GC, is flow cytometry. With this method, we obtained a frequency of DNA aneuploidy of 72%. To our knowledge, this is one of the highest frequencies reported to date in literature, where levels range from 36–89%.[5,6,8,23–29] This may be due to the sampling method, which was multiple in all cases, and to the preservation technique (freezing at −80 °C) of the surgical specimens, which reduces the probability of losing aneuploid clones and gives a better resolution of histograms compared with paraffin-embedded specimens.

In accordance with the findings of several other authors,[8,25,27,28] our study found that DNA aneuploidy and SPF were significantly associated with advanced clinical staging and the presence of lymph-node metastases, which suggests their involvement in tumor progression and aggressiveness.

We did not, however, find any associations between tumor site, grading, and flow cytometry variables, similar to the observations already reported by Victorozon et al.,[8] Ohyama et al.,[23] and Johnson et al.,[24] but dissimilar to the findings of Setala et al.,[25] Abad et al.,[27] Danova et al.,[30] and Nanus et al.[31] Further, neither DNA ploidy nor SPF proved to be associated with tumoral histotype, a fact that had already been observed by others.[26,27,32] To our knowledge, only one study[8] to date has reported an association between DNA ploidy and intestinal histotype. Our data also show a significant association between DNA aneuploidy and high SPF. This might be because a high replicative activity leads to the risk of genetic damage accumulation and the development of clones with aneuploid DNA content.

GCs with DNA aneuploidy and high SPF have a worse prognostic outcome than those with diploid DNA and low SPF;[7,25] nevertheless, at the present time, the prognostic role of SPF in GC still has to be defined more clearly (Table 5). Although they used

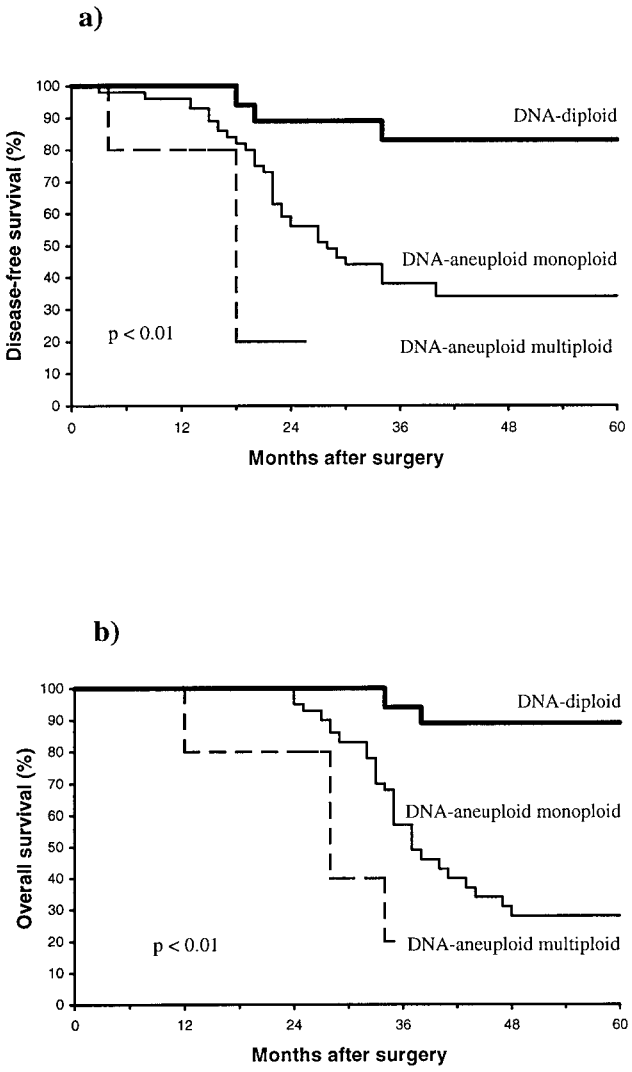

FIGURE 2. a) Disease-free survival and b) overall survival in 69 patients with gastric carcinoma according to DNA-ploidy status.

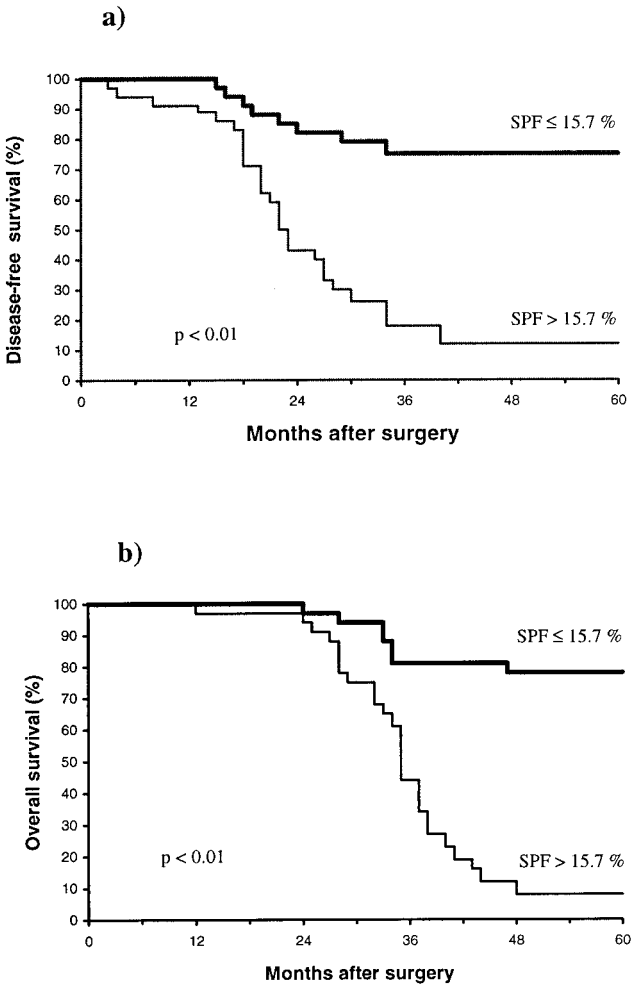

FIGURE 3. a) Disease-free survival and b) overall survival in 69 patients with gastric carcinoma according to S-phase fraction.

different methodological approaches, Lee et al.[5] in a 5-year prospective study on 217 GCs, Yonemura et al.[7] in a retrospective study on 493 GCs, and Ohyama et al.[23] on 172 GCs, have all identified SPF as an independent prognostic variable. Whereas several other authors, such as Victorozon et al.,[8] Setala et al.,[25]) and Abad et al.,[27] do not attribute any predictive value to SPF.

The variable results reported by studies involving SPF evaluation may be partly because of intratumoral heterogeneity, sampling bias, type of tissue used for analysis (fresh or frozen vs. paraffin-embedded), the different methods used by the various investigators, the use of several mathematical models for SPF analysis, and the inclusion of different percentages of tumor subtypes at various stages of disease.

The prognostic value of DNA ploidy in GC seems to be clearer. Published reports almost all agree that this biologic variable has a predictive role. Danesi et al.[6] and Esteban et al.[26] have recently conducted retrospective studies on 137 and 78 GCs and have reported that DNA aneuploidy, together with other clinicopathologic variables, is an independent predictor of survival, a finding in accord with the observations of several other authors.[7,8,23] Whereas Lee et al.[5] have found no association between DNA ploidy and overall survival.

In our study, multivariate analysis showed that depth of invasion, DNA aneuploidy, and high SPF are all independent prognostic factors for disease-relapse and survival, whereas diffuse histotype is associated with only disease relapse.

Therefore, as stated by Yonemura et al.[7] and Ohyama et al.,[23] SPF and DNA ploidy maintain their predictive value even where there are other, well-es-

TABLE 5
DNA Ploidy, S-phase Fraction, and Prognosis in Gastric Carcinoma

Author	Year	No. cases	Type of sample	Method	Follow-up (mos)	SPF Overall Survival		DNA ploidy Overall Survival	
						Univ	Multiv	Univ	Multiv
Yonemura et al.[7]	1990	493	PEB	FCM[a]	36	Yes	Yes	Yes	Yes
Ohyama et al.[23]	1992	172	fresh	FCM[a]	48	Yes	Yes	Yes	No
Rugge et al.[29]	1994	76	PEB	FCM	> 60	—	—	Yes	Yes
Sakusabe et al.[32]	1996[b]	216	PEB	FCM	60	—	—	Yes	Yes
Victorzon et al.[8]	1997	242	PEB	FCM	> 60	Yes	No	Yes	Yes
Setala et al.[25]	1997	289	PEB	FCM	120	Yes	No	Yes	Yes
Abad et al.[27]	1998	76	fresh	FCM	36	No	No	Yes	Yes
Lee et al.[5]	1999	217	PEB	FCM	66, 1	Yes	Yes	No	No
Esteban et al.[26]	1999	78	PEB	FCM	42[c]	—	—	Yes	No
Danesi et al.[6]	2000	137	PEB	FCM	> 60	No	No	Yes	Yes
Our series	2000	69	frozen	FCM	75	Yes	Yes	Yes	Yes

PEB: paraffin-embedded blocks; Univ: univariate analysis; Multiv: multivariate analysis; FCM: flow cytometry.

[a] In vivo Bromodeoxyuridine labeling index.

[b] Stage III gastric cancer.

[c] Lower follow-up.

tablished prognostic variables for GC, such as TNM stage, histologic grade, depth of invasion, lymph-node involvement, and histotype. These data suggest that these biologic variables may be used together with the more common prognostic parameters to reach a better characterization of the tumor and the identification of a subgroup of patients who require more specific therapeutic planning strategies.

Published reports show a low number of mutations (0–12%) of the protooncogene K-*ras*-2 in GC.[34–37] Our own analysis of this gene showed mutations in only 8% (5 out of 63) of the cases examined. This would confirm the nonspecific involvement of K-*ras*-2 in gastric tumorigenesis. Further, its involvement in the development of intestinal and/or diffuse GCs is still not clear. Miki et al.,[38] in fact, have found a higher percentage of mutations in the intestinal type, and Kim et al.[39] in the diffuse type, whereas in Lee et al.[5] and in our study, there were no associations either between mutations in K-*ras*-2 and histotype or with any of the other traditional clinicopathologic variables. Moreover, we found no significant association between DNA ploidy, SPF, and specific mutations in K-*ras*-2. This would probably suggest that mutations of this gene do not cause any apparent changes in cell DNA content or variations in the levels of proliferative activity of the tumor cells. Only a wider prospective study can define more clearly the role of this gene in gastric tumorigenesis.

In conclusion, it seems unlikely that a single biomolecular indicator will be found to be an ideal prognostic variable for GC. The findings of the present prospective study indicate that DNA ploidy and SPF are significant and independent predictive factors in GC patients. These biologic variables, in addition to established clinicopathologic classification, may identify patients at high risk for relapse who may benefit from more specific clinical treatment, (more aggressive surgery, more precise planning of adjuvant or experimental therapy) and who require a more efficient follow-up schedule. In accord with the published data, our study showed a low frequency (8%) of K-*ras*-2 mutations in GC, a result supporting the hypothesis that different GC carcinogenesis pathways may exist that do not involve K-*ras*-2 mutations.

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