Clinical Cancer Research

Prognostic Significance of DNA Ploidy, S-Phase Fraction, and Tissue Levels of Aspartic, Cysteine, and Serine Proteases in Operable Gastric Carcinoma

Antonio Russo, Viviana Bazan, Manuela Migliavacca, et al.

Clin Cancer Res 2000;6:178-184.

Updated version	Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/6/1/178

Cited Articles	This article cites by 32 articles, 6 of which you can access for free at: http://clincancerres.aacrjournals.org/content/6/1/178.full.html#ref-list-1
Citing articles	This article has been cited by 1 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/6/1/178.full.html#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Downloaded from clincancerres.aacrjournals.org on May 28, 2013. © 2000 American Association for Cancer Research.

Prognostic Significance of DNA Ploidy, S-Phase Fraction, and Tissue Levels of Aspartic, Cysteine, and Serine Proteases in Operable Gastric Carcinoma[1]

Antonio Russo,[2] Viviana Bazan, Manuela Migliavacca, Ines Zanna, Carla Tubiolo, Francesca Maria Tumminello, Gabriella Dardanoni, Massimo Cajozzo, Pietro Bazan, Giuseppe Modica, Mario Latteri, Rosa Maria Tomasino, Giuseppe Colucci, Nicola Gebbia, and Gaetano Leto

Laboratory of Oncobiology [A. R., V. B., M. M., I. Z., C. T.], Sections of Chemotherapy [F. M. T., N. G., G. L.] and Surgery [M. C., G. M., P. B., M. L.], Department of Anatomy, Surgery, and Oncology, and Institute of Pathology "R" [R. M. T.], School of Medicine, University of Palermo, 90127 Palermo, Italy; Unit of Medical and Experimental Oncology, National Cancer Institute of Bari, 70126 Bari, Italy [G. C.]; and Epidemiological Observatory, Center of Sicilian Region, 90100 Palermo, Italy [G. D.]

ABSTRACT

A consecutive series of 63 untreated patients undergoing surgical resection for stage I-IV gastric adenocarcinomas (GCs) has been prospectively studied. Our purpose was to analyze the predictive relevance of DNA ploidy, S-phase fraction (SPF), and tissue levels of lysosomal proteinases cathepsin D (CD), cathepsin B (CB), cathepsin L (CL), and urokinase-type plasminogen activator (uPA) and that of the intracellular cysteine proteinase inhibitor stefin A on clinical outcome. All of the patients taking part in this study were followed up for a median of 73 months. DNA aneuploidy was present in 71% of the cases (45/63), whereas 9% of these (4/45) showed multiclonality. Both DNA ploidy and SPF were associated with tumor-node-metastasis (TNM) stage and node status, whereas only DNA ploidy was related to depth of invasion. CB, CL, uPA, but not CD, levels were significantly higher in GC as compared to paired normal mucosa, whereas stefin A levels were lower in tumor tissues. CB levels were significantly associated with TNM stage, nodal status, histological grade, and DNA ploidy. At univariate analysis, only node involvement, advanced TNM stage, DNA aneuploidy, and high SPF proved to be significantly related to quicker relapse and to shorter overall survival, whereas depth of invasion was related only to survival. With multivariate analysis, only high SPF (>15.2%) was related to risk of relapse (RR = 8.50), whereas high SPF and DNA aneuploidy were independently related to risk of death (RR = 1.88 and 2.09, respectively). Our preliminary prospective study has identified SPF and DNA ploidy as important biological indicators for predicting the outcome of patients with GC.

INTRODUCTION

The traditional factors of GC[3] are still inadequate for the prognostic characterization because patients with identical clinical or pathological stages may differ widely in the clinical evolution (1). It is therefore extremely important to define the prognostic factors that may help to recognize the more aggressive types of such tumors. Reliable and reproducible prognostic indicators are being investigated to help clinicians identify high-risk groups and address more rational treatment decisions. Thus, the identification of factors more strictly related to tumor biology may be useful in characterizing patients with a different prognosis. Recently, more and more attention has been focused on the cell kinetics of many human tumors with possible prognostic significance, including gastrointestinal neoplasias (2). At the present time, one of the most widely used approaches is cellular proliferation rate measured by flow cytometric analysis (SPF), proposed as a rapid, simple, and particularly versatile method applicable to fresh, frozen, and paraffin-embedded tissues. In fact, some reports have observed that SPF was an independent prognostic variable in GC (3–5). Previous studies have also identified a DI and/or DNA ploidy as a useful biological factor, which may predict the clinical outcome of GC (4–8). Furthermore, a consistent bulk of investigations has shown that the expression levels of lysosomal aspartic and cysteine proteinase CD, CB, and CL and serine protease uPA may be significantly altered in tumor cells and tissues as compared to their normal counterpart (9, 10). These alterations appear to be associated with the malignant progression of several human neoplastic diseases including GC (10–16). However, recent studies indicate that at least for CB and CL, this

Received 7/20/99; revised 10/1/99; accepted 10/7/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by grants from the Gruppo Oncologico dell'Italia Meridionale special project "Caratterizzazione biomolecolare del carcinoma gastrico resecato," (protocol N.9602 bis), Associazione Italiana per la Ricerca sul Cancro, Consiglio Nazionale della Ricerca, PF Clinical Application of Cancer Research (ACRO), (contracts 92.02199.PF39 and 96.00570.PF39) and Ministero Universitá Ricecca Scientifica e Tecnologica 60%.

[2] To whom requests for reprints should be addressed, at Via Veneto 5, 90144 Palermo Italy. Phone/fax: 39-091-6554529; E-mail: Laboncobiologia@usa.net.

[3] The abbreviations used are: GC, gastric adenocarcinoma; SPF, S-phase fraction; CD, cathepsin D; CB, cathepsin B; CL, cathepsin L; uPA, urokinase-type plasminogen activator; TNM, tumor-node-metastasis; SA, stefin A; DFS, disease-free survival; OS, overall survival; RR, relative risk; DI, DNA index; MoAb, monoclonal antibody.

Downloaded from clincancerres.aacrjournals.org on May 28, 2013. © 2000 American Association for Cancer Research.

phenomenon may very likely be due to an altered regulation and/or decreased affinity of specific intracellular inhibitors of these proteases (17, 18) or also, as recently reported for CB, the amplification of the gene encoding for these enzymes (19, 20), rather than to an increased enzyme activity *per se*.

The aims of this paper were, firstly, to compare DNA ploidy and SPF, their possible relations to the proteinases CD, CB, CL, and uPA, and that of SA, a specific intracellular inhibitor of cysteine proteinases and clinicopathological features; and secondly, to evaluate the possible prognostic role of either DNA ploidy and SPF or proteinases in GC.

MATERIALS AND METHODS

Patient Features. Paired tumor and normal tissue samples collected (January 1992 to June 1996) by the Laboratory of Oncobiology of the University of Palermo from a consecutive series of 63 patients undergoing resective surgery for primary operable GC at a single institution (Department of Anatomy, Surgery, and Oncology) were prospectively studied. Briefly, exclusion criteria included: (*a*) history of previous neoplasias, and (*b*) prior chemotherapy or radiation therapy. Resection of the primary GC was performed in all cases. All patients underwent potentially curative tumor resection, including radical lymph node dissection. To avoid evaluator variability, all resection specimens and microscopic slides had been meticulously examined by a single pathologist (R. M. T.). In addition, the same pathologist assessed tumor size and site, depth of invasion, TNM stage, tumor grade (histological differentiation), and lymph node metastasis. The depth of tumor invasion was also evaluated histologically as reported by Watanabe *et al.* (13). Tumors were staged according to the latest TNM classification (21). Finally, metastatic cases were identified by clinical and histopathological analyses of neoplastic cells in organs such as lymph nodes, liver, and so forth along with the primary tumor. Clinicopathological data of all patients were available (Table 1). Postoperatively, all patients were checked at 3-month intervals for the first 2 years, at 6-month intervals for the next 2 years, and annually thereafter. The follow-up program included a clinical examination, routine check of circulating tumor markers (CEA, TPA, CA19.9, and CA72.4 assay), annual chest radiography, and endoscopy. Abdominopelvic computed tomography scan was also performed every year for the first 2 years. Disease relapse (local recurrence or distant metastasis) was confirmed histologically where possible. All of the patients with relapse disease after surgery received a standard chemotherapeutic regimen for GC (5-fluorouracil, epirubicin, methotrexate, etoposide, doxorubicin, cisplatin).

Tissue Handling. Multiple samples of the primary tumor tissue were taken from different representative areas and processed within 30 min of surgical resection or biopsy. All tissues were carefully trimmed to remove as much nonneoplastic tissue as possible, avoiding the nonviable areas. Furthermore, from each patient, multiple samples of normal mucosa (as confirmed by histology) were taken in a corresponding nontumor area as far as possible from the tumor site to be used as a control for biochemical/cytochemical and flow cytometric analysis. The tissues were bisected, one-half of each sample was processed for pathological examination, and the remaining half of the sample

Table 1 Patient characteristics (n = 63)

	No. patients (%)
Sex	
Males	43 (68)
Females	20 (32)
Age (yr): range (39–79)	
<60	18 (29)
60–69	26 (41)
≤70	19 (30)
Tumor location	
Antrum	15 (24)
Corpus	26 (41)
Fundus or cardia	22 (35)
Tumor size (cm)	
≤5	28 (44)
>5	35 (56)
Depth of invasion[a]	
PS (−)	44 (70)
PS (+)	19 (30)
TNM stage[b]	
I	15 (24)
II	25 (40)
III	18 (28)
IV	5 (8)
Node status	
Node negative	17 (27)
Node positive	46 (73)
Histological grade[c]	
G_1	3 (5)
G_2	25 (40)
G_3	35 (55)

[a] PS (−), invasion into the muscularis propria or into the subserosa without infiltrative growth; PS (+), invasion into the subserosa with infiltrative growth (13).

[b] Stage I: $T_1N_0M_0$; $T_1N_1M_0$; $T_2N_0M_0$. Stage II: $T_1N_2M_0$; $T_2N_1M_0$; $T_3N_0M_0$. Stage III: $T_2N_2M_0$; $T_3N_1M_0$; $T_4N_0M_0$; $T_3N_2M_0$; $T_4N_1M_0$. Stage IV: $T_4N_2M_0$; any T, any N, M_1.

[c] G_1, G_2, and G_3: well-, moderately-, and poorly-differentiated tumors, respectively.

pool was immediately frozen and stored at −80°C until analyzed. The adequacy of the material was checked on frozen tissue sections, and only tissue samples with >80% tumor content were used in subsequent biochemical/cytochemical and flow-cytometric analysis.

Cellular DNA Content and SPF Flow Cytometric Examination. DNA flow cytometry was performed on mechanically disaggregated samples of frozen tumor tissue as in previously described protocols (22). DNA histogram analysis was carried out by the Multicycle Software Program (Phoenix Flow Systems, San Diego, CA), including systematic background substraction (23, 24). DNA ploidy, DI, and SPF were determined as previously reported (22). Briefly, healthy gastric mucosa was used as an internal DNA-diploid control for each sample. Tumors with a DI = 1 were defined as DNA-diploid, and tumors with lower or higher DI values were considered DNA-aneuploid if they contained >10% aneuploid cells.

Tissue Extraction and Determination of CB, CD, CL, uPA, and SA Tissue Content. Paired sets of tumor and normal tissue specimens (70–210 mg) were thawed, minced, and suspended at 25 mg/ml in a tissue homogenization buffer [50 mM Tris-HCl, 5 mM EDTA, 0.5 mM DTT, and 0.2% v/v Triton X-100 (pH 6.9)]. Samples were homogenized at 2°C in

Downloaded from clincancerres.aacrjournals.org on May 28, 2013. © 2000 American Association for Cancer Research.

Table 2 Significative relationships of DNA ploidy and SPF to clinicopathological variables

	DNA ploidy				SPF		
	D[a]	AMon	AMu1	*P*	≤15.2%	>15.2%	*P*
Depth of invasion							
PS (−)[a]	18	23	3				
PS (+)	0	18	1	<0.01			
TNM stage							
I	10	4	1		13	2	
II	8	15	2		14	11	
III–IV	0	22	1	<0.01	4	19	<0.01
Node status							
Node negative	11	5	1		15	2	
Node positive	7	36	3	<0.01	16	30	<0.01
SPF							
≤15.2%	16	15	0				
>15.2%	2	26	4	<0.01			

[a] D, DNA-diploid; AMon, DNA-aneuploid monoclonal; AMul, DNA-aneuploid multiclonal; PS (−), invasion into the muscularis propria or into the subserosa without infiltrative growth; PS (+), invasion into the subserosa with infiltrative growth.

an ice slurry in a Willelms Polytron PT-10 homogenizer (Kinematica, Luzern, Switzerland) set to rotate at 9, with two bursts of 30 s and a 1-min intercooling period. The homogenate was centrifuged at 500 g for 10 min. The supernatant was respun at 13,000 g for 15 min at 4°C in a preparative ultracentrifuge (Beckman Model L80). The 13,000-g supernatant (*i.e.,* cytosol) was stored at −80°C until assays. Total CD content was determined, as previously reported (25), by an antibody-based immunoenzymatic assay kit (Triton, Ciba Corning, Diagnostics, Alameda, CA) according to the manufacturer's instructions. The detection limit was 0.018 pmol/ml. Total CB, CL, and SA tissue levels were determined by commercially available solid phase ELISA kits (KRKA d.d., Novo mesto, Slovenia) in cytosol diluted 1:2 or 1:5 (v/v) according to the manufacturer's protocol. Antibodies used for CB and CL assays recognized mature forms, precursor molecules, and inhibitor-cathepsin complexes. For the determination of SA, MoAbs used in sandwich ELISA were obtained by hybridoma cell lines different from those used to produce other cysteine protease inhibitor MoAbs (26). The minimal detectable concentrations were 0.9 ng/ml for CB, 1.6 ng/ml for CL, and 1.04 ng/ml for SA. uPA levels were determined by a commercially available immunoluminometric assay kit (Byk Sangtec, Cormano, Italy). The MoAbs used bind either to the proenzyme or to the active form as well as to the enzyme bound to its receptor (uPA-R) or to its endogenous inhibitor (PAI-1). The detection limit was reported to be <0.005 ng/ml. The cytosol protein content was determined by a commercially available colorimetric micromethod kit (Sigma, St. Louis, MO).

Statistical Analysis. Because of their asymmetric distribution, the Mann-Whitney *U* test was used to evaluate differences in proteinases and SPF values between the two categories of DNA ploidy. The nonparametric Spearman correlation method was used to evaluate the correlation between the proteinases variables examined. DFS was measured from the day of primary surgery to the date of first relapse (locoregional or metastatic), and OS was measured from the day of surgery to the date of death specifically due to the tumor. Clinical and morphobiological variables were examined univariately by means of the Kaplan-Meier method (27), and significance of differences for each prognostic factor was assessed by the log-rank test and Wilcoxon test or test for trend when appropriate (28). Multivariate analysis was carried out by means of the Cox's logistic regression model using a backward procedure (29). The null hypothesis $\beta = 0$ was tested by the Wald statistic. The relative contribution of clinicopathological and biological 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 RR and the associated 95% confidence limits. The 0.05 level of probability was taken as significant.

RESULTS

Cellular DNA Content and S-Phase Evaluation. Adequate DNA histograms were obtained for all normal and tumoral gastric tissues by means of flow-cytometry. The coefficients of variation of the diploid G_0/G_1 peak ranged from 2.6% to 4.8% (mean, 3.6%). DNA aneuploidy was found in 71% of the cases (45/63), while 9% of these (4/45) showed multiclonality. The SPF ranged from 3.5% to 37.2% (median, 15.2% and interquartile range, 12.1–19.1%). By using the SPF median value as the cutoff point, tumors were accordingly divided into low (≤15.2%) and high (>15.2%) SPF tumors. Table 2 gives a summary of the significant associations of DNA ploidy and SPF to the clinicopathological variables for GC. Both DNA ploidy and SPF were associated with TNM stage and node status, whereas only DNA ploidy was related with depth of invasion. Furthermore, a significant association was found between flow cytometric variables (DNA ploidy and SPF). No association was found between DNA ploidy and SPF and any of the following factors: age, sex, histological grade, tumor size, and tumor site.

CB, CD, CL, SA, and uPA Activity Levels in Gastric Tumor and Normal Tissues. Table 3 reports the tissue levels of CD, CB, CL, uPA, and SA determined in GC and in the corresponding normal mucosa. Only CB, CL, and uPA levels were significantly increased in tumor tissue as compared to the paired normal counterpart ($P < .05$ for CL and $P < .01$ for CB

Downloaded from clincancerres.aacrjournals.org on May 28, 2013. © 2000 American Association for Cancer Research.

Table 3 CB, CD, CL, SA, and uPA levels in GC and paired normal gastric mucosa

	CB (ng/mg protein)	P	CD (pmol/mg protein)	P	CL (ng/mg protein)	P	SA (ng/mg protein)	P	uPA (ng/mg protein)	P
Median										
Mucosa	61.6		27.6		31.0		33.8		0.073	
Tumor	142.0	<0.01	22.5	NS[a]	33.3	<0.05	1.7	NS	0.288	<0.01
Range										
Mucosa	0–408		0–77.4		0–68.6		0–643		0–1.12	
Tumor	0–616		0–104		0–132.5		0–440		0–1.90	

[a] NS, not significant.

Table 4 Correlation between CD, CB, CL, SA, and uPA in GC

	CB	CD	CL	SA	uPA
CB		ro = 0.34 $P < 0.01$	NS[a]	ro = 0.45 $P < 0.01$	ro = 0.28 $P < 0.05$
CD			NS	ro = 0.25 $P = 0.051$	ro = 0.39 $P < 0.01$
CL				NS	NS
SA					ro = 0.36 $P < 0.01$

[a] NS, not significant.

and uPA; Table 3). Moreover, the SA levels were lower in tumor tissue as compared to normal tissue; however, this decrease was not statistically significant. CD tumor levels were significantly correlated with CB, uPA, and SA ($P < .05$), whereas CB levels were correlated with CD, uPA, and SA but not with CL (Table 4). SA levels correlate with all of the enzymes considered, but not with CL. Moreover, CB levels were significantly associated with TNM stage [I (median) = 93.3; II = 142.0; III-IV = 159.8; $P < .05$], node status [node negative (median) = 91.5; node positive = 159.7; $P < .06$], histological grade [G_1 (median) = 63.2; G_2 = 121.7; G_3 = 159.8; $P < .01$] and DNA ploidy [Diploid (median) = 92.4; Aneuploid monoclonal = 156.3; Aneuploid multiclonal = 250.9; $P < .01$].

Impact on Relapse and Survival. The median follow-up of patients was 73 months (range, 12–108 months). At the time of analysis (April 1998), 31 patients had relapsed (2 had local-regional recurrence and 29 had distant metastases) and 30 had died of a tumor-related cause. The overall 5-year survival was 45.8% (SE, 6.9) for the whole series. At the univariate analysis, high stage, lymph-node positivity, DNA aneuploidy, and high SPF proved to be significantly related to quicker relapse and to shorter OS (Table 5), whereas depth of invasion was related only to survival. Univariate analysis failed to reveal any significant association between age, sex, histological grade, tumor location, tumor size, CB, CD, CL, uPA, or SA, and relapse, or survival. Fig. 1 shows the probability of disease-free interval in relation to SPF; Fig. 2 shows the probability of OS by SPF and DNA ploidy. In Cox's model for multivariate analysis, only the significant variables at univariate analysis were considered. The estimated hazards ratio was higher for SPF (>15.2%), which appeared to be the most relevant indicator of relapse (RR = 8.50), whereas multiple regression analysis carried out for OS showed that SPF and DNA ploidy provided independent information for predicting risk of death (Table 6).

DISCUSSION

Many attempts have been made to find new prognostic indicators for patients with GC to identify the subgroup with highly aggressive tumors and a high likelihood of disease relapse or death. Aggressive surgical approaches, such as extended lymph node dissection or at least intensive postoperative therapy, might be options for these poor prognosis patients. There is still some controversy as to whether or not the DNA content and SPF are significant prognostic factors in GC. Several reports have shown that patients with DNA-aneuploid and/or high SPF tumors had a worse prognosis than those with DNA-diploid and/or low SPF tumors (3–7). Recently, in a study performed on fresh tumor specimens taken from 76 patients with GC, Abad *et al.* (6) found that DNA aneuploidy, histological type, and the presence of extranodal metastases were independent predictors of OS. By contrast, SPF did not predict patient outcome. These results agree with those of Setala *et al.* (7) and Victorzon *et al.* (8), whose respective analysis of 289 and 242 GC cases led to the observation that DNA ploidy and TNM stage significantly predicted OS, whereas no significant difference was found between SPF and prognosis. In contrast, Lee *et al.* (3), in a 5-year prospective study of 217 patients with GC, found that SPF was an independent prognostic variable, whereas DNA ploidy was not associated with patient survival. Finally, Yonemura *et al.* (4) and Ohyama *et al.* (5), in their respective series of 493 and 117 patients with GC using multivariate analysis, found that DNA content and SPF (measured by *in vivo* bromodeoxyuridine) were independent prognostic factors for OS. In our prospective study, which was based on univariate and multivariate analyses with established prognostic parameters (depth of invasion, TNM stage, node status, histological grade), we found that SPF was the only predictor of disease relapse. Furthermore, the present study clearly shows that both DNA ploidy and SPF are significant and independent prognostic factors for OS in patients with GC who have undergone surgical resection. Our data also show that DNA multiploidy represents the most powerful predictive indicator for survival. These results suggest that studies of pathological prognostic factors should include these biological variables. Moreover, because DNA analysis of endoscopic biopsy specimens correlates well with surgical specimen DNA analysis (30), preoperative evaluation of flow cytometric variables may be useful for predicting prognosis and choosing an adequate therapeutic modality in patients with GC. The frequency of DNA aneuploidy has been reported in a variable percentage of GC, from 36% to 71% (3–5, 6–8, 31, 32). In the present series, the

Downloaded from clincancerres.aacrjournals.org on May 28, 2013. © 2000 American Association for Cancer Research.

Table 5 Univariate analysis of DFS and OS: distribution of patients for the significant variables according to Kaplan-Meier method

	DFS (n = 63)[a]				OS (n = 59)			
Variable	DFS (%) 24 mo	DFS (%) 60 mo	O/E[a]	P	OS (%) 36 mo	OS (%) 60 mo	O/E	P
Depth of invasion								
PS (−)					69	54	0.80	
PS (+)					44	22	1.80	<0.01
TNM stage								
I	79	71	0.47		79	71	0.44	
II	72	55	0.78		75	57	0.72	
III–IV	40	18	1.94	<0.01	34	11	2.29	<0.01
Node status								
Node negative	81	81	0.29		81	81	0.28	
Node positive	55	34	1.36	<0.01	56	31	1.41	<0.01
DNA ploidy								
Diploid	88	82	0.26		94	88	0.16	
An. monoclonal	54	36	1.30		55	30	1.43	
An. multiclonal	25		3.55	<0.01			3.70	<0.01
SPF								
≤15.2%	87	80	0.31		86	82	0.26	
>15.2%	38	10	2.20	<0.01	38	7	2.36	<0.01

[a] O/E, observed/expected; PS (−), invasion into the muscularis propria or into the subserosa without infiltrative growth; PS (+), invasion into the subserosa with infiltrative growth.

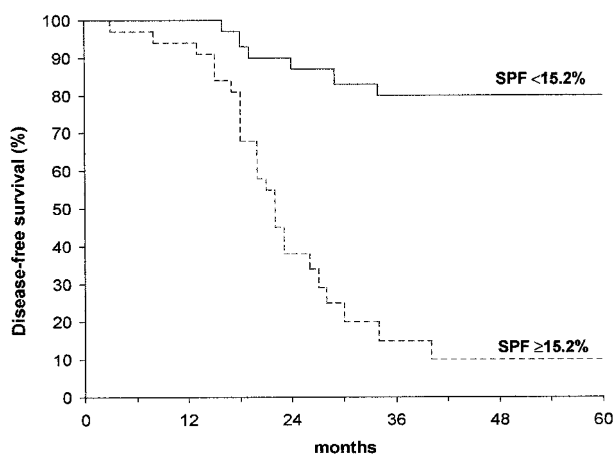

Fig. 1 Probability of DFS according to SPF (≤15.2% *versus* >15.2%; $P < 0.01$) in GC patients.

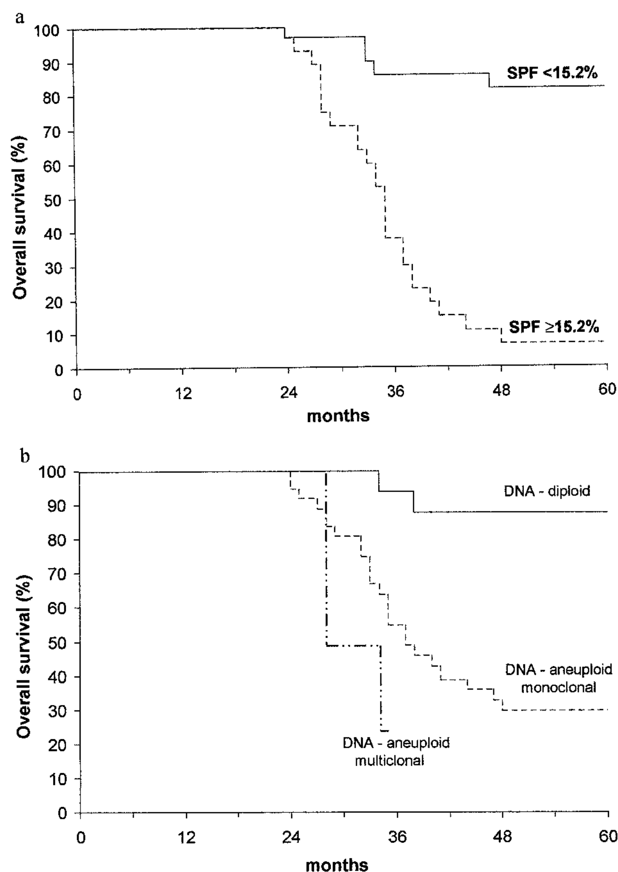

Fig. 2 Probabilty of OS according to (*a*) SPF (≤15.2% *versus* >15.2%; $P < 0.01$) and to (*b*) DNA ploidy status (DNA-diploid *versus* DNA-aneuploid monoclonal *versus* DNA-aneuploid multiclonal; $P < 0.01$) in GC patients.

DNA-aneuploid rate (71%) was among the highest published thus far. This is probably due to the use of multiple tissue sampling of all cases studied, which greatly reduces the probability of missing DNA-aneuploid clones, and to the method chosen to preserve the samples (freezing at −80°C), which makes it possible to obtain a higher histogram resolution compared with paraffin-embedded samples. The present study shows that DNA ploidy and/or SPF are significantly associated to depth of invasion, advanced clinical stage, and the presence of lymph node metastases. Histopathological features are universally considered bad prognostic factors (14). Thus, the association with these variables suggests that flow cytometric indicators are related to tumor aggressiveness. This is in accordance with previous studies (6–8). Moreover, the perturbance of cell cycle due to an increased proliferative activity of aneuploid clones or to a prolongation of the S phase may enhance the risk

Downloaded from clincancerres.aacrjournals.org on May 28, 2013. © 2000 American Association for Cancer Research.

Table 6 Cox proportional hazard analysis to predict the RR of DFS or OS in GC patients

	DFS				OS			
	RR	*P*	CI[a] (95%)	β	RR	*P*	CI (95%)	β
SPF								
(>15.2% *vs.* ≤15.2%)	8.50	<0.01	3.37–21.4	2.14	6.58	<0.01	2.33–18.6	1.88
DNA ploidy								
An. Mon. *vs.* diploid					4.47	NS	0.99–20.2	1.50
An. Mul. *vs.* diploid					8.09	<0.05	1.29–50.6	2.09

[a] CI, confidence interval; NS, not significant.

of genetic alterations. This phenomenon may result in the onset of DNA-aneuploid cells, which would explain the significant relationship between high SPF and aneuploidy observed in the present study. Finally, unlike other investigators (7, 31), we did not observe a significant association between DNA ploidy and tumor site (fundus or cardia) as noted in three other series (3, 8, 33,). The present results confirm, in part, the data obtained from other studies, which have reported elevated content or activity levels of CB, CL, and uPA in GC tissues as compared to normal gastric mucosa (11–13, 15, 16, 32).

However, only CB proved to be significantly associated with most of the clinicobiological parameters of progression of this tumor. Moreover, the intracellular cysteine proteinase inhibitor SA levels were lower in tumor tissue as compared to those in its normal counterpart. These results indicate that the altered regulation of cysteine proteinases/SA ratio at the tumor level may facilitate the progression of GC. In fact, this phenomenon has been observed "*in vitro* " in human cancer cell lines, and it has proved to be associated with a more malignant phenotype (34, 35). Furthermore, decreased levels of SA have also been seen "*in vivo* " in different human tumors of epithelial origin and have proved to be associated with the metastatic potential and differentiation of these tumors (11, 18, 19). These data suggest that the increased level of CB, together with other proteolytic enzyme (metalloproteases), which appear to determine the malignant phenotype of GC, may play a major role in the progression of this tumor (34, 36, 37). However, the recent observations, which showed that proteinase inhibitors at subinhibitor concentrations may act as mitogens thus facilitating cell proliferation (19, 35), indicate that SA may also directly share the malignant progression of GC with a mechanism not related to its proteinase-inhibiting activity. This may in part explain the direct and not the inverse correlation, which might be expected, observed between CB and SA at the tumor level. On the other hand, conflicting results have been obtained regarding the distribution of CD. In fact, unlike the results reported in other studies (14, 16), we did not observe any significant difference in the levels of this protease between GC and normal mucosa or the association with most of the clinicobiological parameters considered, including disease relapse and death. These conflicting results may be partly explained by the different methodological approaches used to assess this parameter (*i.e.,* immunohistochemistry *versus* immunoenzyme assay). Our investigations also showed significantly higher levels of uPA content in tumor tissue as compared to those in normal tissue; no significant association was found, however, between the uPA system and the clinicobiological parameters of GC. These results are partly in agreement with those from other studies (12, 16, 37) and further indicate that the evaluation of the single components of the uPA system, namely the uPA receptor or uPA inhibitors PAI-1 and PAI-2, may be more appropriate for the assessment of the prognostic significance of this serine protease in human cancer (15, 16). However, it cannot be ruled out that the different results we obtained on the prognostic significance of the uPA complex may also be due to the different number of patients evaluated in our study.

In conclusion, DNA ploidy and SPF may be reasonably considered as indicators of biological and clinical aggressiveness. If our results are confirmed in larger prospective studies, it might be possible to add such variables to other prognostic factors for more aggressive strategies such as extended lymph node dissection or intensive postoperative therapy in high risk groups (patients with high SPF and/or DNA-aneuploid tumors) to prevent disease relapse or death. In addition, the clinical impact of the CB/SA system as biochemical prognostic parameters in this tumor has still to be sufficiently assessed.

REFERENCES

1. Allgayer, H., Heiss, M. M., and Schildberg, F. W. Prognostic factors in gastric cancer. Br. J. Surg., *84:* 1651–1664, 1997.

2. Silvestrini, R. Cell kinetics: prognostic and therapeutic implications in human tumours. Cell. Proliferation, *27:* 579–596, 1994.

3. Lee, K. H., Lee, J. S., Lee, J. H., Kim, S. W., Suh, C., Kim, W. K., Kim, S. H., Min, Y. I., Kim, B. S., Park, K. C., Lee, M. S., and Sun, H. S. Prognostic value of DNA flow cytometry in stomach cancer: a 5-year prospective study. Br. J. Cancer, *79:* 1727–1735, 1999.

4. Yonemura, Y., Ooyama, S., Sugiyama, K., Kamata, T., De Aretxabala, X., Kimura, H., Kosaka, T., Yamaguchi, A., Miwa, K., and Miyazaki, I. Retrospective analysis of the prognostic significance of DNA ploidy patterns and S-phase fraction in gastric carcinoma. Cancer Res., *50:* 509–514, 1990.

5. Ohyama, S., Yonemura, Y., and Miyazaki, I. Prognostic value of S-phase fraction and DNA ploidy studied with *in vivo* administration of bromodeoxyuridine on human gastric cancers. Cancer (Phila.), *65:* 116–121, 1990.

6. Abad, M., Ciudad, J., Rincon, M. R., Silva I., Paz-Bouza, J. I., Lopez, A., Alonso, A. G., Bullon, A., and Orfao, A. DNA aneuploidy by flow cytometry is an independent prognostic factor in gastric cancer. Anal. Cell. Pathol., *16:* 223–231, 1998.

7. Setala, L. P., Nordling, S., Kosma, V. M., Lipponen, P. K., Eskelinen, M. J., Hollmen, S. M., Syrjanen, K. J., and Alhava, E. M. Comparison of DNA ploidy and S-phase fraction with prognostic factors in gastric cancer. Anal. Quant. Cytol. Histol., *19:* 524–532, 1997.

8. Victorzon, M., Roberts, P. J., Haglund, C., von Boguslawsky, K., and Nordling, S. Ki-67 immunoreactivity, ploidy and S-phase fraction as prognostic factors in patients with gastric carcinoma. Oncology, *53:* 182–191, 1996.

Downloaded from clincancerres.aacrjournals.org on May 28, 2013. © 2000 American Association for Cancer Research.

9. Rochefort, H., Liaudet, E., and Garcia, M. Alterations and role of human cathepsin D in cancer metastasis. Enzyme Protein, *49:* 106–116, 1996.

10. Lah, T. T., and Kos, J. Cysteine proteinases in cancer progression and their clinical relevance for prognosis. Biol. Chem., *379:* 125–130, 1998.

11. Farinati, F., Herszenyi, L., Plebani, M., Carraro, P., De Paoli, M., Cardin, R., Roveroni, G., Rugge, M., Nitti, D., Grigioni, W. F., D'Errico, A., and Naccarato, R. Increased levels of cathepsin B and L, urokinase-type plasminogen activator and its inhibitor type-1 as an early event in gastric carcinogenesis. Carcinogenesis (Lond.), *17:* 2581–2587, 1996.

12. Plebani, M., Herszènyi, L., Cardin, R., Roveroni, G., Carraro, P., Paoli, M. D., Rugge, M., Grigioni, W. F., Nitti, D., Naccarato, R., and Farinati, F. Cysteine and serine proteases in gastric cancer. Cancer (Phila.), *76:* 367–375, 1996.

13. Watanabe, M., Higashi, T., Watanabe, A., Osawa, T., Sato, Y., Kimura, Y., Tominaga, S., Hashimoto, N., Yoshida, Y., Morimoto, S., Shiota, T., Hashimoto, M., Kobayashi, M., Tomoda, J., and Tsuji, T. Cathepsin B and L activities in gastric cancer tissue: correlation with histological findings. Biochem. Med. Metab. Biol., *42:* 21–29, 1989.

14. Allgayer, H., Babic, R., Grutzner, K. U., Beyer, B. C., Tarabichi, A., Schildberg, W. F., and Heiss, M. M. An immunohistochemical assessment of cathepsin D in gastric carcinoma: its impact on clinical prognosis. Cancer (Phila.), *80:* 179–187, 1997.

15. Ganesh, S., Sier, C. F. M., Heerding, M. M., van Krieken, J. H. J. M., Griffioen, G., Welwaart, K., van de Velde, C. J. H., Lamers, C. B. H. W., and Verspaget, H. W. Prognostic value of the plasminogen activation system in patients with gastric carcinoma. Cancer (Phila.), *77:* 1035–1043, 1996.

16. Allgayer, H., Babic, R., Grutzner, U., Beyer, B. C. M., Tarabichi, A., Schildberg, F. W., and Heiss, M. M. Tumor-associated proteases and inhibitors in gastric cancer: analysis of prognostic impact and individual risk protease patterns. Clin. Exp. Metastasis, *16:* 62–73, 1998.

17. Henskens, Y. M. C., Veerman, E. C. I., and Amerongen, A. V. N. Cystatins in health and disease. Biol. Chem. Hoppe-Seyler, *377:* 71–86, 1996.

18. Scott, G. K. Proteinases and proteinase inhibitors in tumor cell growth and metastasis. Cancer J., *10:* 80–86, 1997.

19. Abdollahi, A., Getts, L. A., Sonoda, G., Miller, P. D., Taguchi, T., Godwin, A. K., Testa, J. H., and Hamilton, T. C. Genome scanning detects amplification of the *cathepsin B* gene (*CTsB*) in transformed rat ovarian surface epithelial cells. J. Gynecol. Invest., *6:* 32–40, 1999.

20. Hughes, S. J., Glover, T. W., Zhu, X. X., Kuick, R., Thornual, D., Orringer, M. B., Beer, D. G., and Hananh, S. A novel amplicon at 8p22–23 results in overexpression of cathepsin B in esophageal adenocarcinoma. Proc. Natl. Acad. Sci. USA, *95:* 12410–12415, 1998.

21. Bears, O. H., Hensen, D. E., Hutter, R. V. P., and Meyers, M. H. Manual for Staging of Cancer, 3rd ed., pp. 69–71. Philadelphia: J. B. Lippincott, 1988.

22. Russo, A., Bazan, V., Morello, V., Tralongo, V., Nagar, C., Nuara, R., Dardanoni, G., Bazan, P., and Tomasino, R. M. Vimentin expression, proliferating cell nuclear antigen and flow cytometric factors. Anal. Quant. Cytol. Histol., *16:* 365–374, 1994.

23. Dean, P. N., and Jett, J. H. Mathematical analysis of DNA distribution derived from flow cytometry. J. Cell Biol., *60:* 523–527, 1974.

24. Rabinovitch, P. S. Numerical compensation for the effects of cell clumping on DNA histograms. Cytometry, *4:* 27, 1990.

25. Tumminello, F. M., Gebbia, N., Pizzolanti, G., Russo, A., Bazan, V., and Leto, G. Cathepsin D content in colorectal cancer. Correlation with cathepsin D activity and other biological parameters. A Preliminary Report. Oncology, *52:* 237–242, 1995.

26. Jerala, R., Zerounik, E., Lohner, K., and Turk, V. Structural basis for the difference in thermodynamic proprieties between the two cysteine proteinase inhibitors human stefin A and B. Protein Eng., *7:* 977–984, 1994.

27. Kaplan, E. L., and Meier, P. Non parametric estimation for incomplete observations. J. Am. Stat. Assoc., *53:* 457–481, 1958.

28. Peto, R., Pike, M. C., Armitage, P., Breslow, N. E., Cox, D. R., Howard, S. V., Mantel, N., McPherson, K., Peto, J., and Smith, P. G. Design and analysis of randomized clinical trials requiring prolonged observation of each patients. II. Analysis and examples. Br. J. Cancer, *35:* 1–39, 1977.

29. Cox, D. R. Regression models and life tables. J. R. Stat. Soc., *34:* 187–220, 1972.

30. Etoh, K., Kaketani, K., Saito, T., Uchino, S., Shimoda, K., Miyahara, M., and Kobayashi, M. DNA stem-line heterogeneity as preoperative prognostic factor in esophageal cancer. Gan to Kagakn Ryoho, *21:* 1–5, 1994.

31. Johnson, H., Jr., Belluco, C., Masood, S., Abou-Azama, A. M., Kahn, L., and Wise, L. The value of flow cytometric analysis in patients with gastric cancer. Arch. Surg., *128:* 314–317, 1993.

32. Danova, M., Riccardi, A., Mazzini, G., Wilson, G., Dionigi, P., Brugnatelli, S., Fiocca, R., Ucci, G., Jemos, V., and Ascari, E. Flow cytometric analysis of paraffin-embedded material in human gastric cancer. Anal. Quant. Cytol. Histol., *10:* 200–206, 1988.

33. Nanus, D. M., Kelsen, D. P., Niedzwiecki, D., Chapman, D., Brennan, M., Cheng, E., and Melamed, M. Flow cytometry as a predictive indicator in patients with operable gastric cancer. J. Clin. Oncol., *7:* 1105–1112, 1989.

34. Scaddan, P. B., and Dufresne, M. J. Characterization of cysteine proteases and their endogenous inhibitors in MCF-7 and Adriamycin-resistant MCF-7 human breast cancer lines. Invasion Metastasis, *13:* 301–313, 1993.

35. Sun, Q. Growth stimulation of 3T3 fibroblast by cystatin. Exp. Cell. Res., *180:* 150–160, 1989.

36. Chambers, A., Colella, R., Denhardt, D. T., and Wilson, S. Increased expression of cathepsin L and B and decreased activity of their inhibitors in metastatic ras transformed NIH 3T3 cells. Mol. Carcinog., *5:* 238–245, 1992.

37. Schwartz, G. K., Wang, H., Lampen, N., Altorki, N., Kelsen, D., and Albino, A. P. Defining the invasive phenotype of proximal gastric cancer cells. Cancer (Phila.), *73:* 22–27, 1994.

Downloaded from clincancerres.aacrjournals.org on May 28, 2013. © 2000 American Association for Cancer Research.