ANNALS OF SURGERY
Vol. 226, No. 6, 736–745

Clinical Value of Extended Biologic Staging by Bone Marrow Micrometastases and Tumor-Associated Proteases in Gastric Cancer

Markus Maria Heiss, M.D.,* Heike Allgayer, M.D.,* Klaus Uwe Gruetzner, M.D.,* Rudolf Babic, M.D.,† Karl-Walter Jauch, M.D.,* and Friedrich Wilhelm Schildberg, M.D.*

*From the *Department of Surgery, Klinikum Grosshadern, and the †Institute of Pathology, Ludwig Maximilians University of Munich, Germany*

Objective
To investigate whether extended staging, including biologic grading and aspects of an early systemic disease component, would give additional prognostic information on patients with curatively resected gastric cancer.

Background
Tumor-associated proteolytic mechanisms have been shown to be essential for invasion and metastasis. The urokinase-type plasminogen activator (uPA) system is of major biologic impact, but different interactive proteases and inhibitors with modulating effects also must be considered. The detection of early tumor cell dissemination indicates the systemic character of a primarily local gastric cancer. The confrontation of the organism with these cells determines the often unpredictable course of an individual tumor after presumed curative primary treatment.

Methods
In a prospective study of 247 consecutive patients with gastric cancer, detection of disseminated tumor cells in bone marrow aspirates was immunocytochemically performed in 180 patients. The expression of uPA, activators of uPA (cathepsin D, antithrombin III), uPA substrates (plasminogen, matrix-metalloproteinase 2 [collagenase IV, 72 kD; MMP-2]), uPA/plasmin inhibitors (plasminogen activator inhibitor type 1 and 2 [PAI-1, PAI-2], α1-antitrypsin, α2-antiplasmin), uPA receptor (uPA-R), and parameters of the uPA-R cycle (α2-macroglobulin, α1-antichymotrypsin) could be determined immunohistochemically and were scored semiquantitatively in 203 patients. Kaplan-Meier statistical techniques and multivariate Cox regression models were used for prognostic analyses.

Results
In multivariate analysis considering all the established risk factors, disease-free survival was independently predicted by PAI-1 (relative risk 2.21, 1.32–3.73) and cathepsin D (relative risk 2.98, 1.28–6.91) besides pT, pN, and extended resection. Tumor cell dissemination was found to be an additional prognostic factor in early tumor stages (pT1, T2) and lymph-

node-negative patients. Stepwise regression analysis revealed an extended staging system with new risk groups. Node-positive, curatively resected pT1/2 patients with low expression of PAI-1 had a favorable prognosis (mean recurrence-free survival [MRT] 54.84 months), similar to that of node-negative patients (MRT 54.76 months). In node-negative, curatively resected pT1/2 patients, detection of bone marrow tumor cells and high expression of PAI-1 defined a subgroup with a steep decrease of prognosis (MRT 36.60 months), which was worse than that of node-positive patients (MRT 45.81).

Conclusion

This new staging model gives better prognostic differentiation of subgroups, which should be considered in future adjuvant therapy protocols. In addition, it indicates that the uPA system might be a future therapeutic target.

Despite improvements and international standardization of the clinical treatment of gastric cancer, the overall outcome of patients with gastric carcinoma has not improved during the last years. However, the description of the exact stage of the tumor disease has led to differential therapeutic concepts with large differences in stage-dependent prognosis.[1] Therefore, in the procedure of staging, it seems desirable to determine the most relevant factors for the prediction of clinical outcome for each patient. Ideally, these factors should also imply options for therapeutic interventions, based on the information they provide about the tumor's biology.

In gastric cancer, the established, conventional prognostic factors describe the anatomic extent of the tumor (TNM, tumor diameter and localization, Borrmann classification) and the histomorphologic aspect of carcinomatous tissue (G, Laurén classification).[1] Unfortunately, these factors could not reach the level of opening new therapeutic options. Until now, they have also been unable to differentiate patients who would probably benefit from adjuvant multimodal protocols.

Recently the approach toward solid tumors has changed, largely because of new knowledge about tumor cell behavior and its functional tools in tumor progression. Tumor cell invasion and metastasis biologically cohere with proteolytic destruction of surrounding matrix components, including basement membranes of vessels, to reach the systemic circulation. Evidence has accumulated that this is achieved by a series of tumor-associated serine-, aspartic-, cysteine-, threonine-, and metalloproteinases.[2–8] Several investigations indicate that some of these protease systems are overexpressed in tumors and that tumors with high evidence of proteolytic parameters are more invasive than others.[2,5,9,10] This has been especially shown for the urokinase-type plasminogen activator (uPA) system (uPA, uPA receptor [uPA-R], plasminogen activator inhibitor type 1 [PAI-1]).[2,9,10] For gastric cancer, our group recently demonstrated an overexpression of the uPA system with strong prognostic impact.[11] Corroborating the results of different groups suggests that, emphasizing PAI-1, the uPA system is one essential biologic measure of invasiveness.[12,13]

However, other proteases and inhibitors are also connected to the uPA system and provide modulating effects and cascade activations.[4,8] Matrix-metalloproteinase 2 (MMP-2), the 72-kD form of collagenase IV, can degrade basement membrane collagen IV and can be activated by uPA.[4,5] Cathepsin D degrades extracellular matrix components and activates cathepsin B, which in turn activates uPA. Active plasmin is inhibited by α2-antiplasmin and α2-macroglobulin.[4] α2-Macroglobulin is further hypothesized to enhance transcellular uPA-receptor circulation.[2] Trypsin is a proteolytic activator of pro-uPA[4] and is inhibited by α1-antitrypsin.[14] In contrast, chymotrypsin is thought to inactivate uPA receptors by cleavage of one of the three uPA-R protein domains.[15] Thus, the chymotrypsin inhibitor α1-antichymotrypsin potentially protects the uPA receptor. Thrombin is thought to cleave uPA proteolytically to an inactive enzyme form.[4] It is antagonized by antithrombin III, but antithrombin III, on the contrary, also can inactivate uPA.[16] In further analyses, we tried to describe these complex patterns with respect to prognostic information.

A comprehensive biologic staging would also consider additional factors such as adhesion molecules, oncogenes, or tumor suppressor genes.[1] However, our approach centers on the uPA system, a primary factor with proven potential for biologic grading in gastric cancer.

Pathophysiologically, the invasiveness of tumor cells is closely associated with detachment and dissemination of tumor cells from the primary tumor, a phenomenon

This study was supported by grant M74/91/Schi 1 from Deutsche Krebshilfe, Dr. Mildred Scheel Foundation. R.B. was supported by the Wilhelm Sander Stiftung, Neustadt/Donau, Germany. Present address: Institute of Pathology and Cytology, Deggendorf, Germany.

Address reprint requests to Markus Maria Heiss, M.D., Department of Surgery, Klinikum Grosshadern, Ludwig Maximilians University of Munich, 81377 Munich, Germany; phone: 00xx-89-7095-1; fax: 00xx-89-7004418.

Accepted for publication May 5, 1997.

with prognostic impact.[17] The detection of early tumor cell dissemination probably indicates the systemic character of gastric cancer, serving as the decisive step from a local tumor toward systemic residual disease despite curative operative treatment. However, more than the sole presence of these cells at the time of surgery, the development of systemic residual disease was strongly associated with clinical prognosis. This implies a biologic autonomy of the systemic disease component in gastric cancer.[18–20]

Both parameters—PAI-1, as representative of biologic grading, and bone marrow tumor cell detection, as representative of systemic disease—were used in this study to determine whether these variables would yield additional prognostic information in curatively resected patients, leading to a better definition of prognostic subgroups with new options for adjuvant therapeutic strategies.

PATIENTS, MATERIALS, AND METHODS

Patients and Operations

Two hundred forty seven patients in a prospective series underwent surgery for gastric cancer between November 1988 and October 1991. After informed consent, 219 of them underwent intraoperative bone marrow aspiration. One hundred twenty-two patients were male, 97 female (ratio 1.27). Mean age was 64.3 years (standard deviation, 11.8).

Of the 219 patients, 139 were curatively (R0) resected (63%). Of these, 72 (52%) underwent total gastrectomy, 43 (31%) subtotal gastrectomy, and 24 (17%) extended resections (colon, pancreas, liver). Of the 80 (36%) palliatively operated patients, 22 (28%) were R1 resected, and 36 (45%) R2. Twenty-two (28%) operations were ceased as explorative laparotomies. Five patients received chemotherapy after palliative resection, and 11 patients with local tumor recurrence underwent radiation therapy.

Tumor Characteristics

Tumors were classified according to the fourth edition of the TNM classification.[21] An overview of tumor characteristics is given in Table 1.

Immunocytochemical staining for tumor-associated proteases and inhibitors could be done on 203 tumors, 139 of them from curatively resected patients.

Clinical Follow-Up

Follow-up was done 6, 12, 18, and 24 months after surgery and at 1-year intervals thereafter. It consisted of the patient's interim history, a physical examination, abdominal ultrasound, gastroscopy, chest x-ray, blood chemistry, differential blood count, and analysis of tumor markers CEA (carcino-embryonal antigen), CA 19-9, and CA 72-4. If recurrence was suspected, additional imaging methods were used and confirmation by biopsy was attempted.

Table 1. TUMOR CHARACTERISTICS

Tumor Classification		Cases	R0 Cases
UICC stage	IA	32	32
	IB	29	29
	II	27	24
	IIIA	38	26
	IIIB	24	19
	IV	69	9
pT stage	pT1	40	40
	pT2	89	67
	pT3	57	26
	pT4	33	6
pN stage	pN0	70	64
	pN1	33	22
	pN2	116	53
M	M0	153	133
	M1	66	6
G	G1	6	6
	G2	67	50
	G3	146	83
Tumor diameter	<20 mm	28	28
	21–50 mm	74	56
	>51 mm	117	55
Laurén classification	Intestinal	109	80
	Diffuse/mixed	101	55
	Unclassified	9	4
Borrmann classification	Polypoid	27	16
	Ulcerated	115	82
	Ulcerative-infiltrative	25	10
	Infiltrative	52	31
Lymphangiosis	Yes	147	87
	No	54	51
	Not investigated	18	1
Tumor localization	Cardia/fundus	38	22
	Corpus	104	59
	Antrum	77	58

Bone Marrow Aspiration Biopsy

Bone marrow aspirations were performed perioperatively. Bone marrow was taken from both iliac crests into a heparinized syringe with a mean volume of 5 mL. After immediate Ficoll-Hypaque density centrifugation (density 1.077; Biochrom, Berlin, Germany) to isolate mononuclear cells (2000*g* for 25 minutes), the interphase fraction was washed twice in phosphate-buffered saline (PBS), resuspended at a concentration of 10^6 cells/mL, and cytocentrifuged to glass slides (10^5 cells per slide). Specimens were air-dried for 12 to 24 hours and stained immediately or stored at −80 C.

Immunocytochemical Alkaline Phosphatase and Antialkaline Phosphatase Staining

Staining was done in a moist chamber according to the established alkaline phosphatase and antialkaline phosphatase (APAAP) method.[22] Bone marrow cytospins were fixed in acetone for 7 minutes and incubated with 20% AB serum and PBS for 25 minutes to reduce unspecific staining. Antibodies were diluted in 10% AB serum and PBS. MaB CK2 against CK18 (Boehringer Mannheim, Germany) was applied for 45 minutes (4 μg/mL, IgG 1), followed by rabbit–antimouse bridging antibody (Dako, Hamburg, Germany; 3 mg/mL, 1:25, 30 minutes) and monoclonal APAAP complex (Dako, 0.17 mg/mL, 1:100, 30 minutes). Each incubation step was followed by thorough washing in PBS. Specifically marked cells were visualized by 0.2 mg/mL naphthol-AS-MX-phosphate (dissolved in dimethylformamide; Sigma, Deisenhofen, Germany), 1% fast blue BB salt (1 mg/mL; Sigma), 0.1 M Tris buffer (pH 8.2), and 0.25 mg/mL levamisole (Sigma). Each assay was negatively controlled, replacing CK2 antibody by nonspecific IgG1 (MOPC 21, Sigma) on one slide and a slide of bone marrow from a healthy donor stained for CK18. Tumor cell lines HT-29 respectively. KATO-III (ATCC, Rockville, MD) served as positive controls.

Immunohistochemical Staining

Tumors were immediately fixed in formalin and embedded in paraffin, cut into 4-μm serial sections, and deparaffinized. Pronase pretreatment (0.1%; Sigma) for 30 minutes was applied as indicated in Table 1. Slides were inactivated with endogeneous peroxidase (0.5% hydrogen peroxide, 20 minutes), followed by rehydration. Staining was performed at room temperature. Each incubation step was followed by thorough washing in 0.001% Brij/PBS (Sigma). Slides were preincubated with 5% horse serum and PBS or 5% swine serum and PBS for 20 minutes.

Monoclonal (source mouse) and polyclonal (source rabbit) antibodies were applied as shown in Table 2. Staining was performed using a highly sensitive avidin biotin elite kit (Vectastain, Burlingame, CA). In case of monoclonal antibodies incubation with horse derived bridging antibody (7.5 μg/mL, 30 minutes) was followed by Vectastain ABC elite complex. In case of polyclonal biotinylated F(ab)2 fragment of affinity-isolated swine antirabbit immunoglobulins (3.3 μg/mL, Dako) incubation was followed by peroxidase-conjugated streptavidin concentrate (Dako, 1:800) for 30 minutes. After washing in PBS, aminoethylcarbazole (Sigma) was added for 15 minutes as enzyme substrate. Counterstaining with hematoxylin completed the procedure.

As negative control served one section of each tumor treated with antibody MLG/7S (Nordic, Tilburg, Netherlands) against murine IgG instead of the primary antibody respectively nonspecific IgGK (MOPC 21) in equimolar protein concentration, the positive control served a routinely processed tumor with known strong expression of the antigens.

All slides were coded and evaluated without knowledge of patient and clinical status by an experienced pathologist (R.B.). Scoring was restricted to tumor cell staining; staining of stromal cells was not considered. Results were classified semiquantitatively into four groups based on the number of positively stained tumor cells: 0, negative; 1, ≤30% positive tumor cells; 2, 30% to 70% positive tumor cells; 3, ≥70% positive tumor cells.

Statistical Analysis

Chi square analysis was performed to determine the correlations between expected and detected frequencies. Group-oriented life-table curves were calculated by Kaplan-Meier analysis and confirmed by Mantel-Cox log rank statistics.[23,24] For multivariate analysis, the Cox proportional hazard model was used, considering established risk factors in gastric cancer.[25] The parameters used for chi square and multivariate analysis were α1-antitrypsin, α1-antichymotrypsin, cathepsin D, antithrombin III, α2-macroglobulin, plasminogen, α2-antiplasmin, MMP-2, uPA, uPA-R, PAI-1 and plasminogen activator inhibitor type 2 (PAI-2) as score 0 to 3. As a diachomised parameter we used Laurén's classification (intestinal *versus* diffuse/mixed), lymphangiosis and vessel infiltration (presence *versus* absence), pT, pN, M, UICC, G, and Borrmann as established, cathepsin D (score 0–1 *vs.* 2–3), intended surgical curability (curative or not curative) and operative procedure (extended or not extended). Tumor localization was considered as cardia or fundus *versus* corpus or antrum, and tumor diameter as a continuous variable as measured.

To determine cutpoints, CART (classification and regression trees hierarchical procedure) analysis was used.[26,27] Stepwise regression analysis was done for developing a new staging model. All statistics were done two-sided at a significance level of $p = 0.05$ using BMDP statistical software[28] and the EDA statistical software package (Department of Medical Information, Biometry and Epidemiology, Klinikum Grosshadern, Munich, Germany).

RESULTS

Biologic Grading of Gastric Cancers

uPA System

Of 219 resected gastric cancer patients, 203 tumors could be stained immunohistochemically for tumor-asso-

Table 2. ANTIBODIES USED FOR IMMUNOHISTOCHEMISTRY

Antibody Against:	Clonality/Isotype/Concentration	Incubation Time/Pronase Pretreatment/Source
uPA	Monoclonal/IgG 1/10 μg/mL	60 min/no/American Diagnostica
uPA-R	Monoclonal/IgG 2a/1 μg/mL	60 min/no/American Diagnostica
PAI-1	Monoclonal/IgG 1/3.3 μg/mL	90 min/no/American Diagnostica
PAI-2	Monoclonal/IgG 1/3.3 μg/mL	90 min/no/American Diagnostica
MMP-2	Monoclonal/IgG 1/1.5 μg/mL	60 min/no/Paesel-Lorei/#14-4012-10004
Cathepsin D	Monoclonal/IgG 1/6.1 μg/mL	60 min/yes/Isotopen Diagnostica CIS/M1G8
tPA	Monoclonal/IgG 1/0.1 μg/mL	60 min/yesAmerican Diagnostica/#373
Plasminogen (Glu/Lys-form)	Monoclonal/IgG 1/10 μg/mL	60 min/no/American Diagnostica/#3642
α2-Antiplasmin	Monoclonal/IgG 1/0.5 μg/mL	60 min/no/American Diagnostica/#3612
α_2-Macroglobulin	Polyclonal/3.0 μg/mL	30 min/yes/Dako/A 033
Antithrombin III	Polyclonal/2.8 μg/mL	30 min/yes/Dako/A 296
α_1-Antitrypsin	Polyclonal/0.05 μg/mL	30 min/yes/Dako/A 012
α_1-Antichymotrypsin	Polyclonal/0.08 μg/mL	30 min/yes/Dako/A 022

ciated proteases. Of these patients, 14 died in hospital (7%). We followed up 189 patients (139 of them curatively resected) prospectively for a median of 31 months (range, 9–56). Ninety-three patients died, 81 of them because of malignancy and 11 without evidence of tumor; in the remaining death, there was evidence of tumor, but death not caused by the tumor. In curatively resected patients (n = 139), 47 recurrences were seen (10 peritoneal carcinoses, 24 locoregional recurrences, 13 distant metastases).

In univariate analysis, there was significant correlation of uPA (p = 0.0032), uPA receptor (p = 0.0306), and PAI-1 (p = 0.0003) with disease-free survival. PAI-2 was not associated with prognosis.

In multivariate analysis considering established risk factors in gastric cancer, PAI-1 was a strong and independent new risk factor. Because of strong correlations with uPA and uPA receptor,[11] these parameters failed to contribute independently. In subgroup analysis, prognostic relevance of the uPA system (PAI-1) was strongest in pT1/pT2, pN01/pN2, and diffuse tumors.[11]

Parameters Modulating the uPA System

Activators of uPA (cathepsin D, antithrombin III), uPA substrates (plasminogen, MMP-2), uPA/plasmin inhibitors (α1-antitrypsin, α2-antiplasmin), and parameters protecting the uPA receptor and promoting the transcellular uPA-R cycle (α2-macroglobulin, α1-antichymotrypsin) were analyzed immunohistochemically, similarly to the investigations of the uPA system.

Cathepsin D (p = 0.0042), α2-macroglobulin (p = 0.0281), and α1-antitrypsin (p = 0.0372) were significantly associated with disease-free survival of the 139 curatively resected gastric cancer patients. No significant association with prognosis could be shown for the other parameters.

Multivariate Cox analysis was performed to correct univariate prognostic associations of the parameters investigated. For disease-free survival, independent prognostic impact could be shown for cathepsin D (p = 0.020, relative risk 2.98), besides PAI-1, pT, pN, and necessity of extended operation. Table 3 gives an overview of univariate and multivariate results on the parameters investigated.

Minimal Residual Tumor Disease in Gastric Cancer

Perioperative Analysis of Disseminated Tumor Cells in Bone Marrow

Of 219 consecutive gastric cancer patients, 180 could be followed prospectively for a median of 44 months (range, 9–60), and disseminated tumor cells in bone marrow were found in 95 patients (53%). In 109 curatively resected patients, 55 (51%) were tumor-cell-positive.

Table 3. OVERVIEW ON PROGNOSTIC IMPACT OF uPA-MODULATING PROTEASES/INHIBITORS OF DISEASE-FREE SURVIVAL

Variable	Univariate Analysis [p(Mantel-Cox)]	Multivariate Analysis [p(Cox proportional hazard)]
Cathepsin D	0.0042	0.0020
Antichymotrypsin	NS	NS
Antitrypsin	0.0372	NS
α_2-Macroglobulin	0.0281	NS

NS = not significant.

Table 4. CORRELATIONS OF POSITIVE TUMOR CELL FINDINGS IN BONE MARROW AT SURGERY

Parameter	Correlation (chi square)
uPA	0.0287
uPA-R	0.3985
PAI-1	0.8668
PAI-2	0.7331
pT	0.0470
pN	0.3140
G	0.9670
UICC	0.1550
Lymphangiosis	0.1930
Laurén	0.3260
Borrmann	0.3260
Tumor size	0.4230

Qualitative detection of perioperatively disseminated tumor cells was not significantly correlated with prognosis. However, quantification of the tumor cell load (negative; 1–3 tumor cells in 10^6; >3 tumor cells in 10^6, groups calculated by CART analysis) revealed significant association with disease-free survival ($p = 0.007$). Multivariate analysis failed to reveal any independent overall impact of perioperatively disseminated tumor cells. However, in the subgroups of pT1/pT2, pN0, and intestinal tumors, the quantity of these cells was an independent prognostic risk factor.[18]

Combining the results on the uPA system in the primary tumors with investigations on disseminated tumor cells (180 patients with immunohistochemistry and bone marrow biopsy at surgery), significant correlation with perioperative evidence of tumor cells could be seen for uPA and pT (Table 4).

Comprehensive Analysis of Biologic Parameters in Gastric Cancer and Proposal of a New Staging Model

An overall multivariate analysis was done considering the uPA system, the uPA-modulating protease systems and detected perioperatively minimal residual disease, in addition to the established risk factors in gastric cancer (Table 5). In this calculation, PAI-1 remained as the dominant new biologic risk parameter, besides cathepsin D, for disease-free survival.

Given our results regarding patient subgroups, we performed stepwise regression tree analysis of established and new risk factors in gastric cancer, starting with surgical curability (R0 *vs.* R1–2) as the strongest risk parameter and proceeding to the next factor most significantly contributing to chi square improvement.[26,27] The resulting model, shown in Figure 1, suggests the sequence of parameters that should be investigated in relevant patient subgroups to specify their individual risk. As the model demonstrates, according to the mean survival times given, among pT1–2 patients the N0 cases with positive tumor cell status in bone marrow at surgery and high levels of PAI-1 in the primary tumor have a worse outcome than pN1–2 patients. Kaplan-Meier analysis revealed that these patients (pT1–2, pN0, tumor cell positive, high PAI-1) have a significantly poorer prognosis than all the other pT1–2, pN0 patients ($p = 0.0306$, Fig. 2). Moreover, the model reveals that among pT1–2 patients, low PAI-1 levels define a patient group of pN1–2 patients with a prognosis similar to that of pN0 patients (Fig. 3).

DISCUSSION

The new staging model we propose, including the parameters PAI-1 (as a representative of the uPA system) and bone marrow tumor cell detection (as a representative of early systemic disease), offers a new approach to differentiate detailed prognostic subgroups of patients with curatively resected gastric cancer. These new prognostic subgroups cannot be defined by established risk factors and thus reflect the clinical impact of the extended staging proposed, including a biologic grading and systemic residual tumor disease.

Until now, grading in gastric cancer was restricted to the morphologic criteria of tumor tissue architecture,[1] the prognostic impact of which has been controversial. Until now, none of the existing histologic classifications (WHO,[21] Laurén,[29] Ming[30]) could demonstrate an independent influence on prognosis.[11,31,32] For tumor cell differentiation (G), results are controversial.[11,33–36] Multivariate prognostic impact of tumor cell dissociation at invasion fronts was reported by Gabbert et al.[33], and Nakane et al.[34] revealed significant relevance of grading. Other studies, including ours, did not find an association with prognosis.[11,35,36]

Research into the biologic mechanisms that underlie the malignant potential of tumor cells assigns a central role to tumor-associated proteases.[2–9] The parameters of the uPA system, especially, are strongly associated with invasiveness and metastatic potential.[2,4,9,10] Their prognostic impact was demonstrated by a strong statistical association with prognosis in gastric cancer,[11–13] and in multivariate analysis, PAI-1 was of dominant independent value.

However, the uPA system should not be seen as an isolated tool of tumor cells for proteolytic activity, but rather as a system with multiple interactions and modulations by other protease and inhibitor systems and cytokines.[4,8] In our studies on modulating effects, we concentrated on distinct representatives of protease interactions

Table 5. COMPREHENSIVE MULTIVARIATE ANALYSIS: DISEASE-FREE SURVIVAL (139 CURATIVELY RESECTED PATIENTS)

	p value		Relative Risk (odds ratio)	95% Confidence Interval
	Univariate	Multivariate		
PAI-1 (score 0–3)	0.0001	0.002	2.21	1.32–3.73
Cathepsin D (score 0.1 *vs.* 2.3)	0.0239	0.020	2.98	1.28–6.91
pT (pT1–pT4)	<0.0001	<0.001	3.03	1.67–5.47
pN (pN0, pN1/2)	<0.0001	0.012	1.81	1.18–2.76
Operation (extended/not extended)	<0.0001	0.042	2.36	1.07–5.18

with the uPA system. Besides activators of uPA (cathepsin D, antithrombin III), inhibitors of uPA (α1-antitrypsin and PAI-2, and also antithrombin III in part has inhibitory functions). Besides activators of uPA substrates (plasminogen, MMP-2), parameters acting on the functional center of the uPA system, and uPA-R (anti-chymotrypsin, α2-macroglobulin), were investigated. Multivariate analysis revealed that of these parameters, cathepsin D contributed independently to prognosis. However, this does not necessarily mean that the other proteases found to be correlated with survival univariately are not involved in modulation of the proteolytic process. It merely shows that in the statistical model applied, these variables, corrected for other parameters, did not contribute independently to the prognostic information.

Proteolytic activity is important for essential mechanisms in the metastatic cascade, especially for detachment from the primary tumor and dissemination of tumor cells, but also for distant implantation and later outgrowth as clinically manifest metastasis.[2–8] This is the link to disseminated tumor cells, a further biologic phenomenon with prognostic impact investigated in our studies. In recent years, it has been shown that these epithelial tumor cells can be detected by the use of anticytokeratin antibodies, due to the exclusively mesenchymal background of bone marrow cells.[17] This detection of tumor cells was found to be of independent prognostic value in breast and colorectal cancer.[37,38]

Also in gastric cancer, early reports showed that these cells can be found at the time of surgery.[39,40] However, in the first large prognostic study, our group observed a correlation of tumor cell detection with prognosis, but not as an independent parameter.[18] The prognostic information increased considering tumor cell quantity. Especially in subgroups of curatively resected gastric cancer patients with early tumor stages (pT1–2) or without evidence of lymph node metastasis (pN0), this disseminated disease was of independent prognostic value.[18]

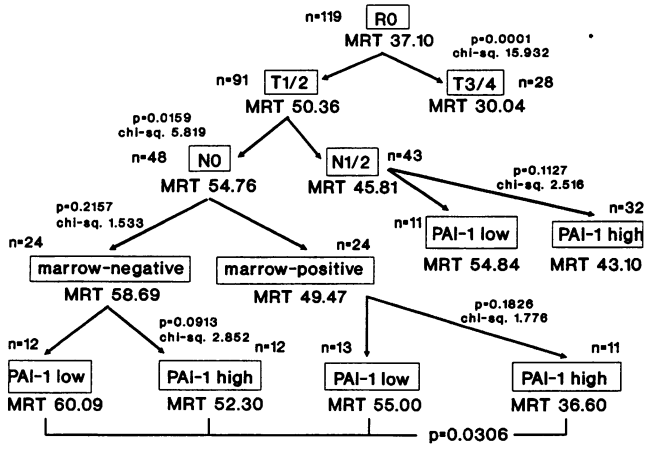

Figure 1. Stepwise regression tree analysis for identification of new, biologically defined clinical patient subgroups (disease-free survival, curatively resected patients). Analysis was done starting with the most significant prognostic parameter and proceeding in stepwise fashion according to the factor that most significantly contributed to chi square improvement. MRT, mean recurrence-free survival time.

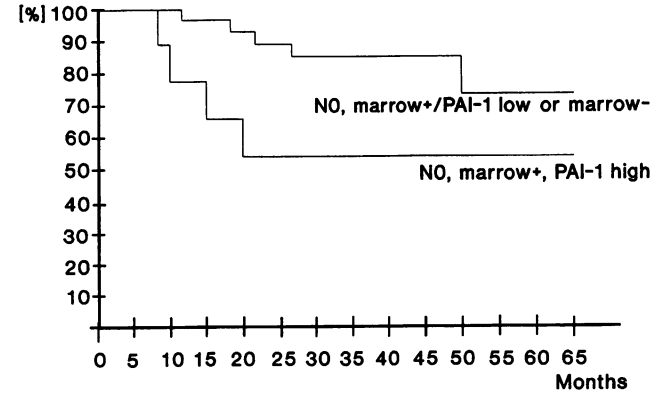

Figure 2. Kaplan-Meier analysis (disease-free survival) of pT1–2 patients with positive tumor cell status and high evidence of plasminogen activator inhibitor type 1 (PAI-1; score 2–3) in their tumors compared to the rest of pT1–2 patients. p(Mantel-Cox) = 0.0306. "Marrow" indicates tumor cells in bone marrow. Positive marrow and high PAI-1: 11 cases, 5 events, mean recurrence-free survival time (MRT) 36.60 months (SD 7.00). Positive marrow and low PAI-1: 37 cases, 4 events, MRT 58.07 months (SD 3.19).

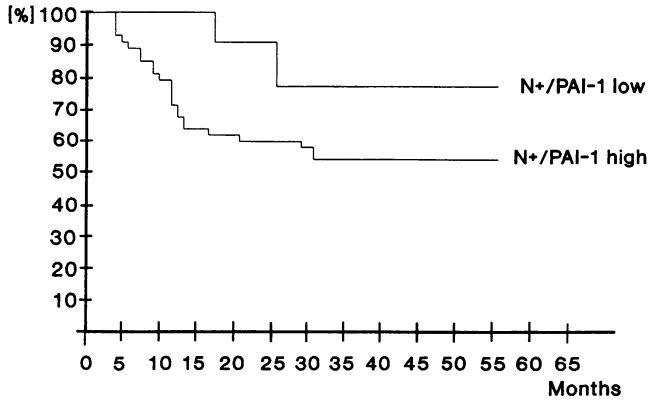

Figure 3. Kaplan-Meier analysis (disease-free survival) of pT1–2, pN1–2 patients according to high (score 2–3) and low (score 0–1) detection of plasminogen activator inhibitor type 1 (PAI-1) in the primary tumor. p(Mantel-Cox) = 0.1127. Low PAI-1: 11 cases, 2 events, mean recurrence-free survival time (MRT) 54.84 months (SD 8.93). High PAI-1: 32 cases, 14 events, MRT 43.10 months (SD 4.70).

It is now believed that these tumor cells represent systemic residual disease, similar to the situation in hematogeneic malignancies, where single clonogenic tumor cells are the reason for later clinical relapse.[17,41] Therefore, the final development of subclinical systemic disease merely reflects the confrontation of the organism's microenvironment with the individual biologic properties of these disseminated tumor cells. In recent investigations, we found evidence that uPA-R might be one of the requisites of these tumor cells, predicting establishment of systemic disease.[19,20] In summary, our investigations led to the conviction that even in early stages of gastric cancer, a systemic disease component can be detected and may play an essential role in clinical outcome.

From these results, we postulated that a new staging system with more emphasis on tumor biology should be developed, considering parameters of biologic grading and minimal residual disease. In the model suggested, we introduced in stepwise fashion the factors that contributed most significantly to the prognosis of our patients. The dominant impact of the R category, which indicates the surgical completeness of tumor removal, has been demonstrated in many clinical studies.[1] This factor is decisive, as it can be influenced by a radical and meticulous operative procedure. Most of the tumors (77%) that could be resected radically (R0) belonged to early infiltration stages (pT1–2). The prognostic difference between these tumors and pT3–4 tumors was impressive, with mean survival times nearly twice as high. The next factor included, histomorphologic diagnosis of lymph node metastasis, divided these patients into groups with survival differences of a mean of 12 months. It is surprising that in patients with node-positive tumors, those with low expression of PAI-1 had a prognosis that was as favorable as that of patients without lymph node metastasis. This demonstrates that biologic aggressiveness of the tumor can be of similar prognostic impact as the preexisting anatomic spread of a tumor to lymph nodes. Until now, this fact, by itself, has been thought to indicate a steep decrease in prognosis in general.

Tumors without lymph node metastasis could be further divided by the detection of disseminated tumor cells. This parameter was selected because of the prognostic impact of bone marrow tumor cells in N0 patients and the prognostic impact of the tumor cell follow-up studies mentioned above.[11] There was a remarkable difference regarding the impact of PAI-1 in stratifying patients according to disseminated tumor cells. In patients without evidence of tumor cells, the prognostic relevance of PAI-1 was only marginal, but in patients with positive tumor cell status, high expression of PAI-1 defined a high-risk group of patients with a prognosis even worse than that of node-positive patients.

For the moment, the clinical value of this biologically enlarged staging can be seen in the more precise definition of risk groups. This might help to stratify adjuvant treatment protocols. The group of node-negative patients with a systemic disease component and upregulation of the uPA system, as indicated by PAI-1, might be suitable for adjuvant systemic therapy. In contrast, adjuvant therapy could potentially be omitted in node-positive patients with tumors of low PAI-1 expression. However, before a definite recommendation can be given, this new biologic staging concept should be verified in a large multiinstitutional trial.

Acknowlegdment

The authors thank Dr. P. Dirschedl (Department of Medical Information, Biometry and Epidemiology, Ludwig Maximilians University of Munich) for essential statistical advice.

References

1. Hermanek P, Maruyama K, Sobin LH. Stomach carcinoma. In Hermanek P, Gospodarowicz MK, Henson DE, et al., eds. Prognostic Factors in Cancer. Berlin, Heidelberg, New York: Springer, 1995:47–64.
2. Blasi F. Urokinase and urokinase receptor: a paracrine/autocrine system regulating cell migration and invasiveness. BioEssays 1993;15:105–111.
3. Dvorak HF. Tumors: wounds that do not heal. N Engl J Med 1986;315:1650–1659.
4. Schmitt M, Jänicke F, Graeff H. Tumor-associated proteases. Fibrinolysis 1992;6(4):3–26.
5. Duffy MJ. The role of proteolytic enzymes in cancer invasion and metastasis. Clin Exp Metastasis 1992;10:145–155.
6. Liotta LA. Tumor invasion and metastases—role of the extracellular matrix: Rhoads Memorial Award Lecture. Cancer Res 1986; 46:1–7.
7. Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and

angiogenesis: an imbalance of positive and negative regulation. Cell 1991;64:327–336.
8. Bond JS. Overview on protease classes, clans, and families. Proceedings AACR Special Conference Proteases and Protease Inhibitors, Panama City, FL, March 1–5, 1996.
9. Ossowski L, Clunie G, Masucci MT, Blasi F. In vivo paracrine interaction between urokinase and its receptor: effect on tumor cell invasion. J Cell Biol 1991;115(4):1107–1112.
10. Schwartz GK, Wang H, Lampen N, et al. Defining the invasive phenotype of proximal gastric cancer cells. Cancer 1994;73(1):22–27.
11. Heiss MM, Babic R, Allgayer H, et al. Tumor-associated proteolysis and prognosis: new functional risk factors in gastric cancer defined by the urokinase-type plasminogen activator system. J Clin Oncol 1995;13(8):2084–2093.
12. Nekarda H, Schmitt M, Ulm K, et al. Prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in completely resected gastric cancer. Cancer Res 1994;54(11):2900–2907.
13. Cho JY, Rha SY, Ahn JB, et al. High levels of urokinase-type plasminogen activator (uPA) and its inhibitor PAI-1 are poor prognostic factors in gastric cancer. AACR Proceedings 1995;36:96.
14. Ichinose A, Fujikawa K, Suyama T. The activation of pro-urokinase by plasma kallikrein and its inactivation by thrombin. J Biol Chem 1986;261:3486–3489.
15. Moller LB. Structure and function of the urokinase receptor. Blood Coagulation and Fibrinolysis 1993;4:293–303.
16. Straight DL, Hassett MA, McKee PA. Structural and functional characterization of the inhibition of urokinase by α-2-macroglobulin. Biochemistry 1985;24:3902–3907.
17. Riethmüller G, Johnson J. Monoclonal antibodies in the detection and therapy of micrometastatic epithelial cancer. Curr Opin Immun 1992;4:647–55.
18. Jauch KW, Heiss MM, Grützner KU, et al. The prognostic significance of early disseminated tumor cells in bone marrow of patients with gastric cancer. J Clin Oncol 1996;14(6):1810–1817.
19. Heiss MM, Allgayer H, Gruetzner KU, et al. Individual development and uPA-R-expression of disseminated tumor cells in bone marrow: a reference to early systemic disease in solid cancer. Nature Med 1995;1(10):1035–1039.
20. Allgayer H, Heiss MM, Riesenberg R, et al. Urokinase-type plasminogen activator receptor (uPA-R): one potential characteristic of metastatic phenotypes in minimal residual tumor disease. Cancer Res 1997;57:1394–1399.
21. Watanbe H, Jass JR, Sobin LH. Histological typing of oesophageal and gastric tumors. In WHO International Histological Classification of Tumors. Berlin, Heidelberg, New York: Springer, 1990.
22. Cordell JL, Falini B, Erber WN, et al. Immunoenzymatic labelling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal antialkaline phosphatase (APAAP complexes). J Histochem Cytochem 1984;32:219–229.
23. Kaplan EL, Meier P. Nonparametric estimation from incomplete observation. J Am Stat Assoc 1958;53:457–481.
24. Peto R, Pike MC, Armitage P, et al. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples. Br J Cancer 1977;37:1–39.
25. Cox DR. Regression models and life tables. J Stat Soc 1972(B); 34:187–220.
26. Breimann L, Friedmann JH, Olsen RA, Stone CJ. Classification and regression trees (CART). Belmont, CA: Wadsworth International Group, 1984.
27. Segal MR. Regression trees for censored data. Biometrics 1988;44:35–47.
28. Dixon WJ, Brown MB, Engelman L, et al. BMDP statistical software. University of California Press, 1985.
29. Laurén R. The two histological main types of gastric carcinoma: diffuse and so-called intestinal type carcinoma. Act Pathol Microbiol Scand 1965;64:31–34.
30. Ming SC. Gastric carcinoma. A pathobiological classification. Cancer 1977;39:2475–2485.
31. Hermanek P. Prognostic factors in stomach cancer surgery. Eur J Surg Oncol 1986;12:241–246.
32. Shiu MH, Perrotti M, Brennan MF. Adenocarcinoma of the stomach: a multivariate analysis of clinical, pathologic and treatment factors. Hepato-Gastroenterology 1989;36:7–12.
33. Gabbert HE, Meier S, Gerharz CD, Hommel G. Tumor-cell dissociation at the invasive front: a new prognostic parameter in gastric cancer patients. Int J Cancer 1992;50:202–207.
34. Nakane Y, Okamura S, Akehira K, et al. Correlation of preoperative carcinoembryonic antigen levels and prognosis of gastric cancer patients. Cancer 1994;73:2703–2708.
35. Hermanek P. Gastric carcinoma—precancerous conditions and lesions, classification, and prognosis. In Hotz J, Meyer HJ, Schmoll HJ, eds. Gastric carcinoma. Berlin, Heidelberg, New York: Springer, 1989.
36. Maruyama K. The most important prognostic factors for gastric cancer patients. A study using univariate and multivariate analyses. Scand J Gastroenterol 1987;22(Suppl. 133):63–68.
37. Diel IJ, Kaufmann M, Goerner R, et al. Detection of tumor cells in bone marrow of patients with primary breast cancer: a prognostic factor for distant metastasis. J Clin Oncol 1992;10:1534–1539.
38. Lindemann F, Schlimok G, Dirschedl P, et al. Prognostic significance of micrometastatic tumor cells in bone marrow of colorectal cancer patients. Lancet 1992;340:685–689.
39. Schlimok G, Funke I, Pantel K, et al. Micrometastatic tumor cells in bone marrow of patients with gastric cancer: methodological aspects of detection and prognostic significance. Eur J Cancer 1991;27:1461–1465.
40. Juhl H, Stritzel M, Wroblewski A, et al. Immunocytological detection of micrometastatic cells: comparative evaluation of findings in the peritoneal cavity and the bone marrow of gastric, colorectal and pancreatic cancer patients. Int J Cancer 1994;57:330–335.
41. Brisco MJ, Condon J, Hughes E, et al. Outcome prediction in childhood acute lymphoblastic leukaemia by molecular quantification of residual disease at the end of induction. Lancet 1994;343:196–199.

Discussion

PROFESSOR B. JEPPSON (Malmö, Sweden): This is an interesting paper and I enjoyed reading it very much. There is not much information available regarding bone marrow micrometastases and tumor-associated proteases in gastric cancer. Your approach of an extended biologic staging in gastric cancer is of clinical importance and much needed. I have one major concern with this study, and that is how certain you are that the cells found in the bone marrow really are tumor cells and not just immature stem cells? Gastric cancer rarely exhibits bone metastasis. How do you envisage the role of the tumor cells in the bone marrow? Thank you.

PROFESSOR I. IHSE (Lund, Sweden): Thank you very much Dr. Heiss. I enjoyed your presentation which I think points to future avenues for staging of malignant disease. Your study is, I must