High Level of Urokinase-Type Plasminogen Activator Is a New Prognostic Marker in Patients with Gastric Carcinoma

**BACKGROUND.** Prognosis of gastric carcinoma is related to invasion and metastasis. Evidence has accumulated that invasion and metastasis in solid tumors require the action of tumor-associated proteases, which promote the dissolution of the surrounding tumor matrix and the basement membrane. The serine protease urokinase-type plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor-1 (PAI-1), appear to have a major function in these processes. Recent reports have demonstrated that expression of these proteolytic enzymes is elevated in breast and colon carcinoma and that it can be associated with invasiveness and poor prognosis. Therefore, the authors evaluated whether the expression and activation of uPA and PAI-1 might be of clinical value as a tumor/biologically defined risk factor in patients with gastric carcinoma.

**METHODS.** Enzyme-linked immunoadsorbent assays were used to test for uPA antigens and PAI-1 in tissue extracts of normal and cancerous tissue from 160 gastric carcinoma patients who were enrolled in the Yonsei Cancer Center Study Group.

**RESULTS.** Both uPA and PAI-1 levels were significantly higher in cancerous tissues than in normal tissues (uPA: 9.4 ± 8.7 vs. 5.3 ± 3.1 ng/mg protein cytosol; PAI-1: 10.9 ± 9.1 vs. 5.8 ± 2.9 ng/mg protein cytosol), (P < 0.001, respectively). Both high uPA and PAI-1 levels were associated with differentiation of the tumor (P = 0.04 and P = 0.004, respectively), and a high PAI-1 level was associated with lymph node metastasis at an advanced stage (P = 0.003 and P = 0.04, respectively). There was a correlation between the levels of uPA and PAI-1 expression in cancerous tissues (correlation coefficient = 0.57). In univariate analysis, a high level of uPA or PAI-1 was associated with a short relapse free survival, but in multivariate analysis only a high level of uPA was an independent prognostic parameter for a short relapse free survival for gastric carcinoma patients.

**CONCLUSIONS.** These data indicate that uPA is a new independent variable for the identification of high risk gastric carcinoma patients. Therefore, therapy targeting uPA can be applied as a new biologic treatment modality for these individuals. Cancer 1997;79:878–83. © 1997 American Cancer Society.

**KEYWORDS:** gastric carcinoma, invasion and metastasis, urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1).

Although the incidence of gastric carcinoma has markedly decreased, it is still the most frequent cancer in the world and is the leading cause of cancer death in Korea. In Japan, the incidence of early gastric carcinoma (a gastric carcinoma that penetrates no deeper than the mucosa or submucosa, regardless of the presence or absence of lymph node metastasis) is approximately 50% but greater than 70% of Korean patients with gastric carcinoma have advanced disease. The overall 5-year survival rate of resectable gastric carcinoma is still dismal (approximately 30%) because most gastric carcinoma patients...
have advanced beyond Stage III.\textsuperscript{1,2} The majority of patients in the treatment failure group die due to the direct effects of the metastasis or complications associated with the treatment of the metastasis.

Cancer invasion and metastasis formation is a multifactorial process and requires the coordinated action of cell-secreted proteolytic enzymes and their inhibitors.\textsuperscript{3–5} Elevated levels of urokinase-type plasminogen activator (uPA) have been implicated in this invasive process. uPA is a Mr 52,000 serine protease, released from cells as an inactive zymogen (pro-uPA) by which limited proteolysis is converted to active 2-chain uPA.\textsuperscript{6,7} A specific receptor binds uPA to cell surfaces, and concomitant cell surface binding of pro-uPA and plasminogen strongly enhances plasmin generation. Plasmin can degrade most components of the extracellular matrix either directly or through the activation of some procollagenases and can activate latent growth factors such as latent transforming growth factor-\(\beta\).\textsuperscript{8–10} Inhibition of uPA activity leads to inhibition of invasion in several experimental systems,\textsuperscript{11–14} and uPA is selectively expressed at invasive foci in some experimental and human cancers.\textsuperscript{15–17} The activity of uPA is controlled not only through synthesis and secretion but also by its specific inhibitors: plasminogen activator inhibitor-1 (PAI-1) and -2 (PAI-2). Recently it has been proposed that PAI-1 plays a role in protecting the tumor against degrading itself and is a potentially important prognostic factor in breast carcinoma.\textsuperscript{18–22}

The purpose of this study was to analyze the antigen levels of uPA and PAI-1 by means of an enzyme-linked immunosorbent assay (ELISA) method and to evaluate their relative value in predicting disease free survival rates in patients with primary gastric carcinoma.

MATERIALS AND METHODS

Patient Characteristics

The 160 patients chosen for this study at the Yonsei University Cancer Center (Seoul, Korea) were diagnosed with primary gastric carcinoma between 1992 and 1995. These patients, whose ages ranged from 27 to 86 years (median, 54 years), all underwent resection with curative intent. Curative resection was defined by the General Rules for the Gastric Cancer Study in Surgery and Pathology of the Japanese Research Society for Gastric Cancer as 1) no involvement of surgical stumps; 2) sufficient lymphatic dissection (R number \(\geq\) N number); 3) no distant metastasis; 4) removal of involved adjacent organs and structures by combined en bloc resection; and 5) no gross residual disease.\textsuperscript{23} Of the 160 patients, 27 had distant lymph node metastasis (\(\geq\) N3 lymph nodes) and were regarded as M1 and Stage IV.

The postoperative adjuvant chemotherapy was performed for advanced stage (Stages II, III, and IV) patients. Stage II and III patients were treated with 5-fluorouracil and doxorubicin with or without nonspecific immune stimulator OK-432, and Stage IV patients were treated with 5-fluorouracil, doxorubicin, and mitomycin C or 5-fluorouracil, etoposide, and cisplatin regimens with or without OK-432.

The median follow-up was 24 months (maximum 48 months) at the time of the study. The follow-up was comprised of a clinical examination every 2–3 months for the first 2 years and every 6 months thereafter. Recurrence, as confirmed by biopsy and/or other relevant diagnostic procedures, was defined as the appearance of new lesions in patients with no previous evidence of disease. At the time of analysis, 53 patients had recurred. Data regarding histopathologic tumor size, lymph node metastasis, and time to relapse were available for nearly all patients (Table 1).

| Characteristic          | Patients (n = 160) |
|-------------------------|--------------------|
| Age (yrs)               | Median (range)     | 55 (27–86) |
| M:F                     | 2.4:1              |
| Location                | Cardia             | 13        |
|                         | Fundus             | 4         |
|                         | Body               | 42        |
|                         | Antrum             | 68        |
|                         | Pylorus            | 32        |
| Lymph node status       | N0                 | 44        |
|                         | N1                 | 60        |
|                         | N2                 | 56        |
|                         | M0                 | 133       |
| Differentiation         | Well               | 17        |
|                         | Moderate           | 51        |
|                         | Poor               | 92        |
| Stage                   | I                  | 1         |
|                         | II                 | 36        |
|                         | IIIA               | 36        |
|                         | IIIIB              | 33        |
|                         | IV                 | 27        |

Methods

Tissue specimens

Immediately after resection, fresh tumor specimens and normal mucosa (both approximately 1 cm\(^3\)) were selected, snap-frozen, and stored in liquid nitrogen. Cryostat sections (5-\(\mu\)m thickness) were prepared from the tumor specimens and normal mucosa, which were then stained with hematoxylin and eosin to confirm the presence or absence of tumor cells. Subsequently, 20 to 30 sections of each tumor and normal tissue sections were cut (total wet tissue weight was between 100 and 150 mg) for tissue extraction. The last section was also stained by hematoxylin and eosin to demonstrate the presence or absence of tumor cells. The cytosolic extracts were prepared using a standard
procedure, including precooling in liquid nitrogen, pulverization with a Microdismembrator, and extraction at 4 °C with a buffer comprised of 10 mM K2HPO4/KH2PO4, 1.5 mM K2 ethylenediamine tetraacetic acid, 10 mM monothioglycerol, 10% glycerol (volume/volume), and 10 mM sodium molybdate (pH 7.5), followed by ultracentrifugation at 100,000 X relative centrifugal force (g) for 1 hour. The supernatant fluids were stored separately at −80 °C until use.

**uPA and PAI-1 ELISAs**

uPA in the cytosolic extracts was measured by a sandwich ELISA, using a polyclonal catching antibody and a mixture of three different biotinylated monoclonal detecting antibodies, which in combination recognize free uPA as well as receptor and PAI-1-bound uPA. The assay was performed as previously described.21,22 PAI-1 was determined using a sandwich ELISA kit (Moenzyme, Horsholm, Denmark) with monoclonal catching and detecting antibodies. Although this assay detects active PAI-1 and PAI-1-complexed uPA, it does not detect structurally related proteins such as PAI-2 and ovalbumin. uPA and PAI-1 were measured in ng/mg cytosolic extracts by calibration with standard preparations. The intra- and interassay variations for both assays were below 10%. Protein concentration of the cytosolic extracts were determined by the Bio-Rad protein assay (Bio-Rad, Richmond, CA) using bovine serum albumin as the standard.

**Statistical Analysis**

Comparison of uPA and PAI-1 levels between normal and cancerous tissue cytosolic extracts was performed by the Student’s t test for paired data. Two-sided P values < 0.05 were considered significant. Distributions were compared using the Wilcoxon test. Comparison of uPA and PAI-1 levels between prognostic parameters was performed by the chi-square test. Life table analysis of relapse free survival was calculated by the product limit method (Kaplan–Meier), and the log rank test was used to determine equality over strata. The Cox proportional hazards model was used for multivariate analysis of relapse free survival. These analysis were performed with the BMDP program. Inclusion and exclusion criteria for the Cox model were set to 0.05.

**RESULTS**

**Distribution of uPA and PAI-1 Levels**

Figures 1 and 2 show the distribution of uPA and PAI-1 levels according to tumor progression in the cytosolic extracts and the relationship between these two parameters. There was a strong positive correlation between uPA and PAI-1 levels in the cytosols (P < 0.001, correlation coefficient = 0.57).

The mean value ± standard deviation of uPA and PAI-1 were 5.3 ± 3.1 and 5.8 ± 2.9 ng/mg protein cytosol, respectively, in normal uninvolved tissue, and 9.4 ± 8.7 and 10.9 ± 9.1 ng/mg protein cytosol, respectively, in cancerous tissue. Both uPA and PAI-1 levels were significantly higher in cancerous tissue than normal tissue (P < 0.001, respectively) (Table 2).

**Prognostic Significance of uPA and PAI-1 Levels**

The prognostic significance of uPA and PAI-1 levels was studied by univariate analysis for each of the pa-
TABLE 3
Comparison of uPA and PAI-1 in Cytosolic Extracts and Other Variables in Gastric Carcinoma Patients

| Variables       | uPA level$^a$ | P value | PAI-1 level$^b$ | P value |
|-----------------|---------------|---------|-----------------|---------|
| Differentiation |               |         |                 |         |
| Well-mod        | 7.2 ± 3.8     | 0.041   | 7.2 ± 6.5       | 0.004   |
| Poor            | 11.2 ± 8.5    |         | 13.3 ± 12.4     |         |
| Tumor stage     |               |         |                 |         |
| T1-T2           | 7.6 ± 4.9     |         | 8.0 ± 4.0       |         |
| T3-T4           | 10.2 ± 8.8    | 0.094   | 11.1 ± 9.0      | NS      |
| Lymph node status |             |         |                 |         |
| N0              | 7.6 ± 3.3     |         | 8.4 ± 8.1       |         |
| N1              | 7.7 ± 5.8     |         | 9.1 ± 8.5       |         |
| N2              | 9.3 ± 8.9     | NS      | 15.0 ± 13.8     | 0.003   |
| Metastasis      |               |         |                 |         |
| M0              | 9.2 ± 9.2     |         | 10.9 ± 10.8     |         |
| M1              | 10.5 ± 6.7    | NS      | 11.3 ± 8.5      | NS      |
| Stage           |               |         |                 |         |
| I-II            | 9.2 ± 4.8     |         | 8.6 ± 7.6       |         |
| III-IV          | 9.5 ± 6.6     | NS      | 12.4 ± 11.9     | 0.041   |

uPA: urokinase-type plasminogen activator; PAI-1: plasminogen activator inhibitor-1; $^a$ Levels of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 are given as mean value ± standard deviation (ng/mg protein cytosol).

Correlation of uPA and PAI-1 with Other Prognostic Variables

The correlation between the cytosolic uPA and PAI-1 levels and other prognostic variables was studied using the chi-square test (Table 3). A high grade of anaplasia was significantly associated with high uPA and PAI-1 ($P = 0.04$ and $P = 0.004$, respectively) and an elevated PAI-1 level was associated with lymph node metastasis and advanced stage ($P = 0.003$ and $P = 0.04$, respectively).

Multivariate Analysis

To compare the prognostic significance of cytosolic uPA and PAI-1 levels with that of other parameters, variables were singly eliminated from the model in a backwards fashion and reincluded only if the $P$ value was $< 0.05$. uPA level was a statistically significant independent variable parameter for relapse free survival, with a relative risk of 3.6, but the PAI-1 level was a stage-dependent prognostic factor (Table 4).

The authors subsequently analyzed the prognostic significance of high uPA expression according to disease stage. Subgroup analysis according to each stage showed statistically significant differences in advanced...
stage patients (Stage II-IV); the relapse free survival of the patients with high uPA expression was lower than those with low uPA expression ($P = 0.037$, $P = 0.048$, and $P = 0.039$, respectively). However, there was no significant difference between the two groups in Stage I patients (survival curves not included).

**DISCUSSION**

The involvement of uPA in the invasion and metastatic mechanisms of gastric carcinoma is well documented, but the exact role of uPA and PAI-1 is less well known. To better analyze their prognostic value, it is important to evaluate the relative importance of these parameters and their interactions. To this end, the uPA and PAI-1 antigen levels were measured in a series of paired 160 gastric normal and tumor specimens and the relationships to their prognostic value studied. The data support the notion that the uPA antigen content of the tumor tissue is an independent and strong prognostic factor in gastric carcinoma patients. Because uPA antigen is an independent prognostic variable, subgroups of patients at high risk for recurrence (even within the risk groups that were until now determined by established prognostic factors) could be further subdivided by uPA level. Subgroup analysis according to each stage in this study showed statistically significant differences in advanced stage patients (Stage II-IV). These data correlate well with the results observed in patients with breast carcinoma. In immunocytochemical and immunofluorescence studies, uPA has been localized to the pericellular area of the tumor cells or to the focal contact sites of fibroblasts. uPA also was demonstrated immunohistochemically in various malignant cancers (e.g., colorectal carcinoma, breast carcinoma, and gastric carcinoma).

Recently reported elevation of uPA plasma levels in patients with Crohn’s disease might be caused by the inflammatory process itself, and uPA has been detected immunohistochemically in tissues obtained from patients with chronic inflammatory bowel disease. Therefore, it is more accurate to test the invasive potential by using a comparison of the uPA level between uninvolved gastric mucosa and cancerous tissue.

Although an elevated uPA level in cancerous tissue cytosolic extracts has prognostic impact, it cannot definitely be predicted when the invasion and metastasis mechanisms activate after curative surgery. To solve this problem, a study is currently underway with the serial check of plasma uPA levels before and after curative surgery. The authors believe completion of this study will answer this question.

The current investigation also reveals that the PAI-1 antigen level of tumor tissue extracts as determined by ELISA is higher in gastric carcinoma than in normal tissues. The presence of PAI-1 in gastric carcinoma at a level correlating with that of uPA suggests that PAI-1 may play a role in the regulation of plasminogen activation, and thereby in the process of gastric carcinoma invasion and metastasis.

Recently, Soff et al. reported that PAI-1 expression in human prostate carcinoma cells reduced primary tumor growth and tumor-associated microvasculature, and resulted in a tenfold inhibition of lung metastasis and a significant inhibition of liver metastasis. However, these findings are quite contradictory to the previous hypothesis of PAI-1 function in vivo. Mapping of the uPA- and PAI-1-producing cells in gastric carcinoma by in situ hybridization will, in combination with immunohistochemical stainings, shed more light on this issue.

In conclusion, uPA can be added to the prognostic markers in gastric carcinoma patients. However, further study with plasma antigen levels for detection of early recurrence or metastasis is needed, suggesting that pharmacologic or molecular inhibition of uPA can be a new target for antiinvasion and antimetastasis therapy.

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