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Original article

Expression of HLA-DR and urokinase-type plasminogen activator in stage IV gastric cancer

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Abstract:
Background. **Some patients with stage IV gastric cancer have a long survival. Host immune response and proteolytic activity in the primary tumor may be associated with outcome in these patients. The purpose of this study was to assess prognostic factors in patients with stage IV far advanced gastric cancer.**
Methods. **Findings in 26 patients who underwent resection of stage IV gastric cancer were retrospectively analyzed for clinicopathological variables, and for the immunohistochemical expression of human leukocyte antigen (HLA)-DR as an index of host immune response and for expression of urokinase type-plasminogen activator (u-PA) as an index of proteolytic activity in the tumor.**
Results. **Of the 13 clinicopathological and immunohistochemical variables tested by univariate analysis surgical curability, lymph node metastasis, HLA-DR expression, and u-PA expression had a significant influence on survival after surgery. Multivariate analysis showed that surgical curability, HLA-DR expression, and u-PA expression independently influenced survival. Patients positive for HLA-DR expression [HLA-DR (+)] and negative for u-PA expression [u-PA (−)] had the best survival: 25-month median survival and 25% 5-year survival rate. Patients who were HLA-DR (+) and u-PA (+), or HLA-DR (−) and u-PA (−) had a median survival of 10 months, a 1-year survival rate of 46.2%, and a 2-year survival rate of 7.7%. HLA-DR (−) and u-PA (+) patients had the worst survival: 4.5-month median survival and 0% 1-year survival.**
Conclusions. **These findings suggest that host immune response and proteolytic activity in the primary tumor may determine malignant potential, and that the combination of positive-HLA-DR and negative-u-PA expression in cancer cells may be a predictor of prolonged survival in stage IV gastric cancer patients.**

Key words: host immune response, malignant potential, serine protease, fibrinolysis, immunohistochemistry

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Introduction

The main factors governing outcome in patients with stage IV gastric cancer are peritoneal dissemination, hepatic metastasis, lymph node metastasis, and direct invasion of adjacent organs. In addition to these clinicopathological factors, host immune response and proteolytic activity in the tumor may also be associated with outcome.

Human leukocyte antigen-DR (HLA-DR) is a membrane-bound glycoprotein encoded by genes located in the HLA-D region of the major histocompatibility complex. It plays an important role in the regulation of the immune response to T-cell-dependent antigen [1]. HLA-DR antigen is mainly expressed on B cells, macrophages, activated T cells, and dendritic cells [2–4]. HLA-DR antigens are detected in some types of cancers associated with lymphocyte infiltration, which can be regarded as an immunological response to the presence of tumor cells [5–9]. It is also thought that tumor cells are passively elicited to express HLA-DR by exposure to cytokines released from lymphocytes [10]. Expression of HLA-DR antigen by cutaneous melanoma cells increases with rate of tumor progression, and is associated with early occurrence of metastasis [11,12]. In contrast, HLA-DR antigen expression in larynx, breast, and colorectal carcinoma cells is reported to be associated with a good prognosis [13–15]. In gastric cancer [16], even in advanced cancer [17], patients whose lesions showed HLA-DR-antigen positivity in cancer cells had a higher 5-year survival rate than those with HLA-DR antigen-negative cancer cells.

Proteolytic enzymes play a role in cancer invasion and metastasis [18,19]. These proteases include collagenases, cathepsins, and plasminogen activators [20–22]. Urokinase type-plasminogen activator (u-PA), a serine protease, may play a central role in these processes. u-PA converts activated plasminogen into plasmin. Plasmin can activate type IV procollagenase,

which then degrades collagen and proteins in the basement membrane [23]. This proteolysis may finally lead to cancer invasion and metastasis. Clinically, high u-PA levels in patients with primary breast cancer are reported to be associated with a high risk of recurrence and short survival [24,25], and in gastric cancer patients, elevated u-PA levels in the primary tumor are associated with a poor outcome [26,27].

In this study we report that positive HLA-DR expression and negative u-PA expression, which may indicate enhanced host immune response and a low grade of proteolytic activity, respectively, identify patients with advanced gastric cancer (stage IV disease) with a better prognosis.

Patients and methods

Patients and clinicopathological factors

We retrospectively analyzed the clinicopathological features of 26 patients who underwent resection of stage IV gastric cancer without preoperative treatment at the First Department of Surgery, Shiga University of Medical Science, Japan, between January 1986 and December 1993. Staging of the tumor was based on the TNM classification of malignant tumors published by International Union Against Cancer (UICC). Features assessed included sex, age, macroscopic type of tumor, depth of tumor invasion, lymph node metastasis (pN), peritoneal dissemination, hepatic metastasis, histopathological grading, lymphatic invasion, blood vessel invasion, and surgical curability (curability).

Adjuvant chemotherapy was administered to 22 patients intraperitoneally during operation or intravenously after surgery, the agents used varying from patient to patient; the agents were mitomycin C (MMC), MMC/cis-platinum (CDDP), 5-fluorouracil (5-FU), CDDP/5-FU, and etoposide/adriamycin/CDDP.

Immunohistochemical study

We tested the tumors of the 26 patients for expression of HLA-DR and u-PA. We used specimens fixed in 10% formalin and embedded in paraffin wax, and immunohistochemical staining was done by the streptavidin-biotin-peroxidase method. In brief, after the sections were dewaxed, endogenous peroxidase activity was inactivated by 100% methanol with 0.3% hydrogen peroxide, and the sections were incubated with 10% non-immune rabbit serum in phosphate-buffered saline for 20 min. LN-3 monoclonal antibody (mAb) (Nichirei, Tokyo, Japan) and u-PA mAb (Biopool, Tokyo, Japan) were used as primary monoclonal antibodies, and were allowed to react for 12 h at 4 °C. This was followed by incubation with a second biotinylated antibody for 20 min. The streptavidin-biotin complex was added, followed by incubation for 15 min. The immunostained areas were visualized using a H_2O_2-supplemented aminoethylcarbazole chromogen, and the sections were counterstained with hematoxylin and mounted. The slides were washed three times with phosphate-buffered saline between each antibody treatment.

In negative control experiments, sections were incubated with non-immune mouse IgG in place of the primary antibody. Positive staining of histiocytes/lymphocytes in the lamina propria provided the necessary positive control for expression of HLA-DR. The vulvar epidermoid carcinoma cell line A431 was provided by Japanese Cancer Research Resources Bank, and cells (1×10^7) were injected under the skin of nude mice, and the subsequent tumor cells were used as a positive control for u-PA antigen. Sections were assessed as positive when more than 10% of the tumor cells were stained with their respective antibodies. The percentage of stained cells in each section was evaluated by two independent observers, each with no knowledge of the clinical outcome or the other observer's determinations. A minimum of ten microscopic fields was counted independently. Areas of obvious tissue necrosis were avoided for counting purposes. In most cases, the two observers agreed; when there were differences, they were resolved by consensus.

Statistical methods

Survival of patients (from time of resection) was determined at the end of March 1997. To analyze results for this relatively small group of patients, several clinicopathologic and immunohistochemical parameters were dichotomized, as shown in the Table 1. Patients were also grouped according to depth of tumor invasion, infiltration of contiguous structures (pT 4) versus tumor confined to within the serosa (pT 1, 2, 3), and no residual tumor (R0) surgery versus microscopic residual tumor (R1) or macroscopic residual tumor (R2) surgery. The survival curves for the patients were calculated by the Kaplan-Meier method [28] and analyzed by the Logrank test [29]. Variables were then entered into the Cox proportional hazards regression model [30] to identify factors which independently influenced survival. The stability of the model was confirmed using a step-backwards and step-forwards fitting procedure. Variables identified as having an independent influence on survival were identical by both procedures. A P value less than 0.05 was considered significant.

Table 1. Clinicopathological and immunohistochemical characteristics and their correlation with survival in stage IV gastric cancer ($n = 26$)

Variable	Number of patients	Median survival (months)	P value
Sex			
Male	17	15	NS[†]
Female	9	8	
Age (years; mean)			
≤65	20	11.5	NS
≥65	6	15.5	
Macroscopic type			
Not diffusely infiltrative	18	14.5	NS
Diffusely infiltrative	8	10	
Histological type			
Differentiated	9	15	NS
Undifferentiated	17	10	
Surgical curability[‡]			
Curative (R0)	7	33	0.0063
Non-curative (R1,2)	19	10	
Lymph node metastasis			
Negative	3	36	0.042
Positive	23	10	
Peritoneal metastasis			
Negative	10	14.5	NS
Positive	16	12.5	
Hepatic metastasis			
Negative	22	14.5	NS
Positive	4	6	
Depth of invasion*			
pT1,2,3	22	15	NS
pT4	4	9	
Lymphatic invasion			
Negative	0	—	—
Positive	26	13.5	
Blood vessel invasion			
Negative	5	18	NS
Positive	21	10	
HLA-DR expression			
Negative	11	10	0.022
Positive	15	16	
u-PA expression			
Negative	15	17	0.006
Positive	11	8	
Combination of HLA-DR and u-PA expression			0.0002
HLA-DR (−) and u-PA (+)	4	4.5	
HLA-DR (+) and u-PA (+) or HLA-DR (−) and u-PA (−)	14	10	
HLA-DR (+) and u-PA (−)	8	23	

u-PA, Urokinase-type plasminogen activator; HLA-DR, human leukocyte antigen
*Depth of tumor invasion, indicated as pT1, invasion of the mucosa or the submucosa; pT2, the muscularis propria or the subserosa, pT3, the serosa, pT4, contiguous structure
[†]NS, not significant
[‡]See text for definition of R0 and R1,2

Results

Clinicopathological characteristics and immunohistochemical results are shown in Table 1. The mean age of the 26 patients was 53.5 years (range, 30–77 years). HLA-DR antigen in cancer cells was detected in 15 of the 26 specimens examined (57.7%) and u-PA antigen in 11 (42.3%). Neither HLA-DR nor u-PA antigen was found in epithelial or stromal cells of normal stomach tissue. There was no relation between expression of HLA-DR or u-PA and whether or not chemotherapy had been given.

Of the 13 variables, curability, pN, HLA-DR expression, and u-PA expression had a significant influence on survival by univariate analysis ($P = 0.0063$; $P = 0.042$; $P = 0.022$; $P = 0.006$, respectively). None of the other nine variables had any influence on survival. Twelve variables were entered into the Cox proportional

hazards regression model to identify factors which independently influenced survival, with lymphatic invasion by the tumor excluded from this analysis, as all patients had positive lymphatic invasion. Multivariate analysis of the 12 variables revealed that curability, HLA-DR expression, and u-PA expression independently influenced survival ($P = 0.010$; $P = 0.0048$; $P = 0.0007$, respectively) (Table 2). "No residual tumor (R0) surgery", "positive for HLA-DR expression [HLA-DR (+)]", and "negative for u-PA expression [u-PA (−)]" were significantly beneficial factors for survival in these stage IV gastric cancer patients.

The overall median survival was 13.5 months (range, 1–167 months). Three patients (11.5%) survived for more than 3 years, and two patients (7.7%) survived for more than 5 years; these two patients are alive, having survived without recurrence for more than 10 years (Fig. 1).

Table 2. Multivariate analysis: Prognostic factors with independent influence on survival in 26 patients with stage IV gastric cancer

Variables	*P* value	Relative risk of death
Surgical curability (R0 vs R1 or R2)	0.01	0.056
HLA-DR expression [HLA-DR (+) vs HLA-DR (−)]	0.045	0.36
u-PA expression [u-PA (−) vs u-PA (+)]	0.0025	0.17

The survival curves in relation to HLA-DR expression and u-PA expression are shown in Fig. 2a,b. The 2-, 3-, and 5-year survival rates for HLA-DR (+) patients were 27.6%, 13.3%, and 13.3%, respectively, and HLA-DR (−) patients had a 0% 2-year survival rate. The 2-, 3-, and 5-year survival rates for u-PA (−) patients were 20%, 13.3%, and 13.3%, respectively, and u-PA (+) patients had a 9.1% 2-year survival rate and a 0% 3-year survival rate.

Combinations of expression of HLA-DR and u-PA antigen were examined. There was a significant difference in survival between three combinations of HLA-

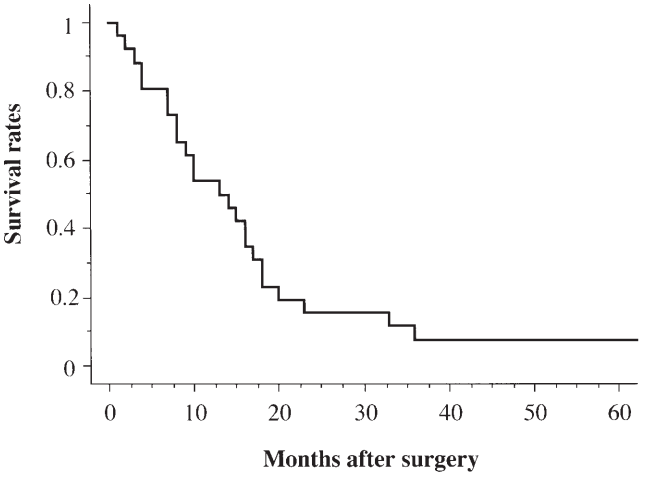
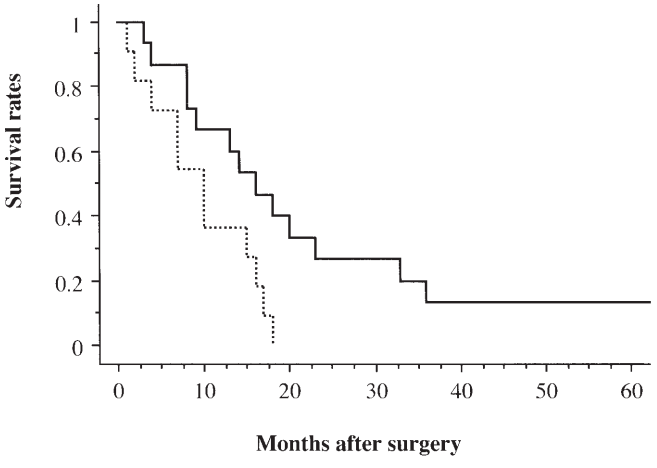
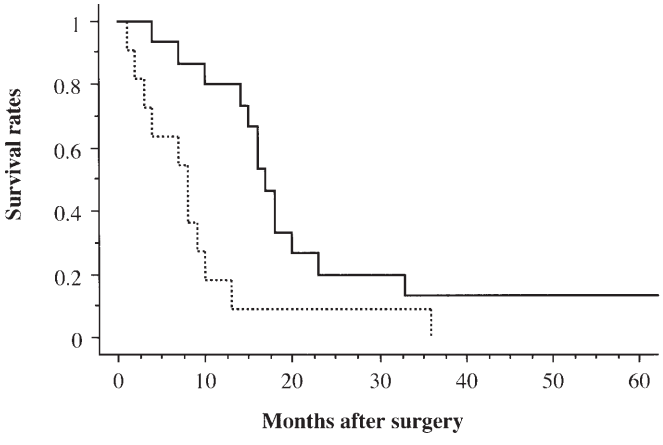


Fig. 1. Overall survival of 26 patients with stage IV gastric cancer. The median survival was 13.5 months, and the 5-year survival rate was 7.7%



a



b

Fig. 2. a Survival of patients with stage IV gastric cancer according to human leukocyte antigen (HLA)-DR expression in the tumor. There was a significant difference in survival ($P = 0.022$) between the positive and negative groups. *Continuous line*, Positive HLA-DR expression [HLA-DR (+)] ($n = 15$); *dashed line*, negative HLA-DR expression [HLA-DR (−)] ($n = 11$). **b** Survival of patients with stage IV gastric cancer according to urokinase type plasminogen activator (u-PA) expression by the tumor. There was a significant difference in survival ($P = 0.006$) between the positive and negative groups. *Continuous line*, Negative u-PA expression [u-PA (−)] ($n = 15$); *dashed line*, positive u-PA expression [u-PA (+)] ($n = 11$)

DR and u-PA expression: (1) HLA-DR (+) and u-PA (−); (2) HLA-DR (+) and u-PA (+) or HLA-DR (−) and u-PA (−); and (3) HLA-DR (−) and u-PA (+) ($P = 0.0002$) (Table 1**)**. HLA-DR (+) and u-PA (−) patients had the best survival: a 25-month median survival and 25% 5-year survival rate. Patients who were HLA-DR (+) and u-PA (+), or HLA-DR (−) and u-PA (−) had a median survival of 10 months and a 46.2% 1-year survival rate and 7.7% 2-year survival rate. HLA-DR (−) and u-PA (+) patients had the worst survival: a 4.5-month median survival and 0% 1-year survival (Fig. 3).

In the HLA-DR (+) and u-PA (−) patients, positive peritoneal dissemination [P(+)], positive hepatic metastasis [H(+)], distant lymph node metastasis [distant pN(+)], and invasion of adjacent organs (pT4) were found in three, one, two, and none of four patients, respectively. In the HLA-DR (−) and u-PA (+) patients, P (+), H (+), distant pN (+), and pT4 were found in three, none, two, and one of four patients, respectively. Therefore, there was no difference in conventional prognostic factors of stage IV gastric cancer between HLA-DR (+) and u-PA (−) and HLA-DR (−) and u-PA (+) patients.

Discussion

Radical procedures such as extensive lymphadenectomy and combined resection of invaded organs are reported to be efficacious in patients with far advanced gastric cancer [31–33]. There are also reports of the limitations of curative surgery in far advanced gastric cancer and an urgent need for adjuvant therapy [34,35]. We found that factors of curability, pN, HLA-DR expression, and u-PA expression, significantly influenced survival on univariate analysis. Multivariate analysis of 12 clinicopathological and immunohistochemical variables revealed that R0 surgery, positive HLA-DR expression, and negative u-PA expression were independently significant beneficial factors for long-term survival in stage IV gastric cancer patients.

Prognosis of malignant tumors is associated with various indexes, such as the expression of p-53 [36–38], c-erbB-2 [39,40], proliferating cell nuclear artigen [41,42] and u-PA [26,27,43]. In the present study, patients negative for u-PA expression in the tumor had a significantly longer survival than those positive for u-PA expression ($P = 0.006$). We confirmed that u-PA expression by the tumor was of prognostic relevance even in far advanced gastric cancer, a finding consistent with observations in primary breast cancer and gastric cancer, in which high u-PA levels are associated with poor patient survival [24–27].

In regard to our other findings, patients positive for HLA-DR expression had a significantly longer survival than those negative for HLA-DR expression ($P = 0.022$), and HLA-DR expression was also confirmed to be of prognostic relevance in far advanced gastric cancer. These observations were in good agreement with the association of HLA-DR expression with good prognosis in larynx, breast, colorectal, and gastric cancer [13–15,17]. However, the relationship between HLA-DR expression and prognosis in melanoma is controversial [11,12,44–46]. Although the mechanisms by which HLA-DR expression in the tumor are associated with prognosis are unclear, and although HLA-DR expression may depend on the type of cancer tissue, it is also possible that tumor-infiltrating lymphocytes may also contribute to these mechanisms. We are now studying infiltrating lymphocytes and cytokines at sites in individual tumors which show HLA-DR expression and at sites which do not.

Malignant potential may be induced by tumor-host interaction. Kakeji et al. [47] examined the relationship between tumor and host in gastric cancer tissues in terms of DNA ploidy and dendritic cell infiltration, and suggested that marked infiltration of dendritic cells in gastric cancer tissue may lead to prolongation of survival in patients with carcinoma that has a low ploidy profile, by preventing widespread nodal involvement. In the present study, HLA-DR and u-PA expression each had an independent influence on survival, according to the results of multivariate analysis of 12 variables. Patients who were both positive for HLA-DR expression

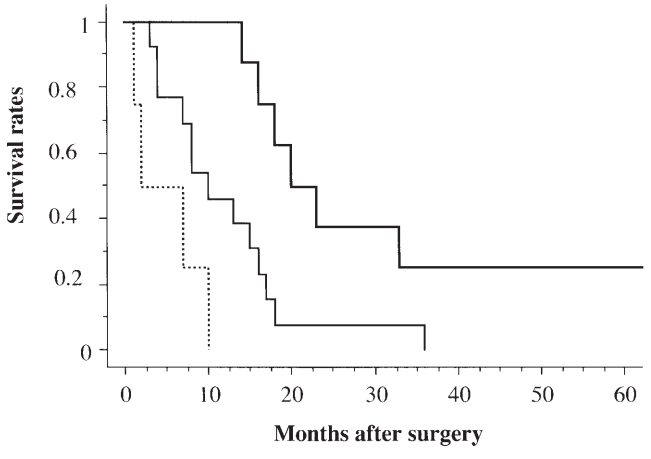


Fig. 3. Survival of patients with stage IV gastric cancer according to combinations of HLA-DR and u-PA expression. There was a significant difference in survival between the three combinations of HLA-DR and u-PA expression shown ($P = 0.0002$). *Thick continuous line*, HLA-DR (+) and u-PA (−) ($n = 8$); *thin continuous line*, HLA-DR (+) and u-PA (+) or HLA-DR (−) and u-PA (−) ($n = 14$); *dashed line*, HLA-DR (−) and u-PA (+) ($n = 4$)

and negative for u-PA expression had a good outcome (23-month median survival; 25% 5-year survival rate), whereas patients who were negative for HLA-DR expression and positive for u-PA expression had an extremely poor outcome (4.5-month median survival; 0% 1-year survival rate), despite there being no differences in conventional prognostic factors [P (+), H (+), distant pN (+), pT4]. Furthermore, even in those patients who underwent residual tumor surgery (R1 or R2), HLA-DR (+) and u-PA (−) patients ($n = 4$) had a 19-month median survival and all survived for more than 1 year. Patients who were HLA-DR (+) and u-PA (+) or HLA-DR (−) and u-PA (−) ($n = 11$) had an 8-month median survival and a 36.4% 1-year survival rate. Patients who were HLA-DR (−) and u-PA (+) ($n = 4$) had only a 4.5-month median survival and none survived for more than 10 months (Murata et al. unpublished observations). These findings suggest that host immune response and proteolytic activity in the tumor may be related to malignant potential and may be of value in identifying patients with a good prognosis. These results should be clarified by studies of larger patient cohorts.

In conclusion, the findings in this study suggest that the host immune response and proteolytic activity in the primary tumor may contribute to malignant potential, even in far advanced gastric cancer, and that the combination of positive-HLA-DR and negative-u-PA expression shows promise as a predictor of prolonged survival in patients with stage IV gastric cancer.

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