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• GASTRIC CANCER •

Ets1 as a marker of malignant potential in gastric carcinoma

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Abstract

AIM: Ets1 proto-oncogene is a transcription factor involved in the activation of several genes of tumor invasion and metastasis. We aimed to determine the relationship between the extent and intensity of Ets1 expression and patients' clinicopathological factors in gastric carcinoma.

METHODS: Immunohistochemical analysis was performed for gastric tumor paraffin-embedded sections, followed by image analysis.

RESULTS: Ets1 was not expressed in the normal gastric epithelium and its surrounding cells. The percentage of Ets1 expressing cells detected increased significantly in both epithelial tumor and stromal cells from high T classification, lymph node metastasis positive, clinical advanced-stage groups ($P<0.001$). The level of Ets1 staining in epithelial tumor cells also reflected the degree of cell differentiation. The percentage of epithelial and stromal cells expressing Ets1 was significantly correlated with the presence of lymph node metastasis ($P=0.014$ and $P<0.001$ respectively). Ets1 expression was not observed in tissue samples from patients with benign gastric ulcers.

CONCLUSION: Ets1 protein expression in epithelial tumor cells reflects the degree of differentiation, and the percentage of Ets1 positive tumor and stromal cells correlates with lymph node metastasis. Thus Ets1 is a valuable marker of malignant potential in terms of invasiveness and metastasis of gastric carcinoma. It is also possible that inhibition of Ets1 is a potential avenue for therapy in gastric cancer.

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INTRODUCTION

Gastric cancer is a leading cause of cancer death in the world. The pathological stage of gastric cancer, including the depth of tumor penetration and the presence of metastases to lymph nodes or distal organs, remains the most important determinant in its prognosis. Usually, these parameters can be determined by microscopic examination of tissue sections from the primary neoplasm and lymph nodes. However, where only primary gastric carcinoma specimens are available for histopathological examination cannot always accurately predict metastasis and disease progression. Recently, from microarray gene expression profiling data[1], the occurrence and progression of cancer were suggested to be related to a series of genetic events affecting the structure and expression of a number of genes. These genes include potential oncogenes, tumor suppressors and those involved in angiogenesis and extracellular matrix (ECM) remodeling. The factors regulating these genes could thus be important markers for disease prognosis and potential therapeutic targets. Since most transcription factors are expressed at very low levels (-10 copies/cell), sensitive assays are required. One suitable approach is to use a specific antibody to detect these factors by immunohistochemical analysis. This allows analysis of the epithelial tumor cells and its surrounding stromal cells. In addition, this approach will enable further understanding of the molecular mechanisms of gastric carcinoma invasion and metastasis.

The Ets family of transcription factors is defined by a conserved DNA binding domain of approximately 85 amino acids. These proteins bind to specific purine-rich DNA sequences, with a core motif of GGAA/T, and have been shown to transcriptionally regulate a number of viral and cellular genes. Ets factors have been shown to be important in a number of biological processes, including cellular proliferation, differentiation, development, transformation, and apoptosis[2-6]. Indeed the Ets1 protein has recently been shown to regulate genes such as vascular endothelial growth factor(VEGF), urokinase plasminogen activator (uPA), matrix-metallopteases (MMPs) and integrin in a variety of cancer cell lines and tissues[7-12]. Many of these genes are involved in angiogenesis and extracellular matrix (ECM) remodeling, important events in the processes of cancer invasion and metastasis.

Microarray analysis from a number of epithelial tumor tissues or cell lines indicate that alterations in Ets1 expression are associated with lung carcinoma[13], breast carcinoma[14], pancreatic carcinoma[15], thyroid carcinoma[16] and oral squamous cell carcinoma[17]. However, it is not clear from these data which cells in the tumor expressed Ets1 or whether this expression was associated with invasive growth. Thus the purpose of this study was to analyze the relationship between Ets1 expression and the malignant behavior in gastric carcinoma, in particular to determine whether Ets1 may have a role in breaking through the muscularis mucosae by invasive tumors and metastasis. This will determine whether Ets1 has potential as a credible marker in predicting the metastatic potential of gastric cancer and assisting in elucidating its role in gastric carcinoma's malignant progression.

MATERIALS AND METHODS

Samples and patients

Since January 2001 to June 2002, we collected biopsies from 43 patients who underwent surgery for gastric neoplasia in the General Surgery Department of Xinhua Hospital, Shanghai Second Medical University. The data set for each patient

included sex, age, histological classification, clinical grade, depth of infiltration (T classification), presence of lymph node metastasis (N classification), distant metastasis (M classification), and clinical stage. Pathological assessments were determined by clinical pathologists according to WHO standards. T1 indicated infiltration of the *lamina propria* or submucosa, T2 infiltration of muscularis propria or the subserosa, T3 penetration of the serosa (visceral peritoneum), and T4 penetration of the serosa and infiltration of adjacent structures. Lymph node (N) classification was divided into no metastasis (N0) and metastasis in 1-6 (N1), 7-15 (N2) or >15 (N3) regional lymph nodes. M classification was M1 for presence or M0 for absence of distant metastasis. Clinical stage was determined by combination of these parameters as stage IA (T1N0M0), stage IB (T1N1M0), stage II (T1N2M0, T2N1M0 or T3N0M0), stage IIIA (T2N2M0, T3N1M0 or T4N0M0), stage IIIB (T3N2M0) and stage IV (TxN3M0, T4NxM0, TxNxM1). In addition, two samples of both benign gastric ulcer and breast cancer were used as controls.

Samples were fixed in formalin immediately after the operation and specially prepared for immunohistochemistry. Briefly, samples of no more than 2×1.5×0.2 cm were dehydrated at 4 ℃, and low temperature paraffin wax (<60 ℃) was used for embedding. 3 μm thick sections were de-waxed immediately before use with xylene (4 ℃) and rehydrated for immunohistochemistry.

Immunohistochemistry

Endogenous peroxidase activity was quenched with 3 % hydroperoxide followed by incubation with 1 % bovine serum albumin to block any nonspecific binding. Primary antibodies used were anti-human Ets1 monoclonal antibody (Transduction Laboratories, Lexington, KY), rabbit anti-human Ets1 polyclonal antibody (Santa Cruz Biotechnology, CA) or rabbit anti-human cytokeratin polyclonal antibody (DAKO, Copenhagen, Denmark). Amplification of the primary antibody reaction was achieved by incubation with an appropriate secondary antibody [Goat anti-Rabbit or anti-mouse IgG, Gene Tech] conjugated to peroxidase. The binding was visualized by DAB and the sections were counterstained with hematoxylin. All incubations and washing were performed at room temperature.

Image analysis

Intensities, percentages, and patterns of immunohistochemical staining for each sample slide were analyzed by KS4000 image analysis system (Zeiss Company, Germany) and recorded individually. For each sample, six representative images were collected at high magnification (×400). Image analysis determined the proportion of epithelial or stromal cells within the tumor expressing Ets1 and the percentage of cells with moderate and intense staining. The data were expressed as proportion of tumor.

Statistic analysis

The data were presented as the mean ± standard deviation (SD) for each group. Statistical analysis was performed using analysis of variance (ANOVA) and Mann-Whitney non-parametric test. Intergroup difference was evaluated by Fisher' s test. $P<0.01$ was considered statistically significant.

RESULTS

Patients characteristics

Samples were obtained from 41 patients, 30 males and 10 females. The age range was from 37 to 87. The 41 patients were diagnosed with malignant tumor. The histological classification included adenocarcinoma (30), signet ring cell carcinoma (6), undifferentiated carcinoma (4) and gastrointestinal stromal tumor (GIST) (1). The samples were divided into two groups according to their depth of infiltration (WHO classification): 15 of T1 or T2, and 25 of T3 or T4. Specimens were also divided according to histological grade: grade I (highly differentiated carcinoma) for 7 samples, grade II (moderately differentiated carcinoma) for 19 samples, grade III (poorly differentiated carcinoma) for 14 samples. 26/40 patients demonstrated lymph node metastasis and only one patient had known distant metastasis (liver).

Expression of Ets1 in gastric carcinoma

In our study, staining of Ets1 was observed in all malignant gastric tumor cells, including adenocarcinoma, signet ring cell adenocarcinoma and undifferentiated carcinoma, and the results are summarized in Table 1. Epithelial tumor cells stained for Ets1 were mainly in the nucleus and only rarely was staining observed in the cytoplasm (Figure 1A). No staining for Ets1 was observed in the non-neoplastic tissue or in tissues from a patient with benign gastric ulcer (Figure 1G). In contrast, antibodies to another Ets factor which is known to be epithelium-specific (Elf5), stained normal gastric epithelium, but lost its expression in gastric cancer (data not shown). Ets1 staining was also observed in newly formed blood vessel endothelial cells (Figure 1B) and interstitial cells in surrounding tissue (Figure 1C). Ets1 protein was localized in both cytoplasm and nucleus of the stromal cells. Furthermore intense staining for Ets1 was observed in invasive cells at the junction of normal tissue and malignant tissue (Figure 1D). Increased Ets1 protein expression observed by immunohistochemistry was confirmed by quantitation of Ets1mRNA levels by RT-PCR and Northern blot (data not shown). Immunohistochemistry of a breast cancer specimen also detected Ets1, but it was predominantly expressed in the cytoplasm (Figure 1E).

Relationship between percentage of Ets1 expressing cells and pathological assessment in gastric carcinoma

Age, sex and histological grade Statistical analysis determined that there was no significant correlation between the expressions of Ets1 in gastric cancer and age, sex or WHO histopathological classifications. Although there was a trend in proportions of Ets1 positively stained epithelial cells from 26.9 % in grade I, increasing to 39.5 % in grade II and 32.8 % in grade III (Table 1), there was no significant correlation ($P=0.366$). A similar trend was also observed for staining of tumor stromal cells ($P=0.486$).

Depth of infiltration (T) classification Considering the importance of breaking through muscularis mucosae, we compared the groups with tumors confined to the mucosa/submucosa (15 samples, T1 or T2) and those tumors which had penetrated the serosa (25 samples, T3 or T4). Our data demonstrated that Ets1 was detected in a higher proportion of both epithelial and stromal cells in tumors from patients with more advanced disease. For example, 17.2±12.0 % of tumor epithelial cells from T1 or T2 tumors were positive for Ets1, whereas 45.6±17.9 % were positive for T3 or T4 tumours ($P<0.001$, Table 1). The proportion of Ets1 expressing tumor stromal cells also significantly correlated with the presence of more advanced disease T classification ($P<0.05$).

Lymph node and distant metastasis Similarly, grouping of patients according to the presence (N123) or absence (N0) of lymph node metastasis showed an increase in Ets1 positive tumor epithelial cells from 24.1±17.4 % (N0) to 40.8±20.8 % (N123, $P<0.05$). In addition, the proportion of Ets1 expressing tumor stromal cells was also significantly higher in patients with lymph node metastasis ($P<0.001$). Only one patient in our samples had known distant metastasis, but Ets1 was detected in both epithelial and stromal cells in tumor sections from this patient (71 % and 61 % respectively).

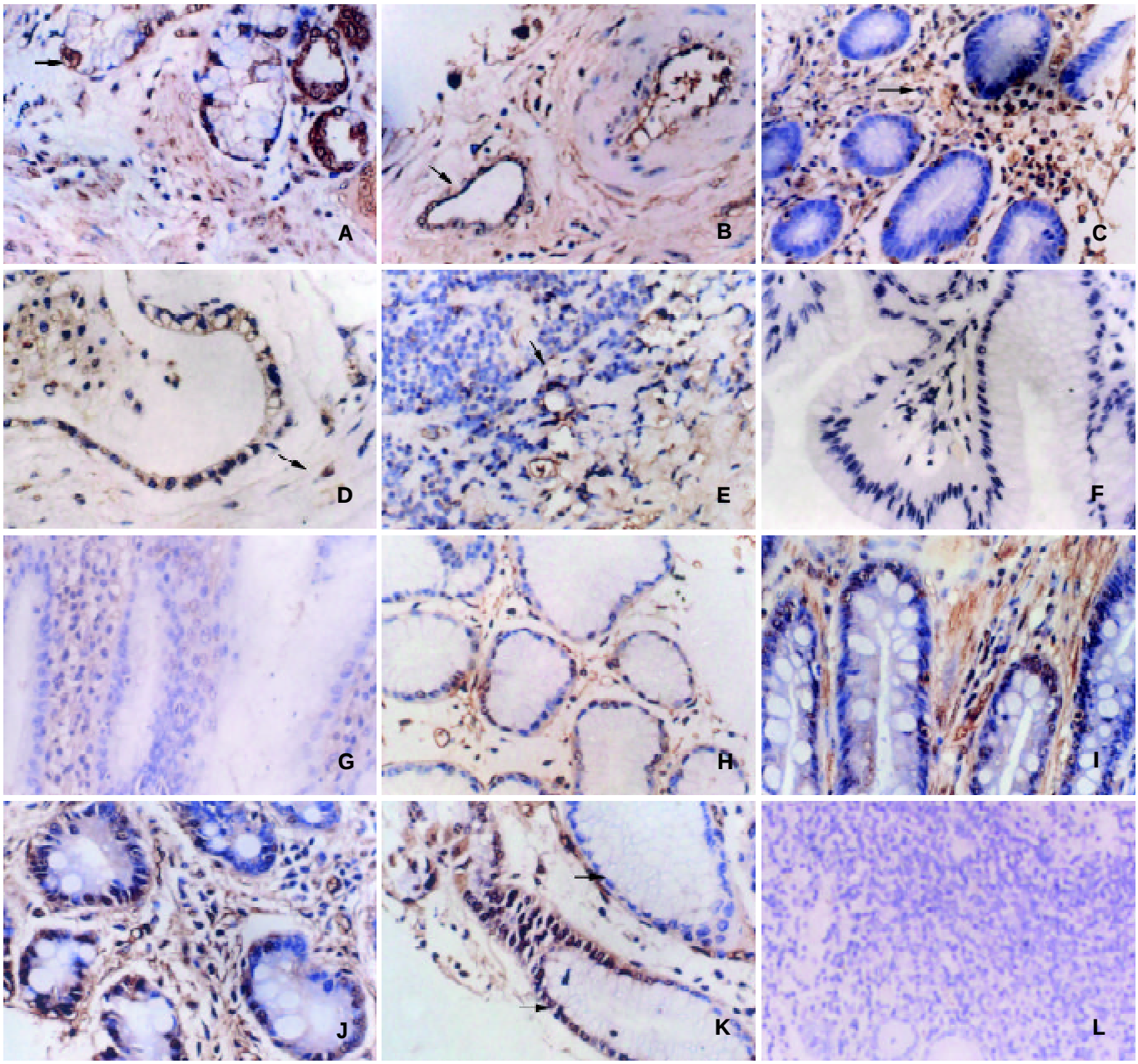

Figure 1 A: Ets1 immunohistochemical stain in gastric cancer cells. The arrowed cell is a typical Ets1 positively stained cell. The area of stain was mainly, located in the nucleus of epithelial tumor cells. Ets1 protein was localized in both cytoplasm and nucleus of the stromal cells. B: Ets1 expression in vascular endothelial cells. This was an example of pooly differentiated gastric adenocarcinoma. Ets1 was present in endothelial cells of blood vessels (arrowed). C: Ets1 is expressed in interstitial cells. Photomicrograph of a typical gastric carcinoma. Ets1 positive stromal cells were detected in the interstitial tissue. D: The expression of Ets1 in polarity. Image of gastric cancer cells infiltrating to the muscular layer. Intense staining of Ets1 was observed at the junction of the infiltrating tumor cells and the serosa. We termed it the polarity expression. E: Ets1 expression in breast carcinoma. Example of breast carcinoma, Ets1 positive cells arrowed. F: Ets1 negative control staining. G: Ets1 is not expressed in glandular cells of benign gastric ulcer. H-J: Ets1 expression in tissues with different histopathological grades. Section of grade I(H), grade II(I) and grade III(J) gastric cancer showing increased positive cells and intensity of Ets1 staining with advanced disease. K: Ets1 expression is high in undifferentiated tumor cell. The image contained well differentiated and poorly differentiated tumor tissues. The number of positive cells and the intensity of the Ets1 staining were increased in undifferentiated tumor cells. L: Ets1 is not expressed in GIST. Photomicrograph of gastrointestinal stromal tumor (GIST). No Ets1 positive cancer cells were observed. (A, H, I, J, K original magnification ×400), (B, C, D, E, F, G original magnification ×100), (L, original magnification ×50).

Clinical stage When the T, N and M classifications were combined into the WHO defined clinical stages, there was a significant correlation between Ets1 staining in epithelial and stromal cells with the presence of more advanced disease ($P<0.001$ and $P<0.005$ respectively).

Relationship between Ets1 levels and clinical disease

Our image analysis also enabled us to compare the signal intensity of Ets1 staining (Table 2-1,2), which reflected the levels of Ets1 (confirmed by RT-PCR, data not shown). When all the patients were considered together, the majority (24/40, 60 %) displayed moderate staining levels in tumor epithelial cells whereas 10/40 (25 %) displayed intense staining. Of these 10 patients with intense staining, however, 9 had tumors penetrating the mucosa (T3 or T4) and 8 had metastasis into the regional lymph nodes. The single patient with distant metastasis also displayed intense staining for Ets1. Similar high levels of Ets1 labelling were observed in stromal cells from

patients with advanced disease. In stromal cells 16/40 (40 %) displayed intense staining. These 16 patients with intense staining included a single patient with distant metastasis, while 11 of these 16 patients had tumors penetrating the mucosa (T3 or T4) and metastasis into the regional lymph nodes. Thus it appeared both the level of Ets1 and the proportion of positive cells were correlated with advanced gastric carcinoma.

Table 1 Ets1 expression in tumor and stromal cells

Factors	No.	Ets1 percentage[T] (mean±SD)	*P* value	Ets1 percentage[S] (mean±SD)	*P* value
Total patients	40	35.0±21.1		44.6±17.5	
Gender					
Male	30	38.0±22.5	0.115	45.2±17.3	0.704
Female	10	25.9±13.1		42.7±18.7	
Age					
>50	32	35.9±20.8	0.572	46.3±17.7	0.206
<50	8	31.1±23.3		37.5±15.7	
Histological classification (WHO)					
Ad	30	35.2±19.9	0.269	43.0±15.4	0.647
Srcc	6	43.3±26.4		49.3±15.2	
Uc	4	21.1±19.6		48.8±34.7	
Grade (WHO)					
I	7	26.9±26.7	0.366	37.3±18.3	0.486
II	19	39.5±19.7		45.7±11.6	
III	14	32.8±19.8		46.6±23.2	
T (tumor)					
T1,2	15	17.2±12.0	<0.001	35.3±17.7	0.008
T3,4	25	45.6±17.9		50.1±15.1	
N (lymph node)					
N0	14	24.1±17.4	0.014	32.4±13.8	0.001
N1,2,3	26	40.8±20.8		51.1±15.8	
M (metastasis)					
M0	39	34.0±20.5	<0.001	44.1±17.5	<0.001
M1	1	71		61	
Clinical stage					
I, II	18	21.8±15.2	<0.001	36.2±18.1	0.005
III, IV	22	45.7±19.2		51.4±13.9	

[T] refers to Ets1 expression in tumor cells [S] refers to Ets1 expression in stromal cells.

Table 2-1 Intensity of Ets1 staining in tumor cell and distribution of patients

Compartment	No.	Weak (+)	Moderate (++)	Intense (⩾+++)	*P* value
Tumor cells	40	6 (15 %)	24 (60 %)	10 (25 %)	
T classification					
T1,2	15	2 (13.3 %)	12 (80 %)	1 (6.7 %)	0.087
T3,4	25	4 (16 %)	12 (48 %)	9 (36 %)	
LN metastasis					
Negative	14	3 (21.4 %)	9 (64.3 %)	2 (14.3 %)	0.438
Positive	26	3 (11.5 %)	15 (57.7 %)	8 (30.8 %)	
Distant metastasis					
Negative	39	6 (15.4 %)	24 (61.5 %)	9 (23.1 %)	0.214
Positive	1	0 (0 %)	0 (0 %)	1 (100 %)	
Clinical stage					
Stage I,II	18	3 (16.7 %)	13 (72.2 %)	2 (11.1 %)	0.183
Stage III,IV	22	3 (13.6 %)	11 (50 %)	8 (36.4 %)	

Table 2-2 Intensity of Ets1 staining in stromal cell and distribution of patients

Compartment	No.	Weak (+)	Moderate (++)	Intense (⩾+++)	*P* value
Stromal cells	40	3 (7.5 %)	21 (52.5 %)	16 (40 %)	
T classification					
T1,2	15	3 (20 %)	7 (46.7 %)	5 (33.3 %)	0.066
T3,4	25	0 (0 %)	14 (56 %)	11 (44 %)	
LN metastasis*					
Negative	14	3 (21.4 %)	6 (42.9 %)	5 (35.7 %)	0.048
Positive	26	0 (0 %)	15 (57.7 %)	11 (42.3 %)	
Distant metastasis					
Negative	39	3 (7.7 %)	21 (53.8 %)	15 (38.5 %)	0.463
Positive	1	0 (0 %)	0 (0 %)	1 (100 %)	
Clinical stage					
Stage I,II	18	3 (16.7 %)	8 (44.4 %)	7 (38.9 %)	0.129
Stage III,IV	22	0 (0 %)	13 (59.1 %)	9 (40.9 %)	

DISCUSSION

Gastric cancer is the most frequent malignancy of the gastrointestinal tract in China and the second most common cause of cancer-related death in the world[18]. The prognosis of patients with gastric cancer has been improving owing to the progress in diagnostic techniques and treatment methods for gastric cancer, but peritoneal dissemination is the main cause of recurrence after curative resection of advanced cancer. The prognosis of gastric cancer which has invaded as far as the gastric serosa was still poor with a 5-year survival of less than 35 %[19]. Among these malignant characteristics of gastric cancer cells, metastasis to the peritoneum is an especially complex phenomenon, which requires the involvement of many different genes in multiple steps for tumor cells. Although many aspects of gastric cancer metastasis await further clarification, adhesion molecules, apoptosis-related genes, and others have been reported to play an important role in peritoneal dissemination of gastric cancers[20], but details of the mechanism involved remain unclear. Since Ets1 has been shown to regulate many genes involved in angiogenesis and extracellular matrix (ECM) remodeling, events important in cancer metastasis, we analyzed the relationship between Ets1 expression and the malignant behavior in gastric carcinoma. This demonstrated that Ets1 expression was related to the pathological stage of gastric cancer, including tumor infiltration and presence of metastases to lymph nodes or distal organs. These data are consistent with other studies that have shown significant correlation between Ets1 expression in tumor cells and the presence of lymph node metastasis. But our data also demonstrated that Ets1 expression in cancer associated stromal cells was significantly correlated with the presence of lymph node metastasis. The proportion of Ets1 expressing cells was also associated with metastasis kinetics, expression of Ets1 within a few epithelial tumor cells in early disease, then expression in stromal, endothelial and other cells in advanced malignant disease where cancer has broken through muscularis mucosae. This suggested that Ets1 had a specific role in the regulation of genes involved in gastric invasion and metastasis, rather than carcinogenesis[2,3] in gastric carcinoma. It also indicated that Ets1 was a promising marker of malignant potential and a potential avenue for therapeutic intervention in gastric cancer.

The level of Ets1 expression in epithelial tumor cells was correlated with the progression of disease, perhaps associated with the degree of cancer cell differentiation. In addition, Ets1 expressing cells were located at the invasive front of the tumor. This is consistent with previous observations that Ets1 was associated with branching morphogenesis and organ formation in embryos[21] and embryonic stem cell differentiation (Xu *et al*.,

unpublished data). Other cell types in gastric cancer, such as stromal cells, significantly increased Ets1 expression which was correlated with penetration through the muscularis mucosae and the presence of lymph node metastasis. These data are important observations since the key approach for advancing our treatment of gastric carcinoma is to clarify the mechanism of infiltration, especially for the first step, namely penetrating the muscularis mucosae. Previously, the interrelated elements concerned with gastric carcinoma' s penetration of the extracellular matrix (ECM), angiogenesis and the surrounding microenvironment have not been connected.

Increased expression of genes encoding enzymes involved in degradation of the extracellular matrix (ECM), such as MMP-1 (collagenase-1), MMP-3 (stromelysin-1), MMP-7 (matrilysin), and MMP-9 (type IV collagenase/gelatinase) has been identified in cancer from recent microarray data[22]. Hence, Ets1 is likely to contribute to tumor invasion and progression through activation of these enzymes. Indeed, expression of these ECM remodeling enzymes was detected concomitant with Ets1 mRNA in tumor cells and/or stroma cells.

Ets1 is also involved in angiogenesis, which is essential for tumor progression. In a non-vascularized tumor the growing tumor becomes hypoxic, thus the observation that Ets1 was induced by hypoxia via hypoxia-inducible factor-1 (HIF-1)[23] is important. VEGF and bFGF also induce Ets1 in endothelial cells. Ets1 was believed to confer an angiogenic phenotype to the endothelial cells through induction of the urokinase-type plasminogen activator (u-PA))[24-26] and MMPs, and also to regulate N-acetylglucosaminyl-transferase V (GnT-V), which has been associated with metastasis of tumors[27]. Thus the microenvironment of a growing tumor may induce Ets1 in tumor cells and/or stroma cells, which subsequently induces angiogenesis-related genes and ECM remodeling enzymes required for tissue invasion and metastasis. It is interesting to note that expression of Ets1 in both tumor and stroma was correlated with poor prognosis in human ovarian carcinoma[28].

Recent development in microarray technologies has resulted in extensive profiling of cancer and cancer metastases[29]. These approaches have generated a vast amount of new data to investigate the molecular mechanisms of cancer metastasis, however, many of these studies have not identified Ets1 as significantly increased. This is surprising given both our data and other data demonstrating increased Ets1 in lung carcinoma, breast carcinoma, pancreatic carcinoma, thyroid carcinoma, and oral squamous cell carcinoma[7-12]. We suggest that this anomaly is due to the sensitivity of microarray analysis, which is commonly used to detect genes with altered expression of more than 2 fold. As a transcription factor, 1-2 fold increased expression could have significant biological effects. It is interesting to note that one microarray study of gastric cancer cell lines reported that it did not find any transcription factor more than 2.0 fold upregulated from 20 k genes examined. However this study identified increased levels of defined Ets regulated genes, such as MMP and VEGF. It is also possible that mRNA status was not consistent with protein expression status or perhaps variation in cell types and Ets1 expression in the tumor sample concealed a more localized Ets1 increase. Thus we believe that our results from immunohistochemistry do not conflict with these microarray data.

In addition to the potential of Ets1 as a diagnostic marker, there is also potential for Ets1 as a potential therapeutic target for metastasis in gastric carcinogenesis. Since increased expression of Ets1 and subsequently its downstream target genes (MMPs, VEGF and HIF) contribute to invasiveness and metastasis of gastric carcinoma, inhibition of its expression is potentially therapeutic. Recently, we reported that inhibition of Ewing' s sarcoma associated EWS/FLI-1 transcription via sequence-specific transcriptional suppressor was sufficient to inhibit the transformed phenotype[30]. Thus specific inhibition of Ets1 function (or induction of Ets1) has potential for reducing metastasis in gastric carcinoma[31].

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