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Phospho-Stat3 expression and correlation with VEGF, p53, and Bcl-2 in gastric carcinoma using tissue microarray

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The signal transducer and activator of the transcription (Stat)-family of proteins are latent cytoplasmic transcription factors that transmit signals from cytokines and growth-factor receptors to the nucleus. Stat proteins, especially Stat3 and Stat5, are constitutively activated in various solid tumors and hematological malignancies. However, the role of Stat3 signaling in gastric carcinoma has not yet been fully determined. This study was conducted to investigate the clinical value of phospho-Stat3 expression in gastric carcinoma. Expression of phospho-Stat3 (Tyr705), vascular endothelial growth factor (VEGF), p53, and Bcl-2 was determined by immunohistochemical staining of tissue microarrays from 137 cases of resected gastric cancer specimens. We evaluated the relationships among phospho-Stat3, VEGF, p53, and Bcl-2 expression and the correlation between expression of these proteins and various clinicopathological factors, including overall survival. Phospho-Stat3 nuclear expression was observed in 18.2% of the cases. Of the total number of cases, 68.6% were positive for VEGF, 40.1% for p53, and 11.7% for Bcl-2. Phospho-Stat3 expression correlated with VEGF ($p=0.021$) and Bcl-2 ($p=0.005$) expression. Positive phospho-Stat3 staining was significantly associated with poor pathological grade. However, there was no significant difference in other clinicopathological parameters, such as tumor stage (T, N, M), pathological type, relapse-free survival, and overall survival between the phospho-Stat3-positive and -negative groups. Co-expression of phospho-Stat3 and VEGF was found in many patients with N3 and Stage IV disease. These results suggest that phospho-Stat3 expression might be associated with angiogenesis, anti-apoptosis, and tumor progression. Further studies are needed to determine the role of phospho-Stat3 in gastric cancer.

Key words: Phospho-Stat3; VEGF; tissue microarray; gastric carcinoma.

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The signal transducer and activator of the transcription (Stat)-family of proteins are latent cytoplasmic transcription factors activated by cytokine receptor-associated tyrosine kinase, growth factor receptor tyrosine kinase, and non-receptor tyrosine kinase, which lead to transmission of their signals into the nucleus (1, 2). Specific Stat family members contribute to the control of normal cellular processes, including immune function, development, differentiation, proliferation, and survival (3–5). Stat proteins, especially Stat3 and Stat5, are constitutively activated in various solid tumors and hematological malignancies. The constitutive activation of Stat3 is frequently detected in advanced breast cancer and in breast cancer cell lines, but not in normal breast epithelial cells

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(6). Both the IL-6-dependent human myeloma cell line U266 and the majority of bone marrow mononuclear cells from multiple myeloma patients possess constitutively activated Stat3 (7). In addition, persistent Stat3 activation has been detected in head and neck cancer, melanoma, prostate cancer, pancreatic cancer, and leukemia. Persistent activation of Stat3 may participate in oncogenesis by promoting tumor angiogenesis, stimulating cell proliferation, mediating immune evasion, and conferring resistance to apoptosis (8). However, little is known about the role of Stat3 signaling in gastric carcinoma.

Tissue microarrays are a method of harvesting cylindrical tissue samples from hundreds of different primary tissue blocks and placing them on a recipient paraffin block, ultimately allowing hundreds of cases to be analyzed simultaneously. Many studies have demonstrated that findings obtained on tissue microarrays are highly representative of their donor tissues. Thus, this technology is an effective tool for examining a large number of cases in a cost-effective manner (9–11).

To determine the effects of Stat3 in tumor angiogenesis, proliferation, and anti-apoptosis, we used tissue microarray to investigate the clinical value of phospho-Stat3 expression in gastric carcinoma and to evaluate its correlation with vascular endothelial growth factor (VEGF), p35, and Bcl-2 expression.

MATERIALS AND METHODS

Tissue samples and tissue microarray construction

We selected patients diagnosed with advanced gastric cancer who underwent curative resection at Hanyang University Hospital between July 1996 and December 2000. We also selected patients with early gastric cancer who underwent curative resection between 1996 and 1997. The patients had well-documented clinical data. We examined a total of 137 specimens (131 cases of advanced gastric cancer and 6 cases of early gastric cancer). Information concerning the date of initial diagnosis, clinical characteristics, relapse, and death were obtained retrospectively. In addition, 30 non-neoplastic stomach tissue samples were used as controls. All of the control gastric tissues had portions with at least some inflammation and 17 cases contained intestinal metaplasia.

Representative areas of the different lesions were carefully selected on H & E-stained sections under the light microscope by a pathologist (CK Park) and marked on individual paraffin blocks. A tissue microarrayer (AccuMax™ Array) was used to isolate three 1-mm diameter tissue core sets from different representative areas for each case. The cores were then transferred to a paraffin recipient block with a 1.9-mm distance between the samples. The tissue microarrays were cut into 3-μm sections and placed onto slides.

Immunohistochemistry

The avidin-biotin complex (ABC) method was used for the immunostaining. The tissue sections were deparaffinized by three 10-min incubations in xylene and then rehydrated in serial graded alcohol. Antigen retrieval was performed for phospho-Stat3, VEGF, p53, and Bcl-2 staining. Endogenous peroxide was blocked with 3% hydrogen peroxide in 45 ml of methanol for 15 min. The slides were washed three times in 1× PBS, 5 min per wash. All slides were preincubated with two drops of normal blocking solution (goat serum) at 37°C for 20 min (100 μl/slide) and shaken. The slides were not allowed to dry. We used the following antibodies: rabbit polyclonal anti-human phospho-Stat3 [tyr-705] antibody (Cell Signaling Technology, Beverly, MA) at a 1:150 dilution, mouse monoclonal IgG anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, USA) at a 1:200 dilution, DO-7 monoclonal anti-p53 antibody (Novocastra Laboratories, Newcastle, United Kingdom) at a 1:100 dilution, and a monoclonal anti-Bcl-2 antibody (DAKO, Carpinteria, CA, USA) at a 1:100 dilution. Each biotinylated secondary antibody was added to the slide and incubated for 30 min at 37°C. This incubation was followed by treatment with the avidin-biotinylated peroxidase complex (Immunotech, Cedex, France) for an additional 30 min at room temperature. After washing with PBS, staining was achieved using 3, 3′-diaminobenzidine (Immunotech, Cedex, France). Counterstaining was performed with hematoxylin for 30 s. Double marker analysis was performed using monoclonal antibodies specific for phospho-Stat3 and VEGF. All slides were coded and evaluated by two experienced pathologists without knowledge of patient identity or clinical status. Each experiment was performed independently twice. The kappa test for concordance between the two pathologists was 0.884. In the discrepant cases, two pathologists reviewed the cases together and reached a consensus.

Positive staining for phospho-Stat3 expression was defined as more than 25% nuclear staining with more than moderate intensity for the tumor cell. Staining in >50% of cells was considered positive for VEGF, staining in >5% of nuclei was considered positive for p53, and staining in >5% of cells was considered positive for Bcl-2.

Statistical analysis

Pearson Chi-square ($\chi 2$) test was used to determine the relationship between phospho-Stat3 (Tyr705),

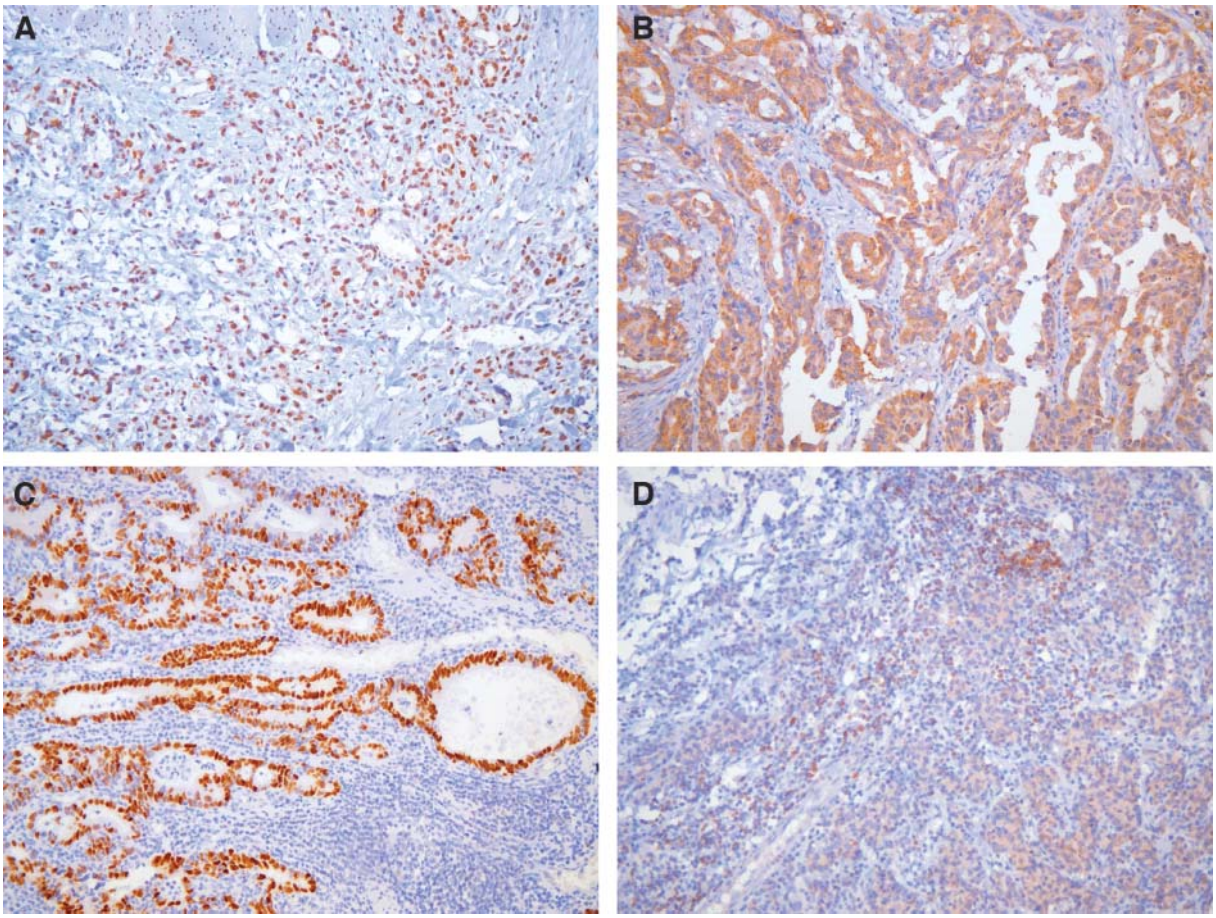

Fig. 1. Immunohistochemical staining of a gastric carcinoma tissue microarray. Each photograph shows representative tissue that is positive for phospho-Stat3 (A), VEGF (B), p53 (C), and Bcl-2 (D) (×200).

VEGF, p53, and Bcl-2 expressions and the correlation of these expressions with various clinicopathological factors. Survival curves were calculated using the Kaplan-Meier method and compared with other prognostic variables using the log-rank test. Stepwise Cox's regression analysis was performed to identify prognostic factors for survival. In all tests, $p<0.05$ was considered to be statistically significant. All statistical analyses were performed using SPSS 10® statistical software.

RESULTS

Patient characteristics

There were 88 male (64.2%) and 49 female (35.8%) patients, with a median age of 56.0 years (range 22–80 years). The most common histological type was tubular adenocarcinoma (43.1%). Tumor stage using AJCC classification was I in 19 patients (13.9%), II in 28 patients (20.4%), III in 54 patients (39.4%), and IV in 36 patients (26.3%). 57 patients received intravenous systemic chemotherapy after surgery and 51 patients received oral 5-fluorouracil. Survival data were available for 114 (83.2%) out of 137 patients. The median follow-up period was 50.9 months (range 0.2–108.0). 37 patients died. The most common cause of death was disease progression (35 patients), while other causes of death included sepsis (1 patient) and gall bladder cancer (1 patient).

Expression of phospho-Stat3, VEGF, p53, and Bcl-2

Phospho-Stat3 nuclear expression of gastric cancer tissue was observed in 25/137 (18.2%) patients. 94/137 (68.6%) patients were positive for VEGF, 55/137 (40.1%) patients were positive for p53, and 16/137 (11.7%) patients were positive for Bcl-2 (Fig. 1). Compared to the control

TABLE 1. *Distribution of different markers in the gastric cancer and control groups*

	Gastric cancer (n=137)	p value	Control (n=30)
Phospho-Stat3			
Negative	112 (81.8%)	0.12	28 (93.3%)
Positive	25 (18.2%)		2 (6.2%)
VEGF			
Negative	43 (31.4%)	0.001	19 (63.3%)
Positive	94 (68.6%)		11 (36.7%)
p53			
Negative	82 (59.9%)	<0.001	30 (100%)
Positive	55 (40.1%)		0 (0%)
Bcl-2			
Negative	121 (88.3%)	0.049	30 (100%)
Positive	16 (11.7%)		0 (0%)

group, gastric cancer tissues more frequently expressed VEGF, p53, and Bcl-2. However, the expression of phospho-Stat3 was not different between the two groups (Table 1).

Phospho-Stat3 expression was significantly correlated with VEGF (p=0.021) and Bcl-2 (p=0.005) expression. Double marker analysis was performed using monoclonal antibodies for phospho-Stat3 and VEGF to confirm whether the immunopositive staining of these two factors co-localized in the tumor specimens. Nuclear expression of phospho-Stat3 was consistent with cytoplasmic expression of VEGF (Fig. 2). Although the expression of p53 was correlated with the expression of phospho-Stat3, it was not statistically significant (p=0.074).

We also analyzed the correlation between these biomarkers and other clinicopathological features. Positive phospho-Stat3 staining was significantly associated with a poor pathological grade. However, there was no significant difference between the phospho-Stat3-positive and -negative groups regarding various other clinicopathological parameters, such as tumor stage (T, N, M) and pathological type (Table 2). 22 cases (16.1%) exhibited phospho-Stat3 and VEGF co-expression and 37 cases (27.0%) did not express phospho-Stat3 and VEGF. When these two groups were compared, we found that the group with phospho-Stat3 and VEGF co-expression had many patients with N3 and stage IV disease. However, there was no significant difference in other variables and overall survival.

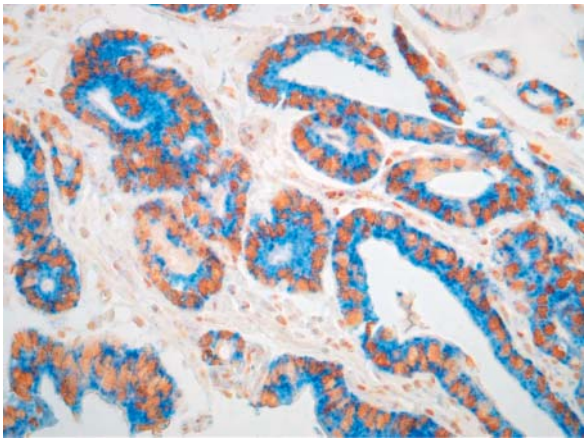

Fig. 2. Double marker analysis shows that the nuclei of gastric cancer cells are positive for phospho-Stat3 (brown) and the cytoplasm of gastric cancer cells are positive for VEGF (blue) (×400).

In the univariate analysis, T, N, M, and stage revealed significant factors for disease-free survival and overall survival. In contrast, the expression of phospho-Stat3, VEGF, p53, and Bcl-2 did not influence overall survival. In multivariate analysis, T and N stage were independent prognostic factors for disease-free survival. In addition, N and M stage were significant factors for overall survival. The expression of phospho-Stat3, VEGF, p53, and Bcl-2 did not influence survival.

DISCUSSION

Gastric adenocarcinoma remains a common cancer with a dismal prognosis; it is the second most common malignancy worldwide (12). *Helicobacter pylori* infection plays a major role in

TABLE 2.*Patient characteristics and expression of phospho-Stat3, VEGF, p53, and Bcl-2*

Factors	Patients (%)	p-Stat3+ (p value)	VEGF+ (p value)	p53+ (p value)	Bcl-2+ (p value)
Age: Median (range)	56 (22–80)				
Sex Male	88 (64.2)	21	62	43	12
Female	49 (35.8)	4	32	12	4
		(0.023)	(0.53)	(0.005)	(0.34)
T1,2	45 (32.8)	10	28	16	9
T3,4	92 (67.2)	15	66	39	7
		(0.4)	(0.26)	(0.44)	(0.03)
N0, 1	79 (58.1)	16	47	29	12
N2, 3	58 (41.9)	9	47	26	4
		(0.48)	(0.007)	(0.34)	(0.14)
M0	125 (91.2)	24	85	47	16
M1	12 (8.8)	1	9	8	0
		(0.35)	(0.62)	(0.05)	(0.19)
Stage I	19 (13.9)	4	12	6	2
II	28 (20.4)	6	16	11	6
III	54 (39.4)	7	35	20	6
IV	36 (26.3)	8	31	18	2
		(0.59)	(0.02)	(0.64)	(0.39)
Pathological type					
Tubular	59 (43.1)	11	47	32	3
Signet ring	48 (35.0)	10	28	11	9
Mixed	25 (18.2)	3	15	9	4
Other	5 (3.6)	1	4	3	0
		(0.83)	(0.08)	(0.008)	(0.12)
Grade					
Well, moderate	50 (36.5)	3	39	26	0
Poor	87 (63.5)	22	55	29	16
		(0.005)	(0.07)	(0.03)	(0.001)

the pathogenesis of gastric carcinoma and the aggressive nature of gastric carcinoma may be related to mutation of various oncogenes and tumor suppressor genes. However, the detailed pathogenetic mechanisms of gastric carcinoma remain unclear. Due to the lack of suitable and specific biomarkers for early detection and prognosis, most cases of the disease are diagnosed at advanced stages. The poor survival rate of gastric carcinoma patients is attributed to late disease diagnosis.

Stat3 protein has a dual role as a cytoplasmic signaling protein and as a nuclear transcription factor. Stat3 is activated by Tyr705 phosphorylation by Janus kinase, SRC tyrosine kinase, or other mechanisms, which leads to dimerization via a reciprocal phosphotyrosine-SH2 interaction (13). Under physiological conditions, the duration of Stat3 activation is temporary, and usually lasts from a few minutes to several hours (14). However, in a variety of human tumors, Stat3 is persistently activated and contributes to oncogenesis (8). Whether abnormal Stat3 expression and activation contributes to gastric cancer development and progression is not known.

A recent study reported that Stat3 was constitutively activated in various human gastric cancer cell lines and phospho-Stat3 was found in 27.5% of human gastric cancer specimens. Furthermore, this report also found that blockade of Stat3 induced apoptosis and growth inhibition of several gastric cancer cell lines (15). Immunohistochemical staining of 86 cases of resected human gastric cancer in a study by Gong et al. showed that phospho-Stat3 expression correlated with VEGF expression. In addition, Gong et al. found that microvessel density and phospho-Stat3 expression were independent prognostic factors of poor survival (16). In our study, we also found that phospho-Stat3 expression was strongly correlated with VEGF expression, suggesting that dysregulated Stat3 activation may play an important role in VEGF overexpression and the elevated angiogenic phenotype found in gastric cancer. Meanwhile, the expression of

phospho-Stat3 did not influence survival in our results. There might be discrepancies in the results between our work and the previous study due to differences in the gastric cancer operation method and race of the patients, and the limitations of tissue microarray.

In our study, although gastric cancer tissues express phospho-Stat3 more frequently than non-neoplastic gastric tissue, phospho-Stat3 expression was not significantly different between the two groups. This might be because the nature of control tissue was affected by phospho-Stat3 expression. Stat3 has been reported to be activated by inflammation and production of pro-inflammatory cytokines (17). Since all control gastric tissue included in our study had some portion of inflammation, further studies including comparisons with normal gastric tissue are needed. Positive phospho-Stat3 staining was significantly associated with poor pathological grade, but we could not find any correlation with tumor stage, disease-free survival or overall survival. However, we also found that phospho-Stat3 expression strongly correlated with VEGF and Bcl-2 expression and that co-expression of phospho-Stat3 and VEGF was related to lymph node metastasis.

Angiogenesis is essential for tumor growth and metastasis. VEGF is one of the most potent stimulators of angiogenesis. Stat3 has been reported to be a direct transcriptional activator of the VEGF gene (18, 19). Transfection of cells with the activated Stat3 mutant (Stat3C) increased VEGF expression and induced angiogenesis. In addition, VEGF expression was downregulated by blocking Stat3 signaling with dominant-negative Stat3 (18). The strong correlation of phospho-Stat3 and VEGF expression in our study confirmed previous results (16). In addition, the finding that phospho-Stat3 and VEGF co-expression is associated with lymph node metastasis suggests that Stat3 plays a role in tumor progression.

In addition, Stat3 signaling contributes to tumor cell proliferation and apoptosis. Several possible downstream targets of Stat3 have been identified, including apoptosis inhibitors and cell cycle regulators, such as Bcl-X_L, Mcl-1, survivin, c-Myc, and cyclin D1 (20–23). According to our results, activated Stat3 signaling in gastric cancer may also induce angiogenesis and suppress apoptosis.

The present study had several limitations. First, the immunohistochemical staining was the only method to determine phospho-Stat3 expression. Immunoprecipitation of cellular extracts from the tumor specimen might be more accurate. Secondly, while it has been established that findings obtained on tissue microarrays are highly representative of the tissues as a whole, some areas of the tissue can still be missing during microarray slide preparation. Furthermore, the clinical data, including relapse or survival data, were analyzed retrospectively. Lastly, a relatively small number of patients were examined in this study.

Nevertheless, we discovered phospho-Stat3 expression in 18.2% of gastric cancers and this expression is strongly related to VEGF and Bcl-2 expression. Furthermore, we found that co-expression of phospho-Stat3 and VEGF is associated with lymph node metastasis. These results suggest that phospho-Stat3 expression might be associated with angiogenesis, anti-apoptosis, and tumor progression in gastric carcinoma. Further studies with the objective of understanding how the Stat3 signaling pathway regulates gastric cancer development and progression are needed to determine the role of phospho-Stat3 in gastric cancer.

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