Expression of p-STAT3 in human gastric carcinoma: Significant correlation in tumour invasion and prognosis

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Abstract. Signal transducers and activators of transcription (STATs) belong to a family of transcription factors activated in response to cytokines and growth factors. Constitutive activation of STAT3 has been observed in a growing number of tumour-derived cell lines, as well as in tumor specimens from human cancers. Our aim was to determine the relationship between the expression of phosphorylated STAT3 (p-STAT3) and clinicopathological factors in human gastric adenocarcinoma. One-hundred and eleven cases of surgically resected human gastric adenocarcinoma were studied by immunohistochemistry. Fifty-five (49.5%) cases showed positive staining for the p-STAT3 proteins. The expression of p-STAT3 was significantly correlated with several clinicopathological factors. Moreover, expression of p-STAT3 was detected by Western blot analysis in 2 different kinds of cultured human gastric carcinoma cell lines and 4 cases of gastric carcinoma tissue obtained at surgery. Western blot analysis confirmed the presence of p-STAT3 in these specimens. In univariate survival analysis, p-STAT3 expression was associated with inferior survival ($p<0.05$). Constitutive activation of STAT3 may play an important role in the tumorigenesis of gastric adenocarcinoma, and the detailed mechanism of STAT3 signaling pathway in gastric adenocarcinoma deserves further investigation.

Introduction

Gastric cancer remains one of the most common cancer types in the world today, despite the fact that the incidence has gradually declined in many countries (1). The occurrence and progression of cancer is related to a series of genetic events affecting the structure and/or expression of a number of oncogenes, anti-oncogenes and growth factors (2,3). Potentially deeply invasive carcinomas, such as gastric carcinoma, have relatively high rates of lymph duct and venous invasion and lymph node metastasis (4). However, the mechanisms of invasion and metastasis of gastric carcinoma have not been fully elucidated.

Signal transducer and activator of transcription (STAT) proteins belong to a family of transcription factors that are normally inactive within the cytoplasm of cells and become activated by tyrosine phosphorylation in response to cytokines and growth factors. In normal cells, STAT activation is transient whereas, in a large number of primary tumors and cancer-derived cell lines, STAT proteins (in particular STAT3) remain activated by persistently activated tyrosine kinases and/or a decrease in the negative regulators of STAT dephosphorylation (5,6). The binding of a cytokine to its cognate receptor rapidly induces the tyrosine phosphorylation of the receptor by Jak kinases. Activated STATs rapidly translocate into the nucleus, bind to recognition sequences in the promoter region of target genes, and regulate their transcription. Recent studies have demonstrated the essential roles of STAT proteins in modulating the process of cell proliferation, differentiation and apoptosis (7-9).

Constitutively activated STAT proteins have been observed in a wide variety of human tumour cell lines and primary tumours including leukemia, multiple myeloma, breast cancer, prostate cancer, and other cancers (10-17). STAT3 has been classified as an oncogene because constitutively activated STAT3 was found to mediate oncogenic transformation in cultured cells and tumour formation in nude mice (18,19). STAT3 activation may not only provide a growth advantage, but also confer resistance to conventional therapies that depend on the mechanism of apoptosis to eliminate tumour cells (20). Previous studies showed that constitutive activation of STAT3 correlated with cyclin D1 expression, possibly providing a prognosis marker in head and neck cancer (21,22). These findings suggest that constitutive activation of STAT3 participates in the development of different human malignancies.

An antibody specific for the activated (phospho-Tyr705) form of STAT3 (p-STAT3) has become available (23). In prostate tissue, the activated form of STAT3 localized predominantly to the nuclei of malignant glands (24). This

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activated form may be a better probe for function than total STAT3, but it has not yet been evaluated in gastric cancer tissues. We evaluated the expression and subcellular localization of phospho-STAT3 (Tyr705) by immunohistochemistry in human gastric cancer.

Materials and methods

We studied 10 cases of gastric adenoma and 111 cases of primary human gastric carcinoma by immunohistochemistry. Of the 111 patients with gastric carcinoma, there were 63 men and 48 women. The median age was 68.9 years (range, 38-89). All tumors were obtained from patients who had undergone surgery or endoscopic resection at Nagasaki University Hospital and Shunkaikai Inoue Hospital between 2000 and 2005. Fifteen specimens of gastric tissue obtained from a normal area adjacent to the tumour specimens (5-10 cm away) were taken and evaluated as normal controls. All samples were snap-frozen within 15 min after surgical removal to ensure preservation of STAT3 activities.

Each tumour was assigned a histological type and a depth grading of infiltration according to the Japanese Classification of Gastric Carcinoma by the Japanese Research Society for Gastric Cancer (25), based on the World Health Organization classification (WHO). Histologically, of the 111 primary human gastric adenocarcinoma cases, 6 were papillary, 20 were tubular of the well-differentiated type, 37 were tubular of the moderately differentiated type, 10 were poorly differentiated adenocarcinoma of the solid type, 16 were poorly differentiated of the nonsolid type, 17 were signet-ring cell carcinoma, and 5 were mucinous adenocarcinoma. A total of 15 normal gastric mucosal tissues were evaluated as normal controls.

Lymphatic and venous invasion was studied on routine hematoxylin and eosin-stained slides. In addition, the Elastica van Gieson stain was used in all cases. Each parameter was defined as 'present' only when invasion was identified with certainty, but defined as 'absent' when not seen at all or not seen with certainty (26,27). Lymph node metastasis was defined as 'present' only when confirmed histologically. The diagnosis was established by two independent pathologists (Y. Yakata and T. Nakayama) and cases with a questionable diagnosis were excluded from the study.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissues, deparaffinized in xylene, and dehydrated in phosphate-buffered saline. Deparaffinized sections were preincubated with an optimal dilution (0.1 μg/ml) of a primary polyclonal goat antibody against human phosphorylated STAT3 (p-STAT3; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The slides were sequentially incubated with a biotinylated and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody, and the reaction products were visualized using diaminobenzidine (DAB; Dako, Carpinteria, CA, USA) with methyl green as a counterstain. Prostatic cancer tissue served as the positive control (24). Two investigators analyzed the immunohistochemical staining (Y. Yakata and T. Nakayama). p-STAT3 expression was classified into two categories, depending on the percentage of cells stained: 0 to 10% positive cells were considered negative (-) and more than 10% positive cells were considered positive (+).

Cell culture. Two human gastric carcinoma cell lines, MKN-28 and SCH, were incubated at 37˚C in a humidified atmosphere of 5% CO_2. MKN-28 and SCH were maintained in RPMI-1640 medium (Invitrogen Corp. NY, USA) supplemented with 10% fetal calf serum. All of these cells were provided by the Japanese Collection of Research Bioresources (Tokyo, Japan). For the induction of p-STAT3, two human gastric carcinoma cell lines were stimulated by 100 ng/ml of interferon-α (IFN-α; Cell Signaling Technology, Beverly, MA, USA) (28).

Immunoblotting. Specimens and cells were resuspended in ice-cold radioimmunoprecipitation (RIPA) buffer and one tablet of complete proteinase inhibitor mixture (Roche Applied Science, Indianapolis, IN, USA) per 50 ml for 10 min, sonicated on ice, and centrifuged (12,000 rpm, 15 min at 4˚C). Protein concentration of the supernatant (protein fraction) was determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA). An aliquot of 10 μg of protein was mixed with an equivalent volume of 2X protein loading buffer containing 2-ß-mercaptoethanol and boiled for 5 min before loading onto a 15% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes using ECL (Amersham Biosciences, Piscataway, NJ, USA) and blocked in TBST containing 5% non-fat dry milk powder. Protein immunoblots were performed using specific antibodies to ß-actin (clone JLA20; Calbiochem, San Diego, CA, USA), phosphotyrosine (Tyr^{705}) STAT3 (Cell Signaling Technology), and STAT3 (Santa Cruz Biotechnology Inc.). The membranes were further incubated with peroxidase-conjugated secondary antibodies, and protein bands were visualized using a commercial chemiluminescence detection kit (ECL Plus; Amersham Biosciences) as described by the manufacturer.

Statistical analysis. The Stat View II program (Abacus Concepts, Inc., Berkeley, CA, USA) was used for statistical analyses. Analyses comparing the expression of p-STAT3 were performed by the Chi-square test for independence and the Mann-Whitney U test. Survival durations were calculated using the Kaplan-Meier method. The log-rank test was used to compare the cumulative survival of patient groups.

Results

The results from immunohistochemical staining are summarized in Table I. Expressed p-STAT3 was heterogeneous in the cytoplasm and nucleus of carcinoma cells. p-STAT3 immunoreactivity in relation to histological type is shown in Table I. Poorly differentiated adenocarcinoma of the solid and nonsolid type and tubular adenocarcinoma of the moderately differentiated type gastric carcinoma stained for p-STAT3. However, tubular adenocarcinoma of the well-differentiated type expressed relatively weak p-STAT3. In signet-ring cell carcinoma, most cases were negative for p-STAT3 (4 of 17, 23.5%). All cases of 5 mucinous carcinoma were negative group-STAT3. All 10 cases of adenoma and all 15 cases of normal mucosa were negative for p-STAT3. There was a statistical difference between total carcinoma and adenoma ($p<0.05$).

Table I. p-STAT3 expression in gastric carcinoma.

	n	-	+	p-value
Tubular adenoma	10	10 (100.0%)	0 (0.0%)	p<0.005[a]
Total carcinoma	111	56 (50.5%)	55 (49.5%)	
Histological differentiation				n.s.
papillary	6	2 (33.3%)	4 (66.7%)	
tub/well	20	14 (70.0%)	6 (30.0%)	
tub/moderate	37	14 (37.8%)	23 (62.1%)	
poor/solid	10	3 (30.0%)	7 (70.0%)	
poor/nonsolid	16	5 (31.3%)	11 (68.8%)	
sig	17	13 (76.5%)	4 (23.5%)	
muc	5	5 (100.0%)	0 (0.0%)	
Lauren's classification				n.s.
Intestinal	63	30 (47.6%)	33 (52.4%)	
Diffuse	48	26 (54.2%)	22 (45.8%)	
Depth of invasion				p<0.001[b]
m	26	24 (92.3%)	2 (7.7%)	
sm	37	21 (56.8%)	16 (43.2%)	
mp	12	4 (33.3%)	8 (667%)	
ss	11	2 (18.2%)	9 (81.8%)	
se	25	5 (28.0%)	20 (80.0%)	
Lymph node metastasis				p<0.005
Present	32	8 (25.0%)	24 (75.0%)	
Absent	79	51 (64.6%)	28 (35.4%)	

[a]Significant difference between adenoma and total carcinoma in the expression of p-STAT3. [b]Significant difference between mucosal carcinomas and other carcinomas. n.s., not significant; tub, tubular adenocarcinoma; sig, signet ring-cell carcinoma; muc, mucinous carcinoma.

Fig. 1 shows a representative example of strong immunohistochemical p-STAT3 staining in a carcinoma invading the subserosa. p-STAT3 protein was detected in both the cytoplasm and the nucleus of almost all carcinomas (Fig. 1B). Fig. 2 shows a mucosal carcinoma with negative staining for p-STAT3.

The presence of immunoreactivity appears to be correlated with the degree of tumour infiltration (Table I). Staining for p-STAT3 was mainly negative for tumours with mucosal infiltration (only 2 of 26 were positive, 7.7%), whereas 43.2% of submucosal infiltrative tumours were p-STAT3 immunoreactive (16 of 37). In tumours infiltrating the proprial muscle layer, the subserosa and the serosal surface, p-STAT3 was frequently expressed (37 of 48, 77.1%). Statistical analysis showed significant differences between mucosal carcinomas and the other carcinomas (p<0.001; Table I). In the invasive carcinomas, the staining intensity in signet-ring cell carcinomas and mucinous adenocarcinomas was statistically lower than in the other type of carcinoma. Also, the invasive front and/or the peripheral parts of the primary tumour were intensely stained compared with superficial and central parts of the tumour in almost all cases.

p-STAT3 expression in primary carcinomas correlated with the presence of lymph node metastasis (p<0.005; Table I). Moreover, p-STAT3 expression in primary carcinoma correlated with the presence of venous invasion (p<0.0001) and lymphatic invasion (p<0.0005) (Table II). There was no correlation between p-STAT3 expression and the differentiation or the pattern of tumour infiltration of primary carcinomas.

The Western blot analyses of p-STAT3 expression in MKN-28 and SCH, human gastric carcinoma cell lines, are shown in Fig. 3. STAT3 expression was detected in both cell lines. P-STAT3 expression after IFN-α stimulation also was up-regulated in both cell lines, whereas a weak expression of p-STAT3 was observed without IFN-α stimulation.

The results of Western blot analysis of p-STAT3 expression in surgical specimens of human gastric cancer are shown in Fig. 4. The STAT3 non-activated type was expressed in all samples. p-STAT3 expression was detected in normal mucosa (N), whereas strong expression of p-STAT3 was observed in the gastric carcinoma tissues (T).

The respective Kaplan-Meier curves are given in Fig. 5. The corresponding log-rank test shows that there is a significant difference between p-STAT3 positive and negative subgroups in gastric cancer patients (p<0.05).

Discussion

We investigated the expression of p-STAT3 in gastric carcinoma using immunohistochemical techniques and

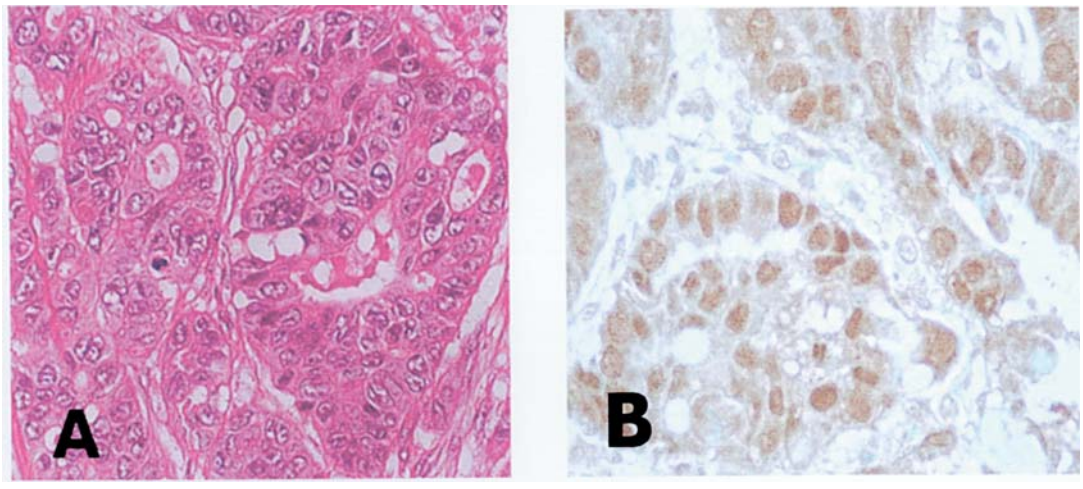

Figure 1. (A) Invasive gastric adenocarcinoma to subserosa (hematoxylin and eosin staining, x400). (B) p-STAT3 reveals positive staining in the cytoplasm and nucleus of carcinoma cells (DAB staining, x400).

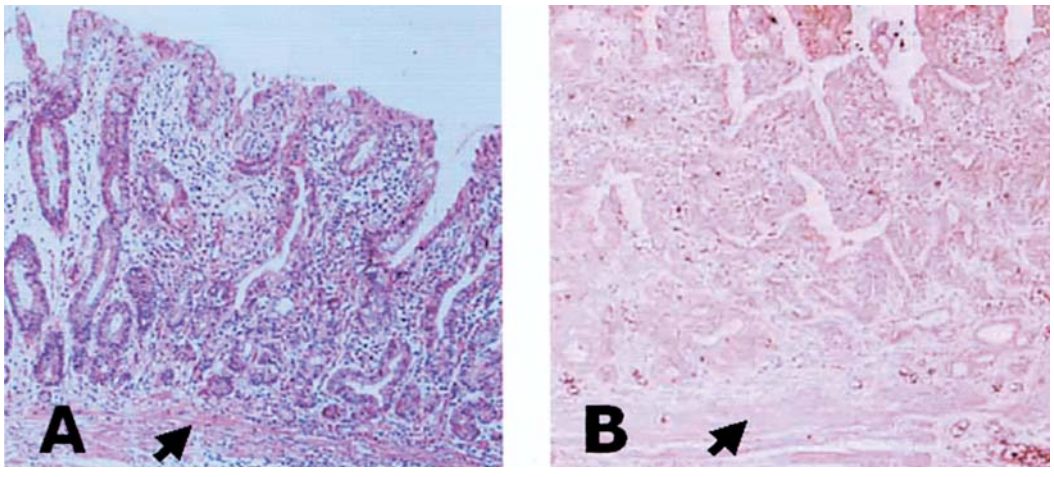

Figure 2. (A) Mucosal gastric adenocarcinoma. Arrow shows the muscularis mucosa (hematoxylin and eosin staining, x100). (B) Carcinoma cells are negative for p-STAT3. Arrow shows the muscularis mucosa (DAB staining, x100).

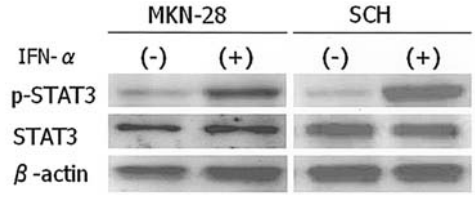

Figure 3. Expression of STAT3 and p-STAT3 in human gastric adenocarcinoma cell lines (MKN28, SCH). Cells were stimulated with 100 ng/ml of IFN-α. ß-actin represents the internal protein control. Two cell lines demonstrate elevated levels of p-STAT3.

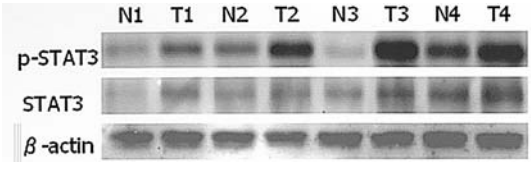

Figure 4. Expression of STAT3 and p-STAT3 in human gastric carcinoma. ß-actin represents the internal protein control. Elevated levels of p-STAT3 in tumour (T) tissues were compared to adjacent normal mucosa (N).

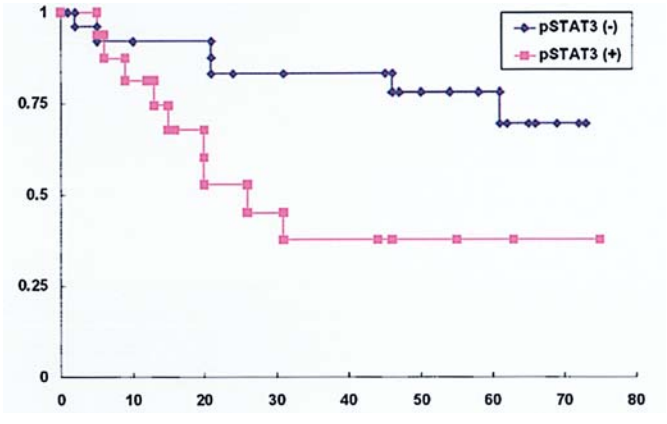

Figure 5. Kaplan-Meier survival curves. The patients with p-STAT3 expressed in carcinoma tissue had significantly worse prognoses (*p<0.05, log-rank test).

Western blot analysis. Statistical analyses of our data showed no correlation between p-STAT3 expression and histological differentiation, but the expression of p-STAT3 correlated

Table II. p-STAT3 expression correlated with vascular invasion in invasive carcinoma (85 cases).

	n	-	+	p-value
Invasive carcinoma	85	35 (43.8%)	50 (58.8%)	
Venous invasion				p<0.0001
Present	45	9 (20.0%)	36 (80.0%)	
Absent	40	26 (65.0%)	14 (21.5%)	
Lymphatic invasion				p<0.0005
Present	62	18 (29.0%)	44 (71.0%)	
Absent	23	17 (73.9%)	6 (26.1%)	

with the degree of tumour invasion, lymphatic and venous invasion and the presence of lymph node metastasis.

Activation of STAT3 signaling is reported to up-regulate the expression of various genes involved in cell survival and proliferation, such as the bcl-2 family, cyclin D1 and c-myc (18,20,29,30). However, induction of apoptosis elicited by STAT3 inhibition was not associated with down-regulation of bcl-2 family expression in gastric cancer cell lines. Thus, bcl-2 family genes might not always be involved in the STAT3-induced inhibition of apoptosis in gastric cancer cells (31). Otherwise, in colorectal carcinoma, protein levels of p-STAT3, cyclin D1 and Bcl-XL were increased (32). In the present study, we did not clarify the downstream components of STAT3 activation. In most tumours, the cause of persistent activation of STAT3 is not known. In some myeloma cells and prostate cancer cells, however, constitutively activated STAT3 signaling is due to abnormally regulated IL6-JAK signaling in an autocrine or paracrine manner (10,33,34). Further studies are needed to clarify the cause of constitutive activation of STAT3 signaling in gastric cancer cells.

In the present study, p-STAT3 showed significantly greater expression in advanced cancer than in 'early' cancer. Our results therefore lend support to previous reports of a correlation between p-STAT3 expression and prognosis. Moreover, our results have shown a correlation between p-STAT3 expression and venous and lymphatic invasion and lymph node metastasis. Notably, there is a significant correlation between p-STAT3 expression and venous invasion. Tumour angiogenesis has been considered the most important predictor of overall survival in gastric cancer (35,36).

The prognosis of patients with gastric carcinoma has conventionally been determined by the extent of the primary tumour and the presence or absence of metastasis (25). However, the mechanism of invasion and metastasis of gastric carcinoma has not been fully elucidated. Some reports have shown that the expression of p-STAT3 is correlated with tumour invasion and prognosis (14,31,32,37,38). The role of the JAK/STAT pathway in human gastric carcinoma has not been fully clarified, particularly the relationship between expression of p-STAT3 and the clinicopathological features of gastric carcinoma. Unlike the situation in normal cells and tissues, constitutively activated STATs are detected in a wide variety of human tumours, including carcinomas of the colorectum (14), ovary (22), prostate (24), breast (39) and leukemia (40). Such aberrant activation of STATs, especially STAT3, is often associated with cell survival, proliferation, and transformation (18,22,41). Thus, as in other types of cancer cells, constitutive activation of STAT3 appears to have an important role in the survival of some kinds of gastric cancer cells by suppressing apoptosis and enhancing cell proliferation (31).

In conclusion, STAT3 is constitutively activated in certain subtypes of human gastric cancer and activation of STAT3 plays an important role in tumour invasion and patient prognosis.

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