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Activation of STAT3 is a marker of poor prognosis in human colorectal cancer

TAKAFUMI KUSABA[1,2], TOSHIYUKI NAKAYAMA[1], KAZUYUKI YAMAZUMI[1,2], YUICHI YAKATA[1], AYUMI YOSHIZAKI[1], KENICHIRO INOUE[3], TAKESHI NAGAYASU[2] and ICHIRO SEKINE[1]

Departments of [1]Molecular Pathology, [2]Translational Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences, [3]Shunkaikai Inoue Hospital, Nagasaki, Japan

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Abstract. It is known that the signal transducer and activator of transcription 3 (STAT3) is a key signaling molecule implicated in the regulation of growth and malignant transformation. Constitutive activation of STAT3 has been observed in a number of tumour-derived cell lines, as well as in a wide variety of human malignancies. The present study was conducted to examine p-STAT3 (activated form of STAT3) expression and its association with clinicopathological factors and prognosis in human colorectal adenocarcinomas. Expression of p-STAT3 was immunohistochemically examined in 108 cases of colorectal adenocarcinoma tissue obtained at surgery. and was found in 57.4% of tumours (62 of 108). p-STAT3 immunoreactivity significantly correlated with the depth grading of tumour invasion ($p<0.001$), lymphatic invasion($p<0.05$), Dukes' classification ($p<0.05$), stage ($p<0.001$) and prognosis after operation ($p<0.001$). Expression of p-STAT3 was a marker of poor prognosis in overall survival ($p<0.01$). Expression of p-STAT3 was detected by Western blot analysis in three colon carcinoma tissue samples obtained at surgery.To our knowledge, this is the first study on the poor prognosis of p-STAT3 in human colorectal adenocarcinomas. These findings suggest that expression of p-STAT3 is an important factor related to tumour invasion and poor prognosis of human colorectal adenocarcinoma.

Introduction

Colorectal cancer is one of the most common malignancies in developed countries (1,2). The incidence of colorectal cancer has been increasing dramatically in Japan (3) and has attracted worldwide attention. The prognosis of colo-rectal cancer patients is based on the depth of tumour cell invasion and the presence of lymph node metastasis (4). Usually, these parameters can be determined by microscopic examination of tissue sections from the primary neoplasm and lymph nodes (5). However, it is not always possible to establish a prognosis based only on the histopathological examination of primary colorectal carcinoma specimens (5). Recently, the suggestion was made that the occurrence and progression of cancer is related to the activation of an intra-cellular signaling pathway (6). However, the mechanism of the invasion of colorectal carcinomas has not been fully determined.

Signal transducers and activators of transcription (STATs) are cytoplasmic transcription factors, and are also key mediators of cytokine and growth factor signaling pathways (7). At present, seven mammalian STAT genes have been identified (8-10). The proteins corresponding to these genes have a conserved structural organization consisting of 750-850 amino acids. The binding of a cytokine to its cognate receptor rapidly induces the tyrosine phosphorylation of the receptor by Jak kinases. Such phosphorylated tyrosines provide docking sites for STATs. The STATs themselves are phosphorylated. The phosphorylated STATs (p-STATs) are released from the receptor, and can then dimerize. The dimeric form can then translocate into the nucleus, where it modulates expression of target genes (11).

A growing number of tumour-derived cell lines, as well as tumour specimens from human cancers, have been reported to express constitutively activated STAT proteins, which are very frequently STAT3 (6). Constitutive activation of STAT3 has been detected in cancers of the pancreas, the prostate, the ovaries, the head and neck, as well as in other cancers (12-15). Our previous study was conducted to explore the relationship between p-STAT3 expression and clinicopathological features in human colorectal adenocarcinoma and adenoma (16). This was the first study to report a significant correlation of p-STAT3 expression with the depth grading of tumour invasion. However, no study had reported a significant association between p-STAT3 expression and prognosis in human colorectal cancer. The present study, therefore, was conducted to explore the relationship between p-STAT3 expression and prognosis in human colorectal cancer.

Correspondence to: Dr Toshiyuki Nakayama, Department of Molecular Pathology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki, 852-8523, Japan
E-mail: toshi-n@net.nagasaki-u.ac.jp

Abbreviations: STAT, signal transducer and activator of transcription

Key words: colorectal cancer, invasion, overall survival, prognosis, p-STAT3

Materials and methods

Cases and tissues. One hundred and eight primary human colorectal adeno-carcinomas were studied by immuno-histochemistry. Of the 108 patients with colorectal carcinoma, there were 66 males and 42 females. The median age was 65.6 years (range, 44 to 86 years). Forty-four tumours were located in the rectum, 38 in the sigmoid colon, 4 in the descending colon, 10 in the transverse colon, 10 in the ascending colon and 2 in the caecum. All tumours were obtained from patients who had undergone surgery at Inoue hospital in Nagasaki between 1994 and 1998.

Each tumour was assigned a histological type according to the World Health Organization classification: well differentiated adenocarcinoma, moderately differentiated adenocarcinoma, poorly differentiated adenocarcinoma, and mucinous adenocarcinoma (17).

According to the TNM staging system of the American Joint Committee on Cancer, the depth grading of tumour invasion in each of the carcinomas was classified into 4 groups: T1, invading submucosa; T2, invading muscularis propria; T3, invading either subserosa or pericolic tissue; and T4, invading through serosa or invading contiguous organs (18). Staging of the tumours was also conducted according to the American Joint Committee on Cancer, after histological studies.

Based on Dukes' classification, the pathological stages of colorectal carcinoma were classified into 4 groups: A, tumour invading submucosa or muscularis propria; B, tumour extending beyond muscularis propria; C, tumour extending with metastases to regional mesenteric lymph nodes without evidence of distant spread; and D, tumour extending with distant metastasis to other sites, commonly the liver and lungs (19-21).

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 observed at all or not observed with certainty (22,23).

Lymph node metastasis was defined as “present” only when confirmed histologically. The diagnosis was established by two independent pathologists (T. Kusaba and T. Nakayama).

Among the 108 patients, 66 remained disease-free for a median follow-up period of 43.7 months, ranging from 0.71 to 60.0 months. In total, 42 patients suffered from local recurrence and/or distant metastasis after the operation, including 16 patients with local recurrence (two also with distant metastasis), and 26 patients with distant metastasis without local recurrence.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissues were cut into 4-mm sections, deparaffinized in xylene and rehydrated in phosphate-buffered saline. Deparaffinized sections were preincubated with normal bovine serum to prevent nonspecific binding, and then incubated overnight at 4˚C with an optimal dilution (1 ug/mg) of a primary goat polyclonal IgG against human p-STAT3 (Tyr705; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The slides were then incubated with alkaline phosphatase-conjugated donkey anti-goat immunoglobulin antibody (Santa Cruz Biotechnology, Inc.). The reaction products were resolved using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; Dako, Carpinteria, CA, USA). Primary antibody preabsorbed with excess recombinant p-STAT3 peptide (Santa Cruz Biotechnology, Inc.) was used for the negative controls. Prostatic tissue served as the internal positive control for p-STAT3 immunostaining (24).

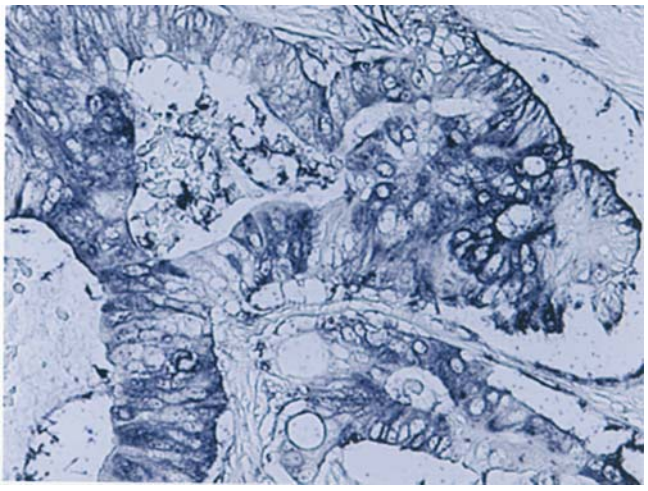


Figure 1. Immunohistochemistry of p-STAT3 in colorectal adeno-carcinomas. p-STAT3 shows strong nuclear and cytoplasmic expression. (immunoalkaline phosphatase staining; original magnification, 400X).

Analysis of the immunohistochemical staining was performed by two investigators (T. Kusaba and T. Nakayama). p-STAT3 expression was classified into two categories, depending on the percentage of cells stained: negative, 0 to 15% positive cells; Positive, >15% positive tumour cells.

Immunoblotting. Specimens and cells were resuspended in ice-cold Radioimmunoprecipitation (RIPA) buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 50 mM NaF, and one tablet of complete proteinase inhibitor mixture (Roche Applied Science, Indianapolis, IN) per 50 ml for 10 min, sonicated on ice, and centrifuged (12,000 x g, 15 min at 4˚C). The protein concentration of the supernatant (protein fraction) was determined by Bradford protein assay (Bio-Rad, Hercules, CA). 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 an SDS-polyacrylamide gel. After electro-phoresis, the proteins were transferred onto nitrocellulose membranes using ECL (Amersham Biosciences, Piscataway, NJ) and blocked in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat dry milk powder. Protein immunoblots were performed using specific antibodies to ß-actin (clone JLA20; Calbiochem San Diego, CA), phosphotyrosine (Tyr^{705}) STAT3 (Cell Signaling Technology, Beverly, MA), STAT3 (Santa Cruz Biotechnology Inc.). The membranes were further incubated with peroxidase-conjugated secondary antibodies, and protein

were visualized using a commercial chemiluminescence kit (ECL Plus; Amersham Biosciences) as described by the manufacturer.

Statistical analysis. The Stat View II program (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analyses. Analyses comparing expression of p-STAT3 were performed with the Chi-square for independence and Mann-Whitney's U test. The Kaplan-Meier method was used in the survival analysis. A log-rank test was used to calculate the significance of differences in the survival analysis. A probability level of less than 0.05 ($p<0.05$) was considered to indicate a significant difference.

Results

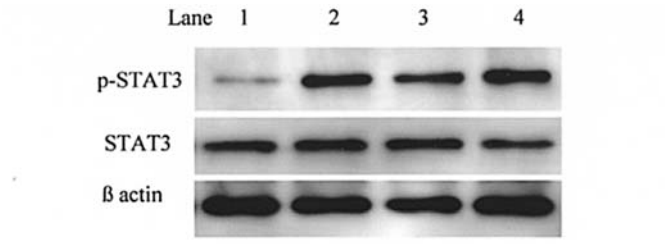
We statistically analyzed the correlation between p-STAT3 immunoreactivity and age, gender and the site of the primary tumour. However, there was no correlation.

Among the 108 adenocarcinomas, histologically, 50 were well differentiated, 50 were moderately differentiated, 4 were poorly differentiated adenocarcinomas and 4 were mucinous carcinomas. There were 30 submucosal infiltrative carcinomas (T1), 14 carcinomas invading proprial muscle layers (T2), 64 carcinomas reaching the subserosa (T3), and none classified as T4. Seventeen cases had lymph node metastasis.

Fig. 1 shows a representative example of strong immunohistochemical p-STAT3 staining in an invasive carcinoma of T3 grade. p-STAT3 protein was detected in both the cytoplasm and the nucleus of almost all carcinomas. The relationships between p-STAT3 immunoreactivity of the tumour cells and pathological features in adenocarcinomas are summarized in Table I. A total of 57.4% (62/108) of colorectal adenocarcinoma cells showed immunoreactivity for p-STAT3. p-STAT3 expression was found in 56.0% (28/50) of well differentiated adenocarcinomas, 64.0% (32/50) of moderately differentiated adenocarcinomas, 50.0% (2/4) of poorly differentiated adenocarcinomas, and 0% (0/4) of mucinous carcinoma. There was no significant correlation between p-STAT3 immunoreactivity and the differentiation of colorectal adenocarcinomas.

p-STAT3 immunoreactivity was compared with the depth grading of tumour invasion. p-STAT3 expression was found in 20.0% (6/30) of T1, in 28.6% (4/14) of T2, and in 81.3% (52/64) of T3. A significant correlation was found between p-STAT3 immunoreactivity and the depth grading of tumour invasion ($p<0.001$). The incidences of lymphatic invasion and venous invasion were 75.9% (82/108), and 46.3% (50/108), respectively. p-STAT3 immunoreactivity significantly correlated with the presence of lymphatic invasion ($p<0.05$).

The incidence of lymph node metastasis was 31.5% (34/108), but p-STAT3 immunoreactivity did not significantly correlate with the presence of lymph node metastasis. p-STAT3 immunoreactivity was compared with Dukes' classification. p-STAT3 expression was found in 29.4% (10/34) of Dukes' A, in 87.5% (28/32) of Dukes' B, in 68.8% (22/32) of Dukes' C, and in 25.0% (2/8) of Dukes' D. Immunoreactivity for p-STAT3 significantly correlated with increasing stages of Dukes' classification ($p<0.001$).



Lane	Patient age (gender)	Histological Differentiation	Invasive grade (Depth)	Dukes classification	Lymph node metastasis	Lymphatic invasion	Venous invasion
1	71(F)	normal human colorectal mucosa					
2	71(F)	wel	T3(ss)	B	+	+	-
3	68(M)	moderate	T4(si)	B	-	-	-
4	80(M)	wel	T3(ss)	B	-	-	-

Figure 2. The expression of phosphorylated (activated) signal transducer and activator of transcription (p-STAT3) is lower in normal mucosa (lanes 1) than in adenocarcinoma (lanes 2–4). There is no difference in the expression of STAT3 between the normal mucosa and carcinoma tissues. ß Actin was used as an internal protein control.

Fig. 2 shows the results of Western blotting for p-STAT3 in surgical specimens of human colorectal cancer. STAT3 expression was detected in all samples. p-STAT3 expression was detected in normal colon mucosa (lane 1), whereas strong expression of p-STAT3 was seen in the colon carcinoma tissues (lanes 2-4).

p-STAT3 immunoreactivity was compared with the stage classification. p-STAT3 expression was found in 20.0% (6/30) of stage I, in 95.0% (38/40) of stage II, in 50.0% (16/32) of stage III, and in 33.3% (2/6) of stage IV. p-STAT3 immunoreactivity was significantly lower in stage I than stage II-IV ($p<0.001$).

p-STAT3 immunoreactivity was compared with prognosis after surgery. p-STAT3 expression was found in 42.4% (28/66) of disease-free and in 81.0% (34/42) of recurrence and/or metastasis. There was a significant difference in the expression of p-STAT3 with prognosis after surgery ($p<0.05$).

Patients with significant constitutively activated STAT3 had worse overall survival than those with negative p-STAT3 (Fig. 3A; log-rank test, $p<0.001$). Moreover, at grade T3 or T4, patients who had p-STAT3 positive staining results had significantly worse overall survival than those who had p-STAT3 negative staining results (Fig. 3B; log-rank test, $p<0.05$). At Dukes' grade A or B, p-STAT3-positive patients had significantly worse overall survival than p-STAT3-negative patients (Fig. 3C; †log-rank test, $p<0.05$). At Dukes' grade C or D, p-STAT3-positive patients had significantly worse overall survival than p-STAT3 negative patients (Fig. 3C; ††log-rank test, $p<0.01$). At stages I or II, p-STAT3-positive patients had significantly worse overall survival than p-STAT3-negative patients (Fig. 3D; †log-rank test, $p<0.01$). At Stage III, p-STAT3-positive patients had significantly worse overall survival than p-STAT3-negative patients (Fig. 3D; ††log-rank test, $p<0.05$). At the presence of lymphatic invasion, p-STAT3-positive patients had significantly worse overall survival than p-STAT3-negative patients (Fig. 3E; log-rank test, $p<0.01$).

We analyzed disease-free survival as a function of the results of p-STAT3 staining, p-STAT3 staining combined

Table I. Relationships between p-STAT3 immunoreactivity of tumor and pathological features.

		p-STAT3 immunoreactivity, n (%)	
	n	+	-
Number of patients	108	62 (57.4)	46 (42.6)
Clinical parameters			
Age (years)			
Range	44-86	44-86	45-85
Mean	65	66	65
Median ± s.d.	65.6±10.5	64.9±12.0	66.1±8.44
Gender			
Male	66	38 (57.6)	28 (42.4)
Female	42	24 (57.1)	18 (42.9)
Primary site			
Caecum/Ascending colon	12	10 (83.3)	2 (16.7)
Transverse colon	10	6 (60.0)	4 (40.0)
Descending colon	4	4 (100)	0 (0)
Sigmoid colon	38	18 (47.4)	20 (52.6)
Rectum	44	24 (54.5)	20 (45.5)
Differentiation			
Well	50	28 (56.0)	22 (44.0)
Moderate	50	32 (64.0)	18 (36.0)
Poor	4	2 (50.0)	2 (50.0)
Mucinous carcinoma	4	0 (0)	4 (100)
Depth grading of tumour invasion[a]			
T1	30	6 (20.0)	24 (80.0)
T2	14	4 (28.6)	10 (71.4)
T3	64	52 (81.3)	12 (18.7)
T4	0	0 (0)	0 (0)
Lymph node metastasis			
Absent	74	46 (62.2)	28 (37.8)
Present	34	16 (47.1)	18 (52.9)
Lymphatic invasion[b]			
Absent	26	10 (38.5)	16 (61.5)
Present	82	52 (63.4)	30 (36.6)
Venous invasion			
Absent	58	36 (62.1)	22 (37.9)
Present	50	26 (52.0)	24 (48.0)
Dukes' classification[c]			
A	34	10 (29.4)	24 (70.6)
B	32	28 (87.5)	4 (12.5)
C	32	22 (68.8)	10 (31.2)
D	8	2 (25.0)	6 (75.0)
Stage[d]			
I	30	6 (20.0)	24 (80.0)
II	40	38 (95.0)	2 (5.0)
III	32	16 (50.0)	16 (50.0)
IV	6	2 (33.3)	4(66.7)
Prognosis after surgery[e]			
Disease-free	66	28 (42.4)	38 (57.6)
Recurrence and/or metastasis	42	34 (81.0)	8 (2.0)

[a]Significant difference ($p<0.001$), Mann-Whitney's U test. [b]Significant difference ($p<0.05$), Chi-square for independence test. [c]Significant difference ($p<0.05$), Mann-Whitney's U test. [d]Significant difference between Stage I vs II-IV($p<0.001$), Chi-square for independence test. [e]Significant difference ($p<0.001$), Chi-square for independence test.

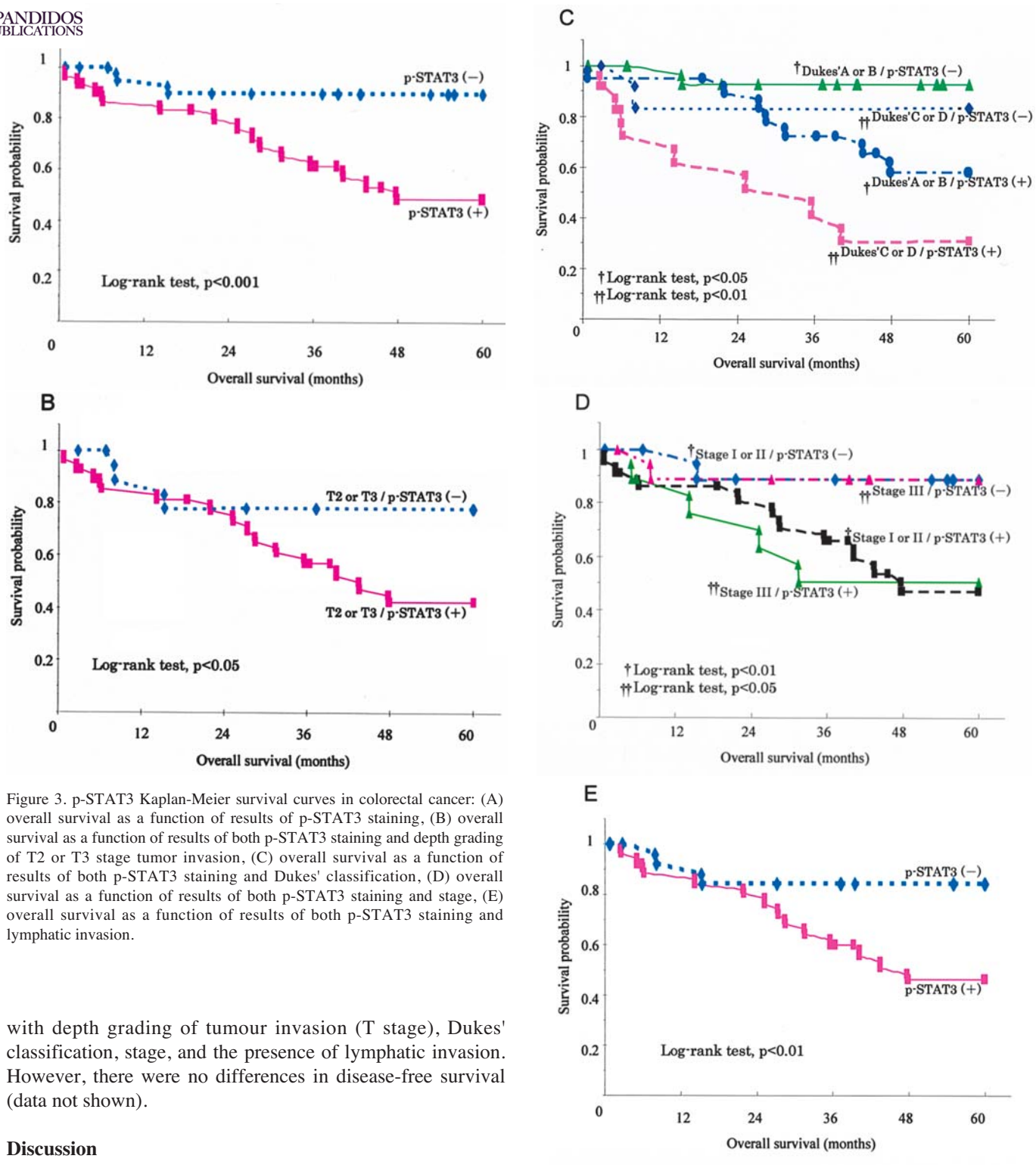


Figure 3. p-STAT3 Kaplan-Meier survival curves in colorectal cancer: (A) overall survival as a function of results of p-STAT3 staining, (B) overall survival as a function of results of both p-STAT3 staining and depth grading of T2 or T3 stage tumor invasion, (C) overall survival as a function of results of both p-STAT3 staining and Dukes' classification, (D) overall survival as a function of results of both p-STAT3 staining and stage, (E) overall survival as a function of results of both p-STAT3 staining and lymphatic invasion.

with depth grading of tumour invasion (T stage), Dukes' classification, stage, and the presence of lymphatic invasion. However, there were no differences in disease-free survival (data not shown).

Discussion

Recently, many reports have suggested an important role for constitutively activated STAT3 in malignant transformation and tumour progression (25). In our previous study, we reported on the correlation between the expression of p-STAT3 and the depth grading of tumour invasion in human colorectal adenocarcinomas (16). In the present study, the expression of p-STAT3 was found to be more intense in the deeper invasive areas, as shown in Table I. However, there was no correlation between p-STAT3 immunoreactivity and the presence of lymph node metastasis and venous invasion. The main purpose of this study was to examine p-STAT3 expression and its potential role in the prognosis for human colorectal cancer.

We found a correlation between the expression of p-STAT3 and a poor prognosis in human colorectal cancer. In previous studies, the expression of p-STAT3 was found to be more intense in the deeper invasive areas (16,26,27) and so was thought that there might be a correlation between expression of p-STAT3 and prognosis in human malignancies. However, only a few studies have reported such a correlation (26-29).

Dolled-Filhart *et al* and Hsiao *et al* found that good prognoses were related to p-STAT3 expression in node-negative breast cancer and nasopharyngeal cancer, respectively (28,29). In contrast, Horiguchi *et al*, Masuda *et al*, and Gong *et al* found that poor prognoses were related to the expression of p-STAT3 in renal cell cancer, head and neck squamous cell carcinoma, and gastric cancer, respectively (26,27,30). We suggest that these discrepancies in prognosis prediction may have occurred because the examined cancers originated in different organs. Our study on human colorectal cancer is, to our knowledge, the first one to report a significant correlation of p-STAT3 expression with poor prognosis in this type of cancer.

Prognosis has been considered to correlate with tumour invasion and metastasis (16,26-31). Constitutively activated STAT3 regulates tumour growth and invasion by affecting the expression of genes related to tumour invasion and cell survival (32-39). The present study showed a significant correlation between p-STAT3 expression and poor prognosis. Our results may reflect the importance of p-STAT3 in tumour invasion.

Although downstream events from constitutively activated STAT3 are not adequately revealed, many studies have indicated that its expression in human malignancies is responsible for malignant progression because of the upregulation of cell cycle progression and the prevention of apoptosis (36-40).

Overexpression of p-STAT3 was frequently found in human colorectal carcinoma and its expression was associated with depth grading of tumour invasion and poor prognosis. We considered a potential correlation between the expression of p-STAT3 and prognosis for each T grade but found no correlation. The expression of p-STAT3 may have an important function in invasion rather than tumour formation. Although the expression of p-STAT3 correlated with a poor prognosis in this study, the prognostic significance of p-STAT3 must be confirmed in additional studies.

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