Expression of p-STAT3 in human colorectal adenocarcinoma and adenoma; correlation with clinicopathological factors

T Kusaba, T Nakayama, K Yamazumi, Y Yakata, A Yoshizaki, T Nagayasu, I Sekine

Background: The signal transducer and activator of transcription 3 (STAT3) is a key signalling molecule implicated in the regulation of growth and malignant transformation. Constitutive activation of STAT3 is seen in several tumour derived cell lines, and in a wide variety of human malignancies. Aims: To examine the relation between p-STAT3 (activated form of STAT3) expression and clinicopathological factors in human colorectal adenocarcinoma and adenoma. Methods: Immunohistochemical analyses were carried out on tissues from 44 colorectal adenomas and 95 colorectal adenocarcinomas, comprising 18 intramucosal carcinomas and 77 invasive carcinomas. Results: Seventy-seven of these 139 samples (55.4%) showed immunoreactivity for p-STAT3. Positive staining for p-STAT3 was seen in 69 of the 95 carcinomas. Only eight of the 44 adenomas showed immunopositivity for p-STAT3, resulting in a significant difference between total adenocarcinomas and adenomas (p < 0.001). Among the 95 cases of colorectal adenocarcinoma, p-STAT3 immunoreactivity was significantly correlated with the depth of tumour invasion (p < 0.05), venous invasion (p < 0.05), lymph node metastasis (p < 0.05), and increasing stages of the Dukes' classification (p < 0.01). Expression of p-STAT3 was detected by Western blot analysis in two different cultured human colorectal carcinoma cell lines and six colon carcinoma tissue samples obtained at surgery. Conclusion: This is the first study to report a significant correlation of p-STAT3 expression with the depth of tumour invasion. These findings suggest that p-STAT3 expression is an important factor related to carcinogenesis and/or tumour invasion of colorectal adenocarcinoma.

MATERIALS AND METHODS

Cases and tissues
Forty-four human colorectal adenomas and 95 primary human colorectal adenocarcinomas were studied by immunohistochemistry. Of the 95 patients with colorectal carcinoma, there were 52 men and 43 women. The median age was 65.6 years (range, 32–87). Twenty-four tumours were located in the rectum, 27 in the sigmoid colon, seven in the descending colon, 19 in the transverse colon, and three in the caecum. All tumours were obtained from patients who had undergone endoscopic resection or surgery at Nagasaki University Hospital, Japan between 2000 and 2004. Fifteen specimens of normal colon mucosal tissue, taken from patients without colorectal cancer, were evaluated as normal controls.

Each tumour was assigned a histological type according to the World Health Organisation classification as follows: well differentiated adenocarcinoma, moderately differentiated adenocarcinoma, poorly differentiated adenocarcinoma, and mucinous adenocarcinoma.14

Abbreviations: IFNα, interferon α; IL-6, interleukin 6; p-STAT, phosphorylated (activated) signal transducer and activator of transcription; STAT, signal transducer and activator of transcription
According to the TNM staging system of the American Joint Commission on Cancer, the depth of tumour invasion in each of the carcinomas was classified into five groups, as follows: Tis, carcinoma in situ or limited to mucosa; T1, invading the submucosa; T2, invading the muscularis propria; T3, invading either the subserosa or pericolic tissue; and T4, through the serosa or invading contiguous organs. Based on the Dukes’ classification, the pathological stages of colorectal carcinoma were classified into four groups as follows: A, tumour invading the submucosa; B, invading the muscularis propria; C, with metastases to regional mesenteric lymph nodes but without evidence of distant spread; and D, with distant metastasis.

Lymphatic and venous invasion were studied on routine haematoxylin 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. Lymph node metastasis was defined as “present” only when confirmed histologically.

The diagnosis was established by two independent pathologists (T Kusaba and T Nakayama).

**Immunohistochemistry**

Formalin fixed, paraffin wax embedded tissues were cut into 4 μm sections, dewaxed in xylene, and rehydrated in

**Figure 1** (A) Immunohistochemistry for phosphorylated (activated) signal transducer and activator of transcription (p-STAT3) in colorectal adenocarcinomas. p-STAT3 shows strong nuclear and cytoplasmic expression. (Immunooalkaline phosphatase staining; original magnification, ×400). (B) Expression of p-STAT3 was seen in all layers of this adenocarcinoma (immunooalkaline phosphatase staining; original magnification, ×10).

**Figure 2** In almost all of the carcinomas studied, the intensity of phosphorylated (activated) signal transducer and activator of transcription (p-STAT3) expression was stronger in the deeper parts of invasion than in the superficial areas. (A) Expression of p-STAT3 in a carcinoma invading the submucosa (original magnification, ×60). (B) Expression of p-STAT3 in a carcinoma invading the muscularis propria. (C) Expression of p-STAT3 in a carcinoma invading the subserosa (original magnification, ×60).
phosphate buffered saline. Dewaxed sections were preincubated with normal bovine serum to prevent non-specific binding, and then incubated overnight at 4°C with an optimal dilution (1 μg/ml) of a primary goat polyclonal IgG against human p-STAT3 (Tyr705; Santa Cruz Biotechnology Inc, Santa Cruz, California, 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, California, 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.21

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, 0–15% positive cells; positive, > 15% positive tumour cells.

Cell culture

Two human colorectal adenocarcinoma cell lines, DLD-1 and Colo320DM, were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air. DLD-1 was maintained in DMEM/F-12 (Invitrogen Corp, Carlsbad, California, USA) supplemented with 10% fetal calf serum.22 Colo320DM was maintained in RPMI 1640 medium (Invitrogen Corp) supplemented with 10% fetal calf serum.22 Both of these cell lines were provided by the Health Science Research Resources Bank (Osaka, Japan). For the induction of p-STAT3, two human colorectal adenocarcinoma cell lines were stimulated by interferon-α (IFNα; Cell Signaling Technology, Beverly, Massachusetts, USA).24

Immunoblotting

Specimens and cells were resuspended in ice cold radioimmunoprecipitation buffer (1% phosphate buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1mM phenylmethylsulfonyl fluoride, 1mM Na3VO4, 50mM NaF, and one tablet of complete proteinase inhibitor mixture (Roche Applied Science, Indianapolis, Indiana, USA)/50 ml) for 10 minutes, sonicated on ice, and centrifuged (12,000 xg for 15 minutes at 4°C). The protein concentration of the supernatant (protein fraction) was determined with the Bradford protein assay (Bio-Rad, Hercules, California, USA). An aliquot of 10 μg of protein was mixed with an equivalent volume of 2x protein loading buffer containing β-mercaptoethanol and boiled for five minutes before loading on to a sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, proteins were transferred on to nitrocellulose membranes using the ECL system (Amersham Biosciences, Piscataway, New Jersey, USA) and blocked in Tris buffered saline with Tween (50mM Tris/HCL, pH 7.5, 150mM NaCl, 0.05% Tween 20) containing 5% non-fat dry milk powder. Protein immunoblots were performed using specific antibodies to β actin (Santa Cruz Biotechnology Inc), phosphotyrosine (Tyr705) STAT3 (Cell Signaling Technology), and STAT3 (Santa Cruz Biotechnology Inc). The membranes were further incubated with peroxidase conjugated secondary antibodies, and protein bands were visualised 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, California, USA) was used for statistical analyses. Analyses comparing the expression of p-STAT3 were performed with the χ2 test for independence and the Mann-Whitney U test.

RESULTS

We analysed the correlation between p-STAT3 immunoreactivity and sex, age, and site of the primary tumour, but there were no significant associations.

Histologically, there were 18 intramucosal carcinomas and 77 invasive carcinomas. All 18 cases of intramucosal carcinoma were well differentiated adenocarcinomas (data not shown). Of the 95 invasive carcinomas, 45 were well differentiated adenocarcinomas, 41 were moderately differentiated adenocarcinomas, three were poorly differentiated adenocarcinomas, and six were mucinous carcinomas. Among the invasive carcinomas, there were eight submucosal infiltrative carcinomas (T1), five carcinomas invading proper muscle layers (T2), 61 carcinomas reaching the subserosa (T3), and three carcinomas through the serosa or invading contiguous organs (T4). Sixty five patients had lymph node metastasis.

Figure 1A shows a representative example of strong immunohistochemical p-STAT3 staining in an invasive carcinoma, T3 grade. p-STAT3 protein was detected in both the cytoplasm and the nucleus of almost all carcinomas. The Stat View II program (Abacus Concepts Inc, Berkeley, California, USA) was used for statistical analyses. Analyses comparing the expression of p-STAT3 were performed with the χ2 test for independence and the Mann-Whitney U test.

Table 1

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Relations between p-STAT3 immunoreactivity and the pathological features of the tumours</th>
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<tbody>
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<td>N</td>
<td>p-STAT3 immunoreactivity (%</td>
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<td></td>
<td>Normal epithelium</td>
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<td>T4</td>
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<td>Lympthatic invasion</td>
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<td>Absent</td>
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<td>Present</td>
<td>63</td>
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<td>Venous invasion</td>
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<td>Lymph node metastasis</td>
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<td>Present</td>
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<td>A</td>
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<td>B</td>
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<td>C</td>
<td>31</td>
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*See Materials and Methods for classification of staining intensity; †Significant difference between total adenocarcinomas and adenomas (p<0.001), χ2 for independence test; ‡p<0.01, Mann-Whitney’s U test; §p<0.05, χ2 for independence test; †‡p<0.001, Mann-Whitney’s U test; p-STAT, phosphorylated (activated) signal transducer and activator of transcription.
Table 1 shows p-STAT3 immunoreactivity of human colorectal tumour cells. p-STAT3 was not expressed in the 15 cases of normal colorectal epithelium. In the adenomas, only eight of 44 showed immunoreactivity for p-STAT3. Immunoreactivity was noted in nine of the 18 intramucosal carcinomas, whereas strong immunoreactivity was found in 60 of the 77 invasive carcinomas. Statistical analysis showed a significant difference between adenocarcinomas and adenomas (p < 0.001).

Table 1 summarises the associations between p-STAT3 immunoreactivity and the pathological features of the adenocarcinomas. p-STAT3 expression was found in 29 of the 45 well differentiated adenocarcinomas, 34 of the 41 moderately differentiated adenocarcinomas, one of the three poorly differentiated adenocarcinomas, and five of the six mucinous carcinomas. There was no significant correlation between p-STAT3 immunoreactivity and the differentiation of colorectal adenocarcinomas.

p-STAT3 immunoreactivity was compared with the depth of tumour invasion. p-STAT3 expression was found in nine of the 18 Tis tumours, in four of the eight T1 tumours, in two of the five T2 tumours, in 51 of the 61 T3 tumours, and in all three T4 tumours. A significant correlation was found between p-STAT3 immunoreactivity and the depth of tumour invasion (p < 0.01).

Lymphatic invasion and venous invasion were found in 30 of 95 and 58 of 95 tumours, respectively. p-STAT3 immunoreactivity was significantly correlated with the presence of venous invasion (p < 0.05).

Immunoreactivity at the primary site of colorectal carcinoma significantly correlated with the presence of lymph node metastasis (p < 0.05). Immunoreactivity for p-STAT3 also correlated with increasing stages of the Dukes’ classification (p < 0.001).

Figure 3 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 (lanes 1–2), whereas strong expression of p-STAT3 was seen in the colon carcinoma tissues (lanes 3–8).

Figure 4 shows the results of western blotting for p-STAT3 expression in human colorectal carcinoma cell lines. STAT3 expression was detected in both of the colorectal carcinoma cell lines tested. p-STAT3 expression after IFNα stimulation was detected in both colorectal carcinoma cell lines, whereas weak expression of p-STAT3 without IFNα stimulation was seen in one of colorectal carcinoma cell lines.
DISCUSSION

In recent years, many reports have suggested a crucial role for the STAT3 signalling pathway in malignant transformation and tumour progression.\(^2\) In our study, we found a correlation between the expression of p-STAT3 and the depth of tumour invasion in human colorectal adenocarcinomas. The expression of p-STAT3 was found to be more intense in the deeper invasive areas, as shown in Fig 2. Therefore, it is possible that the phosphorylation of STAT3 is upregulated in the advanced stages of invasion. The invasion of tumour cells induces lymph node metastasis and venous invasion. This is consistent with our results, which showed a significant correlation between p-STAT3 immunoreactivity and the presence of lymph node metastasis and venous invasion. Ma et al reported that there was a significant correlation between the expression of p-STAT3 and the presence of lymph node metastasis and invasion in human colorectal carcinoma.\(^26\) Moreover, Masuda et al also reported that the expression of p-STAT3 significantly correlated with stage in human squamous cell carcinoma of the head and neck.\(^27\) This suggests that the activation of STAT3 might play an important role in the invasion and metastasis of carcinomas.

STAT3 is activated by phosphorylation at Tyr705, which induces dimerisation, nuclear translocation, and DNA binding.\(^28\) We used an antibody to p-STAT3 (Tyr705), which detects STAT3 only when phosphorylated at Tyr705. Small amounts of p-STAT3 (Tyr705) can be detected in the cytoplasm without stimulation,\(^29\) although certain cytokines, such as interleukin 6 (IL-6), upregulate the phosphorylation of STAT3, which then moves from the cytoplasm into the nucleus.\(^29\) In our study, normal mucosa faintly expressed p-STAT3 in the cytoplasm. However, colorectal adenocarcinoma cells expressed p-STAT3 intensely in both the cytoplasm and the nucleus. Although no data are available on the expression of cytokines in colorectal adenocarcinoma, some cytokines may contribute to the activation of STAT3 in colorectal adenocarcinoma.

"Activated STAT3 may regulate tumour growth and metastasis by affecting the expression of genes related to tumour invasion, angiogenesis, the cell cycle, and cell survival"\(^30\)

The STAT transcription factor family has recently been reported to regulate directly the genes of the Bcl-2 family, which are key regulators of apoptosis in normal mucosa and adenocarcinoma.\(^31\) Concentrations of the antiapoptotic proteins of this family are increased in response to gp130 growth factor,\(^36\) cyclin D1,\(^38\) and the c-myc oncogene.\(^39\) Thus, activated STAT3 may regulate tumour growth and metastasis by affecting the expression of genes related to tumour invasion, angiogenesis, the cell cycle, and cell survival.

STATs are tyrosine phosphorylated transcription factors activated by JAK family kinases.\(^40\) Various ligands, including interferons and growth factors, induce the activation of the JAK kinases, which in turn induce the activation of the STATs.\(^41\) STAT3 is activated by IL-6 or IFN\(\alpha\).\(^42\) IFN\(\alpha\) upregulated the expression of p-STAT3 in one of the cell lines used in our study, Colo 320DM cells (Fig 4). Some reports have shown that IL-6 expression correlates with carcinogenesis and/or tumour progression.\(^43\) Although we did not examine the expression of cytokine receptors, the constitutive activation of STAT3 might be induced by the stimulation of certain cytokines or interferons that activate the JAK kinases, such as IL-6 or IFN\(\alpha\). Such a possibility awaits further studies for confirmation.

Take home messages

- Phosphorylated (activated) signal transducer and activator of transcription (p-STAT3) expression significantly correlated with the depth of tumour invasion, venous invasion, lymph node metastasis, and increasing stages of the Dukes' classification.
- To the best of our knowledge, this is the first study to report a significant correlation between p-STAT3 expression and the depth of tumour invasion in colorectal adenocarcinoma.
- p-STAT3 expression appears to be an important factor in the carcinogenesis and/or tumour invasion of colorectal adenocarcinoma.

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