SPANDIDOS PUBLICATIONS

Significance of p-STAT3 expression in human colorectal adenocarcinoma

JAE-KYOUNG PARK[1], RAN HONG[1], KYUNG-JONG KIM[2], TAE-BUM LEE[3] and SUNG-CHUL LIM[1,3]

Departments of [1]Pathology and [2]Surgery, [3]Research Center for Resistant Cells, College of Medicine, Chosun University, Gwangju, Korea

Received April 17, 2008; Accepted May 28, 2008

DOI: 10.3892/or_00000047

Abstract. To examine the relationship between p-STAT3 and the clinicopathological parameters of colorectal adenocarcinoma (CRA), we initially conducted immunohistochemical (IHC) analyses on formalin-fixed tissues. A total of 127 invasive CRA, 20 colorectal adenomas and 20 normal mucosae were obtained. To clarify the validity of p-STAT3 as determined by the IHC analysis, quantitative real-time PCR was performed on fresh samples from 51 CRA-4 carcinomas *in situ*, 47 invasive CRA and on 51 normal mucosae. IHC analyses were conducted after formalin fixation from 51 CRA for comparison. The statistically significant difference of immunoreactivity for p-STAT3 between the CRA and adenoma, and between the CRA and normal mucosae was identified. Among the 174 CRA, p-STAT3 immunoreactivity significantly correlated with the T- and clinical stage. Among the 47 invasive CRA, the expression of STAT3 as determined by real-time PCR significantly correlated with tumor size, M stage and clinical stage. The overall findings of the real-time PCR analyses correlated with the findings of the IHC analyses. These findings suggest that p-STAT3 expression has an important role related to the tumorigenesis and tumor progression of CRAs. Moreover, IHC analysis is a reliable and useful modality of assessing the status of p-STAT3 expression in formalin-fixed samples.

Introduction

The prognosis of colorectal cancer patients varies depending on the depth of the invasion of tumor cells and the lymph nodal status, and these factors are assessed only by a histopathological examination of surgically resected tissues (1). Colorectal cancer undergoes the adenoma-carcinoma sequence or *de novo* pathway, and multiple steps of genetic changes such as the loss or mutation of adenomatous polyposis coli (APC), alteration of DNA methylation, chromosome loss, mutation of the p53 gene and/or mutation of the *K-ras* gene occur sequentially (2).

STAT3, signal transducer and activator of transcription 3, is a cytoplasmic transcription factor that has been shown to be a mediator of the IL-6 receptor signal transduction pathway. It has also been demonstrated to have diverse functions, and its activation is induced not only by cytokine or growth factor receptors, but also by oncogenes such as *src and sis* (3-5). Immediately after the binding of cytokines to their receptor, the receptor is phosphorylated by Janus kinase (JAK) at tyrosine at the binding site of STAT. STAT bound to the receptor undergoes phosphorylation, and detaches from the receptor in the p-STAT form. Subsequently, it dimerizes, translocates to the nucleus and controls the expression of various target genes (6).

In some cancer cell lines, the activation of STAT3 has been induced, and the blocking of STAT3 action induces apoptosis (7,8). After dimerization, STAT3 acts as an oncoprotein and induces tumors in nude mice (9). The transformation of cells by the activation of STAT3 is associated with factors accelerating cell cycle progression, such as cyclin D1 and c-myc, and/or the up-regulation of genes suppressing apoptosis, such as *bcl-xL*, *mcl-1* or *survivin* (9-13). In many cancers, the dysregulation of STAT3 develops, and it has been shown to be closely associated with poor prognosis and metastasis (14-18). However, the function of STAT3 is not always clear and further studies on its action mechanism are required. For example, in head and neck tumors, the cell cycle may be stimulated directly by the expression of cyclin D1 with STAT3 expression (16). In malignant melanoma cells, STAT3 has been shown to down-regulate cell proliferation (19).

In this study, we examined the role of STAT3 in the formation and progression of colorectal adenocarcinoma in terms of the adenoma-carcinoma sequence by measuring the expression of phosphorylated active STAT3 in the colorectal cancer tissues of patients by immunohistochemical (IHC) methods. By analysis of the various clinicopathological parameters pertinent to prognosis, a comparison with the benign precancerous lesion adenoma and the normal mucosa was

Correspondence to: Dr Sung-Chul Lim, Department of Pathology, Chosun University Hospital, 588 Seosuk-dong, Dong-gu, Gwangju 501-140, Korea
E-mail: sclim@chosun.ac.kr

Key words: colorectum, adenocarcinoma, STAT-3, immunohistochemistry, real-time PCR

examined. The role of STAT3 in the formation and progression of colorectal cancer was assessed. The validity of the IHC analysis for STAT3 was verified by using quantitative real-time PCR.

Materials and methods

Case selection and tissue sampling. For quantitative real-time PCR, we randomly collected fresh surgical specimens from 51 patients who underwent curative surgery for sporadic colorectal adenocarcinoma (including 4 cases of carcinoma *in situ* in the background of adenoma) at our University Hospital between March 2004 and October 2005. In each case, adjacent normal mucosa was also collected. These specimens were cleaned with normal saline, placed in an Eppendorf tube and stored at -80˚C until analysis. These procedures were performed within 15 min. For IHC analysis, we used colorectal adenocarcinoma tissue, stored as paraffin-embedded blocks, and corresponding to 51 cases.

Informed consent was obtained from each subject according to the institutional guidelines, and the research protocols were approved by the ethics committee of our hospital.

Among the patients that underwent curative surgery for colorectal adenocarcinoma at our hospital from January 1992 to December 2004, 127 non-consecutive patients were selected with relatively well-preserved paraffin-embedded tissues, complete medical records and a known follow-up status. Patient survival was confirmed through telephone calls and by mail. Those patients who underwent preoperative chemoradiotherapy and emergency surgery, and those that had evidence of hereditary non-polyposis colorectal cancer or familial adenomatous polyposis were excluded from the study. The various clinicopathological parameters of the patients were confirmed by a review of the patient charts and pathology files. The relationship of the clinicopathological parameters, quantitative real-time PCR or IHC data with survival was investigated for the 127 patients.

Twenty cases of colon adenoma (16 low-grade and 4 high-grade adenomas) and 20 cases of normal-looking colon mucosa, which were all obtained endoscopically, were subjected to IHC analysis for a comparison study.

Clinical and histopathological analysis

Microscopic examination. Each tumor was re-evaluated by a retrospect analysis of the medical records and the tissue slide file of the pathology department. Age, gender, tumor size, histological subtype and the degree of differentiation, the depth of tumor invasion, the status of lymph node metastasis, lymphovascular invasion and distant metastasis, were assessed.

According to the WHO classification, the histological grade was classified as a well-, moderately- and poorly-differentiated adenocarcinoma, and mucinous adenocarcinoma (20). The stage was defined according to the TNM staging system of the American Joint Committee on Cancer (21).

Tissues to be examined were fixed in 10% neutral formalin, and the prepared paraffin-embedded tissues were sectioned 4-5 μm in thickness, and hematoxylin and eosin staining was performed. The cells were examined under a light microscope, the representative area suitable to the study purpose was selected, and slides were prepared for IHC analysis.

Immunohistochemical staining. The samples investigated in this study were analyzed using goat polyclonal p-STAT3 (Tyr 705: sc-7993) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunolocalization was performed using the goat ImmunoCruz™ staining system: sc-2053 (Santa Cruz Biotechnology), according to the manufacturer's protocol.

Briefly, the 4-μm sections that were obtained after formalin fixation and paraffin embedding were deparaffinized in xylene and rehydrated with distilled water through a graded series of ethanol solutions. The sections were placed in a glass jar with 10 mM citrate buffer (pH 6.0) and irradiated in a microwave oven for 15 min and then allowed to cool down in the jar at room temperature for 20 min. The slides were rinsed with Tris-buffered saline (TBS). Blocking reagent was added for 10 min after quenching the endogenous peroxidase activity in 0.3% hydrogen peroxide for 10 min. The slides were washed as before, and subsequently subjected to the primary antibody reaction. p-STAT3 (dilution 1:200) was applied to the tissue section and allowed to incubate in a moist chamber overnight at 4˚C. After washing with TBS, a biotinylated link antibody was applied for 10 min, followed by treatment with streptavidin peroxidase for an additional 10 min. After washing out the excess complex, the localization of antibodies was visualized by incubating the sections for 15 min in an UltraVision Plus detection system (Lab Vision, Fremont, CA, USA) and counterstaining was performed with Mayer's hematoxylin. The positive control for p-STAT3 was prostatic adenocarcinoma (22). Instead of the primary antibody, TBS was used for the negative control.

Analysis and interpretation of the staining. To exclude subjectivity a pathologist who did not know the clinical course of the subjects evaluated the staining results. As a result of the staining for STAT3, nuclear and cytoplasmic staining were divided, and nuclear staining was reclassified according to the percentage of positive cells within the tumor as negative (0-15% positive) and positive (>15% positive).

The cases in which only the cytoplasm was stained were considered negative, and only a case where the cytoplasm and the nucleus were stained simultaneously, was determined to be an intracytoplasmic positive case.

Clinicopathological findings. Among 174 cases of invasive colorectal cancer used for the IHC analysis and excluding 47 cases that underwent real-time PCR analysis, the survival status of 127 invasive adenocarcinoma cases until December 31, 2004 was assessed. The result revealed a survival record of 156 months, which is the longest thus far.

In the 51 cases where quantitative real-time PCR was performed, the time after surgery was relatively short and the cases were excluded from the follow-up observation subjects. Among the 51 cases, 4 were carcinoma *in situ* (CIS), and the remaining 47 were grouped as the subjects to be compared with invasive adenocarcinomas.

The invasive adenocarcinomas were subdivided again according to histological differentiation, nodal status, T-, M- and clinical stage, as shown in Table I.

Quantitative real-time PCR. Extraction of mRNA from the colorectal cancer tissues and the corresponding normal tissues was performed by using the RNeasy system and following the manufacturer's protocol (Qiagen, Hilden, Germany).

Table I. Relationship between p-STAT3 immunoreactivity or STAT3 real-time PCR and the clinicopathological features of the tumors (%).

		Immunoreactivity[a]		Intracytoplasmic			PCR T/N ratio[b]	
	N	Positive	Negative	Positive	Negative	Positive cell rate (mean ± SD, %)	>1	≤1
Normal mucosa	20	0	20 (100)	0	20 (100)	0	-	-
Adenoma	20	4 (20)	16 (80)	0	20 (100)	9.50±9.265	-	-
CIS	4	2 (50)	2 (50)	0	4 (100)	12.00±11.518	4 (100)	0
Adenocarcinoma	174	135 (78)	39 (22)	26 (15)	148 (85)	37.59±26.242	37 (79)	10 (21)
Differentiation							c	
Well	35	25 (71)	10 (29)	5 (14)	30 (86)	30.00±25.000	9 (75)	3 (25)
Moderate	111	92 (83)	19 (17)	17 (15)	94 (85)	40.72±25.716	22 (76)	7 (24)
Poor	12	8 (67)	4 (33)	1 (8)	11 (92)	36.67±27.661	4 (100)	0
Mucinous	16	10 (63)	6 (38)	3 (19)	13 (81)	33.13±29.770	2 (100)	0
T stage		f						
1	7	4 (57)	3 (43)	0	7 (100)	30.00±33.665	2 (100)	0
2	33	22 (67)	11 (33)	2 (6)	31 (94)	23.79±21.066	7 (88)	1 (12)
3	130	107 (82)	23 (18)	24 (19)	106 (82)	41.81±25.767	24 (73)	9 (27)
4	4	2 (50)	2 (50)	0	4 (100)	27.50±32.016	4 (100)	0
Nodal status				d		f		
Negative	109	74 (68)	35 (32)	10 (9)	99 (91)	30.55±26.223	19 (83)	4 (17)
Positive	65	61 (94)	4 (6)	16 (25)	49 (75)	49.38±21.804	18 (75)	6 (25)
M stage				d		e	f	
0	167	128 (77)	39 (23)	22 (13)	145 (87)	36.35±25.966	31 (76)	10 (24)
1	7	7 (100)	0	4 (57)	3 (43)	67.14±11.852	6 (100)	0
Clinical stage		d		f		f	c	
I	32	20 (63)	12 (38)	1 (3)	31 (97)	24.84±24.838	4 (80)	1 (20)
IIA	71	50 (70)	20 (30)	7 (10)	64 (90)	32.68±26.670	12 (80)	3 (20)
IIB	23	19 (83)	4 (17)	2 (9)	21 (91)	36.09±23.010	14 (70)	6 (30)
IIIA	8	7 (88)	1 (13)	5 (63)	3 (38)	59.38±24.559	7 (100)	0
IIIB	27	27 (100)	0	6 (22)	21 (78)	52.41±19.532	-	-
IIIC	13	12 (92)	1 (8)	5 (39)	8 (62)	54.23±18.579	-	-
IV	0	-	-	-	-	-	-	-

CIS, carcinoma *in situ*; negative, no metastasis; positive, metastasis. [a]Immunoreactivity; positive cell rate >15%, positive; ≤15%, negative; [b]ratio of PCR intensities of the tumors vs. normal mucosae. Four cases of CIS and 47 cases of adenocarcinoma were conducted by PCR analysis. [c]P<0.05, [d]P<0.01, [e]P<0.005 and [f]P<0.0001.

cDNA was prepared in a 20 μl reaction containing total RNA (1 μg), avian myeloma leukemia virus (AMV) reverse transcriptase and oligo dT primers (Promega, Madison, WI, USA).

The real-time PCR measurement of STAT3 cDNA was performed with a Light Cycler Instrument (Roche) and by using Fast Start DNA Master SYBR-Green I Kit (Roche Diagnostics, Mannheim, Germany). For verification of the correct amplification product, the PCR products were analyzed on a 2% agarose gel stained with ethidium bromide.

The specific primer pair for STAT3 was the sense primer 5'-CAT GTG AGG AGC TGA GAA CGG-3' and the antisense primer 5'-AGG CGC CTC AGT CGT ATC TTT-3' (synthesized by Bioneer, Daejeon, Korea). Each reaction (20 μl) contained 2 μl cDNA, 2.5 mM $MgCl_2$, 1 pmol of each primer and 2 μl Fast Starter Mix (containing buffer, dNTPs, SYBR-Green dye and Taq polymerase).

The amplification consisted of one cycle at 95˚C for 30 sec followed by 40 cycles at 95˚C for 5 sec, a 65˚C annealing step for 10 sec, and a step at 72˚C for 20 sec. Amplification was followed by a melting curve analysis to verify the correct size of the amplicon. A negative control without cDNA was run with every PCR to assess the specificity of the reaction. An analysis of the data was performed using Light Cycler software version 3.5. The PCR efficiency was determined by analyzing a diluted series of cDNA solutions (the external standard curve). The identity of the PCR product was confirmed by comparing its melting temperature (Tm) with the Tm of the amplicons from the standards or the positive controls.

Analysis and interpretation of PCR. The level of expression of STAT3 as determined by PCR for tumor tissue samples (T) was divided by the level of expression of STAT3 determined by PCR for normal mucosa (N). This ratio was classified for cases >1 (PCR T/N ratio >1) and for cases ≤1 (PCR T/N ratio ≤1), and compared with the various clinicopathological factors.

For the statistical analysis of the expression of STAT3 in colorectal cancer, its association with various clinicopathological factors, survival rate or survival length, and the difference of the expression of STAT3 in the normal mucosa, adenoma and colorectal cancer, the χ^2 test, Fisher's exact test, ANOVA and Student's t-test were used. The statistical significance was set at $p<0.05$. For statistical analysis, the Stat View software package (Abacus Concepts, Berkeley, CA, USA) was used.

Results

Clinicopathological significance of p-STAT3

Immunoreactivity for p-STAT3. Immunoreactivity of p-STAT3 by staining in the normal mucosa was not detected. A statistically significant difference between normal mucosa (0%) and adenocarcinoma (78%) ($p<0.0001$), adenoma (20%) and adenocarcinoma ($p<0.005$), as well as CIS (25%) and invasive adenocarcinoma ($p<0.05$) was detected (Figs. 1 and 2). Among the 20 adenoma cases, 3 out of the 16 low-grade adenomas (18.8%) and 1 out of the 4 high-grade adenomas (25%) demonstrated immunoreactivity for p-STAT3. High-grade adenomas revealed a higher frequency of p-STAT3 immunoreactivity than did low-grade adenomas, but the level was not statistically significant because of the limited number of samples. Therefore, a positive expression of p-STAT3 staining is speculated to be involved in the formation of malignant tumors.

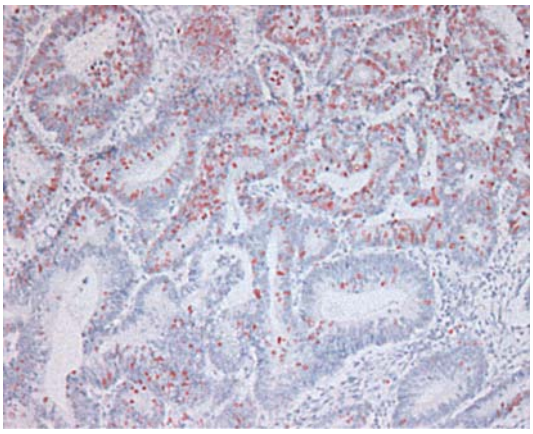

Figure 1. Immunohistochemical staining for p-STAT3 in colon cancer cells. The rate of positive nuclear staining in the area of overt adenocarcinomatous change (top) is significantly higher than that of the adenomatous area (bottom). The LSAB method was utilized and the cells were counterstained with hematoxylin.

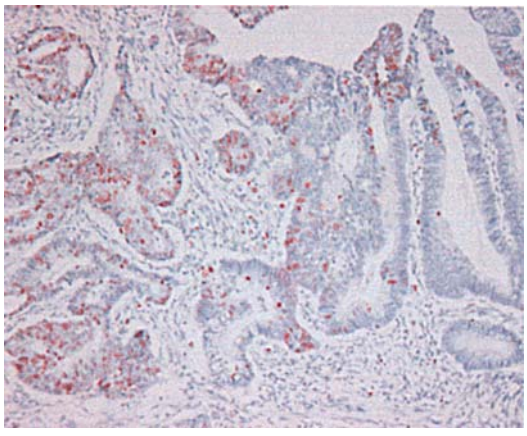

Figure 2. Immunohistochemical staining for p-STAT3 in colon cancer cells. The rate of positive nuclear staining in the area of invasive adenocarcinoma (left) is significantly higher than that of the non-invasive adenocarcinoma area (right). The LSAB method was utilized and the cells were counterstained with hematoxylin.

According to the T stage, the result of a comparative analysis showed that as the tumor stage was more advanced, immunoreactivity was significantly enhanced ($p<0.0001$). In addition, concerning the difference according to the clinical stage, as the stage became higher, immunoreactivity was enhanced significantly ($p<0.01$).

A statistically significant association of immunoreactivity for p-STAT3 with the differentiation of tumor, nodal status and distant metastasis was not detected. In addition, in comparison with survival length (months), a significant difference for the p-STAT3-negative group of patients (64.35±33.99 months) and the p-STAT3-positive group of patients (52.29±30.27 months) was not detected.

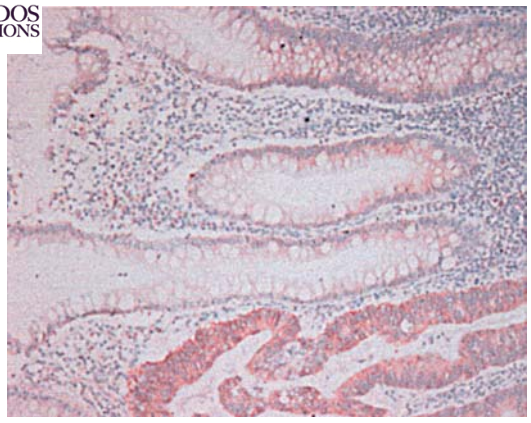

Figure 3. Immunohistochemical staining for p-STAT3 in a colon adenoma. Cytoplasmic staining without nuclear staining is demonstrated in the adenoma (bottom). Faint intracytoplasmic staining without nuclear staining is identified in the adjacent cryptal epithelia of the normal mucosa (top). The LSAB method was utilized and the cells were counterstained with hematoxylin.

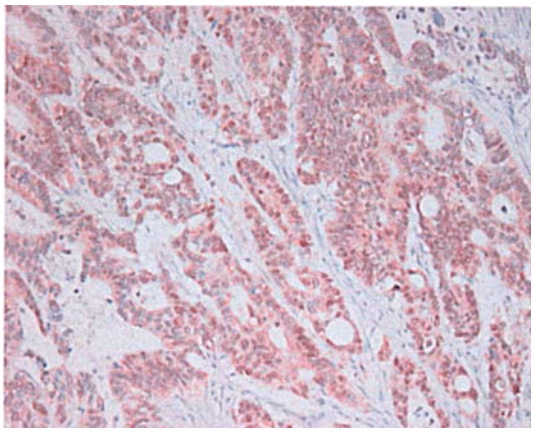

Figure 4. Immunohistochemical staining for p-STAT3 in colon cancer cells. Nuclear staining as well as intracytoplasmic staining is identified in the adenocarcinoma. The LSAB method was utilized and the cells were counterstained with hematoxylin.

Intracytoplasmic staining of p-STAT3. The intracytoplasmic staining of p-STAT3 was not detected in normal mucosa, adenoma and CIS, and was detected only in 26 out of 174 invasive adenocarcinomas (15%). All cases (100%) of intracytoplasmic staining were invasive adenocarcinomas and showed a statistically significant difference compared with the CIS cases that did not show intracytoplasmic staining (0%) ($p<0.05$) (Figs. 3 and 4).

An analysis examining the nodal status and M stage showed a statistically significantly higher intracytoplasmic staining in the patients with lymph node and distant metastasis ($p<0.01$). An analysis examining the clinical stage showed that as this stage became higher, its association with intracytoplasmic staining was very significant ($p<0.0001$).

In a comparison with the survival length (months), patients with tissue samples that did not show intracytoplasmic staining survived longer (57.08±28.86 months) than those with tissue samples that showed intracytoplasmic staining (43.29±44.42 months), and thus a significant difference was detected ($p<0.05$). However, a statistically significant difference in the differentiation of tumor or the T stage with the level of intracytoplasmic staining was not detected. Therefore, the intracytoplasmic expression of p-STAT3 is speculated to be associated with tumor progression, invasion and metastasis.

The positive cell rate of p-STAT3. In the IHC staining of p-STAT3, the association of the percentage of the stained tumor nuclei to the nuclei of total cells (positive cell rate) with clinicopathological factors was compared and analyzed.

The p-STAT3 positive cell rate between normal mucosa (0%) and adenoma (9.50±9.27%) ($p<0.005$), normal mucosa and adenocarcinoma (37.59±26.24) ($p<0.0001$), adenoma and adenocarcinoma ($p<0.0005$), CIS (12.0±11.52) and invasive adenocarcinoma ($p<0.05$), and between normal mucosa and CIS ($p<0.005$) showed a significant difference. Among the 20 adenoma cases, the p-STAT3 positive cell rate between low-grade (8.31±7.74) and high-grade adenomas (13.17±5.04) was not statistically significant because of the limited number of high-grade adenomas. It was speculated that p-STAT3 may be involved in the process of tumor formation (Fig. 5).

The p-STAT3 positive cell rate showed a statistically and markedly significant association with the nodal status as well as the clinical stage ($p<0.0001$). As lymph node metastasis and the clinical stage became higher, the positive cell rate also increased, showing a significant association with the M stage ($p<0.005$). Thus, in cases where distant metastasis developed, the positive cell rate was elevated.

Compared with the survival status, the p-STAT3 positive cell rate did not demonstrate a statistical significance. A longer survival time was associated with a lower p-STAT3 positive cell rate ($p<0.005$). A significant association with the differentiation of tumor or the T stage was not detected.

An analysis of the correlation of p-STAT3 positive cell rate to the T/N ratio by quantitative real-time PCR showed that as the p-STAT3 positive cell rate increased, the STAT3 PCR product within the tumors increased significantly ($p<0.0001$). This finding suggests that examination of the expression of p-STAT3 by the IHC method is very accurate and sensitive.

Clinicopathological significance of STAT3 by real-time PCR. A significant difference in the amount of STAT3 PCR product for the CIS and invasive adenocarcinoma cases was not detected. In a comparison of the STAT3 T/N ratio for the CIS and invasive adenocarcinoma cases, a statistically significant difference was not detected.

A comparison of the amount of STAT3 PCR product with tumor size showed that, compared with a 3-cm maximal long axis of the tumor, a significant difference was detected ($p<0.05$). For the STAT3 T/N ratio, compared with a 5-cm maximal long axis of the tumor, a significant difference was detected ($p<0.05$).

The amount of the STAT3 PCR product or the STAT3 T/N ratio compared with the T- or N-stage did not show a statistically significant difference. A comparison of M stage and the amount of STAT3 PCR product showed a statistically significant difference, and the amount of the STAT3 PCR product for cases with distant metastasis (0.24±0.33) was higher than in cases without distant metastasis (0.09±0.07) ($p<0.0001$).

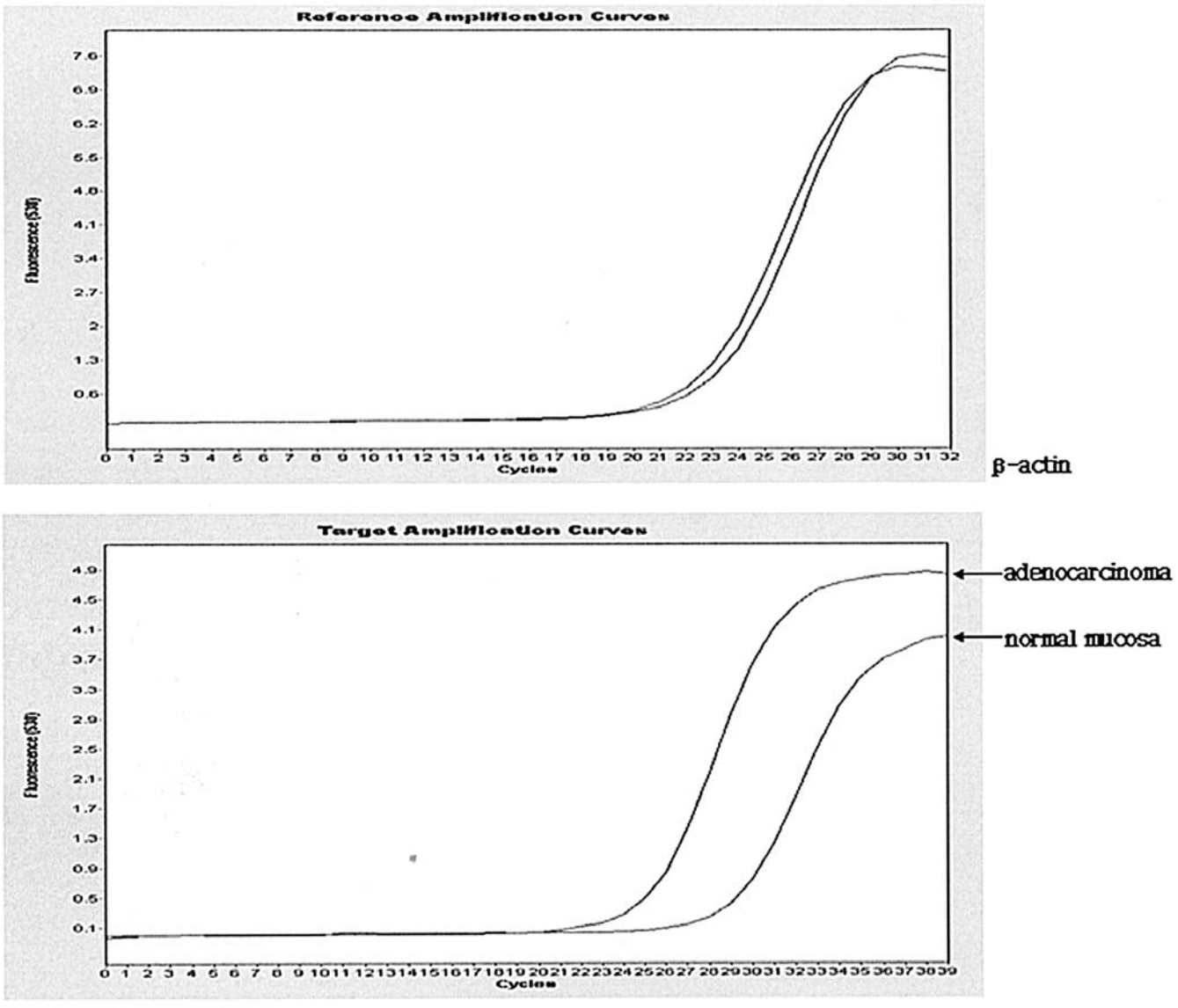

Figure 5. Representative quantitative real-time PCR curves for STAT3 in a colon adenocarcinoma and in normal mucosa. Upper panel: reference amplification curves of ß-actin for the adenocarcinoma and normal mucosa. Lower panel: target amplification curves of STAT3 for the adenocarcinoma and normal mucosa.

Similarly, a difference of the amount of STAT3 PCR product according to the clinical stage was statistically significant, and as the clinical stage increased, so did the amount of the STAT3 PCR product ($p<0.05$). A comparison of the amount of the STAT3 PCR product ($p<0.01$) and the STAT3 T/N ratio ($p<0.05$) to the differentiation of tumor showed a significant difference, and the poorer the differentiation grade, the higher the amount of the STAT3 PCR product, as well as the STAT3 T/N ratio, observed.

Discussion

STAT3 have been shown to accelerate cellular proliferation, suppress apoptosis, and act on tumor progression directly (7). In this study, the result of the comparison and analysis of p-STAT3 (Tyr705) among the normal mucosa, adenoma and adenocarcinoma showed that it was involved not only in neoplastic but also in malignant transformation. Its expression was significantly correlated to the T stage, the nodal status, the M stage and the clinical stage. Thus, its involvement in tumor progression was found.

Kusaba *et al* (23) have reported that the expression of p-STAT3 significantly correlated to lymph node metastasis and venous invasion. However, in our case, it did not correlate directly to vascular invasion (data not shown). Nonetheless, it correlated significantly to lymph node and distant metastasis.

With the advantage that a retrospective analysis is relatively simple and readily performed, an analysis of the alteration of proteins in a signal transduction system by IHC staining has frequently been performed. However, its reliability is typically questioned. In this study, for 51 cases of colorectal cancer, the amount of p-STAT3 determined by IHC staining correlated with the overall level of STAT3 expression as determined by the real-time PCR analysis. With a larger

2 or higher clinical stage, or when distant metastasis 1 or differentiation was poor, the expression level of p-STAT3 as determined by PCR analysis significantly increased. This suggests that the specific assessment of the expression of p-STAT3 by IHC staining is very accurate and sensitive.

In p-STAT3 (Tyr705) staining, 15% of the colorectal cancer cases showed intracytoplasmic staining for p-STAT3 expression which was associated with the advanced clinical and N stage, occurrence of distant metastasis, and a decrease of survival length. STAT3 is activated by the phosphorylation of Tyr705 (24). Following phosphorylation, STAT3 is stabilized by dimerization and translocates to the nucleus (25). Therefore, on the conversion of STAT3 presenting in the cytoplasm to p-STAT3 and following the induction of its activation, it then resides in the nucleus. In IHC staining studies on invasive breast cancer, Berclaz *et al* (26) have reported that in the non-tumor area, STAT3 is expressed only in the cytoplasm. However, in the malignant tumor area, it is expressed in the cytoplasm and nucleus.

Once the anti-p-STAT3 antibody specific to the active form of STAT3 phospho-Tyr705 became available (27), in a study on the prostate, the active form of STAT3 was found to be localized in the nucleus of prostate cancer cells, and it is anticipated that p-STAT3 (Tyr705) expression may serve as a superior probe than the expression of total STAT3 (20). Dolled-Filhart *et al* (28) have reported that in breast cancer, a comparison of the simultaneous staining of p-STAT3 (Tyr705) in the nucleus and cytoplasm on the survival length was investigated, and a significant difference was not detected. With the recognition that the transcriptional activity of STAT3 is regulated by a second phosphorylation in serine (residue 727) (29-31), studies on the function of p-STAT3 (Ser727) have also been reported. For intraepithelial neoplasia of the uterine cervix, the expression of p-STAT3 (Ser727) was detected in the nucleus and cytoplasm of cells showing dysplasia. As the level of dysplasia increased, the number of cells showing a positive expression in the nucleus and/or the cytoplasm also increased. p-STAT3 (Ser727) is considered to be involved in the progression of tumors in the cervical epithelium, and may be useful as a biomarker (32).

It has been reported that in normal colon crypt epithelial cells, proteins belonging to the Bcl-2 family are expressed (33), and when the cells are treated with IL-6, the Bcl-2 concentration increased in a dose-dependent manner (34). In addition, IL-6 induces the phosphorylation of STAT3 in normal mucosa (35). Therefore, the activation of STAT3 may prevent apoptosis of the colorectal epithelial cells.

STAT3 increases the expression of matrix metalloproteinase 2, which is involved in cancer metastasis and invasion (36). In addition, activated STAT3 directly induces the transcription of *VEGF*, *cyclin D1* and *c-myc* oncogene (9,37,38), and decreases expression of the anti-apoptotic genes such as *Bcl-xL* and *Mcl-1* (39,40). Consequently, it is thought that p-STAT3 mediates an effect on the expression of genes pertinent to tumor invasion, angiogenesis, cell cycle and cell survival, thus regulating tumor growth and metastasis.

In this study, p-STAT3 (Tyr705) was involved in not only the formation of a colorectal tumor but also in the tumor progression process as is shown by T stage, nodal status, and distant metastasis, and thus its expression mediated an effect on their survival length. p-STAT3 may be valuable as a biomarker that may predict the poor prognosis of a patient. Furthermore, it has been demonstrated that for p-STAT3 (Tyr705) the IHC staining method was reliable and accurate.

Acknowledgements

This study was supported by research funds from Chosun University, 2004. We are grateful to Dr So-Yeon Ryu, and the Medical Research Institute, Chosun University, for statistical analysis.

References

1. Newland RC, Dent OF, Lyttle MN, Chapuis PH and Bokey EL: Pathologic determinants of survival associated with colorectal cancer with lymph node metastases. A multivariate analysis of 579 patients. Cancer 15: 2076-2082, 1994.
2. Burt RW, DiSario JA and Cannon-Albright L: Genetics of colon cancer: impact of inheritance on colon cancer risk. Annu Rev Med 46: 371-379, 1995.
3. Akira S, Nishio Y, Inoue M, *et al*: Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. Cell 8: 63-71, 1994.
4. Bowman T, Garcia R, Turkson J and Jove R: STATs in oncogenesis. Oncogene 15: 2474-2488, 2000.
5. Levy DE and Lee CK: What does Stat3 do? J Clin Invest 109: 1143-1148, 2002.
6. Zhong Z, Wen Z and Darnell JE Jr: Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science 264: 95-98, 1994.
7. Bromberg JF: Stat proteins and oncogenesis. J Clin Invest 109: 1139-1142, 2002.
8. Garcia R, Bowman TL, Niu G, *et al*: Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene 3: 2499-2513, 2001.
9. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C and Darnell JE Jr: Darnell, Stat3 as an oncogene. Cell 6: 295-303, 1999.
10. Epling-Burnette PK, Liu JH, Catlett-Falcone R, *et al*: Inhibition of STAT3 signaling leads to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression. J Clin Invest 107: 351-362, 2001.
11. Epling-Burnette PK, Zhong B, Bai F, *et al*: Cooperative regulation of Mcl-1 by Janus kinase/stat and phosphatidylinositol3-kinase contribute to granulocyte-macrophage colony-stimulating factor-delayed apoptosis in human neutrophils. J Immunol 15: 7486-7495, 2001.
12. Oving IM and Clevers HC: Molecular causes of colon cancer. Eur J Clin Invest 32: 448-457, 2002.
13. Shen Y, Devgan G, Darnell JE Jr and Bromberg JF: Constitutively activated Stat3 protects fibroblasts from serum withdrawal and UV-induced apoptosis and antagonizes the proapoptotic effects of activated Stat1. Proc Natl Acad Sci USA 13: 1543-1548, 2001.
14. Benekli M, Xia Z, Donohue KA, Ford LA, Pixley LA, Baer MR, Baumann H and Wetzler M: Constitutive activity of signal transducer and activator of transcription 3 protein in acute myeloid leukemia blasts is associated with short disease-free survival. Blood 99: 252-257, 2002.
15. Horiguchi A, Oya M, Shimada T, Uchida A, Marumo K and Murai M: Activation of signal transducer and activator of transcription 3 in renal cell carcinoma: a study of incidence and its association with pathological features and clinical outcome. J Urol 168: 762-765, 2002.
16. Masuda M, Suzui M, Yasumatu R, *et al*: Constitutive activation of signal transducers and activators of transcription 3 correlates with cyclin D1 overexpression and may provide a novel prognostic marker in head and neck squamous cell carcinoma. Cancer Res 15: 3351-3355, 2002.
17. Kusaba T, Nakayama T, Yamazumi K, *et al*: Activation of STAT3 is a marker of poor prognosis in human colorectal cancer. Oncol Rep 15: 1445-1451, 2006.

18. Lassmann S, Schuster I, Walch A, *et al*: STAT3 mRNA and protein expression in colorectal cancer: effects on STAT3-inducible targets linked to cell survival and proliferation. J Clin Pathol 60: 173-179, 2007.
19. Kortylewski M, Heinrich PC, Mackiewicz A, *et al*: Interleukin-6 and oncostatin M-induced growth inhibition of human A375 melanoma cells is STAT-dependent and involves upregulation of the cyclin-dependent kinase inhibitor p27/Kip1. Oncogene 24: 3742-3753, 1999.
20. Jass JR and Sobin LH: Histological Typing of Intestinal Tumours. 2nd edition, Springer-Verlag, Berlin, 1989.
21. Greene FL, Page DL and Fleming ID: AJCC Cancer Staging Manual. 6th edition, Springer-Verlag, New York, 2002.
22. Campbell CL, Jiang Z, Savarese DM and Savarese TM: Increased expression of the interleukin-11 receptor and evidence of STAT3 activation in prostate carcinoma. Am J Pathol 158: 25-32, 2001.
23. Kusaba T, Nakayama T, Yamazumi K, Yakata Y, Yoshizaki A, Nagayasu T and Sekine I: Expression of p-STAT3 in human colorectal adenocarcinoma and adenoma; correlation with clinicopathological factors. J Clin Pathol 58: 833-838, 2005.
24. Guschin D, Rogers N, Briscoe J, *et al*: A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. EMBO J 3: 1421-1429, 1995.
25. Turkson J and Jove R: STAT proteins: novel molecular targets for cancer drug discovery. Oncogene 27: 6613-6626, 2000.
26. Berclaz G, Altermatt HJ, Rohrbach V, Siragusa A, Dreher E and Smith PD: EGFR dependent expression of STAT3 (but not STAT1) in breast cancer. Int J Oncol 19: 1155-1160, 2001.
27. Bartoli M, Gu X, Tsai NT, Venema RC, Brooks SE, Marrero MB and Caldwell RB: Vascular endothelial growth factor activates STAT proteins in aortic endothelial cells. J Biol Chem 27: 33189-33192, 2000.
28. Dolled-Filhart M, Camp RL, Kowalski DP, Smith BL and Rimm DL: Tissue microarray analysis of signal transducers and activators of transcription3 (Stat3) and phospho-Stat3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. Clin Cancer Res 9: 594-600, 2003.
29. Ram PA, Park SH, Choi HK and Waxman DJ: Growth hormone activation of Stat 1, Stat 3, and Stat 5 in rat liver. Differential kinetics of hormone desensitization and growth hormone stimulation of both tyrosine phosphorylation and serine/threonine phosphorylation. J Biol Chem 8: 5929-5940, 1996.
30. Sadowski HB, Shuai K, Darnell JE Jr and Gilman MZ: A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science 24: 1739-1744, 1993.
31. Wen Z, Zhong Z and Darnell JE Jr: Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell 28: 241-250, 1995.
32. Yang SF, Yuan SS, Yeh YT, Wu MT, Su JH, Hung SC and Chai CY: The role of p-STAT3 (ser727) revealed by its association with Ki-67 in cervical intraepithelial neoplasia. Gynecol Oncol 98: 446-452, 2005.
33. Harnois C, Demers MJ and Bouchard V: Human intestinal epithelial crypt cell survival and death: Complex modulations of Bcl-2 homologs by Fak, PI3-K/Akt-1, MEK/Erk, and p38 signaling pathways. J Cell Physiol 198: 209-222, 2004.
34. Leu CM, Wong FH, Chang C, Huang SF and Hu CP: Interleukin-6 acts as an antiapoptotic factor in human esophageal carcinoma cells through the activation of both STAT3 and mitogen-activated protein kinase pathways. Oncogene 30: 7809-7818, 2003.
35. Hierholzer C, Kalff JC, Chakraborty A, Watkins SC, Billiar TR, Bauer AJ and Tweardy DJ: Impaired gut contractility following hemorrhagic shock is accompanied by IL-6 and G-CSF production and neutrophil infiltration. Dig Dis Sci 46: 230-241, 2001.
36. Xie TX, Wei D, Liu M, Gao AC, Ali-Osman F, Sawaya R and Huang S: Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. Oncogene 29: 3550-3560, 2004.
37. Nakajima K, Yamanaka Y, Nakae K, *et al*: A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells. EMBO J 15: 3651-3658, 1996.
38. Wei LH, Kuo ML, Chen CA, Chou CH, Lai KB, Lee CN and Hsieh CY: Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via STAT3 pathway. Oncogene 13: 1517-1527, 2003.
39. Catlett-Falcone R, Landowski TH, Oshiro MM, *et al*: Constitutive activation of Stat3 signaling confers resistance to apoptosis inhuman U266 myeloma cells. Immunity 10: 105-115, 1999.
40. Niu G, Bowman T, Huang M, *et al*: Roles of activated Src and Stat3 signaling in melanoma tumor cell growth. Oncogene 10: 7001-7010, 2002.