

## The Immunohistochemical Expression of STAT3, Bcl-xL, and MMP-2 Proteins in Colon Adenoma and Adenocarcinoma

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**Background/Aims:** Signal transducers and activators of transcription (STATs) are a family of transcription factors that are activated in response to cytokines and growth factors. STAT3 activation has been implicated in modulating the activity of downstream mediators, such as Bcl-xL and matrix metalloproteinase-2 (MMP-2). The aim of this study was to investigate the immunohistochemical expression of STAT3, B-cell lymphoma-extra large (Bcl-xL), and MMP-2 proteins according to histopathological parameters in colon adenocarcinomas, including lymph node metastasis, tumor differentiation, the TNM stage and the tumor size. **Methods:** Immunohistochemical staining with monoclonal STAT3, Bcl-xL, and MMP-2 antibodies was performed on paraffin-embedded specimens from 20 colon adenomas and 39 adenocarcinomas. **Results:** The expression of STAT3, Bcl-xL, and MMP-2 was increased in the adenocarcinomas as compared with the adenomas ( $p < 0.001$ ). STAT3 expression was stronger in tumors with a distant metastasis than in tumors without a distant metastasis ( $p = 0.012$ ). A larger tumor size was related to an increase in STAT3 expression ( $p = 0.035$ ). **Conclusions:** STAT3, Bcl-xL, and MMP-2 may play important roles in the tumorigenesis of colorectal carcinoma. STAT3 may be indicative of a poor prognosis due to its correlation with distant metastases and a larger tumor size. (*Gut Liver* 2012;6:45-51)

**Key Words:** Colon; Signal transducers and activators of transcription 3; B-cell lymphoma-extra large; Matrix metalloproteinase-2

### INTRODUCTION

Colorectal cancer is the third most common malignancy and the incidence of colorectal cancer is high in Western countries.<sup>1</sup>

The theory of adenoma-carcinoma sequence is now accepted as the mechanism of colorectal cancer.<sup>2</sup> While many of the oncogenes and tumor suppressor genes that are involved in the tumorigenesis of colorectal cancer have been identified, the molecular mechanisms of colorectal carcinoma are still poorly understood. With the description of the signal transduction cascades, it has recently become clear that the signal transducers and activators of transcription (STATs) signaling pathway may play an important role in the malignant transformation of human malignancies.<sup>3</sup> Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family and these proteins are transcription factors in the cytoplasm and important mediators of cytokines and growth factors.<sup>4</sup> Seven STATs have currently been identified in mammals and STAT3 is located on chromosome 17.<sup>5</sup>

Recent studies have demonstrated the essential roles of STATs proteins in modulating the process of cell proliferation, differentiation, and apoptosis.<sup>6-8</sup> There have been some studies showing that the activation of STAT3 contributed to the process of apoptosis in ovarian cancer cells by regulating the expression of B-cell lymphoma-extra large (Bcl-xL),<sup>9</sup> and activated STAT3 regulates the tumor invasion of melanoma cells by regulating the gene transcription of matrix metalloproteinase-2 (MMP-2).<sup>10</sup>

Bcl-xL is a member of the Bcl-2 family protein first identified by Boise *et al.*<sup>14</sup> This protein is known to be an important factor that regulates apoptosis.<sup>12</sup> As a mitochondrial membrane protein, it prolongs the survival of cells by modulating the electri-

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cal and osmotic homeostasis of the mitochondria in response to various stimuli.<sup>13,14</sup>

As a family of zinc-dependent endopeptidases, MMPs can degrade most of the components of the basement membrane and the extracellular matrix (ECM).<sup>15</sup> MMP-2 is a 72 kDa type IV collagenase and it is also known as gelatinase A. The role of MMP-2 in colorectal cancer was suggested for the first time in 1992 and it is known to participate in the destruction of the basement membrane at the early stage of colorectal cancer.<sup>16,17</sup>

We investigated the expressions of STAT3, Bcl-xL, and MMP-2 in colorectal adenoma and adenocarcinoma by performing immunohistochemistry and we evaluated the relationship between these proteins and the clinicopathologic parameters.

## MATERIALS AND METHODS

### 1. Materials

We studied 20 cases of colorectal adenomas and 39 cases of primary human colorectal adenocarcinomas that were obtained from patients who had undergone endoscopic resection or surgery at Daejeon St. Mary's Hospital. There were 14 men and 6 women in the adenoma group and 17 men and 22 women in the adenocarcinoma group. The median age of the adenoma group and the adenocarcinoma group was 56 and 70 years, respectively. The colorectal adenomas were divided into low grade dysplasia and high grade dysplasia according to the World Health Organization (WHO) classification.<sup>18</sup> There were 10 low grade dysplasias and 10 high grade dysplasias in the adenoma group. We classified the stage of the adenocarcinomas according to the tumor, node, metastasis (TNM) staging system of the American Joint Committee on Cancer,<sup>19</sup> and we assigned a histological type according to the WHO classification as follows: well differentiated adenocarcinoma, moderately differentiated adenocarcinoma, and poorly differentiated adenocarcinoma.<sup>18</sup> We summarized the basic demographic characteristics of the adenocarcinoma patients in Table 1.

### 2. Methods

#### 1) Immunohistochemical staining

The formalin fixed, paraffin embedded tissues were cut into 4  $\mu$ m sections and they were mounted onto poly-L-lysine coated glass slides. The sections were deparaffinized in xylene and rehydrated in a graded series of alcohol solutions. These sections were then subjected to an antigen retrieval procedure; the slides were soaked in citrate buffer (10 mM, pH 6.0), heated for 10 minutes twice (for 20 minutes overall) at 60°C in an oven, washed in phosphate buffered saline (PBS) 3 times and then treated with 3% H<sub>2</sub>O<sub>2</sub> solution for 10 minutes.

We used the EnVision-HRP detection system (DAKO, Carpinteria, CA, USA) and the primary antibodies were anti-STAT3 (dilution 1:100; Cell Signaling Technology, Beverly, MA, USA), anti-Bcl-xL (dilution 1:300; Cell Signaling Technology), and

anti-MMP-2 (dilution 1:100; Abcam, Cambridge, MA, USA). The primary antibody was incubated with the slides for 60 minutes at room temperature. The slides were washed with PBS and reacted with EnVision reagent for 10 minutes and then they were stained with diaminobenzidine. The slides were counterstained with 10% Meyer's hematoxylin and then they were observed using an optical microscope. Incubation without the primary antibody was used as a negative control.

#### 2) Analysis of the immunohistochemical staining

The immunoreactivity of STAT3, Bcl-xL, and MMP-2 proteins was classified as follows: negative (-); weak staining (1+):  $\leq$ 25% of the cells staining positive; moderate staining (2+): 25% to 50% of the cells staining positive and strong staining (3+):

**Table 1.** The Basic Characteristics and Clinicopathologic Parameters of the 39 Patients with Colorectal Adenocarcinoma

Clinicopathologic parameter	Characteristic	No. (%)
Gender	Male	17 (43.6)
	Female	22 (56.4)
Age, yr	Range	36-89
	Mean	66.6
	Median	70
Depth of invasion	T1	2 (5)
	T2	1 (3)
	T3	34 (87)
	T4	2 (5)
Lymph node involvement	N0	17 (43.6)
	N1	22 (56.4)
Distant metastasis	M0	28 (71.8)
	M1	11 (28.2)
TNM stage	I	3
	II	12
	III	13
	IV	11
Histologic grade	Well differentiated	6 (15.4)
	Moderately differentiated	28 (71.8)
	Poorly differentiated	5 (12.8)
Tumor size	$\geq$ 5 cm	16 (41)
	<5 cm	23 (59)
Primary site	Right colon	13 (33)
	Left colon	10 (26)
	Rectum	16 (41)

T1, tumor invades the submucosa; T2, tumor invades the muscularis propria; T3, tumor invades through the muscularis propria into the subserosa or perirectal tissues; T4, tumor directly invades other organs or structures and/or perforates the visceral peritoneum; N0, no regional lymph node metastasis; N1, lymph node metastasis; M0, no distant metastasis; M1, distant metastasis; TNM, tumor, node, metastasis.

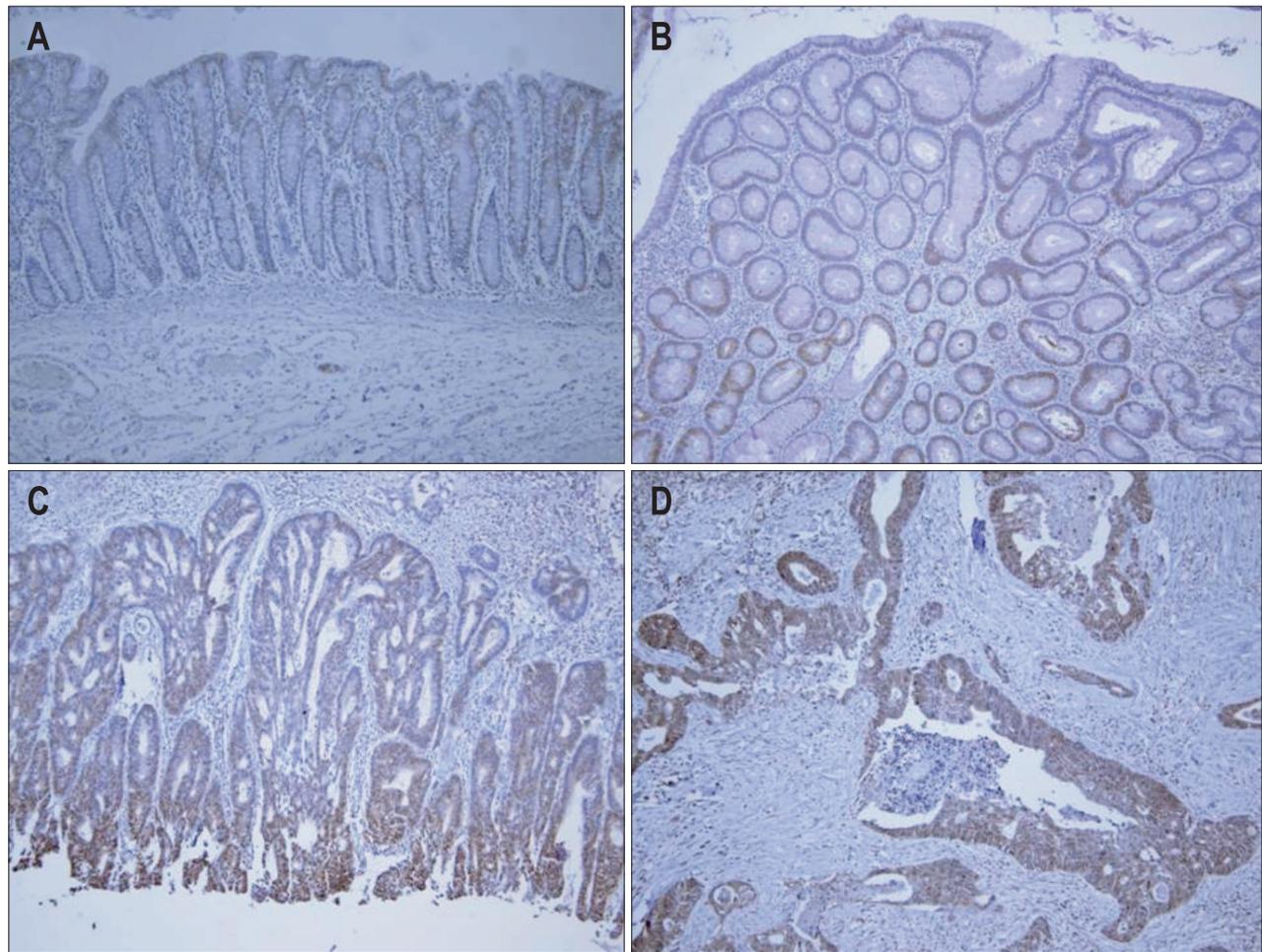
**Table 2.** The Staining Intensity of STAT3, Bcl-xL, and MMP-2 in Colon Adenoma and Adenocarcinoma

	STAT3		Bcl-xL		MMP-2	
	No. (%)	Intensity	No. (%)	Intensity	No. (%)	Intensity
Adenoma (n=20)	18 (90)	1.45±0.75	18 (90)	1.5±0.76	9 (45)	0.5±0.6
Low grade (n=10)		1.7±0.67		1.2±0.63		0.4±0.51
High grade (n=10)		1.2±0.79		1.8±0.78		0.6±0.69
Adenocarcinoma (n=39)	38 (97)	1.92±0.74*	38 (97)	2.3±0.78*	34 (87)	1.7±0.92*

Data are presented as mean±SD or number (%).

STAT3, signal transducer and activator of transcription 3; Bcl-xL, B-cell lymphoma-extra large; MMP-2, matrix metalloproteinase-2.

\*p-value<0.05 compared to the adenoma.



**Fig. 1.** Immunohistochemical staining for STAT3 in colon adenoma and adenocarcinoma. (A) Normal tissue (×100, negative). (B) Adenoma (×100, weakly positive). (C) Adenocarcinoma (×100, moderately positive). (D) Adenocarcinoma (×100, strongly positive).

>50% of the cells staining positive. Each observer estimated the percentage of cells stained and they graded the intensity of immunostaining based on a visual assessment of the intensity of the brown reaction product within the cell cytoplasm. The final immunostaining score reported was the average of two observers.

### 3) Statistical analysis

SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used for

the statistical analyses. The analyses that compared the expressions of STAT3, Bcl-xL, and MMP-2 between adenoma and adenocarcinoma were performed using independent t-tests. The data are presented as means±standard deviations. The relationships between the levels of STAT3, Bcl-xL, and MMP-2 and the various clinicopathological parameters in adenocarcinoma were determined by chi-square tests. p-values <0.05 were considered statistically significant.

## RESULTS

### 1. The expressions of STAT3, Bcl-xL, and MMP-2 in colorectal adenoma

The expression of STAT3, Bcl-xL, and MMP-2 was seen in 90% (18/20), 90% (18/20), and 45% (9/20) of the colorectal adenoma, respectively. The staining intensity of STAT3, Bcl-xL, and MMP-2 was  $1.45 \pm 0.75$ ,  $1.5 \pm 0.75$ , and  $0.5 \pm 0.6$ , respectively. When the staining intensity between low grade dysplasia and high grade dysplasia was compared, there was no statistically significant difference (Table 2).

### 2. The expressions of STAT3, Bcl-xL, and MMP-2 in colorectal adenocarcinoma

The expression of STAT3, Bcl-xL, and MMP-2 was seen in 97%, 97%, and 87%, respectively, of the colorectal adenocarcinomas. The staining intensity of STAT3, Bcl-xL, and MMP-2 was  $1.92 \pm 0.74$ ,  $2.3 \pm 0.78$ , and  $1.7 \pm 0.92$ , respectively. The staining intensity of STAT3, Bcl-xL, and MMP-2 was significantly higher in the colorectal adenocarcinoma as compared with that of the adenoma respectively ( $p < 0.001$ ) (Fig. 1, Table 2).

The status of the STAT3 expression in colorectal adenocarcinoma is summarized in Table 3. The STAT3 expression was stronger in the colorectal adenocarcinoma with distant metastasis than that in the colorectal adenocarcinoma without distant metastasis ( $p = 0.012$ ), and it was also stronger in the larger size tumor ( $\geq 5$  cm) than that in the smaller size tumor ( $p = 0.035$ ).

We classified the adenocarcinoma group according to the histologic grade as well, moderate and poorly differentiated. The expression of STAT3 was stronger in the differentiated group of tumors (well and moderate differentiated adenocarcinoma) than that in the poorly differentiated group ( $p = 0.031$ ). The expressions of Bcl-xL and MMP-2 were also stronger in the differentiated group (well and moderate differentiated adenocarcinoma) than that in the poorly differentiated group ( $p = 0.034$ ,  $p = 0.01$ , respectively, the data is not shown). The expression of each protein was not significantly different according to age, gender, the TNM staging and the lymph node status.

## DISCUSSION

STAT3 is a member of the Janus activated kinase (JAK)/STAT signaling pathway and it was first identified as a DNA binding factor that selectively binds to the interleukin-6 (IL-6)-responsive element in the promoter of acute phase genes from IL-6 stimulated hepatocytes.<sup>6,20</sup> STAT3 is activated by many cytokines and growth factors, including epidermal growth factor, platelet-derived growth factor and IL-6 as well as by oncogenic proteins, such as Src and Ras.<sup>6,21</sup>

The biologic functions of STAT3 are very broad. STAT3 plays a crucial role in the regulation of cell proliferation, survival, apoptosis, and differentiation. Constitutively activated STAT

**Table 3.** The Relationship between Clinicopathologic Parameters and STAT3 Expression in Colorectal Adenocarcinoma

Clinicopathologic parameter	STAT3				p-value
	Negative (n=1)	Weak staining (n=9)	Moderate staining (n=21)	Strong staining (n=8)	
Age					0.089
$\geq 70$	0	3	10	7	
$< 70$	1	6	11	1	
Gender					0.415
Male	1	5	7	4	
Female	0	4	14	4	
Stage					0.556
I+II	0	3	10	2	
III+IV	1	6	11	6	
Lymph node involvement					0.465
Negative	0	4	11	2	
Positive	1	5	10	6	
Distant metastasis					0.012*
M1	1	3	2	5	
M0	0	6	19	3	
Histologic grade					0.031*
Well	0	0	6	0	
Moderate	0	7	14	7	
Poor	1	2	1	1	
Size					0.035*
$\geq 5$ cm	0	0	6	5	
$< 5$ cm	1	9	15	3	

STAT3, signal transducer and activator of transcription 3; M0, no distant metastasis; M1, distant metastasis.

\* $p$ -value  $< 0.05$ .

proteins have been observed in a wide variety of human cell lines and primary tumors including leukemia, multiple myeloma, breast cancer, prostate cancer, and other cancers.<sup>22-25</sup>

In this study, we found that the STAT3 expression was significantly higher in the tumors with distant metastasis and a larger size. The staining intensity of STAT3 was significantly higher in the adenocarcinomas than that in the adenomas. Ma *et al.*<sup>26</sup> reported that there was a significant correlation between the expression of the phosphorylated or activated form of STAT3 (p-STAT3) and the presence of lymph node metastasis and invasion in human colorectal carcinoma. Although Kusaba *et al.*<sup>27</sup> reported on the correlation between p-STAT3 and lymph node metastasis or the Duke stage, there was no significant correlation of STAT3 with lymph node metastasis and the TNM stage in our current study.

Although tyrosine phosphorylation is required for STAT3 to bind to specific DNA target sites, the nuclear import of STAT3

takes place constitutively and independently of tyrosine phosphorylation<sup>28</sup> and phosphorylation is not a prerequisite for STAT3 nuclear import. Thus, the requirement of phosphorylation for the transcriptional activity of STAT3 remains a controversial issue.<sup>29-31</sup>

Xie *et al.*<sup>32</sup> reported that STAT3 upregulated the expression of MMP-2, and that this was correlated with metastasis and invasion of carcinoma. Horiguchi *et al.*<sup>33</sup> reported that the activation of STAT3 in renal cell carcinoma is associated with distant metastatic disease and the roles and mechanisms of activated STAT3 in metastases have been discovered.<sup>21</sup>

Numerous downstream genes of STAT3 signaling have been identified. p-STAT3 has been shown to protect tumor cells from apoptosis and promote cell proliferation by regulating the genes encoding antiapoptotic associated proteins and proliferation associated proteins such as Bcl-xL and cyclin D1.<sup>21,31</sup>

Since the discovery of Bcl-2, several homologs of this gene and its encoded protein have been identified. Some of these homologs function as blockers of cell death (Bcl-2, Mcl-1, Bcl-xL), whereas others are promoters of apoptosis (Bax, Bak, Bcl-xS).<sup>34</sup> Two distinct forms of Bcl-x (Bcl-xL, Bcl-xS) have been identified and they have different functions and molecular weights. Bcl-xL is a 21-kD protein and it suppresses apoptosis, whereas Bcl-xS is 19-kD and it promotes apoptosis.<sup>11,35</sup> Studies of Bcl-xL protein in gastric, colorectal and pancreatic cancers have been reported. The expression rate of Bcl-xL was related with the prognosis of patients with pancreatic cancer and Bcl-xL was increased in colorectal cancer.<sup>26,36-38</sup>

In this study, the expression of Bcl-xL was more increased in adenocarcinoma than that in adenoma, with significance. Zhang *et al.*<sup>39</sup> reported that the expression of Bcl-xL was associated with lymph node metastasis, the pathologic grade and Duke's stage of colorectal carcinoma. Krajewska *et al.*<sup>37</sup> suggested that the expression of Bcl-xL was increased in undifferentiated primary colorectal cancers. Kusaba *et al.*<sup>40</sup> reported that there was no significant correlation between p-STAT3 immunoreactivity and the differentiation of colorectal adenocarcinomas.

Our result showed a decreased expression of STAT3, Bcl-xL, and MMP-2 in undifferentiated adenocarcinoma. However, our results have limitations due to the small number of subjects. Therefore, further studies are needed to identify the relationship between these proteins and the differentiation of adenocarcinoma.

There is substantial evidence of STAT3's involvement in tumor cell migration and invasion. STAT3 has been shown to transcriptionally activate the expression of genes that promote tumor cell migration and invasion. Specifically, activated STAT3 regulates tumor invasion of melanoma cells by regulating the gene transcription of MMP-2.<sup>32</sup> The MMP family consist of roughly 20 members, and they are chiefly involved in the dissemination of cancer cells by breaking down the ECM and creating an environment that supports the initiation and maintenance

of tumor growth.<sup>41</sup> There have been several recent reports that the expression of MMP-2 protein was increased according to the depth of invasion and it was increased in adenocarcinoma rather than in adenoma.<sup>42,43</sup> Shin *et al.*<sup>44</sup> reported that the expression of MMP-2 is correlated with lymph node metastasis in colorectal cancer.

In this study, the MMP-2 expression was significantly higher in the adenocarcinomas than that in the adenomas, and the correlation with lymph node metastasis and the pathologic stage was not shown. It is known that the MMP-2 protein expression is related with the distant metastatic process.<sup>45</sup> In this study, the MMP-2 expression was not related with distant metastasis.

Our results suggested that the expressions of STAT3, Bcl-xL, and MMP-2 were increased in both adenoma and adenocarcinoma as compared with the expressions in the normal mucosa, and the staining intensity was increased with the progression from adenoma to adenocarcinoma. The results showed that these proteins may be related to the development of colorectal adenocarcinoma with the progression from adenoma to carcinoma. Especially, our results showed that STAT3 had correlation with distant metastasis and a larger tumor size in colorectal adenocarcinoma, so we suggest that STAT3 might be related with a poor prognosis for patients with colorectal adenocarcinoma. However, further studies are needed for understanding the detailed relationships of these proteins in colorectal carcinogenesis.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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