

• LARGE INTESTINAL CANCER •

# Transforming growth factor- $\beta$ 1 in invasion and metastasis in colorectal cancer

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## Abstract

**AIM:** To investigate the role of TGF  $\beta$ 1 in invasion and metastasis in colorectal cancer by analysing TGF $\beta$ 1 correlated with depth of tumor invasion, stage and metastasis.

**METHODS:** Serum TGF $\beta$ 1 levels were determined in 50 patients with colorectal cancer and 30 healthy volunteers using a TGF $\beta$ 1 enzyme-linked immunosorbent assay. TGF $\beta$ 1 expression in primary and lymph node metastatic lesions were detected in 98 cases of colorectal cancer by immunohistochemical staining and *in situ* hybridization.

**RESULTS:** Serum levels of TGF $\beta$ 1 in patients with colorectal cancer ( $40 \pm 18 \text{ mg} \cdot \text{L}^{-1}$ ) were significantly higher than those in the healthy control group ( $19 \pm 8 \text{ mg} \cdot \text{L}^{-1}$ ),  $P < 0.05$ . Elevated levels of serum TGF $\beta$ 1 were found in 60% of patients with colorectal cancer when the mean  $+2 \text{ s}$  was used as the upper limit of the normal range ( $35.1 \text{ mg} \cdot \text{L}^{-1}$ ). Increases in serum TGF $\beta$ 1 levels were significantly associated with Duke's stage ( $P < 0.05$ ), but there was no significant difference between Duke's stage B patients and Duke's stage C patients. In the cytoplasm of cancer cells, TGF $\beta$ 1 was immunostained in 37.8% (37/98) of colorectal cancer, and this expression was confirmed by *in situ* hybridization. Among 35 cases of colorectal cancer with lymph node metastatic lesions, TGF $\beta$ 1 positive staining was found in 18 (51.4%) cases of primary tumor, and 25 (71.4%) cases with lymph node metastatic lesions, respectively. Of 17 cases with no staining in the primary lesion, 7 (41.2%) cases showed TGF $\beta$ 1 staining in the metastatic lesion. Serum TGF $\beta$ 1 levels and TGF $\beta$ 1 expression in colorectal cancer tissues were correlated significantly with depth of tumor invasion, stage and metastasis. Patients in stage C-D, T3-T4 and with metastasis had significantly higher TGF $\beta$ 1 levels than patients in stage A-B, T1-T2 and without metastasis ( $P < 0.05$ ).

**CONCLUSION:** These results suggest that transforming growth factor- $\beta$ 1 is closely related to the invasion and metastasis of colorectal cancer. It increased the

**invasive and metastatic potential of tumor by altering a tumor microenvironment. TGF $\beta$ 1 may be used as a possible biomarker.**

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## INTRODUCTION

The incidence of colorectal cancer has become high in China and its biology is a hot topic of research<sup>[1-44]</sup>. Tumor invasion and metastasis are complex processes in which cancer cells detach from the original tumor mass to establish metastatic foci at distant sites. Metastatic cells characteristically lose growth inhibitory responses, undergo alteration in adhesiveness and demonstrate enhanced production of enzymes that can degrade extracellular matrix components. Since it is the development of metastatic disease that is primarily responsible for cancer mortality, an understanding of the mechanisms that facilitate metastatic tumor progression is of great importance<sup>[1]</sup>. Transforming growth factor (TGF)- $\beta$ 1 is a 25-kd polypeptide. This growth factor regulates cell growth and differentiation in both normal and transformed cells. TGF- $\beta$ 1 was found to inhibit the growth of normal and neoplastic cells. Resistance to the negative growth-regulating properties of TGF- $\beta$ 1 has been observed in epithelial and mesenchymal tumors. Tumor cell lines that lack TGF- $\beta$  receptors lose responsiveness to TGF- $\beta$ 1, and the escape of cells from TGF- $\beta$ 1 mediated negative regulation is linked to tumor progression<sup>[45]</sup>. Colorectal cancer is one of the most malignant neoplasms. TGF- $\beta$ 1 plays a crucial role in tumor extension. We examined the expression of TGF- $\beta$ 1 in primary and lymph node metastatic lesions in colorectal cancer, as well as serum TGF- $\beta$ 1 levels in the peripheral veins. Our objective is to determine the clinical significance of TGF- $\beta$ 1 in advance of colorectal cancer.

## MATERIALS AND METHODS

### Patients

Serum TGF- $\beta$ 1 assays were performed in 50 patients treated from July 1999 to June 2000. There were 32 men and 18 women, and their age ranged from 23 to 74 years (means,  $53 \pm 11$  years). According to Duke's staging criteria, 9 cases were stage I, 18 stage II, 18 stage III and 5 stage IV. Thirty healthy volunteers were selected as control group among whom there were 17 men and 13 women. Their age ranged from 20 to 56 years (means,  $45 \pm 8$  years).

A total of 98 colorectal adenocarcinoma patients (including the 50 patients above) who had undergone surgical resection in the Affiliated Zhongnan Hospital of Wuhan University (Wuhan, China) from July 1998 to December 2000, TGF $\beta$ 1 and TGF $\beta$ R II immunohistochemical staining and *in situ* hybridization were performed. There were 53 men and 45 women, and their age ranged from 23 to 74 years (means,

56 $\pm$ 11 years). Among 98 patients, 17 were well differentiated adenocarcinoma, 47 moderately differentiated adenocarcinoma and 34 poorly differentiated adenocarcinoma. According to Duke' s staging criteria, 34 cases were stage I , 29 stage II , 30 stage III and 5 stage IV .

### Methods

**Preparation of serum sample and TGF  $\beta$  1 assay** Two mL of blood sample, collected from the peripheral vein before surgery, were stored for approximately 3 h at 4 °C until the samples were centrifuged. Blood samples were centrifuged at 3 000 g for 20 min. The serum was separated and stored frozen at -70 °C until the time of analysis. TGF $\beta$ 1 was assayed using human TGF $\beta$ 1 enzyme-linked immunoabsorbent assay kits. The ELISA kits were obtained from Quantikine Co. of USA. The TGF $\beta$ 1 assay was performed according to the methods outlined in the package insert. Standard samples of 200  $\mu$ g were added to each well, and incubated for 3 h at room temperature. After complete wash of each well, 200  $\mu$ l TGF $\beta$ 1 conjugate was added to each well and these were incubated for 1.5 h at room temperature. We repeated the aspiration/wash and added 200  $\mu$ l of substrate solution to each well and incubated for 20 min. Finally, we added 50  $\mu$ l of stop solution and absorbances in each well were measured using a spectrophotometric plate reader at a wavelength of 490 nm. To determine the TGF- $\beta$ 1 concentration in each sample, we first calculated the average absorbance value in each set of duplicates. Serum levels of TGF- $\beta$ 1 were calculated from linear regression equation.

**Immunohistochemistry** All the tissue specimens were fixed in 100 mL  $\cdot$  L<sup>-1</sup> neutral formalin and embedded in paraffin. Five-um thick sections were xylene dehydrated in ethanol. Tissue sections were washed three times in 0.05 mol $\cdot$  L<sup>-1</sup> PBS, and incubated in endogenous peroxidase blocking solution. Non-specific antibody binding was blocked by pretreatment with PBS containing 5g $\cdot$  L<sup>-1</sup> bovin serum albumin. Sections were then rinsed in PBS and incubated overnight at 4 °C with diluted anti-TGF $\beta$ 1 and anti-TGF $\beta$ R II protein polyclonal antibody. The steps were performed using Immunostain kit according to the manufacturer' s instructions. PBS was used as substitutes of protein antibody for negative control groups. The sections were examined under light microscopy. Anti-TGF $\beta$ 1 and anti-TGF $\beta$ R II protein polyclonal antibody were purchased from Bosden Comp. (Wuhan, China). S-P detection kit was purchased from Fuzhou Maixin Comp. (Fuzhou, China). Anti-TGF $\beta$ 1 and anti-TGF $\beta$ R II protein polyclonal antibody were diluted to 1:100.

**In situ hybridization** All the tissue specimens were fixed in 100 mL  $\cdot$  L<sup>-1</sup> neutral formalin and embedded in paraffin. Six-um thick sections were xylene dehydrated in ethanol, and digested with 10 mg $\cdot$  L<sup>-1</sup> proteinase for 10 min. Sections were washed in 0.5 mol $\cdot$  L<sup>-1</sup> PBS for 15 min. They were incubated overnight at 37 °C with the 500  $\mu$ g $\cdot$  L<sup>-1</sup> digoxigenin-labeled RNA probe in hybridization buffer. After hybridization, sections were washed in 2 $\times$  SSC for 10 min at 37 °C and finally in 0.2 $\times$  SSC for 15 min at 37 °C. Sections were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody for 60 min at 37 °C. The steps were performed using in situ hybridization kit according to the manufacturer' s instructions. The kits were purchased from Bosden Comp. (Wuhan, China).

TGF $\beta$ 1 *in situ* hybridization probe sequences were:

- (1)5-CGTTTCACCAGCTCCATGTCGATGGTCTTGCAAT-3'
- (2)5-CTTGATTTTAATCTCTGCAAGCGCAGCTCTGCACG-3'

(3)5-TTGGTATCCAGGGCTCTCCGGTGCCGTGAGCTGTG-3'

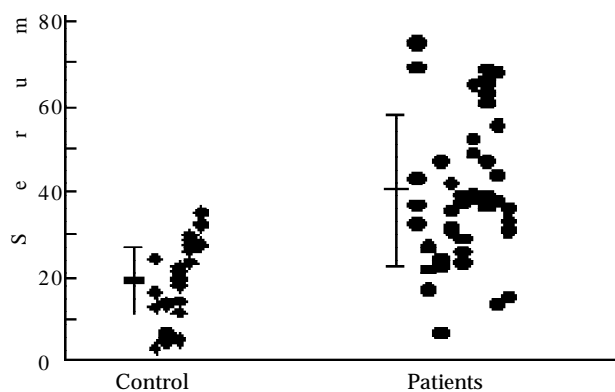
### Statistical analysis

The difference between each group was analyzed by Chi-square test and correlativity. The limit of significant difference was  $P < 0.05$ .

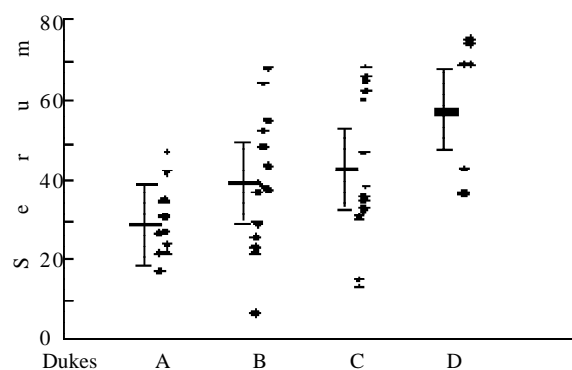
## RESULTS

### Serum TGF $\beta$ 1 levels in patients with colorectal cancer

Serum TGF $\beta$ 1 levels in patients with colorectal cancer (40.4  $\pm$ 17.6  $\mu$ g $\cdot$  L<sup>-1</sup>) were significantly higher than in normal controls (19.2 $\pm$ 8.0  $\mu$ g $\cdot$  L<sup>-1</sup>),  $P < 0.01$ . Elevated levels of serum TGF $\beta$ 1 were found in 60 % of patients with colorectal cancer when the mean +2 s was used as the upper limit of the normal range (35.1  $\mu$ g $\cdot$  L<sup>-1</sup>, Figure 1). Increased in serum TGF $\beta$ 1 levels were significantly associated with Duke' s stage ( $P < 0.05$ ), but there was no significant difference between Duke' s stage B patients and Duke' s stage C patients. Serum levels of TGF $\beta$ 1 were 29.2  $\pm$ 7.3  $\mu$ g $\cdot$  L<sup>-1</sup> in Duke' s stage A patients, 39.5 $\pm$ 11.9  $\mu$ g $\cdot$  L<sup>-1</sup> in Duke' s stage B patients, 43.1 $\pm$ 15.8  $\mu$ g $\cdot$  L<sup>-1</sup> in Duke' s stage C patients, and 57.8 $\pm$ 16.2  $\mu$ g $\cdot$  L<sup>-1</sup> in Duke' s stage D patients. Serum levels of TGF $\beta$ 1 in each stage were significantly higher than those in the control group (Figure 2). Serum levels of TGF $\beta$ 1 were not correlated with age, gender, tumor size and differentiation degree of tumor .



**Figure 1** Serum TGF $\beta$ 1 in patients with colorectal cancer. Bars represent mean  $\pm$  standard deviation.



**Figure 2** Serum TGF $\beta$ 1 in patients with colorectal cancer according to Duke' s stage. Bars represent mean  $\pm$  standard deviation.

TGF $\beta$ 1 and TGF $\beta$ R II Expression in colorectal cancer tissue The TGF $\beta$ 1 and TGF $\beta$ R II protein were stained mainly in the cytoplasm and cell membrane of cancer cells, as shown in Figures 3 and 4. Staining was classified as negative if less than 10 % of the cells were positive and as positive if more than 10 % were positive<sup>[2]</sup>. Thirty-seven (37.8 %) of 98 tissues from colorectal cancer patients were positive for TGF $\beta$ 1

staining and forty-six(46.9 %) were positive for TGF $\beta$ R II staining. The expression of TGF $\beta$ 1 and TGF $\beta$ R II was correlated significantly with the depth of invasion, stage of disease and metastasis (lymph node and distant metastasis). Patients in T<sub>3</sub>-T<sub>4</sub>, stage C-D and with metastasis had significantly higher expression of TGF $\beta$ 1 than patients in T<sub>1</sub>-T<sub>2</sub>, stage A-B and without metastasis ( $P<0.05$ ). Patients in T<sub>3</sub>-T<sub>4</sub>, stage C-D and with metastasis had significantly lower expression of TGF $\beta$ R II than patients in T<sub>1</sub>-T<sub>2</sub>, stage A-B and without metastasis ( $P<0.05$ ). The expression of TGF $\beta$ 1 and TGF $\beta$ R II was not correlated with age, gender, tumor size and differentiation degree of tumor (Table 1).

**Table 1** Clinicopathologic characteristics of colorectal cancer with expression of TGF $\beta$ 1 and TGF $\beta$ R II

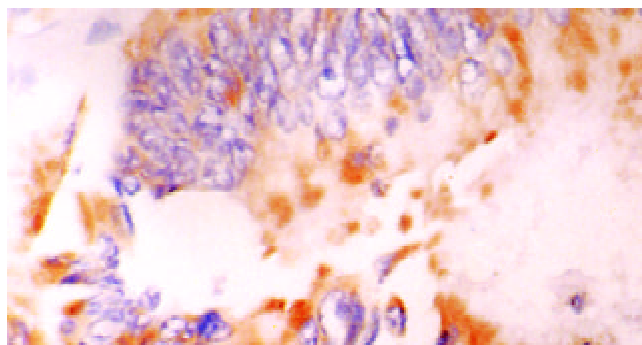
Variables	<i>n</i>	TGF $\beta$ 1-positive		TGF $\beta$ R II -positive	
		<i>n</i> (%)		<i>n</i> (%)	
Age( yrs)	$\geq 50$	60	24(40.0)	30(50.0)	
	$< 50$	38	13(34.2)	16(42.1)	
Sex	Male	53	20(41.5)	25(53.7)	
	Female	45	17(31.0)	21(37.9)	
Tumor size	$< 5\text{cm}$	56	21(37.5)	24(42.9)	
	$\geq 5\text{cm}$	42	16(38.1)	22(52.4)	
Histology					
Well-diff. ade	17	9(52.9)	11(64.7)		
Mode-diff. ade	47	15(31.9)	19(40.4)		
Poor-diff. ade	34	13(38.2)	16(47.1)		
Depth of invasion					
T <sub>1</sub> -T <sub>2</sub>	60	17(28.3)	33(55.0)		
T <sub>3</sub> -T <sub>4</sub>	38	20(52.6) <sup>a</sup>	13(34.2) <sup>a</sup>		
Metastasis	Present	35	18(51.4)	11(31.4)	
	Absent	63	19(30.2) <sup>a</sup>	35(55.6) <sup>a</sup>	
Stage	A	34	8(23.5)	23(67.7)	
	B	29	9(31.1)	13(44.8)	
	C+D	35	20(57.1) <sup>a</sup>	10(28.6) <sup>a</sup>	

<sup>a</sup> $P<0.05$ , T<sub>1</sub>-T<sub>2</sub> vs T<sub>3</sub>-T<sub>4</sub>, Present vs Absent, A, B vs C+D

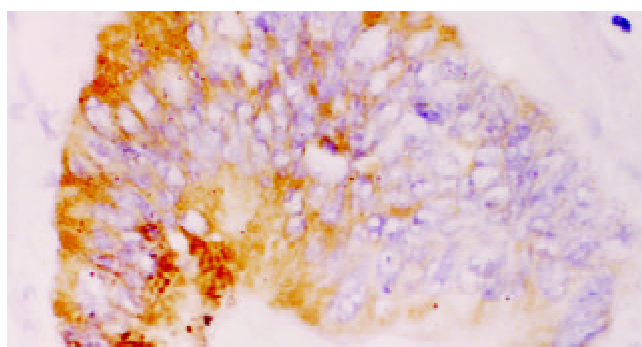
#### TGF $\beta$ mRNA expression in colorectal cancer tissue

The expression of TGF $\beta$ 1mRNA in 50 colorectal cancer tissues was examined. The degree of staining was prominent in cases of TGF $\beta$ 1 positive immunohistochemical staining, but it was rare for negative cases of immunohistochemical staining. The pattern of distribution of TGF $\beta$ 1mRNA is the same as immunohistochemical staining (Figure 5).

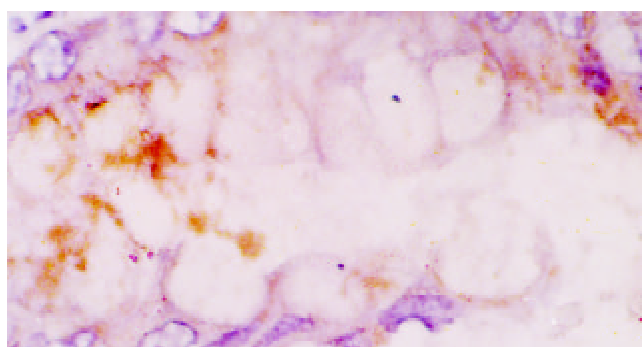
**Relationship of TGF $\beta$ 1 expression between primary and lymph node metastatic lesions** Among 35 cases of colorectal cancer with lymph node metastatic lesions, the TGF $\beta$ 1 positive rate was 51.4 % (18/35) for primary lesions and 71.4 % (25/35) for metastatic lesions in the lymph nodes. Of 17 cases with no staining in the primary lesion, 7 cases (41.2 %) showed TGF $\beta$ 1 staining in the metastatic lesion (Figure 6).



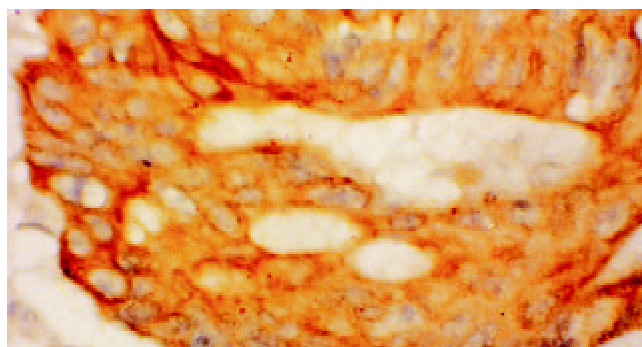
**Figure 3** TGF $\beta$ 1 staining in cytoplasm of cancer cells.  $\times 400$



**Figure 4** TGF $\beta$ R II staining in cytoplasm of cancer cells.  $\times 400$



**Figure 5** TGF $\beta$ 1mRNA expression in colorectal cancer *in situ* hybridization.  $\times 400$



**Figure 6** TGF $\beta$ 1 Expression in lymph node metastatic lesions.  $\times 400$

#### DISCUSSION

TGF $\beta$ 1 has been found to be overexpressed locally in many tumors, and is believed to play a role in tumor transformation and progression, as well as in tumor regression<sup>[46-48]</sup>. Although

TGF $\beta$ 1 acts as a potent inhibitor of cell growth and tumor progression, loss of this negative regulation can lead to tumor development. TGF $\beta$ 1 switches to a growth stimulator with tumor progression. Growth inhibition is replaced in many tumors by the opposite response, growth stimulation. This paradoxical switch in the responsiveness of tumor cell to TGF $\beta$ 1 during neoplastic progression may be due to the inactivation of the TGF $\beta$ 1 signaling pathway such as mutations in the type 2 TGF $\beta$  receptor gene or through reduced expression and increase of blood supply to a tumor mass and inhibition of immunologic mechanisms involved in tumor identification and cytolysis. In cases of breast cancer, expression of TGF $\beta$ 1 was positively associated with invasion and metastasis. Maehara *et al.*<sup>[45]</sup> reported that the expression of TGF $\beta$ 1 in gastric cancer cells was closely related to infiltrative growth of the cancer and to the higher rate of lymph node metastasis.

We found that TGF $\beta$ 1 levels in the serum of patients with colorectal cancer were significantly elevated compared with normal controls. TGF $\beta$ 1 was overexpressed in colorectal cancer tissue compared with normal colorectal mucosa. Elevated serum levels of TGF $\beta$ 1 and over-expression of TGF $\beta$ 1 in colorectal cancer tissue were correlated significantly with invasion and metastasis of colorectal cancer. Patients in T<sub>3</sub>-T<sub>4</sub>, stage C-D and with metastasis had significantly higher expression of TGF $\beta$ 1 in tumor tissue and TGF $\beta$ 1 levels in the serum than patients in T<sub>1</sub>-T<sub>2</sub>, stage A-B and without metastasis ( $P < 0.05$ ). The expression of TGF $\beta$ 1 in tumor tissue and TGF $\beta$ 1 levels in the serum were not correlated with age, gender and differentiation degree of tumor. Shim *et al.*<sup>[49]</sup> reported that patients of colorectal cancer in stage C-D had significantly higher expression of TGF $\beta$ 1 in tumor tissues and TGF $\beta$ 1 levels in the serum than patients in stage A-B ( $P < 0.05$ ). TGF $\beta$ 1 in colorectal cancer patients may be associated with disease progression. Among 35 cases of colorectal cancer with lymph node metastasis lesions, the TGF $\beta$ 1 positive rate was 51.4 % (18/35) for primary lesions and 71.4 % (25/35) for metastatic lesions in the lymph nodes. Of 17 cases with no staining in the primary lesion, 7 cases (41.2 %) showed TGF $\beta$ 1 staining in the metastatic lesion. The preferential expression of TGF $\beta$ 1 in lymph node metastases suggests a clonal selection of tumor cells with TGF $\beta$ 1 expression, specific for the higher potential of lymph node metastasis in tumor advance, and TGF $\beta$ 1 plays a role related to the malignant progression of colorectal cancer. We also found that TGF $\beta$ R II expression was significantly lower in colorectal cancer tissues than in normal mucosa. The expression of TGF $\beta$ R II in tumor tissues of Patients in T<sub>3</sub>-T<sub>4</sub>, stage C-D and with metastasis was significantly lower than that in patients in T<sub>1</sub>-T<sub>2</sub>, stage A-B and without metastasis ( $P < 0.05$ ). The expression of TGF $\beta$ R II in tumor tissues was not correlated with age, gender and differentiation degree of tumor. The lower expression of TGF $\beta$ R II in colorectal cancer may be associated with disease progression. In our previous study<sup>[50,51]</sup>, we found that TGF $\beta$ 1 expression was correlated significantly with angiogenesis in colorectal cancer tissues and inhibition of immunologic mechanisms involved in tumor identification and cytolysis. TGF $\beta$ 1 is closely related to the invasion and metastasis of colorectal cancer, and it may be used as a possible biomarker.

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