

## Expression of Smad4 and TGF- $\beta$ 2 in Colorectal Carcinoma

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**Abstract.** *Background:* TGF- $\beta$ , a potent natural antiproliferative agent, is believed to play an important role in suppressing tumorigenicity. This effect is mediated through Smad4, a tumour-suppressor gene, at chromosome 18q21, which affects gene transcription and controls cell growth. The aim of the study was to investigate the expression of Smad4 and TGF- $\beta$ 2 in colorectal carcinomas and to correlate them with pathological parameters and patient survival. *Materials and Methods:* Formalin-fixed paraffin-embedded tissue from 49 cases of colon carcinoma was stained by immunohistochemistry for TGF- $\beta$ 2 and Smad4 protein. *Results:* Smad4 nuclear and cytoplasmic staining was absent in 9/49 (18.3%) or reduced in 18/49 (36.7%) colorectal carcinoma, while in the remaining 22 (44.8%) Smad4 expression comparable with colonic mucosa was observed. TGF- $\beta$ 2 cytoplasmic staining was expressed in all cases and was overexpressed in 24/49 (48.9%) carcinoma. A statistically significant correlation was found between Smad4 expression and tumour grade ( $p=0.02$ ) and between TGF- $\beta$ 2 expression and Dukes' stage ( $p=0.03$ ). A slight tendency for a relationship between Smad4 and TGF- $\beta$ 2 ( $p=0.25$ ) was also observed. No statistically significant relationship between the above markers and survival was detected. *Conclusion:* In poorly-differentiated carcinoma, Smad4 protein expression was retained and may be linked to TGF- $\beta$ 2 overexpression, due to the activation or deregulation of the TGF- $\beta$  signalling pathway. Inactivation of the TGF- $\beta$  gene occurs at an early stage of colorectal carcinogenesis, while inactivation of Smad4 is probably a late event.

The chromosome region 18q21 is deleted in over 70% of sporadic colorectal cancer. Three candidate tumour suppressor genes, DCC, Smad4 and Smad2, map to this region (1, 2). The Smad4 gene is involved in signal transduction of TGF- $\beta$ , a potent inhibitor of the proliferation of epithelial cells (3). After

phosphorylation and activation by receptor kinases, hetero-oligomeric Smad complexes migrate into the nucleus and, either directly or in complex with other proteins, induce the expression of growth-regulatory genes (4). Inactivation of Smad4 can occur by intragenic mutation of one allele coupled with loss of the other allele, or by deletion of both alleles (homozygous deletions) (5). In addition to these mechanisms, the hypermethylation of the promoter region may contribute to inactivating the Smad4 gene (6, 7) Approximately 50% of pancreatic carcinoma, 20% of colon carcinoma and 10% of lung cancer exhibit mutations in Smad4 (8-11).

TGF- $\beta$  inhibits cells from entering the S-phase by suppressing the expressions of cyclin A and cyclin E and by increasing the expression of Cdk inhibitors such as p16, p15, p21 or p27 (12). TGF- $\beta$  is able to down-regulate c-myc expression, which can regulate, at least indirectly, the expression of G1 cyclins (13, 14). TGF- $\beta$  induces the accumulation of hypophosphorylated Rb, which prevents progression into the S-phase (15). TGF- $\beta$  also regulates the formation of stroma and the depositing of extracellular matrix (16). TGF- $\beta$  inhibits the generation of cytotoxic T lymphocytes and the production of interferon  $\gamma$  and tumour necrosis factor- $\alpha$  by T lymphocytes (17). Reduced responsiveness to TGF- $\beta$  seems to be an important event in colorectal carcinogenesis (18). TGF- $\beta$  resistance is associated with mutations in, or the down-regulation of, the expression of TGF- $\beta$  receptors I and II, or signal transducers of the Smad family. Mutations of the TGF- $\beta$  receptors have been described in colorectal carcinoma with mismatch repair deficiency (19, 20).

The above data prompted us to investigate, through immunohistochemistry, the expressions of Smad4 and TGF- $\beta$ 2 in colorectal carcinoma and to correlate them with pathological parameters and patient survival.

### Materials and Methods

*Patients and tissues.* A search for cases of colorectal carcinoma in the 1990-2000 files of the Department of Pathology, Elpis Hospital, Athens, Greece, yielded 200 patients, but only 49 cases had data on therapy and clinical outcome and, thus, were selected. The

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Table I. *Smad4* expression in relation to clinicopathological findings.

	Total	Smad4				P
		-	+	++	+++	
Gender						
Male	24	5	7	5	7	0.57 (NS)
Female	25	4	11	5	5	
Age						
>60	34	6	13	8	7	1.0 (NS)
≤60	15	3	5	2	5	
Differentiation grade						
Well	1	0	0	0	1	0.02
Moderate	37	9	15	7	6	
Poor	11	0	3	3	5	
Dukes' stage						
A	1	0	1	0	0	0.07 (NS)
B	15	4	7	2	2	
C	22	2	6	6	8	
D	11	3	4	2	2	

NS: Not statistically significant at the 5% level.

Note: For the statistical tests, *Smad4* was categorized as a 2-level variable: -/+ vs. ++/+++.

patients included 24 males and 25 females (M:F ratio, 0.96), with ages ranging from 28-80 years old (mean, 63 years). The diagnosis and staging according to the Dukes' classification were assessed from formalin-fixed paraffin-embedded sections, stained with haematoxylin and eosin. All the patients had received adjuvant chemotherapy (5FU/FA). The mean survival time had been 41 months (range, 2 to 145+ months).

**Immunohistochemistry.** Immunostaining for the presence of TGF-β2 and *Smad4* proteins was performed on formalin-fixed, paraffin-embedded tissue sections, using the streptavidin-biotin peroxidase-labelling procedure. The TGF-β2 (polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the *Smad4* (clone B8, Santa-Cruz Biotechnology) antibodies were used at dilutions of 1:200 and 1:100, respectively, for 45 min. A step of microwave heating in a solution of sodium citrate, pH 6, was performed prior to incubation with both the antibodies, as described in our previous study (21). The negative control slides were prepared by omitting the primary antibody.

*Interpretation of the staining*

a) *Smad4*: The percentage of *Smad4*-positive cells was evaluated and scored as follows: (-) <5%, (+) 5-9%, (++) 10-34% and (+++) >35%. Non-neoplastic colonic epithelium, stromal fibroblasts and lymphoid aggregates showed strong to moderate immunoreactivity and served as the positive control.

b) *TGF-β2*: The intensity of the staining was scored as reduced (+), maintained (++) or increased (+++) in comparison with normal colonic mucosa.

**Statistical analysis.** The statistical analysis was performed using the SAS 9.0 software package. The correlation of categorical variables (*Smad4* (-/+ vs. ++/+++), TGF-β2 (+/+ vs. +++), gender, age (>60 vs. ≤60), grade and Dukes' stage) was evaluated with the

Table II. *TGF-β2* expression in relation to clinicopathological findings.

	Total	TGF-β2			P
		+	++	+++	
Gender					
Male	24	7	6	11	0.77 (NS)
Female	25	5	7	13	
Age					
>60	34	9	10	15	0.36 (NS)
≤60	15	3	3	9	
Differentiation grade					
Well	1	0	1	0	0.40 (NS)
Moderate	37	11	9	17	
Poor	11	1	3	7	
Dukes' stage					
A	1	0	0	1	0.03
B	15	6	6	3	
C	22	3	5	14	
D	11	3	2	6	

NS: Not statistically significant at the 5% level.

Note: For the statistical tests TGF-β2 was categorized as a 2-level variable: +/+ vs. +++.

Fisher's exact test. Survival curves for the patients were calculated using the Kaplan-Meier method and analysis was performed using the log-rank test.

**Results**

**Histology.** One of the carcinoma was well-differentiated, 37 moderately- and 11 poorly-differentiated. There was one Dukes' stage A, 15 stage B and 22 stage C. Eleven patients had liver metastases (stage D) at the time of diagnosis.

**Immunohistochemistry.** The results are summarized in Tables I-II.

a) *Smad4* protein expression: *Smad4* nuclear and cytoplasmic staining was absent (-) in 9/49 (18.3%) or reduced (+) in 18/49 cases (36.7%) (Figure 1). In the remaining 22 cases (44.8%), expression at levels comparable to colonic mucosa (++ to +++) was observed (Figure 2). In five mucinous carcinomas (three stage C and two stage D), strong expression of *Smad4* was retained.

b) *TGF-β2* expression: TGF-β2 cytoplasmic staining was observed in all cases. In 12/49 (24.5%) the staining was reduced (Figure 3), in 13/49 (26.5%) it was maintained, while it was increased in the remaining 24 cases (48.9%) (Figure 4).

**Statistics and survival analysis.** A statistically significant correlation was found between *Smad4* expression and tumour grade (p=0.02) and between TGF-β2 expression and Dukes' stage (p=0.03). A slight correlation tendency

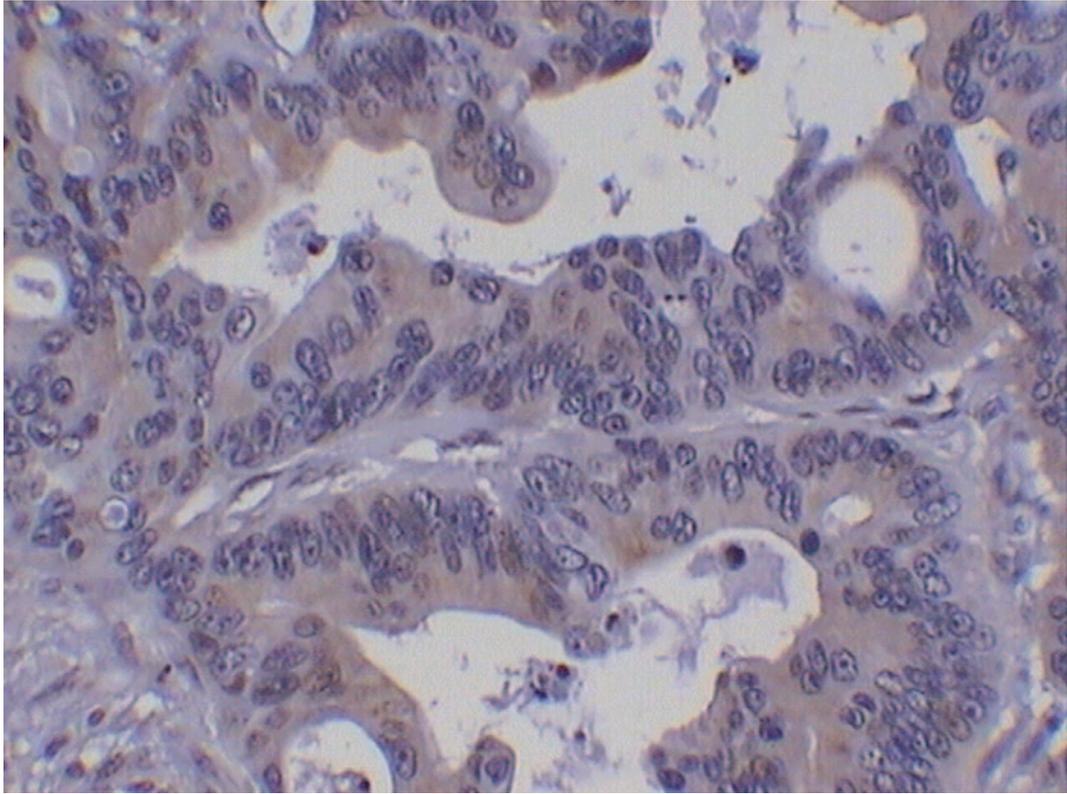


Figure 1. *Reduced Smad4 expression in colorectal carcinoma.*

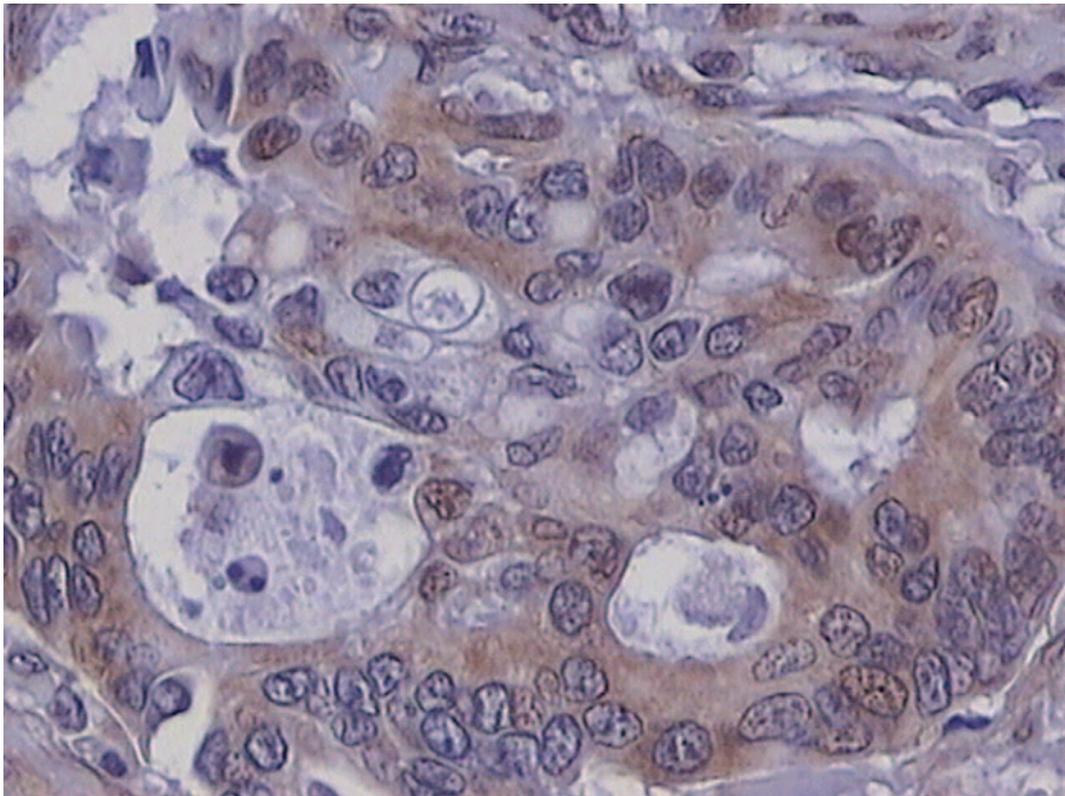


Figure 2. *Retained Smad4 expression in colorectal carcinoma.*

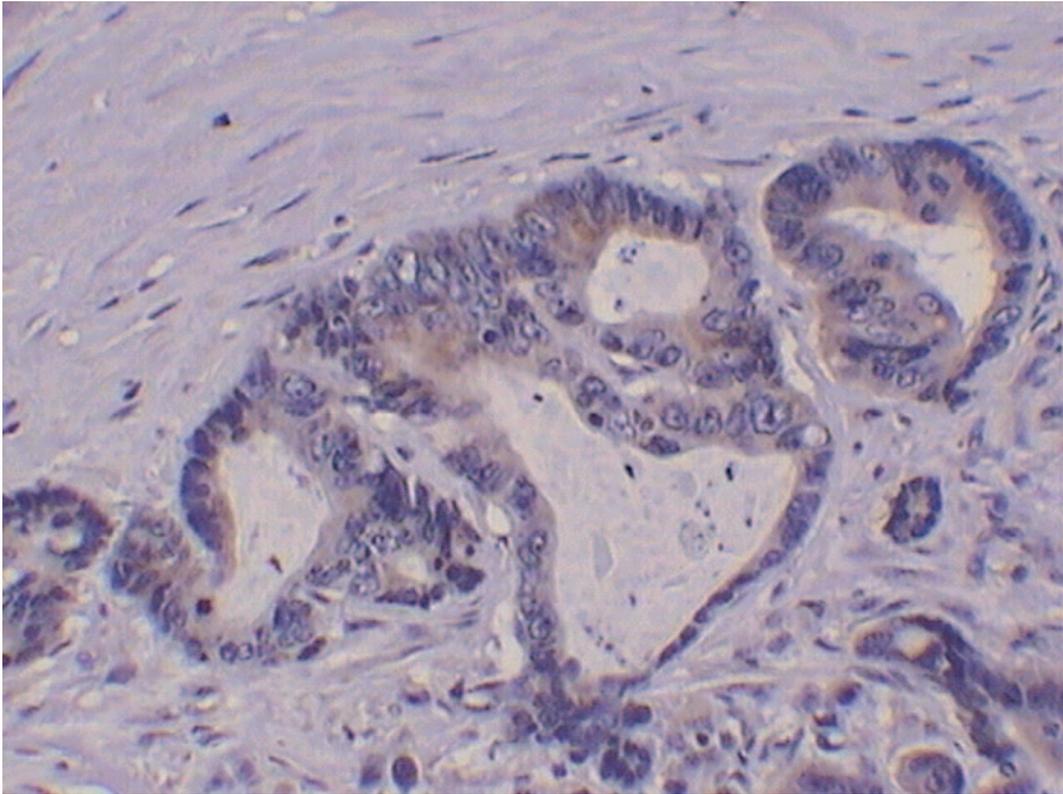


Figure 3. *Reduced TGF-β2 expression in colorectal carcinoma.*

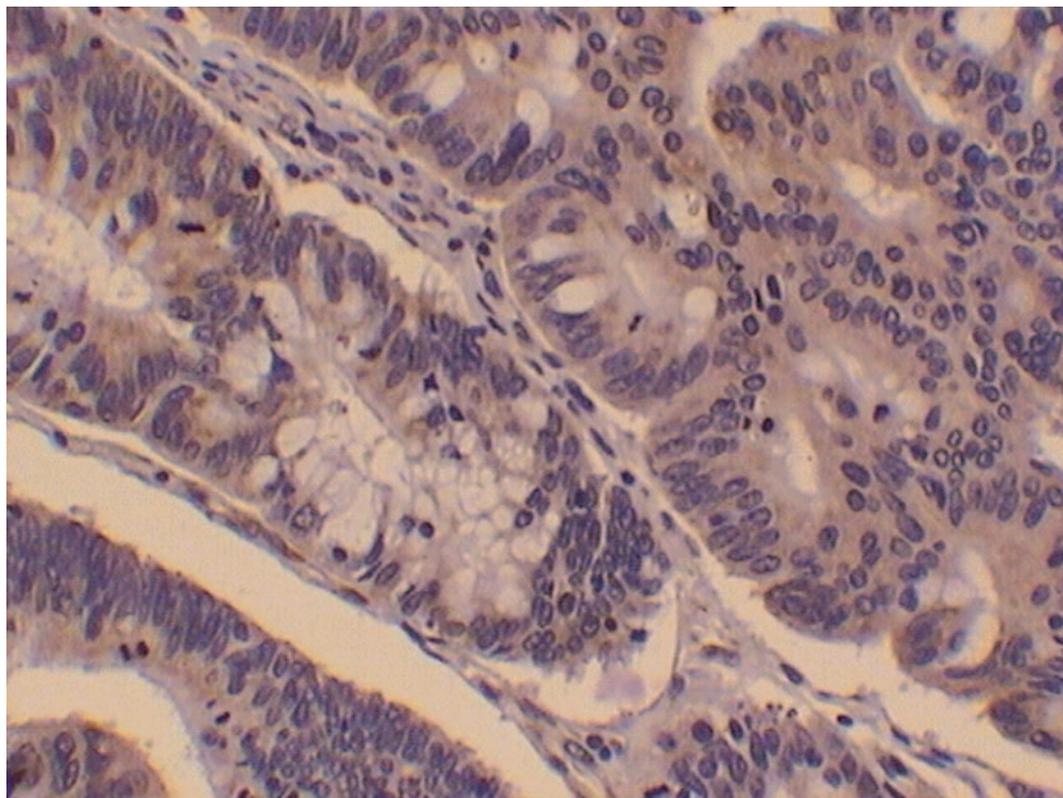


Figure 4. *Increased TGF-β2 expression in colorectal carcinoma.*

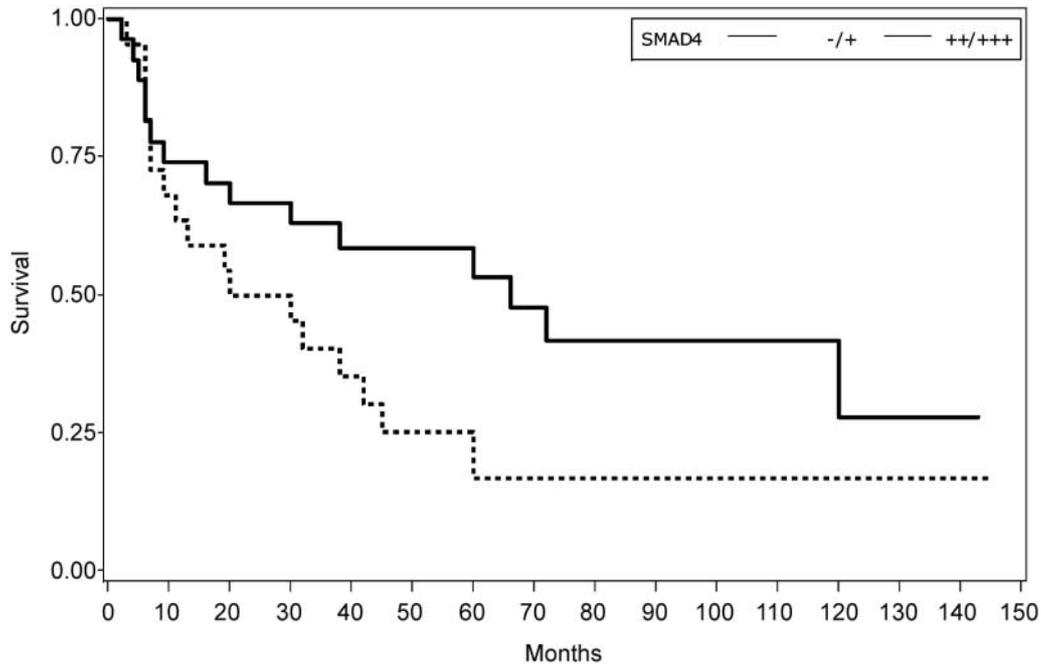


Figure 5. The postoperative survival curves of patients according to Smad4 expression.

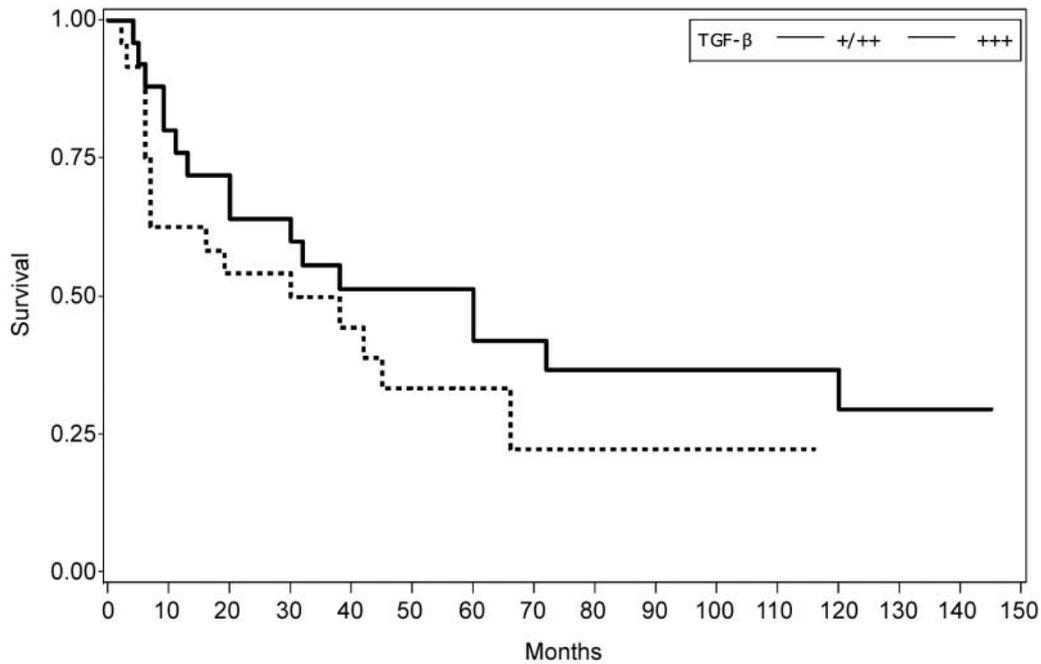


Figure 6. The postoperative survival curves of patients according to TGF- $\beta$ 2 expression.

was observed between TGF- $\beta$ 2 overexpression and retained Smad4 expression ( $p=0.25$ ) (Table III).

The survival curves did not differ significantly according to Smad4 ( $p=0.09$ ) or TGF- $\beta$ 2 ( $p=0.25$ ) expressions (Figure 5 and Figure 6, respectively).

## Discussion

One of the most important events in colon carcinogenesis appears to be the loss of genetic material in chromosome 18q21. A gene termed DCC (deleted in colorectal

Table III. Correlation of TGF-β2 with Smad4 expression.

TGF-β2	Total	Smad4	
		-, +	++, +++
+, ++	25	16	9
+++	24	11	13

p=0.25.

carcinoma) has been identified and proposed to be a candidate tumour suppressor gene (22). More recently, the gene DPC4 (deleted in pancreatic carcinoma 4, also called Smad4) was identified in the same region (3). Mutations of Smad4 have been found in a high proportion of pancreatic carcinoma and less frequently in colorectal, prostatic, head, neck, biliary tract and lung cancer (23, 24). Although loss of heterozygosity (LOH) at the 18q21 locus has been detected in 60% of colorectal carcinoma, only 14% showed mutations or homozygous deletions of Smad4 (9). The incidence of Smad4 inactivation was low in adenomas and localised carcinoma, but seemed to increase with tumour progression (25). Inactivating mutations in Smad4 have been reported in more than 30% of carcinoma with distant metastases (26).

Smad4 expression was reduced in 36.7% of our cases. This finding is in accordance with other studies (27), and may be of significance as it is possible that decreased expression of Smad4 contributes to malignant growth (28-30). The loss of Smad4 expression in 18.3% of our cases, most of them moderately- or poorly-differentiated carcinoma, suggests that inactivation of Smad4 is a late event in colorectal carcinogenesis (31). Strong expression of Smad4 was retained in mucinous carcinoma, suggesting that loss of Smad4 may play a less important role in this cancer subset. This finding is in agreement with that of Reinacher-Schick *et al.* (32). In the present study, the loss or reduced expression of Smad4 was also statistically significantly correlated with the grade of differentiation of the carcinoma. Miyaki *et al.* (25) found that the frequency of Smad4 mutations was also correlated with the stage of the carcinoma, a finding not confirmed in our study. In addition, the authors of the only study to correlate Smad4 expression and clinical outcome found that loss or reduced expression was associated with significantly shorter overall survival (33). In our study, the survival curves did not differ significantly according to Smad4 expression.

The overexpression of TGF-β has been reported in various types of carcinoma (34-38). In the present study, TGF-β2 increased in 48.9% of the cases. TGF-β2 overexpression was more frequently detected in poorly-differentiated carcinoma and was statistically significantly

correlated with the Dukes' stage. There was a slight tendency for TGF-β2 and Smad4 expressions to correlate. The unexpected retained expression of Smad4 in poorly-differentiated carcinoma may be due to TGF-β2 overexpression, which was also observed in these cases. Several studies have suggested that increased TGF-β expression appears to stimulate the metastatic potential (39-41) and to correlate with decreased survival (42, 43). The statistical analysis of our cases, however, revealed no statistically significant correlation between the expression of TGF-β2 and the clinical outcome.

The escape of tumour cells from TGF-β-mediated growth inhibition, as well as the ability of TGF-β to suppress the immune system, to induce angiogenesis and tumour stroma, have implied a possible role of TGF-β in the maintenance and progression of transformed cells in the host (44). Further studies are required to clarify the significance of the Smad4 / TGF-β signalling pathway to colorectal carcinogenesis.

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