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RESEARCH ARTICLE

Prognostic significance of transforming growth factor beta (TGF-β) signaling axis molecules and E-cadherin in colorectal cancer

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Abstract The transforming growth factor beta (TGF-β) signaling pathway has been considered both a tumor suppressor and a cancer promoter. Additionally, downregulation of cell adhesion molecules such as E-cadherin plays an important role in the metastatic potential of colorectal cancer (CRC). The aim of the present study was to evaluate TGF-β, TGF-β type I receptor (TGF-βR1), TGF-β type II receptor (TGF-βR2), Smad4, pSmad2/3, and E-cadherin expression in colorectal carcinoma and to correlate the obtained data with other standard prognostic parameters, such as disease stage, metastases, and patient survival. TGF-β, TGF-βR1, TGF-βR2, Smad4, pSmad2/3, and E-cadherin expression was evaluated immunohistochemically in 195 unrelated CRC specimens and the results subjected to various statistical analyses. TGF-β was expressed in 71.28%, TGF-βR1 in 61.0%, TGF-βR2 in 54.4%, Smad4 in 61.5%, pSmad2/3 in 71.3%, and E-cadherin in 50.26% of the colorectal carcinoma samples tested. The correlation of immunoexpression with the clinicopathological parameters of CRC revealed that the high expression of TGF-β and low expression of TGF-βR1, TGF-βR2, Smad4, pSmad2/3, and E-cadherin were correlated with tumor–node–metastasis (TNM) stage of disease. High TGF-β expression and low TGF-βR1, TGF-βR2, Smad4, and pSmad2/3 expression were also correlated with lymph node metastasis. The Kaplan–Meier survival curves demonstrated a clear association of cancer-specific overall survival with high TGF-β, as well as low TGF-βR1, TGF-βR2, Smad4, pSmad2/3, and E-cadherin expression. Our results suggest that TGF-β, TGF-βR1, TGF-βR2, Smad4, pSmad2/3, and E-cadherin are closely related to TNM stage of CRC. Moreover, TGF-β, TGF-βR2, Smad4, pSmad2/3, and E-cadherin emerge as valuable independent biomarkers of prognosis in CRC patients.

Keywords TGF-β signaling · Smads · E-cadherin · Colorectal cancer (CRC)

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Introduction

Colorectal cancer (CRC) remains the most common cancer and the second leading cause of cancer mortality in Europe (212,000 deaths, 12.3% of the total) [1]. Several mechanisms have been implicated in colorectal carcinogenesis, including chromosomal and microsatellite instability, serrated pathway and epigenetic mechanisms, different genes related to invasion and metastasis as well as the transforming growth factor beta (TGF-β)/Smad signaling pathway. The TGF-β pathway

is engaged in a number of biological processes, including cell proliferation, differentiation, migration, and apoptosis [2].

The initiation of TGF-β signaling begins with the binding of TGF-β ligands to TGF-β type II receptor (TGF-βR2), which in turn attracts and activates TGF-β type I receptor (TGF-βR1). TGF-β1 ligand is the most abundant isoform of the three (TGF-β1, TGF-β2, and TGF-β3) expressed in mammalian epithelium. Smad2 and Smad3 are phosphorylated at the C-terminal serines by the activated TGF-βR1 and form heteromeric complexes with Smad4. Ultimately, Smad2/3–4 complex translocates into the nucleus and binds to specific regulatory elements on target genes [3, 4].

The TGF-β signaling pathway is thought to be both a tumor suppressor and a cancer promoter [5]. TGF-β switches from an inhibitor of tumor cell growth to a stimulator of growth and invasion during human colon carcinoma progression. At present, the precise mechanism by which TGF-β pathway is able to switch from an inhibitor to a stimulator of carcinogenesis is unclear.

Furthermore, TGF-β acts as a major inducer of epithelial to mesenchymal transition (EMT) [6]. EMT is a crucial process during embryonic development that allows differentiation of multiple tissues and organs [7]. However, EMT also plays a role in pathological states and cancer progression [8]. Cell–cell adhesions are reduced in cancer. This leads to a loss of contact inhibition of proliferation and escape from growth arrest signaling. In order to metastasize, cancerous cells must escape adhesion from the primary site and proliferate elsewhere. Loss of the cell adhesion molecule E-cadherin is often the primary cause of the scattering of the cells to invasive carcinoma [9]. E-cadherin is one of the cadherins present in epithelial cells, and its binding via catenins to the cytoskeleton forms the functional component of adherens junctions [10]. It has been suggested that there is no relationship between loss of E-cadherin expression and tumor stage or survival in CRC patients [11, 12]. Nevertheless, other studies show an association between loss of E-cadherin expression and decreased survival [13–15].

The aim of this study was to evaluate the association among TGF-β signaling cascade molecules expression, including TGF-βR1, TGF-βR2, Smad4, activated (i.e., phosphorylated) Smad2/3 (pSmad2/3), and E-cadherin, in a well-characterized series of CRC population, and to determine whether they could be used as molecular biomarkers in CRC. Furthermore, we investigated whether the expression of these molecules correlates with recognized clinical and histopathological parameters, such as age, tumor location, stage of the disease, lymph node metastases, and patient survival, in order to define their potential prognostic role.

Material and methods

Patients and samples

Clinical data and pathologic tissue samples were retrieved from "Tzaneio" General Hospital, Pireaus, Greece. A total of 195 unrelated patients with CRC, who were surgically treated with the intention to cure between the years 2005 and 2006, were included in this study. No patient received neoadjuvant chemotherapy or radiotherapy. All patients were born in and living in Greece. All patients gave their informed consent, and the Hospital Review Board approved the protocol of the study.

All samples were formalin-fixed and paraffin-embedded, and the diagnosis of the tumor was established after observing sections stained with hematoxylin and eosin. Clinical and pathological information were entered into a database according to the criteria of tumor–node–metastasis (TNM) classification of the International Union Against Cancer [16]. All patients were followed up immediately after discharge from the hospital, and survival was measured from the date of surgery to the date of death or status at the last follow-up (December 2010). Patients who failed to appear for scheduled appointments during follow-up, or died from other causes unrelated to cancer, were censored and were not included in our study as no data or histological preparations were recorded for them. Surgical mortality was defined as death within 1 month from surgery. Table 1

Table 1 Clinical and histopathological characteristics of colorectal cancer patients (n=195)

Characteristics	Number (%)
Gender	
Male	103 (52.8)
Female	92 (47.2)
Age at diagnosis (years)	
Mean±SD	68.6±10.4
Range	36–90
Tumor location	
Cecum	20 (10.3)
Ascending	24 (12.3)
Transverse	11 (5.6)
Descending	29 (14.9)
Sigmoid	39 (20.0)
Rectum	72 (36.9)
Clinical stage	
I	37 (19.0)
II	76 (39.0)
III	58 (29.7)
IV	24 (12.3)
Lymph node metastasis	
Yes	75 (38.46)
No	120 (61.54)

summarizes the clinical and histopathological characteristics of the 195 CRC patients that were included in our study with a follow-up (median±SD and range) of 56.0±16.7 and 1–72 months, respectively. Sixty-three patients died from cancer-related causes during follow-up.

Immunohistochemical staining and evaluation

Sections from the colorectal adenocarcinoma and normal mucosa at the resection margins were obtained after surgical resection. For every case, one paraffin block containing both cancerous and normal mucosa tissue was selected for the immunohistochemical detection of TGF-β, TGF-βR1, TGF-βR2, Smad4, pSmad2/3, and E-cadherin protein expression.

Three-micrometer-thick sections were mounted onto glass slides coated with aminopropylmethoxysilane, dewaxed in xylene, and rehydrated with graded alcohols. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min. Before application of the primary antibody, sections were immersed in 10 mM citrate buffer (pH 6.0), rinsed in Tris-buffered saline (TBS), and heated in a microwave oven (650–800 W) for 3 cycles of 5 min each. In order to reduce nonspecific binding of antisera, sections were washed with TBS before application of the following primary antibodies (all purchased from Santa Cruz Biotechnology): antihuman polyclonal TGF-β1 (V) (sc-146, dilution 1:50), rabbit polyclonal IgG TGF-βR1 (V-22) (sc-398, dilution 1:50), rabbit polyclonal IgG TGF-βR2 (L-21) (sc-400, dilution 1:50), mouse monoclonal IgG Smad4 (B-8) (sc-7966, dilution 1:100), goat polyclonal IgG pSmad2/3 (Ser 423/425) (sc-11769, dilution 1:50), and anti-rabbit polyclonal E-cadherin (H-108) (sc-7870, dilution 1:200) against both wild and mutated forms. Sections were subsequently treated with the secondary antibody for 30 min and incubated with avidin–biotin–peroxidase complex for 30 min. Diaminobenzidine was used as a chromogen followed by slight hematoxylin counterstaining.

A TGF-β staining score was determined based on previously published methods [17–19]. TGF-β expression was considered positive whenever >10% of cancer cells were stained and negative if ≤10% of cancer cells were stained, after investigating the number of cytoplasmic TGF-β staining in ten fields (100 cells/field) under light microscopy.

For the immunohistochemical evaluation of TGF-βR1 and TGF-βR2, a semiquantitative method was used taking into account the intensity of staining and the percentage of cells stained. Thus, a score of 2 (≥50% of cells stained with high intensity) was considered positive, while a score of 1 (≥5% and <50% cells stained regardless of the intensity of staining, or >50% of cells stained with low or moderate intensity) and a score of 0 (≤5% cells stained regardless of the intensity) were considered negative.

The expression of Smad4 was assessed by comparing staining in tumor cells and normal epithelial cells. Tumorous cells which stained as strongly as the normal epithelial cells were considered positive (+). Weaker staining of tumor cells compared with the normal epithelial cells was considered weak (±), whereas absence of staining was considered negative (−). A preserved expression of Smad4 was classified if >50% of cells were positive and a reduced expression in all other cases [17].

Similarly, a staining score of 0 (≤10% cells stained regardless of the intensity) was considered negative for Smad4 and pSmad2/3, while a staining score of 1 (≥10% and <50% cells stained regardless of the intensity, or ≥50% cells stained with low or moderate intensity) and a staining score of 2 (≥50% cells stained with high intensity) were considered positive.

An E-cadherin staining score was considered positive whenever >25% of neoplastic cells had a preserved membranous expression as strong as the normal epithelial cells. A negative score was considered whenever 25% or less of neoplastic cells had preserved expression [20]. Assessments were performed by two independent pathologists who were blinded to the patient's data.

Statistical analyses

All statistical analyses and graphs were performed using MedCalc version 11.5.1.0 (www.medcalc.be) for Windows. Categorical variables were assessed by Student's *t* test or one-way analysis of variance and chi-squared test for continuous and noncontinuous variables as appropriate. Kaplan–Meier survival curves were constructed, and differences in survival between groups were compared using the log-rank test. Survival analysis was carried out by using cancer-specific death as the end point. Multivariable survival analysis was performed using a backward stepwise Cox proportional hazards regression model. A p value of 0.05 was considered as a limit for inclusion of a variable.

Results

Expression of TGF-β, TGF-βR1, TGF-βR2, Smad4, and pSmad2/3 in tumor and normal tissue

The 195 CRC specimens displayed a positive cytoplasmic staining reaction for TGF-β in 139 (71.3%) patients, while 56 (28.7%) had a negative staining (Table 2). TGF-β was found mainly in the cytoplasm (Fig. 1), and occasionally, some inflammatory cells were stained in the tumor stroma. In normal mucosa, the intensity of TGF-β staining was weakly positive in cells situated deep within the crypts and became gradually stronger towards the tip of the villi. Both

Table 2 Expression of TGF-β in comparison to clinicopathological parameters

	TGF-β$^+$ n=139	TGF-β$^-$ n=56	P value
Gender			0.7704
Male	72	31	
Female	67	25	
Age at diagnosis, years			0.9960
≤60	28	12	
>60	111	44	
Tumor location			0.5747
Cecum	14	6	
Ascending	18	6	
Transverse	6	5	
Descending	23	6	
Sigmoid	26	13	
Rectum	52	20	
TNM stage			0.0013
I	24	13	
II	46	30	
III	45	13	
IV	24	0	
Lymph node metastasis			0.0089
Yes	62	13	
No	77	43	

Table 3 Expression of TGF-βR1 in comparison to clinicopathological parameters

	TGF-βR1$^+$, n=119	TGF-βR1$^-$, n=76	P value
Gender			0.4368
Male	66	37	
Female	53	39	
Age at diagnosis, years			0.4473
≤60	27	13	
>60	92	63	
Tumor location			0.6544
Cecum	13	7	
Ascending	11	13	
Transverse	7	4	
Descending	21	8	
Sigmoid	24	15	
Rectum	43	29	
TNM stage			0.0408
I	8	29	
II	28	48	
III	29	29	
IV	11	13	
Lymph node metastasis			0.0282
Yes	38	37	
No	81	39	

normal mucosa and cancer cells exhibited a diffuse cytoplasmic pattern of staining. The receptors TGF-βR1 and TGF-βR2 were expressed in cell cytoplasms in 119 (61%) and 106 (54.4%), respectively, of the cancerous specimens (Tables 3 and 4; Figs. 2 and 3). Immunoexpression of these receptors in stroma could be detected in cells apparently representing fibroblasts and myofibroblasts.

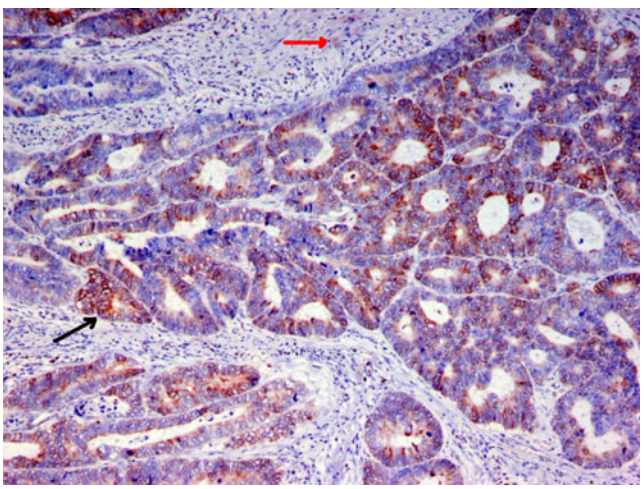

Fig. 1 Representative results of TGF-β immunostaining in CRC tissue. The image was taken at ×200 magnification. *Black arrow* demarcates cell cytoplasm stained by TGF-β antibody, and *red arrow* demarcates occasional staining of inflammatory cells by TGF-β antibody

The common mediator Smad4 displayed a positive staining in the tumor cytoplasm in 120 (61.5%) of the patients, while 75 (38.5%) had a negative staining (Table 5; Fig. 4). Tumor nuclear staining for Smad4 was rarely observed, while normal epithelial mucosa tissue displayed staining but not the lamina propria. pSmad2/3 exhibited a positive nuclear immunoexpression in 121 (62.1%) of the patients (Table 6; Fig. 5). pSmad2/3 was also detected in stromal cells of cancerous tissue.

Expression of E-cadherin in tumor and normal tissue

Altogether, 98 patients (50.26%) were positive and 97 patients (49.74%) were negative for normal E-cadherin staining (Table 7; Fig. 6). The normal colonic epithelium displayed a characteristic evenly distributed staining along the intercellular borders. No nuclear staining could be detected in the normal epithelial cells. In cancerous tissues, abnormal expression of E-cadherin was manifested as weak immunoexpression at the intercellular borders, faint cytoplasmic staining, or complete absence of staining. Occasionally, both membranous and cytoplasmic stainings of weak intensity were observed in cancerous cells.

Table 4 Expression of TGF-βR2 in comparison to clinicopathological parameters

	TGF-βR2$^+$, n=106	TGF-βR2$^-$, n=89	P value
Gender			0.8878
Male	56	47	
Female	50	42	
Age at diagnosis, years			0.5317
≤60	60	16	
>60	46	73	
Tumor location			0.4263
Cecum	12	8	
Ascending	12	12	
Transverse	9	2	
Descending	14	15	
Sigmoid	21	18	
Rectum	38	34	
TNM stage			0.0001
I	25	12	
II	58	18	
III	19	39	
IV	4	20	
Lymph node metastasis			0.0001
Yes	23	52	
No	83	37	

Expression of TGF-β, TGF-βR1, TGF-βR2, Smad4, pSmad2/3 and clinicopathological features

A significant correlation between TGF-β expression and other variables such as age, gender, and tumor location was not determined. However, a significant association between TGF-β expression and conventional prognostic

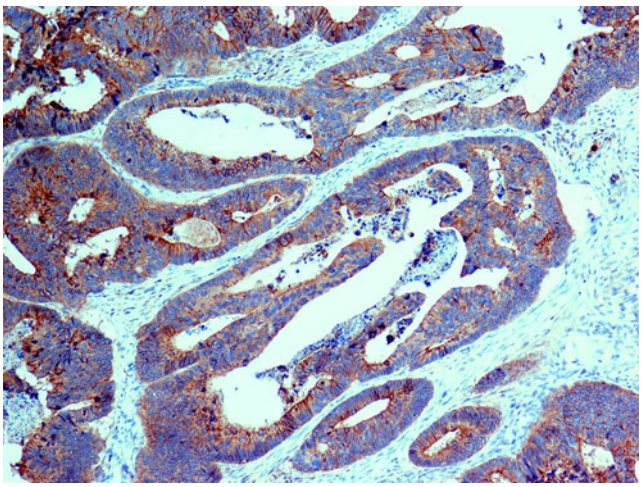

Fig. 2 Representative results of TGF-βR1 immunostaining in CRC tissue. The image was taken at ×200 magnification

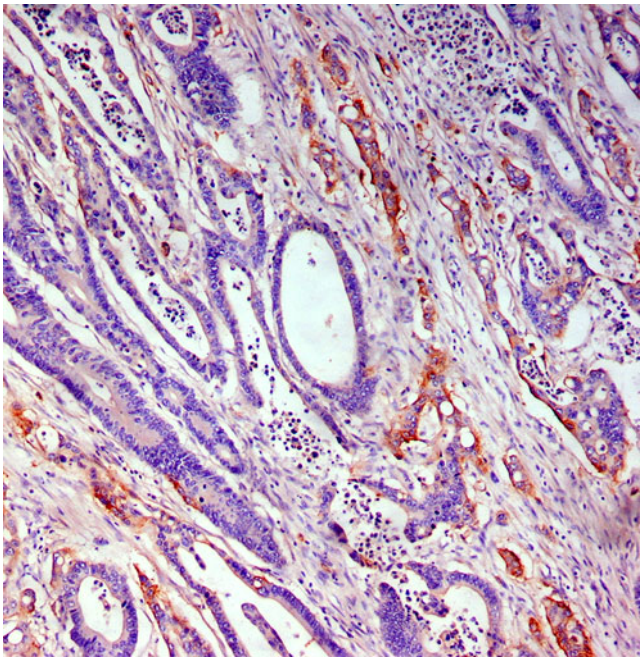

Fig. 3 Representative results of TGF-βR2 immunostaining in CRC tissue. The image was taken at ×200 magnification

Table 5 Expression of Smad4 in comparison to clinicopathological parameters

	Smad4$^+$, n=120	Smad4$^-$, n=75	P value
Gender			0.9729
Male	90	65	
Female	30	10	
Age at diagnosis, years			0.0750
≤60	30	10	
>60	90	65	
Tumor location			0.1876
Cecum	14	6	
Ascending	15	9	
Transverse	9	2	
Descending	21	8	
Sigmoid	23	16	
Rectum	38	34	
TNM stage			0.0299
I	26	11	
II	53	23	
III	31	27	
IV	10	14	
Lymph node metastasis			0.0441
Yes	39	36	
No	81	39	

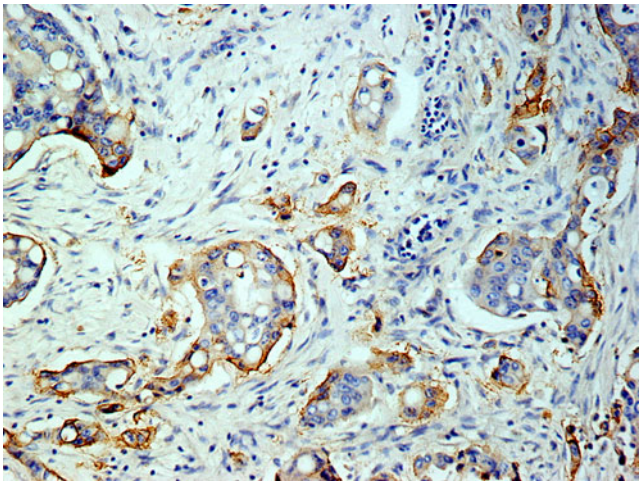

Fig. 4 Representative results of Smad4 immunostaining in CRC tissue. The image was taken at ×200 magnification

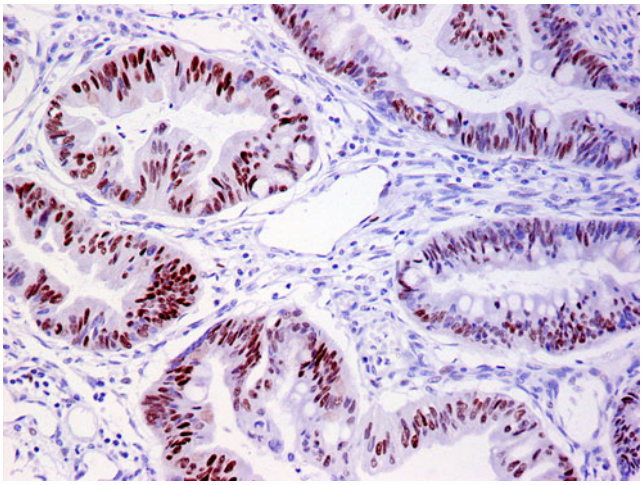

Fig. 5 Representative results of pSmad2/3 immunostaining in CRC tissue. The image was taken at ×200 magnification

factors such as TNM stage ($p<0.0013$) and lymph node metastasis ($p<0.0089$) was observed (Table 2). TGF-β immunopositivity was correlated with increasing clinical stage and the presence of lymph node metastasis. Both receptors of the TGF-β signaling pathway, namely TGF-βR1 and TGF-βR2, were not associated with any clinicopathological parameters other than TNM stage and presence of lymph node metastasis (Tables 3 and 4). Increasing TGF-βR1 and TGF-βR2 immunopositivity were correlated with earlier stage carcinomas ($p=0.0408$ and $p<0.0001$, respectively) and no lymph node metastasis ($p=0.0282$ and $p<0.0001$, respectively). Similarly, Smad4 and pSmad2/3 were correlated with the presence of lymph node metastasis and clinical stage (Tables 5 and 6). Further analysis revealed

Table 6 Expression of pSmad2/3 in comparison to clinicopathological parameters

	pSmad2/3$^+$, $n=139$	pSmad2/3$^-$, $n=56$	*P* value
Gender			0.7704
Male	72	31	
Female	67	25	
Age at diagnosis, years			0.9960
≤60	28	12	
>60	111	44	
Tumor location			0.5747
Cecum	14	6	
Ascending	18	6	
Transverse	6	5	
Descending	23	6	
Sigmoid	26	13	
Rectum	52	20	
TNM stage			0.0013
I	24	13	
II	46	30	
III	45	13	
IV	24	0	
Lymph node metastasis			0.0089
Yes	62	13	
No	77	43	

Table 7 Expression of E-cadherin in comparison to clinicopathological parameters

	E-cadherin$^+$, $n=98$	E-cadherin$^-$, $n=97$	*P* value
Gender			0.5160
Male	49	54	
Female	49	43	
Age at diagnosis, years			0.8308
≤60	19	21	
>60	79	76	
Tumor location			0.5310
Cecum	13	7	
Ascending	13	11	
Transverse	6	5	
Descending	13	16	
Sigmoid	16	23	
Rectum	37	35	
TNM stage			0.0253
I	24	13	
II	41	35	
III	26	32	
IV	7	17	
Lymph node metastasis			0.0683
Yes	31	44	
No	67	53	

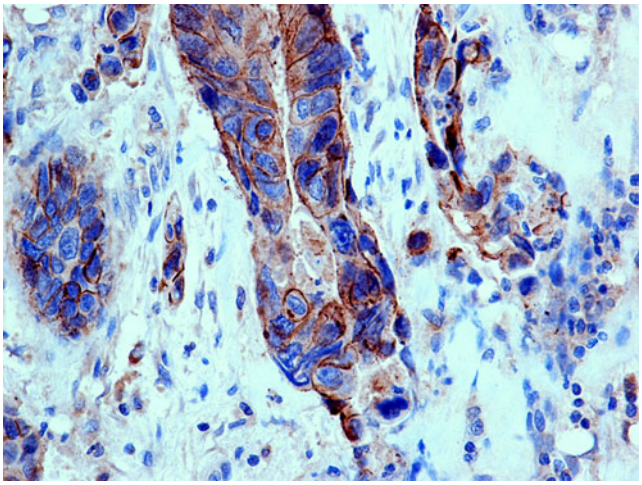

Fig. 6 Representative results of E-cadherin immunostaining in CRC tissue. The image was taken at ×400 magnification

that the patients with preserved Smad4 and/or pSmad2/3 immunoexpression had earlier stage CRC (p=0.0299 and p=0.0013, respectively) and absence of lymph node metastasis (p=0.0441 and p=0.0089, respectively). However, Smads were not associated with age, tumor location, and gender.

Expression of E-cadherin and clinicopathological features

No significant association between E-cadherin expression and age, gender, tumor location, and lymph node metastasis was noted. E-cadherin expression was significantly correlated with TNM staging (p=0.0253) and showed a trend for lymph node metastasis but did not reach statistical importance (p=0.0683; Table 7). The absence or reduced E-cadherin expression correlates with advanced-stage colorectal carcinoma.

Clinical outcome and the expression of TGF-β, TGF-βR1, TGF-βR2, Smad4, pSmad2/3, and E-cadherin

During the studied period, there were 63 cancer-related deaths. The univariate analysis demonstrated a significant association between increasing stages of disease (p<0.0001), lymph node metastasis (p<0.0001), and decreased overall cancer-specific survival (data not shown). Survival analysis for TGF-β was performed by grouping patients into two categories (negative expression and positive expression). The estimated cancer-specific overall survival rates for these groups were 86.20% and 55.40%, respectively (p=0.0003). The Kaplan–Meier cancer-specific curve by TGF-β expression demonstrated a clear association of poor cancer-specific overall survival after diagnosis of CRC with high TGF-β expression (Fig. 7a).

There was a significant difference in overall survival and the presence of receptors TGF-βR1 and TGF-βR2 (p=0.0001 and p<0.0001, respectively). Patients with lower receptors' immunoexpression had a worse clinical outcome (Fig. 7b, c). Similarly, patients with lower Smad4 and/or pSmad2/3 expression had a worse prognosis (Fig. 7d, e).

The prognostic value of E-cadherin was analyzed by grouping patients into two groups (positive score/negative score as discussed earlier). The cancer-specific survival rates with respect to these groups were 55.60% for patients with a negative score and 72.90% for patients with a positive score. The Kaplan–Meier graph is provided to show the association of negative immunoexpression of E-cadherin and poor prognosis (Fig. 7f). At multivariate regression analysis, TGF-β, TGF-βR2 (but not TGF-βR1), Smad4, pSmad2/3, and E-cadherin immunoexpression emerge as independent prognostic factors in CRC (Table **8**).

Discussion

EMT via TGF-β/Smad pathway has been studied in a variety of cancers [21–24] and other diseases [25, 26]. TGF-β is a tumor suppressor in the early stages of carcinogenesis, and in later stages, it is believed to play a dual role: in a paracrine and in an autocrine manner [27, 28]. In the latter, tumorous cells undergo EMT through TGF-β signaling, and as a result, these cells become more invasive with greater metastatic potential. TGF-β suppresses immune responses of non-transformed cells of tumor masses and augments angiogenesis, leading to progression and metastasis [27, 29].

In the present study, histological sections from a series of colorectal carcinomas were immunostained and underwent TGF-β, TGF-βR1, TGF-βR2, Smad4, pSmad2/3, and E-cadherin microscopical examination. TGF-β expression was maintained in 71.3%, TGF-βR1 in 61.0%, TGF-βR2 in 54.4%, Smad4 in 61.5%, pSmad2/3 in 71.3%, and E-cadherin in 50.26% of all cases. Increased TGF-β serum levels have been found to augment the metastatic potential of tumorous cells [30, 31] and to correlate with decreased survival. At the same time, E-cadherin reduced expression correlates with metastasis and poor clinical outcome [32, 33]. However, previous studies have explored the prognostic impact of E-cadherin and reported no significant correlation with survival [34].

Our results demonstrate that TGF-β and its downstream molecules TGF-βR1, TGF-βR2, Smad4, pSmad2/3, and E-cadherin were negatively correlated. TGF-β showed a positive correlation with advanced TNM stage and lymph node metastasis, while TGF-βR1, TGF-βR2, Smad4, pSmad2/3, and E-cadherin showed a negative correlation with advanced-stage and lymph node metastasis. These findings are in agreement with the work of other researchers [35, 36]. However, previous studies suggest that TGF-β has no apparent correlation with stage and lymph node metastasis [37,

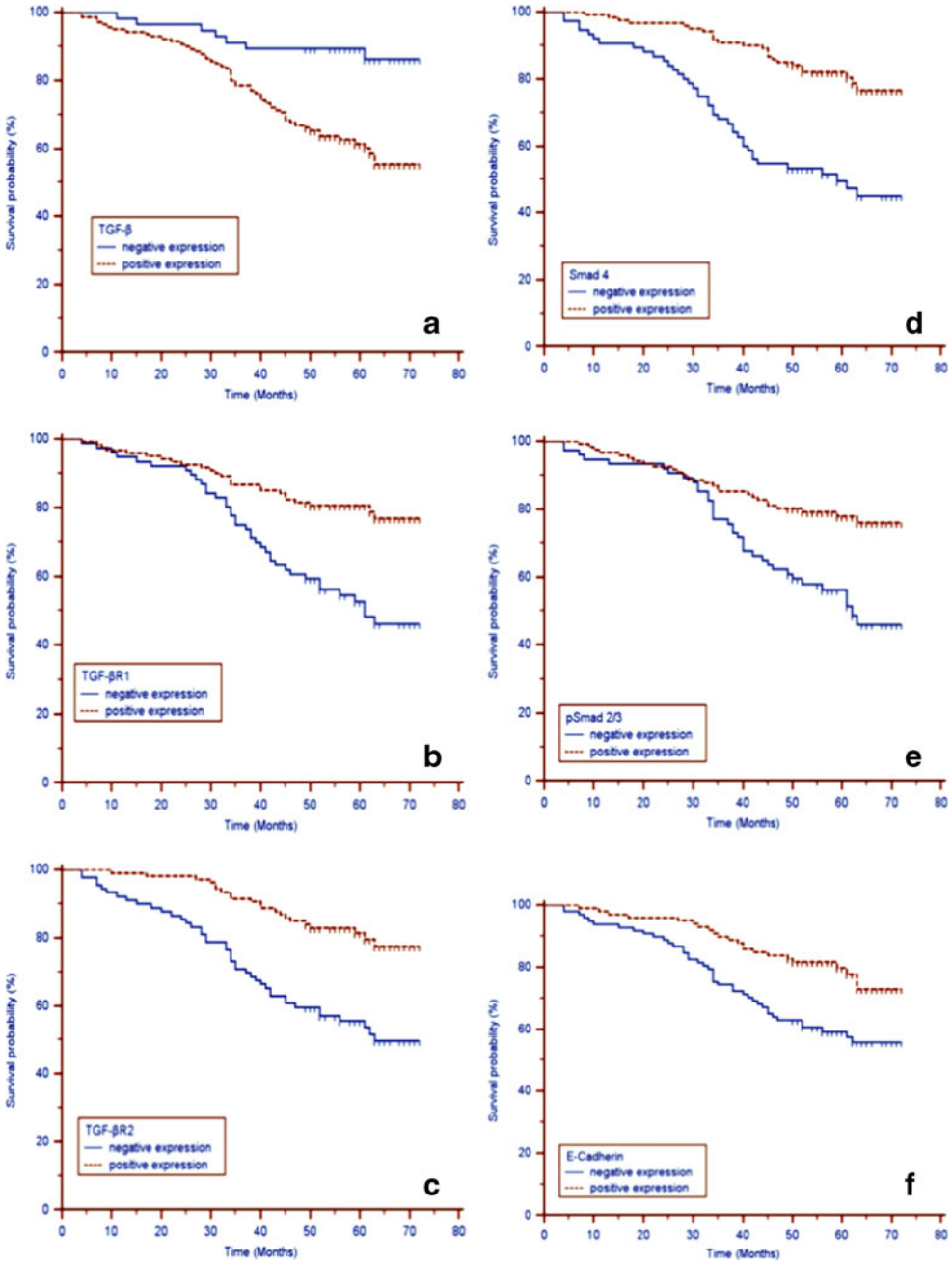

Fig. 7 Cancer-specific overall survival of patients after diagnosis of CRC by TGF-β (**a**), TGF-βR1 (**b**), TGF-βR2 (**c**), Smad4 (**d**), pSmad2/3 (**e**), and E-cadherin (**f**) immunoexpression

Table 8 Cox proportional hazard estimation analysis

Covariate	*b*	SE	*P*	Exp(b)	95% CI of Exp(b)
TGF-β	1.5423	0.4141	0.0002	4.6753	2.0852 to 10.4827
TGF-βR1	−0.5694	0.2959	0.0543	0.5659	0.3178 to 1.0076
TGF-βR2	−0.6051	0.2741	0.0273	0.5460	0.3199 to 0.9318
Smad4	−0.7870	0.3014	0.0090	0.4552	0.2529 to 0.8193
pSmad2/3	−0.7215	0.2625	0.0060	0.4860	0.2913 to 0.8109
E-cadherin	−0,6376	0.2655	0,0163	0.5286	0.3150 to 0.870

The analysis for disease status was performed by grouping patients into four groups (TNM stage I–IV), for markers (TGF-β, TGF-βR1, TGF-βR2, Smad4, pSmad2/3, and E-cadherin) by grouping patients according to positive and negative expression

38], and that TGF-β1 is related to an increased disease-free and overall survival. We have also shown that there is no significant correlation between TGF-β, TGF-βR1, TGF-βR2, Smad4, pSmad2/3, E-cadherin and other clinicopathological parameters. It was recently demonstrated that Smad4 expression is a common feature in colorectal cancer, in all age group patients, including early-onset colorectal cancer [39].

Our data pose that cancer cells of colorectal tumors in the earlier stages of carcinogenesis are modulated by TGF-β signaling pathway in a tumor suppression fashion; hence, normal activation of the TGF-β cascade occurs with the resulting suppression of EMT and normal expression of E-

cadherin at the intercellular borders of the cells. This signaling maintains the polarity and the integrity of the cells, thus blocking invasion and metastasis. In the advanced stages of carcinogenesis, tumor cells escape the suppressor effects of TGF-β. Indeed, the increased expression of TGF-β observed in our study does not lead to activation of its downstream receptors and effectors in a canonical manner, and a decreased expression of TGF-βR1, TGF-βR2, Smad4, and pSmad2/3 is noted. Conceivably, abnormal TGF-β signaling induces EMT which, in turn, promotes invasion and metastasis of tumor cells through reduced or altered expression of E-cadherin.

The switching of TGF-β signaling from a tumor suppressor to a tumor promoter is crucial in the process of carcinogenesis. TGF-βR2, the receptor which attracts TGF-β at first, has been found to be mutated in 30% of CRC cases [40], and restoration of TGF-βR2 expression results in suppression of tumorigenicity [41]. Recently, a new polymorphic allele of TGF-βR1 with a three-alanine deletion from a nine-alanine stretch in the N-terminus has been described and confirmed to be associated with CRC [42]. Furthermore, the frequency of mutational events of *Smad4* gene increases with the progression of carcinogenesis, being 0% in adenomas, 10% in intramucosal carcinomas, 7% in invasive carcinomas without distant metastasis, 35% in primary invasive carcinomas with distant metastasis, and 31% in carcinomas which metastasized to the liver and distant lymph nodes, or disseminated [43]. Moreover, other data revealed that mutations in *Smad2* gene are associated with colorectal carcinoma of the sporadic form and suggested that *Smad2* could be a candidate tumor suppressor gene [44]. Interestingly enough, there are no *Smad3* mutations identified so far in human colon cancer. All the above could be potential mechanisms that drive the TGF-β signaling pathway from a tumor suppressor to a tumor promoter. Another important consideration is the role of TGF-β in colorectal carcinogenesis through pathogenic inflammation. Since colon cancer is driven by inflammation, the role of TGF-β in cancer-associated inflammation remains to be clarified. Nevertheless, additional studies are required to elucidate these important issues.

In summary, TGF-β, TGF-βR2, Smad4, pSmad2/3, and E-cadherin appear to be useful biomarkers of prognosis for patients with CRC. The escape of TGF-β-mediated growth inhibition of tumorous cells as well as the ability of TGF-β to induce EMT through E-cadherin might play a role in tumor progression and metastasis. In the era of targeted anticancer treatment, research efforts in this area might be helpful for future therapeutic approaches with regimens targeted against TGF-β signaling components.

Conflicts of interest None

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