Abnormal expression of Endoglin and its receptor complex (TGF-ß1 and TGF-ß receptor II) as early angiogenic switch indicator in premalignant lesions of the colon mucosa

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Received April 16, 2010; Accepted June 21, 2010

DOI: 10.3892/ijo_00000767

Abstract. The precise timing of the angiogenic switch in colorectal cancer development is still unclear. The simultaneous expression of Endoglin (CD105), transforming growth factor (TGF)-ß1 and TGF-ß receptor (R) II were quantified in surgical specimens comprising normal human colon, pre-malignant dysplastic tissue, *in situ*, and invasive colon cancer specimens, at mRNA and protein levels, respectively by real-time PCR and immunohistochemistry. Serum concentrations of soluble Endoglin and TGF-ß1 were evaluated. mRNA and CD105$^+$-microvessel density (MVD) increased significantly in dysplastic colon and carcinoma versus normal tissues; values correlated respectively with dysplasia degree and Dukes' stages. TGF-ß1 expression was significantly upregulated in most severe dysplastic adenoma specimens, while TGF-ß1 transcript and protein signals were intense in carcinoma, positively-correlated with tumor progression. TGF-ß1 RII was overexpressed in adenoma and carcinoma versus normal samples, but unrelated with dysplasia or Dukes' stage. Soluble Endoglin serum levels were equivalent in adenoma and normal tissues; in carcinoma the highest levels were in invasive tumor. Circulating TGF-ß1 levels were increased in severe dysplasia and progressed with tumor progression. Correlations between adenoma dysplasia degree and TGF-ß RII and CD105$^+$-MVD, and between tumor Dukes' staging and TGF-ß1 and CD105$^+$-MVD, were significant. TGF-ß1 and Endoglin and TGF-ß1 serum levels, TGF-ß1 staining and CD105$^+$-MVD were significantly and inversely associated with disease-free survival. TGF-ß1 levels were an independent and significant prognostic factor of disease-free survival. These findings suggest active angiogenesis occurs in many pre-malignant colon cases and supports more careful evaluation of different chemopreventive agents.

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Key words: Endoglin, TGF-ß1, TGF-ß receptors, colon adenoma, colorectal carcinoma

Introduction

Colon carcinoma is the third commonest form of cancer and the second cause of cancer-related death in the Western world, leading to 655,000 deaths worldwide per year (1). Despite the emergence of new targeted agents and the use of various therapeutic combinations, none of the treatment options available is curative in patients with advanced cancer.

Neoplastic conversion of human colorectal cells occurs in a stepwise fashion, leading from normal colonic epithelium to cancer (2). Despite the model adenoma-carcinoma sequence associated with an accumulation of genetic events (3), there are still no absolute criteria that can predict adenoma progression to cancer. Tumor angiogenesis is a critical step in the development, metastatic spread and regrowth of cancer. Tumors promote angiogenesis by secreting factors, including vascular endothelial growth factor (VEGF)-A, basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-ß (4-6). Several reports suggest that an angiogenic switch may be activated in the premalignant stage of several human cancers (7-14).

TGF-ß is a multifunctional family of cytokines, which play a dual and paradoxical role in cancer: they can function as tumor suppressors in the early stages of epithelial tumorigenesis, having strong anti-proliferative, pro-apoptotic and tumor-growth-inhibiting effects; however, they can subsequently act as a tumor promoter factor, stimulating the epithelial-to-mesenchymal transition (EMT), invasiveness of cancer cells and inhibiting immune surveillance (15). Importantly, TGF-ß also plays an important role in angiogenesis by promoting proliferation and migration of endothelial cells at low concentrations, and leading to vessel maturation at high concentrations (6,16). Furthermore, TGF-ß is known to induce VEGF expression (17). Genetic studies and knockout mice for the different components of the TGF-ß signaling pathway have shown that TGF-ß is indispensable for angiogenesis (18). TGF-ß1 is over-expressed in most human cancers (19).

Three isoforms of the cytokine are recognized: TGF-ß1, TGF-ß2, and TGF-ß3; each is encoded by a separate gene and has distinct *in vivo* functions (20). The core TGF-ß signaling pathway comprises type I [also known as the activin receptor-like kinase-5 (ALK5)] and type II receptors, where type I acts downstream of type II, and the combinatorial heterodimeric association of these receptors determines the specificity of the ligand signaling (21). Type III receptors (22) include the auxiliary molecules Endoglin and betaglycan.

Endoglin, designated as CD105, is a homodimer cell-membrane protein capable of fine-tuning the availability of TGF-ß to the signaling receptors, thereby determining the outcome of TGF-ß's biological activity (23). It has been demonstrated that Endoglin requires association with TGF-ß receptor (R) type II to bind ligands, and that it can interact with TGF-ß RI or RII in the absence of ligand (24). Based on different experimental data, it has been shown that Endoglin binds with high affinity to TGF-ß1 and TGF-ß3 and, through its interactions with the TGF-ß R type I and type II, regulates their phosphorylation status and subsequently their signalling ability (25). Moreover, considerable experimental evidence shows that Endoglin is expressed at low levels in resting mature and immature endothelial cells, and that it is overexpressed in vascular endothelial cells of tissues undergoing active angiogenesis, such as regenerating and inflamed tissues, or tumors (26,27). Furthermore, levels of Endoglin positively correlate with the extent of endothelial cell proliferation (28) and with the expression of proliferation markers in tumor endothelia (29). In addition, Endoglin/CD105 has been suggested to be the most suitable marker available to quantify tumor angiogenesis (30).

The expression profiles of Endoglin in association with TGF-ß1 and its receptor have not been studied in colon pre-malignant lesions. We decided to analyze comparatively the expression of functional receptor complexes Endoglin/TGF-ß1/TGF-ß RII, at successive stages of the colon normal-adenoma-carcinoma sequence, to verify their role in tumor development and progression, providing the rationale for precautional therapeutic targeting opportunities.

Patients and methods

Patients. The study group comprised 75 patients who had received a diagnosis of colon adenoma [n=32, 22 men, 10 women, aged median (range) 57 (32-77)], or adenocarcinoma [n=50, 25 men, 25 women, aged median (range) 61.5 (24-87)] at the Department of Clinical Physiopathology, Division of General Surgery (University of Turin, Turin, Italy) between January 2004 and June 2008. None had undergone anti-cancer treatment before entering the study, which was conducted under strict observance of the principles of the Declaration of Helsinki. Adenoma tissue specimens were obtained from endoscopic maneuvers and cancer specimens from surgical resections. All cancer patients had histopathologically-confirmed primary colorectal adenocarcinomas and were staged by Dukes' system, revised by Astler and Coller (31). Table I shows details of the patients and their diseases. Adenoma specimens were graded by patho-logists for degree of dysplasia (n=10 low, L; n=12 moderate, M; n=10 high, H). Histopathologically-confirmed normal colon biopsies (n=10) were used as controls. Complete follow-up data were available for all patients. Among patients with polyps, the median follow-up was 44 months (range 35-96); none developed tumor during this period. At conclusion of the study, 12 (24%) carcinoma patients had died and 38 (76%) were still alive of whom 22 (44%) remained disease-free, whereas 28 (56%) had a recurrence. The median time to recurrence was 10 months (range 1-50). The median follow-up period for survivors was 11 months (range 2-55 months).

Tissue and serum specimens. Aliquots of fresh normal, pre-malignant and malignant colon tissue were fixed in formalin- and paraffin-embedded for immunohistochemical analysis, or placed in liquid nitrogen prior to mRNA extraction. Fresh blood samples from patients and normal subjects were centrifuged at 700 g for 20 min at 4˚C. The upper phase (serum) was removed, divided into aliquots and stored at -20˚C until use.

RNA extraction and reverse transcription. Frozen sections (6 μm) were taken from blocks of tissue and, starting with the first section, every fifth section was routinely stained with hematoxylin and eosin and evaluated histopathologically by an experienced pathologist (Francesco A. Mauri). Sections from areas estimated to have at least 80% malignant cells were pooled for *Endoglin* mRNA analysis. Similarly, colon mucosa specimens from patients free of neoplastic or inflammatory diseases were examined by the pathologist and defined as 'normal' tissue. Total RNA was isolated using TRIzol Reagent (Invitrogen, Life Technologies, Gaithersburg, MD) following the manufacturer's instructions, and the RNA concentration was determined by spectrophotometry. Total RNA was treated with DNase I (Invitrogen, Life Technologies) and reverse-transcribed using ImProm-II Reverse Transcription System (Promega, Corp., Madison, WI). Briefly, 360 ng of DNase I treated RNA were incubated with 0.5 μg of random primer at 70˚C for 5 min, then added to 15 μl of reverse transcription reaction mix containing 1X ImProm-II reaction buffer, 3 mM $MgCl_2$, 0.5 mM dNTPs, 20 U RNasin Ribonuclease Inhibitor and 1 μl ImProm-II Reverse Transcriptase and incubated at 42˚C for 60 min.

Real-time quantitative RT-PCR. Real-time quantitative PCR analysis was performed on iCycler iQ system (Bio-Rad, Hercules, CA) via SYBR Green I dye detection. Complementary DNA (cDNA) corresponding to 5 ng of total RNA was amplified in a reaction (25 μl total volume) containing 1X iQ SYBR Green Supermix (Bio-Rad) as recommended by the manufacturer. Primers were added to the reaction mix at a final concentration of 300 nM. The primer sequences used were as follows: *rRNA 18S*, forward primer CTG CCC TAT CAA CTT TCG ATG GTA G, and reverse primer CCG TTT CTC AGG CTC CCT CTC (GenBank accession no. X03205); *Endoglin*, forward primer GCC AGC ATT GTC TCA CTT CAT G, and reverse primer GCA ACA AGC TCT TTC TTT AGT ACC A (GenBank accession no. AH006911); *TGF-ß1*, forward primer GAC ACC AAC TAT TGC TTC AG, and reverse primer CAG GCT CCA AAT GTA GGG (GenBank accession no. X02812); *TGF-ß RII*, forward primer AGC ATC ACG GCC ATC TGT G and reverse primer: TGG CAA

Table I. Main pathological features of the colon carcinoma population studied.

Carcinoma Dukes' stages	A[1]	B1[1]	B2[2]	C1-C3[3]	D[4]	Total
N.	8 (16%)	7 (14%)	13 (26%)	13 (26%)	9 (18%)	50 (100%)
Grading						
G1	2	1	0	0	0	3 (6%)
G2	5	5	11	9	6	36 (72%)
G3	2	1	1	4	3	11 (22%)
Tumor site						
Right colon	2	3	5	3	4	17 (34%)
Transverse colon	0	0	1	1	0	2 (4%)
Descending and Sigmoid colon	3	2	5	5	7	22 (44%)
Rectum	1	2	1	2	3	9 (18%)

Data are presented as medians (range) for continuous variables, and as frequencies (%) for categorical variables.

ACC GTC TCC AGA GT (GenBank accession no. M85079). Negative controls omitted cDNA from specific PCR amplification. For each patient the PCR was run in triplicate. Specificity of the amplicons was confirmed from the melting curve of the PCR product at the end of the reaction. PCR efficiency (E) was determined using the iCycler iQ software and the method described by Ramakers *et al* (32). For each sample the Ct was acquired using the Fit point Method (33). The relative expression ratio of the target cytokine genes was computed using the relative expression software tool (REST) (34): this software calculates an expression ratio relative to the control group (normal tissue) normalized to rRNA 18S. The expression ratio (R) is: $R=E_{target}^{\Delta Ct \text{ target (mean control-mean sample)}}/E_{reference}^{\Delta Ct \text{ reference (mean control-mean sample)}}$.

Immunohistochemical detection of CD105, TGF-ß1 and TGF-ß RII. Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded serial sections. The anti-CD105 (SN6h, Dako, Carpinteria, CA, USA) reacting with both forms of Endoglin (the smaller form with M_r 160,000, termed S-EDG and the larger form with M_r 170,000, termed L-EDG) (35), anti-TGF-ß1, and -TGF-ß RII monoclonal antibodies (Santa Cruz Biotech., Santa Cruz, CA) were diluted 1:10, 1:100, and 1:70, respectively, in phosphate-buffered saline (PBS) in accordance with the manufacturer's recommendations. Briefly, sections were dewaxed and rehydrated through decreasing alcohol series up to distilled water. Endogenous peroxidase activity was suppressed by incubation with 3% (v/v) hydrogen peroxide in phosphate-buffered saline (PBS) for 10 min. Heat-induced epitope retrieval was performed with Target Retrieval Solution (Dako) using an electric pressure-cooker for 20 min at 120˚C, with cooling before immunostaining. Non-specific binding was blocked using 1% (v/v) normal horse serum for 30 min, and the slides were then incubated with diluted primary antibodies for 1 h followed by PBS wash. Antibody binding was localized using a secondary biotin-labeled anti-mouse antibody and the streptavidin-conjugated horseradish peroxidase LSAB-2 kit (Dako), and revealed using 3,3'-diaminobenzidine (DAB) as the chromogenic substrate for peroxidase. The slides were then rinsed with PBS, counterstained with Mayer's hematoxylin and mounted with mounting medium.

All the sections were interpreted by a pathologist (F.A. Mauri) who was blind to clinical findings. Positive and negative controls were included in all reactions. MVD were quantified by the method proposed by Weidner *et al* (36). Briefly, the sections were scanned at magnification x100 to select the three areas with the most vascularization (hot-spots), in which the microvessels were counted at magnification x200 and their density expressed as the mean number of microvessels/mm^2. Any brown-stained single cell or cell cluster, with or without a discernible lumen, which was clearly separated from the adjacent microvessels, tumor cells or other elements of connective tissue was considered to be a countable vessel. TGF-ß1 and TGF-ß RII immunostaining was scored by the immunoreactive score (IRS) system proposed by Rammele and Stegner (37) in which IRS = SI (staining intensity) x PP (percentage of positive cells). SI was classified as 0, negative; 1, weak; 2, moderate; and 3, strong. PP was defined as 0, negative; 1, 1-20% positive cells; 2, 21-50% positive cells; and 3, 51-100% positive cells. Ten visual fields from different areas of each specimen were chosen at random for IRS evaluation, and the average IRS was calculated.

Determination of Endoglin and TGF-ß1 concentrations in sera. Soluble Endoglin and TGF-ß1 concentrations were determined in serum samples collected from adenoma and cancer patients and control subjects, using a commercial ELISA kit (R&D Systems, Abingdon, UK), following the

manufacturer's instructions. For TGF-ß1 determination, serum samples were tested after transient acidification (reduction of the pH to 1.5 by addition of 1 N HCl for 10 min at room temperature and neutralization with 1.2 N NaOH in 0.5 M HEPES). All samples were evaluated in duplicate. The minimum detectable concentrations of Endoglin ranged from 0.001 to 0.030 ng/ml, for TGF-ß1 from 1.7 to 15.4 pg/ml.

Statistical analysis. For statistical analysis the non-parametric Mann-Whitney test was used to compare median values with clinico-pathological variables. The correlation between different parameters was analyzed using the non-parametric Spearman correlation coefficient. Disease-free survival rates were analyzed using Kaplan-Meier curves and the related log-rank test. Calculation of the relative hazard ratios and the relative 95% confidence intervals using Cox proportional hazard model was employed. Differences were considered significant when $p<0.05$. All of the statistical analyses were performed using the SPSS for Windows (13.0) package (SPSS, Inc., Chicago, IL).

Results

Expression of Endoglin, TGFß-1 and TGF-ß RII mRNA in normal, dysplastic and malignant colorectal tissues. Endoglin, TGFß-1 and TGF-ß RII gene expression was quantitatively assessed in normal colon, adenoma at various degrees of dysplasia and adenocarcinoma at different Dukes' stages, using real-time RT-PCR. Normalized gene expression was compared between the different groups, and expression ratios were calculated using the REST software. As shown in Fig. 1, in general *Endoglin* mRNA was significantly up-regulated in adenoma and tumors versus (vs) control normal mucosa ($p\leq0.001$ and <0.001, respectively) and in carcinoma vs adenoma ($p=0.002$). Interestingly, when the different degrees of colon polyp dysplasia (mild, moderate, high) were associated with *Endoglin* expression mRNA ratio, a progressive increase in *Endoglin* transcript accumulation in adenomatous tissue was observed (Table II). When cancer patients were divided into Dukes' stages A, B, C or D, these different disease stages were not associated with significant variations in *Endoglin* mRNA expression ($p>0.05$), excepting Dukes' A vs Dukes' B ($p=0.046$) (Table II).

TGF-ß1 mRNA levels in adenoma and tumors were significantly more abundant than in normal colon mucosa ($p=0.006$ and <0.001, respectively) and in carcinoma vs adenoma ($p<0.001$) (Fig. 1). There were no significant differences in *TGF-ß1* mRNA levels between the different degrees of colon polyp dysplasia (Table II). By contrast, when cancer patients were categorized on the basis of tumor stage of the Dukes' classification, a progressive increase in *TGF-ß1* transcript accumulation was observed ($p<0.05$) (Table II).

TGF-ß RII mRNA accumulation was similar in normal, adenoma and carcinoma. Moreover, the different degree of displasia of adenoma and Dukes' staging in carcinoma could not be distinguished on the basis of *TGF-ß RII* transcript (Table II).

Endoglin, TGF-ß1 and TGF-ß RII proteins in situ expression in normal, dysplastic and malignant colorectal tissues. Since

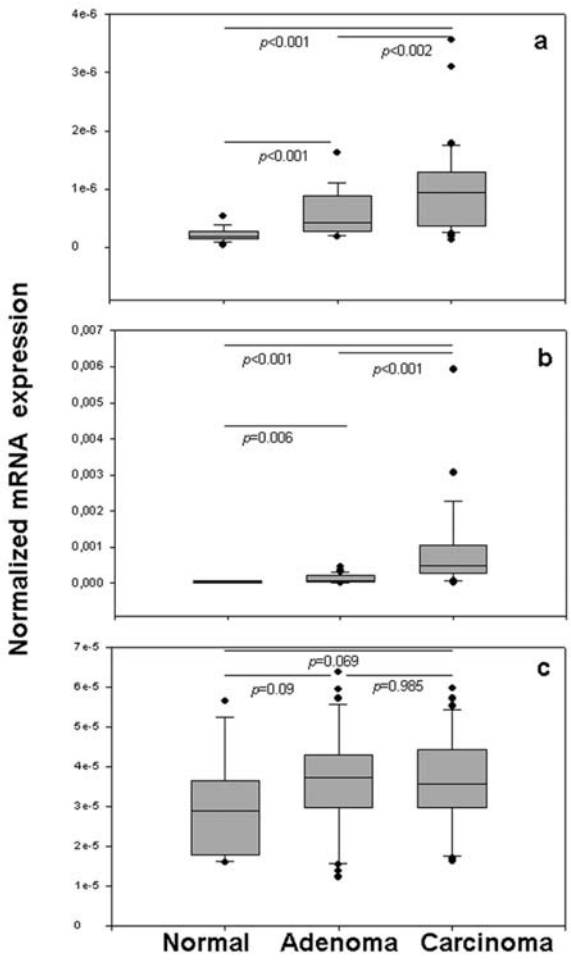

Figure 1. Expression levels of *Endoglin*, *TGF-ß1* and *TGF-ß1 RII* mRNA in normal mucosa, polyps and carcinoma of the colon were assessed via real-time RT-PCR, and normalized to rRNA 18S levels. Normalized *Endoglin*, *TGF-ß1* and *TGF-ß1 RII* mRNA expression values are presented in (a), (b) and (c), respectively. Median, 10th, 25th, 75th and 90th percentiles are presented as vertical boxes with error bars. Dots indicate outliers. P-values obtained by the Mann-Whitney U test.

mRNA expression does not necessarily correspond to protein expression, and because the presence of varying amounts of normal counterpart in specimens cannot be excluded, we next analyzed adenoma and tumor tissues to establish, by immunohistochemistry, *in situ* expression status of Endoglin, TGF-ß1 and TGF-ß RII in comparison with normal colon mucosa.

Fig. 2 shows representative Endoglin/CD105 differential expression patterns for normal, dysplastic and malignant colorectal tissues. The microvessels in colon mucosa from normal controls were usually minimally positive or totally negative to CD105. In adenoma, CD105 was expressed by 27% of vascular endothelial cells, preferentially observed in the surface area. In the tumor tissues, CD105 stained positively only in a subset of microvessels (64%). Preferentially, the anti-CD105 mAb stained endothelial sprouts and small vessels with a restricted lumen. The median (range) $CD105^+$-MVD-calculated for normal, adenoma and carcinoma cases were $0/mm^2$ (0-1), $1.8/mm^2$ (0-4), and $3.8/mm^2$ (2.12-9.760), respectively. As shown in Fig. 3, a significant increase in $CD105^+$-MVD occurred in the normal-adenoma-carcinoma sequence (normal vs adenoma, $p=0.001$, normal vs carcinoma,

Table II. *Endoglin, TGF-ß1* and *TGF-ß RII* gene expression ratios in relation to colon adenoma and carcinoma specimens.

	Gene expression ratio median (range)[a]		
	Endoglin	*TGF-ß1*	*TGF-ß RII*
Adenoma (n=32)	2.010 (0.867-7.646)	1.824 (0.190-10.170)	1.237 (0.409-2119)
Mild dysplasia (n=10)	1.154 (0.867-1.252)	1.806 (0.590-10.170)	1.243 (0.409-3557)
	0.003[b,c]	0.531	0.489
	<0.001[d]	0.602	0.687
Moderate dysplasia (n=12)	2.011 (1.058-2.760)	1.507 (0.296-7.800)	1.390 (0.933-3.999)
	<0.001[e]	0.156	0.385
Severe dysplasia (n=10)	4.392 (3.559-7.646)	3.569 (0.190-7.128)	2.248 (0.461-2.902)
Carcinoma (n=50)	4.392 (0.601-16.734)	10.602 (0.555-131.600)	1.337 (0.537-2.989)
Dukes' A (n=8)	1.644 (1.235-7.971)	2.353 (0.555-5.797)	1.261 (0.744-2.789)
	0.654[f]	<0.001	0.586
	0.046[g]	<0.001	0.927
	0.207[h]	<0.001	0.913
Dukes' B1/B2 (n=20)	4.017 (0.601-7.807)	8.437 (5.859-17.666)	1.783 (0.537-2.586)
	0.082[i]	0.043	0.409
	0.324[j]	0.001	0.579
Dukes' C (n=13)	5.151 (1.546-16.734)	12.643 (7.424-67.576)	1.471 (0.590-2.466)
	0.449[k]	0.016	0.943
Dukes' D (n=9)	5.467 (0.850-8.367)	33.875 (16.537-131.600)	1.190 (0.565-2.989)

[a]Calculated according to the REST-software. [b]P-value obtained by Randomization test. [c]Mild dysplasia vs moderate dysplasia. [d]Mild dysplasia vs severe dysplasia. [e]Moderate dysplasia vs severe dysplasia. [f]Dukes' stage A vs Dukes' stage B. [g]Dukes' stage A vs Dukes' stage C. [h]Dukes' stage A vs Dukes' stage D. [i]Dukes' stage B vs Dukes' stage C. [j]Dukes' stage B vs Dukes' stage D. [k]Dukes' stage C vs Dukes' stage D.

p<0.001, adenoma vs carcinoma, p<0.001). As shown in Table III, an increment of $CD105^+$-MVD from mild-grade dysplasia to high-grade dysplasia was statistically significant. Moreover, $CD105^+$-MVD increased in the process of tumor progression from Dukes' A to Dukes' C.

Representative TGF-ß1 differential expression patterns for normal, dysplastic and malignant colorectal tissues are shown in Fig. 2. Moderate or strong immunoreactivities were observed in 81.1% of cases (27/38) of colorectal cancer, as compared with no positivity or faint staining in 20% cases of normal colon mucosa. TGF-ß1 in adenoma and tumor samples was stained more intensely than it was in normal epithelial cells [IRS median (range): 3 (1-4) vs 0.25 (0-1) and 2(0.5-9) vs 0.25 (0-1), respectively, p<0.001]. No difference was observed between adenoma and carcinoma (p=0.573) (Fig. 3). Moreover, as shown in Table III, among the different degrees of adenoma dysplasia only high dysplasia could be distinguished on the basis of TGF-ß1 staining. By contrast, when tumor samples were analyzed by Dukes' classification, staining intensity with anti-TGF-ß1 antibody gradually increased in successive stages of colorectal tumor progression. This trend was statistically significant.

Representative TGF-ß RII immunostaining patterns for normal, dysplastic and malignant colorectal tissues are shown in Fig. 2. A uniform distribution of TGF-ß RII was found in normal colon epithelial cells as well as in vascular endothelial cells and fibroblasts in the stroma [median (range) IRS: 2 (1-4)]. In general, TGF-ß RII showed a patchy distribution of staining in adenoma, more intense than in normal tissues [median (range) IRS: 3 (0.5-6) vs 2 (1-3), p=0.031]. TGF-ß RII was overexpressed in tumors compared with dysplastic and normal tissues [median (range) IRS: 4 (1-9) vs 3 (0.5-6) and 2 (1-4), p=0.002 and p=0.005, respectively]. In a few neoplastic samples, a low content of receptor was observed in individual cells within the malignant epithelial clusters (6%). The immunoreactivity of the endothelial and stromal compartments was similar in normal, adenoma and tumor tissues (Fig. 3). As shown in Table III the different degrees of adenoma dysplasia and the Dukes stage in colorectal carcinoma could not be distinguished on the basis of TGF-ß RII expression.

Soluble Endoglin and TGF-ß1 serum levels in patients with colon adenoma and carcinoma versus healthy donors. Since in certain tumors soluble Endoglin concentrations are increased (38), using specific ELISA we measured soluble Endoglin levels in sera from adenoma and colon carcinoma patients vs normal controls. As shown in Fig. 4a, in general

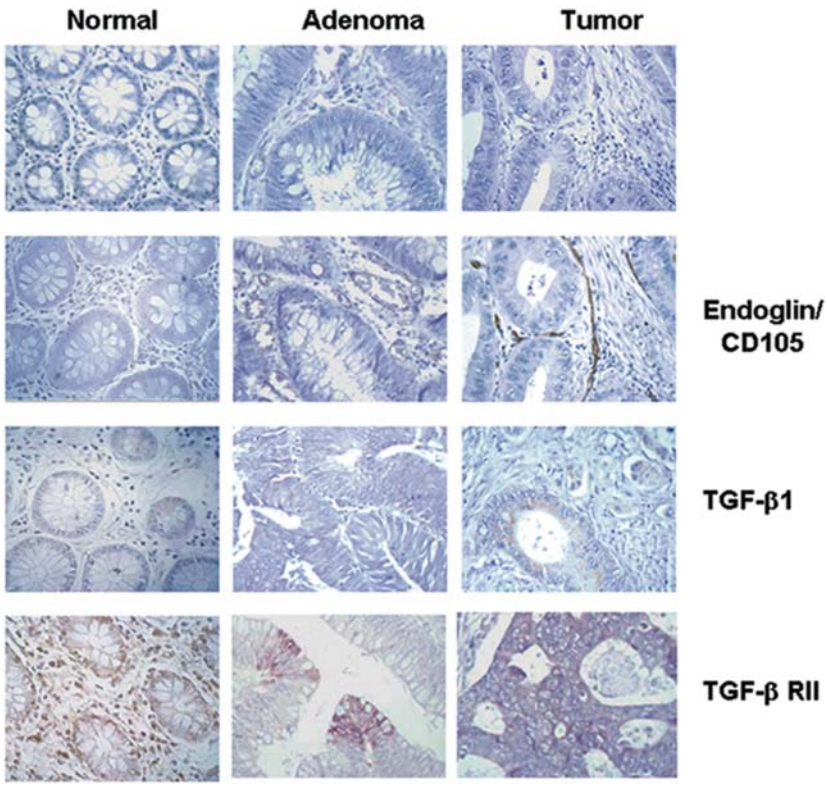

Figure 2. Patterns of typical Endoglin/CD105, TGF-ß1 and TGF-ß RII immunostaining in normal, dysplastic and malignant colorectal tissues, using a representative example of a moderate dysplasia adenoma and Dukes' D colon carcinoma (original magnification x250).

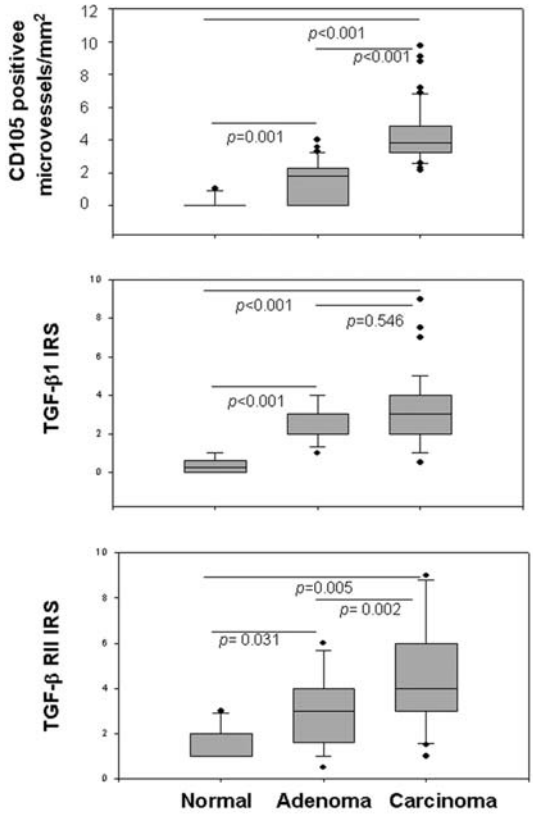

Figure 3. Quantitative analysis of immunohistochemical staining of normal, dysplastic and neoplastic colonic mucosa with anti-CD105, -TGF-ß1, TGF-ß RII antibodies. The microvessel density (MVD) and immunoreactive scores (IRS) were obtained as described in Materials and methods. Median, 10th, 25th, 75th and 90th percentiles are presented as vertical boxes with error bars. Dots indicate outliers. P-values obtained by the Mann-Whitney U test.

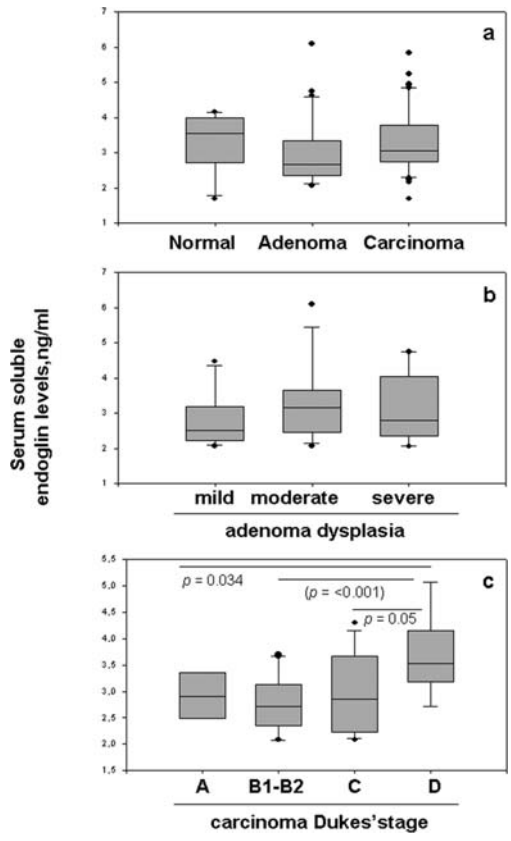

Figure 4. Serum Endoglin concentration by ELISA in normal, adenoma and carcinoma (a), in adenoma patients categorized by degree of dysplasia (b), and in carcinoma patients categorized by Dukes' stage (c). Median, 10th, 25th, 75th and 90th percentiles are presented as vertical boxes with error bars. Dots indicate outliers.

Table III. Endoglin, TGF-ß1 and TGF-ß RII protein expression in normal tissue, colon adenoma and carcinoma specimens.

	Immunoreactivity		
	Endoglin (CD105+-MVD)[a]	TGF-ß1 (IRS)[b]	TGF-ß RII (IRS)
Adenoma			
Mild dysplasia (n=10)	0 (0-2.3)	2 (1-3)	2 (0.5-6)
	0.009[c]	0.074	0.845
	0.001[d]	0.008	0.1
Moderate dysplasia (n=12)	2 (0-3)	3 (2-4)	2.75 (0.5-6)
	0.134[e]	0.321	0.09
Severe dysplasia (n=10)	2.2 (1-4)	3.5 (2-4)	4 (2-5)
Carcinoma (n=50)			
Dukes' A (n=8)	2.660 (2.170-3.670)	1 (0.5-2)	3.5 (1.5-9)
	0.006[f]	0.006	0.623
	0.001[g]	<0.001	0.882
	<0.001[h]	<0.001	0.633
Dukes' B1/B2 (n=20)	3.535 (2.120-4.50)	2.5 (1-4)	3.5 (1-9)
	<0.001[i]	0.026	0.593
	<0.001[j]	<0.001	0.321
Dukes' C (n=13)	4.670 (3.20-9.09)	3 (2-5)	3.5 (1-9)
	0.125[k]	0.025	0.472
Dukes' D (n=9)	5.30 (4.230-9.760)	5 (3-9)	5 (1.5-9)

[a]MVD calculated as reported in Materials and methods. [b]IRS calculated as reported in Materials and methods. [c]Mild dysplasia vs moderate dysplasia. [d]Mild dysplasia vs severe dysplasia. [e]Moderate dysplasia vs severe dysplasia. [f]Dukes' stage A vs Dukes B. [g]Dukes' stage A vs Dukes C. [h]Dukes' stage A vs Dukes D. [i]Dukes' stage B vs Dukes C. [j]Dukes' stage B vs Dukes D. [k]Dukes' stage C vs Dukes' stage D.

no statistically-significant differences were observed between normal subjects and patients with benign or neoplastic disease [(median (range) values 3.547 (1.689-4.165) ng/ml, 2.052 (2.530-6.100) and 3.059 (1.689-5.834) ng/ml, respectively (p>0.05)]. Moreover, although serum Endoglin concentrations were not correlated with the degree of dysplasia (Fig. 4b), all of the highest Endoglin values were detected in serum samples from Dukes' D carcinoma patients (Fig. 4c). Post-operative Endoglin serum measurement, performed approximately one week after surgery in colorectal carcinoma patients, showed no significant difference compared to pre-surgery values [median (range) 3.059 (1.689-5.834) ng/ml vs 3.035 (2.080-5.050) ng/ml, p=0.266)].

As shown in Fig. 5A, serum levels of TGF-ß1 in patients with colorectal carcinoma median (range) [40.5 (24-60) pg/ml] were significantly higher than those in the healthy control group [34.7 (18.3-45.6) pg/ml, p=0.017] and than those in adenoma patients [34 (20-45) pg/ml, p<0.001]. TGF-ß1 serum concentrations were significantly higher in high dysplastic adenoma (p>0.05) [high dysplasia: 40.5 (32-45) pg/ml vs low dysplasia: 25.5 (21-41) pg/ml, p=0.001] (Fig. 5B). Serum levels of TGF-ß1 also increased from Dukes' tumor stages A to C and D (p<0.01) (Fig. 5C). The median serum level of TGF-ß1 in patients with colorectal carcinoma before surgery dropped significantly, to 34 (22-49) pg/ml, after curative surgical resection of the tumor (p<0.001). Serum levels of TGF-ß1 after tumor resection decreased more significantly in Dukes' D patients with higher preoperative levels of TGF-ß1, from 53.5 (43-60) pg/ml to 36 (28-49) pg/ml (p<0.001) which was within the normal range.

Correlation between CD105+-MVD, TGF-ß1 IRS, TGF-ß RII IRS and circulating Endoglin and TGF-ß1 levels in patients with colon adenoma and carcinoma. To investigate the relationship between *in situ* Endoglin, TGF-ß1 and TGF-ß RII expression and circulating Endoglin and TGF-ß1 levels, we used the Spearman's rank correlation test to correlate the findings obtained by immunohistochemistry and the serum concentrations of the proteins detected by ELISA. In adenoma patients, no significant association was found between any of the variables considered. By contrast, statistically significant positive correlations were found in carcinoma patients: CD105+-MVD vs serum Endoglin levels (r=0.308, p=0.03), TGF-ß1 IRS vs circulating TGF-ß1 levels (r=0.567, p=0.0001), TGF-ß1 IRS vs TGF-ß RII IRS (r=0.281, p=0.04), circulating TGF-ß1 levels vs CD105+-MVD (r=0.623, p=0.0001), TGF-ß1 IRS vs serum Endoglin levels (r=0.332, p=0.02), and TGF-ß1 IRS vs CD105+-MVD (r=0.494, p=0.0001).

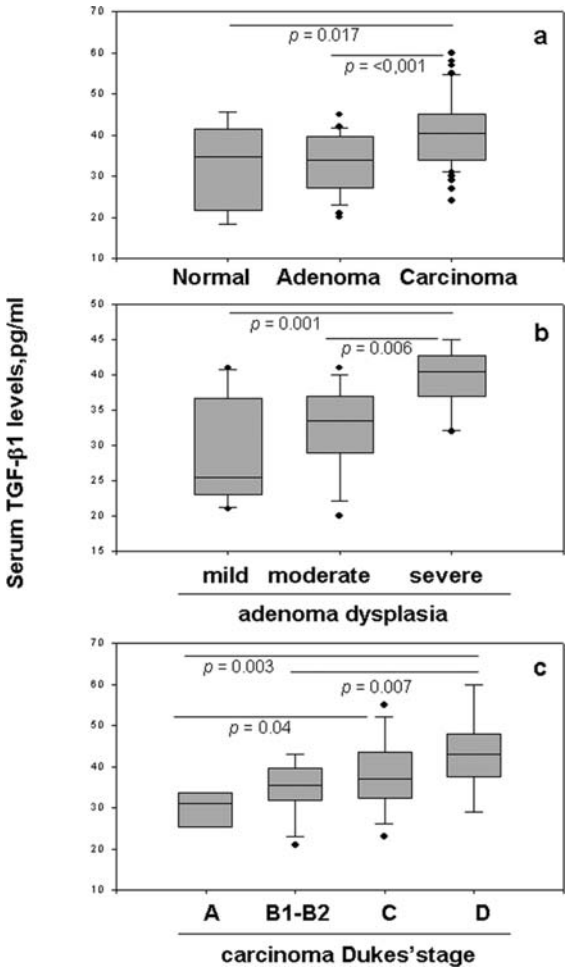

Figure 5. Serum TGF-ß1 concentration by ELISA in normal, adenoma and carcinoma (a), in adenoma patients categorized by degree of dysplasia (b), and in carcinoma patients categorized by Dukes' stage (c). Median, 10th, 25th, 75th and 90th percentiles are presented as vertical boxes with error bars. Dots indicate outliers.

Association of CD105⁺-MVD, TGF-ß1 IRS, TGF-ß RII IRS and circulating Endoglin and TGF-ß1 levels with degree of dysplasia in adenoma, Dukes' stage in carcinoma, and disease-free survival. To clarify the clinical significance of our study, we examined the correlation between the degree of dysplasia in adenoma, the Dukes' stage in carcinoma, and *in situ* expression and circulating levels of the parameters considered. As shown in Table IV, when Endoglin and its receptor complex components were compared with the degree of dysplasia in adenoma patients (n=32, of whom 10, low dysplasia; 12, moderate dysplasia; 10, high dysplasia), a statistically significant correlation was found between dysplasia degree and TGF-ß RII and CD105⁺-MVD (p=0.039, and p=0.0001, respectively). When CD105⁺-MVD, TGF-ß1 IRS, TGF-ß RII IRS and circulating Endoglin and TGF-ß1 levels were compared with carcinoma patients' Dukes' stages (n=50) of whom 8 Dukes' A, 20 Dukes' B, 13 Dukes' C, and 9 Dukes' D, a statistically-significant correlation was found between Dukes' stage and circulating TGF-ß1 and Endoglin levels (p=0.0001, and p=0.005, respectively) and with CD105⁺-MVD (p=0.0001).

Kaplan-Meier curves for disease-free survival in carcinoma patients, using median values of different variables as cutoff point between low and high patient groups, combined with univariate analysis, found no difference in disease-free survival for the two groups in the case of TGF-ß RII (p=0.44), whereas TGF-ß1 and Endoglin plasma levels, TGF-ß1 IRS and CD105⁺-MVD were significantly and inversely associated with patient disease-free survival: patients with high TGF-ß1 (≥40.5 pg/ml) and Endoglin (≥3.05 ng/ml) plasma levels, TGF-ß1 IRS (≥3) and CD105⁺-MVD (≥3.8) had significantly shorter disease-free survival (p=0.009, 0.02, 0.002, and 0.0002, respectively) (Fig. 3). Multivariate analysis indicated that only plasma TGF-ß1 levels were an independent and significant prognostic factor of disease-free survival (p=0.005), being associated with increased risk of poor prognosis (Cox proportional hazard model (HR)=1.11, 1.03-1.20; 95% CI)).

Discussion

Most colon adenomas remain benign and asymptomatic lesions. However, a small proportion may evolve to malignancy, and there is evidence indicating that almost all colorectal carcinomas develop from adenomatous polyps (39). The angiogenic switch, the process of developing a high-density

Table IV. Correlation of Endoglin and TGF-ß1 serum levels, CD105⁺-MVD and TGF-ß1 and TGF-ß RII immunostaining with polyp dysplasia degrees and carcinoma Dukes' stages.

	Dysplasia degree		Dukes' stage	
	r[a]	P-value	r[a]	P-value
TGF-ß1 (serum levels, pg/ml)	-0.0837	0.647	0.751	0.0001
TGF-ß1 (IRS)	0.000	0.999	0.733	0.0001
TGF-ß RII (IRS)	0.367	0.039	0.152	0.291
	0.36		0.36	
Endoglin (serum levels, ng/ml)	0.152	0.403	0.388	0.005
CD105⁺-MVD	0.633	0.0001	0.839	0.0001

[a]r, correlation Spearman's coefficient.

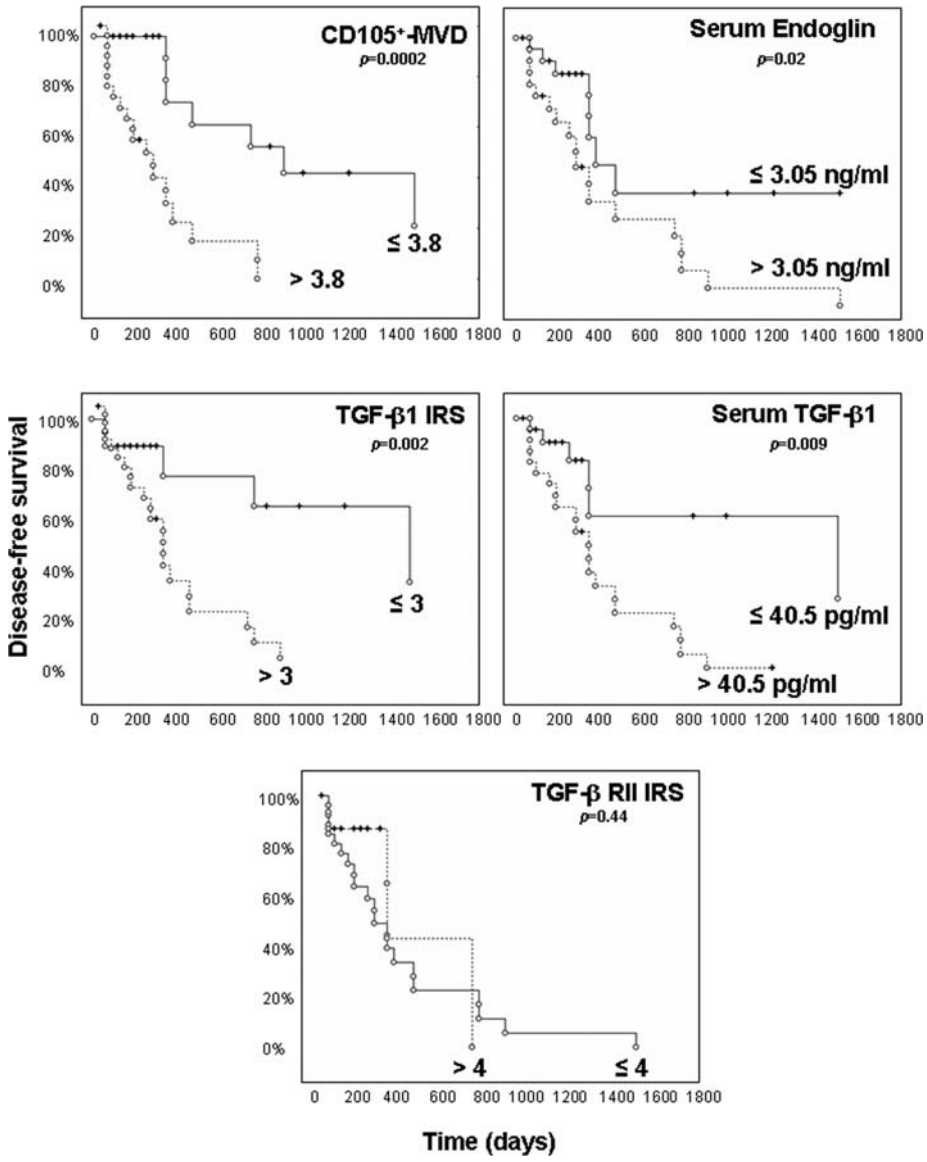

Figure 6. Disease-free survival (analyzed by the Kaplan-Meier method) for patients with high (> median value) and low (≤ median value) CD105+-microvessel density (MVD), Endoglin and TGF-ß serum levels, TGF-ß1 immunoreactive scores (IRS) and TGF-ß RII IRS. The CD105+-MVD and IRS were obtained as described in the Materials and methods. The significant differences between the two groups are reported (log-rank test).

vascular network that connects tumor and host circulation, is a crucial step for the progression of a tumor from a benign to malignant state (7). Since it was originally believed that the initiation of angiogenesis occurs simultaneously with tumor invasion (40), few studies have investigated angiogenesis at the individual stages of the spectrum from dysplasia to *in situ* and invasive colon carcinoma (41,42). In this study, we evaluated for the first time the simultaneous expression of the Endoglin/TGF-ß RII functional complex and its ligands TGF-ß1 in the colon adenoma-carcinoma sequence, together with its clinical significance. Our data suggest that angiogenesis is initiated early in this sequence, although the greatest increase occurs with tumor invasion. Endoglin, the most reliable marker of proliferating endothelial cells (43), is weakly expressed in normal colon tissue but up-regulated, at both mRNA and protein levels, in adenoma. A progressive increase in Endoglin transcript accumulation and CD105+-MVD occurred in adenomatous tissue, from mild to severe dysplasia. These data suggest that tumor angiogenesis is not necessarily a characteristic of invasive tumor, as originally thought, but may be an early event during colon cancer development. Since the expression of CD105 significantly differed from low-grade to high-grade adenoma and to carcinoma whereas, as has been reported, the MVD for a pan-endothelial markers (CD34, CD31) does not (42), it is possible that the very early stages of dysplastic transformation already acquire their vasculature, not only by incorporation of pre-existing normal vessels (recognized by pan-endothelial markers), but also by induction of new blood vessels (recognized by CD105); this ability is characteristic of tumor behavior. Based on experimental evidence, it has been demonstrated that angiogenesis precedes overt tumor formation during chemically-induced carcinogenesis, suggesting that tumor progression depends on a switch from a prevascular to a vascular phase (43). Furthermore,

MVD has been found significantly increased in a relatively large spectrum of pre-malignant lesions, including oral mucosa (44), bronchial epithelium (45), skin (46), colon dysplasia (41), Barrett's epithelium (47), and the uterine cervix (9).

Our study also shows that accumulation of mRNA for Endoglin is upregulated in colon carcinoma tissues, in comparison with normal and dysplastic colon mucosa. As expected, we found overexpression of Endoglin protein in the endothelium of colon carcinoma tissue, positively correlated with disease progression. These results are in agreement with studies showing that the $CD105^{+}$-MVD value is a prognostic marker in colon carcinoma (41,48-50), as well as in prostate (51), breast (52,53), kidney (54), esophageal (55,56), head and neck (57,58), uterus (59) and non-small cell lung cancers (60).

Endoglin is not only expressed on the cell surface, but can also be released after proteolytic shedding of the membrane-associated extracellular domain, and may be detected in the blood (62). Quantification of circulating Endoglin levels revealed, for the first time, no significant difference between patients with colon adenoma and normal subjects. Moreover, soluble Endoglin levels were not correlated with the degree of dysplasia. We also found that, in general, serum Endoglin levels in colon carcinoma patients did not differ from those of healthy controls and patients with adenoma. However, all of the highest Endoglin values were detected in serum samples from advanced, metastatic carcinoma patients. This is in line with findings relating to colon carcinoma (48,62,63), breast cancer, and other solid tumor patients (62,64). Therefore, circulating Endoglin levels may be useful as an indicator for disease progression and to identify patients at risk of recurrence and metastasis. The preferential expression of Endoglin in endothelial cells of the tumor vasculature versus neoplastic cells has led to the suggestion that soluble Endoglin originates from the neovasculature (65). Thus, as reported for several severe vascular diseases (66), also in the cancerogenic process soluble Endoglin may prevent binding of TGF-ß1 to its signaling receptors, impairing downstream signaling activity. *In vitro* studies have demonstrated the importance of regulating the availability of active TGF-ß. Low extracellular TGF-ß1 concentrations promote the cell proliferation and migration that is associated with the active proliferation of new vessels in angiogenesis (28). By contrast, high levels of extracellular TGF-ß1 lead to cytostasis and synthesis of ECM proteins that are associated with mature or differentiating vessels (67).

The lack of a significant difference between pre- and post-operative Endoglin serum levels may be explained by the fact that lowered soluble CD105 levels following removal of the tumor are overshadowed by postoperative angiogenesis during the process of wound healing.

Membrane associated Endoglin can bind TGF-ß1 with high affinity and regulates its access to the signaling type I and type II receptors (68). Of the three mammalian TGF-ß isoforms, TGF-ß1, localized to endothelial cells during embryogenesis (69), is the most likely to be involved in angiogenesis. Our results demonstrate that colon adenoma and cancer cells express higher levels of TGF-ß1 mRNA and protein than their normal counterparts. Moreover, whereas TGF-ß1 *in situ* expression was significantly higher only in high dysplastic polyps, a progressive increase in TGF-ß1 transcript and protein accumulation was observed in successive stages of the colorectal tumor progression. TGF-ß1 has been found to be overexpressed locally in various types of solid tumors, including cancer of the breast (70), colon (71,72), esophagus (73), stomach (74), pancreas (75), liver (76), lung (77), prostate (78), brain (79), and malignant melanoma (80). Overexpression is frequently correlated with advanced tumor stage, metastases and a poorer overall prognosis. In addition, circulating TGF-ß1 levels of patients with high dysplastic adenoma were significantly higher in comparison with those of patients with low dysplasia, whereas in carcinoma patients TGF-ß1 levels progressively increased with disease progression. It has been shown that malignant tumors can not only activate latent TGF-ß derived from other sources in the microenvironment, but also produce TGF-ß1 itself (81). Consistent with the idea that tumor-derived TGF-ß1 is released into the circulation, post-operative serum levels of TGF-ß1 decreased significantly.

TGF-ß has been found to have bidirectional functions in the progression of solid cancers: in the early stages it acts as a tumor suppressor by inhibiting epithelial cell proliferation, while in later stages it functions as a pro-oncogenic factor through stimulation of matrix deposition, perturbation of immune function, activation of angiogenesis and induction of EMT (82). The mechanisms whereby TGF-ß switches from being a tumor suppressor to a promoter facilitating tumorigenesis are not fully understood. The effects of the TGF-ß1 protein are exerted through specific receptor complexes present on the cell surface: its binding to the type II receptor activates and transphosphorylates the type I receptor, which subsequently propagates the signal by phosphorylating the receptor-regulated Smad (R-Smad) family of proteins that, upon activation, form heteromeric complexes with a co-operative homologue named Co-Smad, that translocate into the nucleus where regulate the transcriptional activity of target genes (21).

Our results show that the TGF-ß RII protein, present in epithelial cells but also in mesenchymal cells such as fibroblasts and endothelial cells, is overexpressed in adenoma and carcinoma versus the normal colon. However, the different degrees of adenoma dysplasia and Dukes stages in colorectal carcinoma could not be distinguished on the basis of TGF-ß RII expression. Membrane trafficking defects of RII have been proposed as one mechanism accounting for the loss of growth inhibition by TGF-ß. However, our results provide no argument in favor of reduced or undetectable amounts of cell-surface TGF-ß RII, as observed in a majority of colon cancer cases showing microsatellite instability (83), since the antibody used for detection is directed against the intracellular portion of the receptor. Moreover, the TGF-ß pathway could still be impaired through mutation of RII, as reported, or through post-receptor event alterations (84).

The functional interaction between TGF-ß1/CD105 should be taken into consideration. In adenoma patients no significant association was found between any of the variables considered. By contrast, a statistically-significant positive cross-relationship was found between local and circulating levels of Endoglin and TGF-ß1 in carcinoma patients. Studies conducted in

experimental models provide direct evidence that highly expressed CD105 antagonizes the inhibitory effects of TGF-ß1 and thus contributes to the proliferation, migration, and capillary formation of endothelial cells, the three key events in the angiogenic process (85). On the other hand, interestingly, TGF-ß potently stimulates Endoglin expression via Smads (68) and in synergy with the hypoxia pathway (86). Therefore, the progressive loss of TGF-ß growth inhibition and increased expression of TGF-ß with the concomitant induction of Endoglin could be the crucial event associated with malignant conversion and progression in colon cancer. However, it cannot be ruled out that, as reported, Endoglin/CD105 can also act independently of the TGF-ß signaling pathway (68).

To clarify the clinical significance of our study, we examined the correlation between the degree of dysplasia of adenomas, the Dukes' stage of carcinomas, and *in situ* expression and circulating levels of the parameters considered. A statistically-significant correlation was found between dysplasia degree and TGF-ß RII and $CD105^+$-MVD. By contrast, a statistically-significant correlation was observed between Dukes' classification and circulating TGF-ß1 and Endoglin levels as well as $CD105^+$-MVD.

TGF-ß1 and Endoglin plasma levels, TGF-ß1 IRS and MVD were significantly and inversely associated with patient disease-free survival. Multivariate analysis indicated that only plasma TGF-ß1 levels were an independent and significant prognostic factor of disease-free survival, associated with increased risk of poor prognosis. Both TGF-ß1 and Endoglin act as valuable indicators of prognosis in several tumors (56,70,87-97).

Currently, as alternative or complementary strategies to traditional chemotherapy, drugs targeting angiogenesis have been developed to block tumor growth and metastases by interrupting, at different levels, the signaling pathways of angiogenic factors. Some of these novel approaches have already been approved by health authorities and are now integrated into cancer care, including that of colon carcinoma (98).

Adenomatous polyps are well-demarcated masses of epithelial dysplasia, with uncontrolled crypt cell proliferation. Some seem to be reversible, but others progress to carcinoma *in situ* and to invasive carcinoma. Predictive factors of reversibility or progression are virtually unknown. The increased expression of angiogenesis markers Endoglin, TGF-ß RII and TGF-ß1 appears to be an early event in the adenoma-carcinoma sequence, in accordance with previous reports showing a close association between proangiogenic VEGF and tissue factor (TF) in the early stages of colorectal development (14). We assume that overexpression of this receptors/ligand complex may participate in colorectal carcinogenesis and may possibly possess prognostic value. It is thought that angiogenesis-targeted chemoprevention may prevent or delay the progression from pre-malignant condition to carcinoma. Thus anti-angiogenic therapy may become an interesting strategy not only in patients with carcinoma, but also in chemoprevention in patients with high risk of developing such a tumor, otherwise submitted to the 'watchful waiting' strategy. However, further studies are necessary to clarify the molecular mechanisms that regulate angiogenesis in pre-malignant conditions in order to provide evidence that support the clinical application of anti-angiogenic drugs in these patients. In this context, preclinical and correlative studies should continue to explore the mechanisms of action as well as predictive markers for efficacy and toxicity.

Acknowledgements

This work was supported by grants from the Piedmontese Regional Government to G.B.

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