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Connective Tissue Growth Factor Inhibits Metastasis and Acts as an Independent Prognostic Marker in Colorectal Cancer

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Background & Aims: **Connective tissue growth factor (CTGF) has been shown to be implicated in tumor development and progression. The aim of this study was to investigate the role of CTGF in progression of colorectal cancer (CRC).** ***Methods:*** **Immunohistochemical staining of specimens from 119 patients with CRC was performed. Liposome-mediated transfection was used to introduce a CTGF expression vector into CRC cell lines. Transfectants were tested in invasive ability and experimental hepatic metastasis in BALB/c mice. Furthermore, a FOPflash/TOPflash reporter assay was performed to investigate CTGF on the β-catenin/T-cell factor signaling pathway.** ***Results:*** **Patients with stage II and stage III CRC whose tumors displayed high CTGF expression had a significantly higher overall survival and a disease-free advantage over patients with CRC with low CTGF expression. Alterations in the CTGF level in CRC cell lines modulated their invasive ability with an inverse correlation. In addition, a reduction in the CTGF level of CT26 cells after stable transfection with antisense CTGF resulted in increased liver metastasis in BALB/c mice. The activity of the β-catenin/T-cell factor signaling pathway and its downstream effector gene matrix metalloproteinase 7 in these CTGF-transfected cells was strongly attenuated. Blockage of matrix metalloproteinase 7 with its neutralizing antibodies inhibited increased invasiveness in antisense CTGF-transfected CT26 cells.** ***Conclusions:*** **Our results implicate CTGF as a key regulator of CRC invasion and metastasis, and it appears to be a useful and better prognosis factor for patients with stage II and stage III CRC.**

Colorectal cancer (CRC) is the second leading cause of death from cancer in the United States, with approximately 130,000 new cases and 50,000 deaths per year.[1] In Taiwan, CRC is the third leading cause of death from cancer, with nearly 7000 new cases and 3200 deaths per year.[2] The overall 5-year survival rate of CRC in the United States is about 55%.[3] The major reason for this poor prognosis is the propensity of CRC to invade adjacent tissues and to metastasize to distant organs. The rate of local recurrence and metastasis of CRC ranges from about 25% to 50%.[4] Relapses often occur in the liver, regional colon, or lung as well as in the ovaries, bone, anastomosis, or brain.

Connective tissue growth factor (CTGF/CCN2) is a member of the CCN family, which comprises CTGF, cysteine-rich 61 (Cyr61/CCN1), nephroblastoma overexpressed (Nov/CCN3), Wisp-1/elm1 (CCN4), Wisp-2/rCop1 (CCN5), and Wisp-3 (CCN6). CCN proteins exhibit diverse cellular functions in areas such as regulation of cell division, chemotaxis, apoptosis, adhesion, motility, and ion transport.[5–9] Human *CTGF* messenger RNA (mRNA), a single transcript of 2.4 kilobases, is expressed in various tissues such as the heart, brain, placenta, lung, liver, muscle, kidney, and pancreas.[5] Although the *CTGF* transcript is commonly expressed in most human adult tissues, its physiologic function in these tissues remains unclear. Recently, CTGF expression has been shown to be associated with tumor development and progression.[10–17] For example, the level of CTGF expression is positively correlated with bone metastasis in breast cancer,[10] glioblastoma growth,[11] poor prognosis in esophageal adenocarcinoma,[12] aggressive behavior of pancreatic cancer cells,[13] invasive melanoma,[14] and chondrosarcoma.[15] In contrast, overexpression of CTGF has been shown to suppress the tumor growth of oral squamous cell carcinoma cells transplanted into mice.[16] Supportively, other CCN family members such as Cyr61

Abbreviations used in this paper: CRC, colorectal cancer; CTGF, connective tissue growth factor; MMP, matrix metalloproteinase; PCR, polymerase chain reaction; RT, reverse transcription; Tcf, T-cell factor.

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(CCN1) and Nov (CCN3) have also been found to display lower expression in certain tumors compared with their normal counterparts. Overexpression of Cyr61 or Nov in cancer cells can inhibit their growth either in cell cultures or in animal models.[5,8,17] The varying role of CTGF or other CCN members in different tumors seems important, but their exact mechanism has not yet been clarified.

In this study, we assessed the expression of CTGF in samples of normal adjacent epithelium, premalignant lesions, and CRC specimens by immunohistochemistry. Our data show that low expression of CTGF was statistically significantly correlated with lymph node metastasis, easier recurrence, and shorter survival. The in vitro invasion abilities of several human CRC cell lines and in vivo experimental hepatic metastasis were determined. The underlying mechanism of how CTGF affected the capacity of invasion/metastasis was also investigated.

Materials and Methods

Patients

Our study included 119 consecutive patients with CRC treated at National Taiwan University Hospital between December 1996 and July 1999. There were 61 men and 58 women, and the average age was 62.7 ± 13.4 years (median, 63 years; range, 27–89 years). All patients underwent complete surgical resection, and their clinical and pathologic data were available. Patients with familial adenomatous polyposis, hereditary nonpolyposis CRC syndrome (according to Amsterdam criteria), or inflammatory bowel diseases or who had had a malignant tumor within 5 years were excluded from this study. Tumor stage was based on the postoperative pathology report and a preoperative clinical evaluation including chest radiograph, carcinoembryonic antigen level, and abdominal ultrasonography or computed tomography. Information about clinical outcome was obtained from a hospital chart review or a direct telephone interview with the patient's personal physician. All patients were followed up, and this involved periodic examinations comprising serum blood chemistry panels, carcinoembryonic antigen level, endoscopy, and abdominal ultrasonography and radiographs of the thorax. Computed tomography or magnetic resonance imaging was also performed in cases in which there was a suspected tumor recurrence. The overall survival time was calculated from the date of surgery to the time of the last visit or death and the disease-free survival time from the date of resection to relapse. The median follow-up time was 58.9 months. Tumor distribution according to primary site was 29 in the right colon, 53 in the left colon (from splenic flexure to end of sigmoid colon), and 37 in the rectum. Fourteen patients had stage I, 37 had stage II, 51 had stage III, and 17 had stage IV disease. The 5-year survival rates were 90%, 74.5%, 55.0%, and 5.9% for stages I–IV, respectively.

Immunohistochemistry

After rehydration, sections (4 μm) of a paraffin-embedded tissue block that had been cut on glass slides were incubated in 3% hydrogen peroxide to block endogenous peroxidase activity. Following trypsinization, the sections were blocked by incubation in 3% bovine serum albumin in phosphate-buffered saline (PBS). The primary antibody, a polyclonal goat anti-human CTGF antibody (R&D Systems, Minneapolis, MN), was applied to the slides at a dilution of 1:50 and incubated at 4°C overnight. After washes in PBS, the samples were treated with biotin-labeled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:250 for 1 hour at room temperature. Detection was performed with an ABC kit (DakoCytomation, Glostrup, Denmark). The slides were stained with diaminobenzidine, washed, counterstained with Delafield's hematoxylin, dehydrated, treated with xylene, and mounted. The pathologist assessing immunostaining intensity was blinded to the patients' information. The results of immunohistologic staining were classified using extent of cell stained; these were level 0 (negative staining), level 1 (<5% of tumor cells stained), level 2 (<50% of tumor cells stained), and level 3 (>50% of tumor cells stained).

Cell Culture

HCT116, Caco-2, and NIH3T3 cells were maintained in Dulbecco's modified Eagle medium (Life Technologies, Inc, Carlsbad, CA), with the addition of 4 mmol/L L-glutamine and 10 mmol/L sodium pyruvate (Sigma Chemical Co, St Louis, MO). In addition, the medium used for Caco-2 cells was supplemented with 10 μg/mL transferrin. COLO205 and HT-29 cells were cultured in RPMI 1640 (Life Technologies), and the medium used for CT26 cells contained an additional 10 mmol/L HEPES, 4.5 g/L glucose, and 10 mmol/L sodium pyruvate. All media used for cell culture were supplemented with 10% fetal bovine serum and a 1% penicillin (10,000 U/mL)/streptomycin (10,000 mg/mL) solution (Life Technologies, Inc). Cells were maintained at 37°C in the presence of 5% CO_2 in air. All cells were passaged into new medium every 2–3 days and before confluence.

Western Blotting

Cells were washed with PBS containing 5 mmol/L EDTA and 1 mmol/L sodium orthovanadate, scraped into lysis buffer (20 mmol/L Tris-HCl [pH 8.0], 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μmol/L leupeptin, and .15 U/mL aprotinin), and stored for 30 minutes on ice. Tumor parts of tissues from patients with CRC were also homogenized with the lysis buffer. The lysed cells or tissues were centrifuged at 14,500 *g* for 30 minutes at 4°C, and the supernatant was collected. Proteins in the supernatant were quantified by spectrophotometry. Proteins in the cell, tissue lysate (40 μg of protein), or after trichloroacetic acid precipitation of condition medium collected after 48 hours in confluent condition were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis on a 12% gel and electrotrans-

ferred to a polyvinylidene difluoride membrane (Immobilon-P membrane; Millipore Corp, Bedford, MA). After the blot was blocked in a solution of 3% bovine serum albumin, .1% Tween 20, and PBS, the membrane-bound proteins were probed with primary antibodies against β-actin (Sigma Chemical Co), CTGF (R&D Systems), or β-catenin (BD Transduction Laboratories, BD Biosciences, Woburn, MA). The membrane was washed and then incubated with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) for 30 minutes. Antibody-bound protein bands were detected with enhanced chemiluminescence reagents (Amersham Bioscience, Piscataway, NJ) and photographed with Kodak X-Omat Blue autoradiography film (Perkin Elmer Life Science, Boston, MA).

Reverse-Transcription Polymerase Chain Reaction

Reverse transcription (RT) of RNA isolated from cells was performed in a final reaction volume of 20 μL containing 5 μg of total RNA in Moloney murine leukemia virus reverse-transcriptase buffer (Promega, Madison, WI), which consists of 10 mmol/L dithiothreitol, all 4 deoxynucleoside triphosphates (each at 2.5 mmol/L), 1 μg of $(dT)_{12–18}$ primer, and 200 U of Moloney murine leukemia virus reverse transcriptase (Promega). The reaction mixture was incubated at 37°C for 2 hours, and the reaction was terminated by heating at 95°C for 5 minutes. One microliter of the reaction mixture was then amplified by polymerase chain reaction (PCR) with the following pairs of primers: CTGF primers, 5′-GCTTACCGACTGGAAGACACGTT-3′ (sense) and 5′-TCATGCCATGTCTCCGTACATC-3′ (antisense), to produce a 500–base pair fragment of the CTGF gene; matrix metalloproteinase (MMP)-7 primers, 5′-GGTCACCTACAGGATCGTATCATAT-3′ (sense) and 5′-CATCACTGCATTAGGATCAGAGGAA-3′ (antisense), to produce a 500–base pair fragment of the MMP-7 gene; MMP-9 primers, 5′-CAACATCACCTATTGGATCC-3′ (sense) and 5′-GGGTGTAGAGTCTCTCGCTG-3′ (antisense), to produce a 480–base pair fragment of the MMP-9 gene; and β-actin primers, 5′-GATGATGATATCGCCGCGCT-3′ (sense) and 5′-TGGGTCATCTTCTCGCGGTT-3′ (antisense), to produce a 320–base pair fragment product of the β-actin gene, used as the internal control. The PCR amplification was performed in a reaction buffer containing 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, all 4 deoxynucleoside triphosphates (each at 167 μmol/L), 2.5 U of *Taq* DNA polymerase, and 10 μmol/L primers. The reactions were performed in a Biometra Thermaoblock (Biometra Inc, Miami, FL) with the following program: denaturing for 1 minute at 95°C, annealing for 1 minute at 56°C, and elongating for 1 minute at 72°C for a total of 28 cycles; final extension took place at 72°C for 10 minutes. Equal volumes of each PCR sample were subjected to electrophoresis on a 1.5% agarose gel, which was then stained with ethidium bromide and photographed under UV illumination.

Construction of CTGF (Sense and Antisense) Expression Plasmids

The cloning process of CTGF expression plasmids was described previously.[18] Briefly, total RNA was extracted from lung adenocarcinoma cell lines (CL1-0 cells), and CTGF complementary DNA was cloned and amplified by RT-PCR with the primers 5′-ATGACCGCCGCCAGTATGG-3′ and 5′-TCATGCCATGTCTCCGTACATCTT-3′ (PubMed accession number XM-037056) and subcloned into a pcDNA3/V5-His TOPO TA vector (Invitrogen, San Diego, CA) in forward (sense) or reverse (antisense) direction. The CTGF-expressing vectors are used in transient and stable transfections of colorectal carcinoma cell lines in vitro.

Plasmid, Transient Transfection, and Reporter Gene Assay

The CTGF (sense) expression vectors were transiently transfected into HCT116 cells with TransFast transfection reagents (Promega). Briefly, 3 μg of plasmid DNA (CTGF [sense, antisense] or pcDNA3) and 8 μg of transfection reagents were mixed, and the transfection protocol was performed according to the manufacturer's instructions (Promega). Reporter gene assay was performed in the same way. A total of 2×10^5 cells were cotransfected with 1.5 μg of CTGF-expressing vector and 1.5 μg of luciferase reporter constructs TOPflash or FOPflash (kind gifts from Dr Deran Tsuo). TOPflash contains 3 copies of the T-cell factor (Tcf)/LEF-binding site (AAGATCAAAGGGGGT) upstream of a TK minimal promoter. FOPflash contains a mutated Tcf/LEF-binding site (AAGGCC AAAGGGGGT). One hour after transfection, the cells were transferred and cultured in normal complete medium for another 8 hours. The transfected cells were harvested and subjected to RT-PCR and Western blot analysis. Luciferase activity was measured according to the manufacturer's instructions.

HCT116 and CT26 Cell Lines Express Sense and Antisense CTGF

HCT116 cells expressing CTGF (sense) or CT26 cells expressing CTGF (antisense) were established by transfection with the following expression vectors: CTGF sense or CTGF antisense. After 48 hours of transfection, cells were trypsinized and replated in RPMI 1640 with 10% fetal calf serum and 1000 μg/mL G418. G418-resistant clones were selected and expanded.

Boyden Chamber Assay

For invasion assays, we used modified Boyden chambers with filter inserts (pore size, 8 μm) coated with Matrigel (40 μg; Collaborative Biomedical, Becton Dickinson Labware, San Jose, CA) in 24-well dishes (Nucleopore Corp, Pleasanton, CA). Approximately 1×10^6 cells in 100 μL of complete medium were placed in the upper chamber, and 1 mL of the same medium was placed in the lower chamber. After 48 hours of culture, the cells were fixed in methanol

for 15 minutes. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells were then stained with .05% crystal violet in PBS for 15 minutes. Cells on the underside of the filters were viewed and counted using a Leica Microsystems (type 090-135.001, Wetzlar, Germany) microscope system. Each clone was plated in duplicate in each experiment, and each experiment was repeated at least 3 times.

In Vitro Cell Growth Assay

HCT116 and CT26 transfectants were added to 6-cm dishes initially containing 5×10^4 cells per well. NIH3T3 cells (1×10^4 cells per well) were treated with condition medium collected from CTGF transfectants for 48-hour culture. NIH3T3 cells were then trypsinized and resuspended, and cell numbers were counted using a hemocytometer at varying time points.

Experimental Metastasis

CT26 cells or transfectants were washed, and 5×10^5 cells were suspended in 1 mL of PBS. Syngeneic 6- to 8-week-old BALB/c female mice were injected with .1 mL of these cells into the portal vein via intrasplenic injection, which was performed by a minilaparotomy (.5 cm in length) over the left flank.[19] Autopsies were performed when animals were moribund or after 6 weeks with macroscopic and microscopic examination for the presence of metastasis. These experiments were approved by the institute's Animal Welfare Committee.

Fractionations of Cell

Cells were scraped in ice-cold PBS, recovered by centrifugation at 500*g* for 3 minutes, resuspended in 200 μL of CER I buffer (NE-PER nuclear and cytoplasmic extraction reagents; Pierce, Rockford, IL), and then vigorously vortex mixed for 15 seconds. After incubation on ice for 10 minutes, CER II buffer was added to the tube. The tube was then vortexed for 5 seconds and the cytoplasmic fraction was pelleted at 4°C by centrifugation for 5 minutes at 16,000*g*. The insoluble fraction, which contains nuclei, was resuspended in 100 μL of ice-cold NER. After vortexing for 15 seconds, the sample was returned to ice. Vortexing for 15 seconds was continued at 10-minute intervals for a total of 40 minutes. Finally, the tube was centrifuged at maximum speed for 10 minutes and the nuclear fraction immediately transferred to a prechilled tube and stored.

MMP-7 Invasive Function Blocked With Specific Antibodies

CT26 transfectants of 100 μL serum-free medium were placed in the upper Boyden chamber. A total of 1 mL of the same medium and anti–MMP-7 monoclonal antibody (MAB3322; Chemicon, Temecula, CA) or immunoglobulin (Ig) G control antibody (at 2, 5, and 10 μg/mL) were placed in the lower chamber. After 48 hours of culture, the cells were fixed in methanol for 15 minutes. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells were then stained with .05% crystal violet in PBS for 15 minutes. Cells on the underside of the filters were viewed and counted using a Leica Microsystems (type 090-135.001) microscope system. Each clone was plated in duplicate in each experiment, and each experiment was repeated at least 3 times.

Statistical Analysis

A comparison of the background data was performed between the low-CTGF and the high-CTGF groups. This was done for scale variables (expressed as mean ± SD) by a Mann–Whitney test and for nominal variables by a Fisher exact test. Analysis of the survival data was by the Kaplan–Meier method. Kaplan–Meier curves were compared by a log-rank test. Regression analysis was utilized using an extended Cox regression model for age as the continuous time-varying covariate and dummy variables for nominal covariates. A multivariate model was used for significant covariates detected in the univariate models. *P* values were 2 sided, and the significant level was .05.

Results

CTGF Is an Independent Prognostic Factor in Patients With CRC

The expression level of CTGF in CRC was determined by immunohistochemistry using a CTGF-specific antibody. This antibody does not cross-react with other CCN members. The results of the immunohistologic staining were classified as level 0 (negative staining), level 1 (<5% of tumor cells stained), level 2 (<50% of tumor cells stained), and level 3 (>50% of tumor cells stained). A high level of immunoreactivity for CTGF (level 3) was detected in normal colon epithelium (Figure 1*A*) and a colon polyp specimen (Figure 1*B*). The CTGF protein was predominantly localized in the cytoplasm or the membrane of normal or tumor epithelial cells. Interestingly, differentiated and noninvasive colorectal tumors also showed high expression for CTGF (Figure 1*C*). In contrast, very weak immunoreactivity (levels 0 and 1) for CTGF was observed in poorly differentiated and metastatic colorectal tumors (Figure 1*D*). Negative staining for CRC was shown when immunostaining using an IgG control (Figure 1*A*, inset). Of the 119 CRC specimens analyzed, low expression level (levels 0 and 1) and high expression level (levels 2 and 3) of CTGF in CRC specimens was 44% (53 of 119) and 56% (66 of 119), respectively. The incidence of low CTGF expression among the 4 TNM stages was 29% (4 of 14) in stage I, 35% (13 of 37) in stage II, 47% (24 of 51) in stage III, and 71% (12 of 17) in stage IV disease ($P = .059$). The relationship between

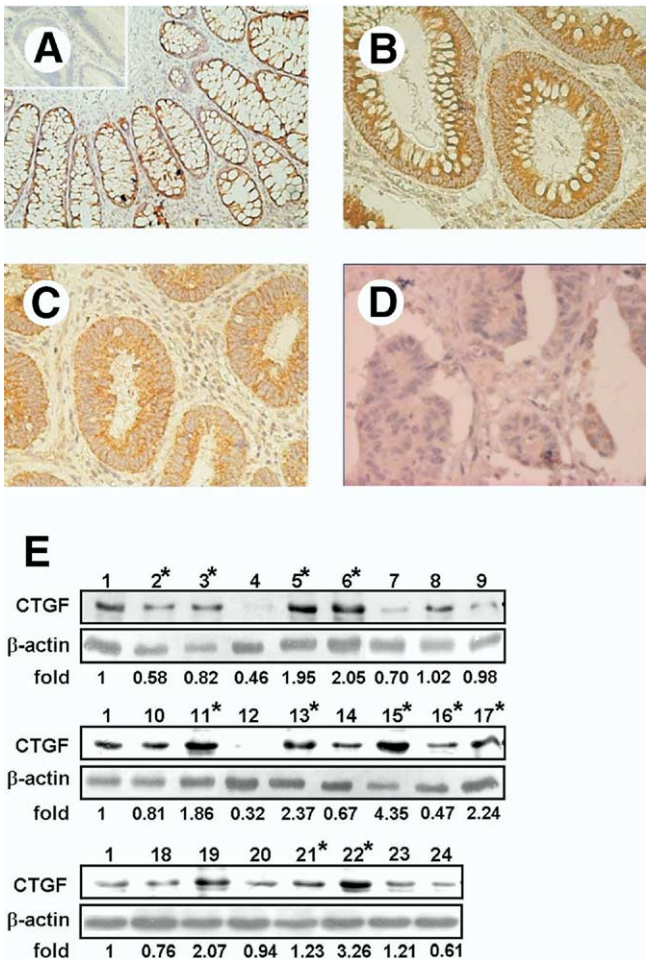

Figure 1. Representative CTGF immunohistochemical staining of normal human colon tissue, polyp, and colorectal carcinomas. High CTGF expression is shown in (*A*) normal colon tissue and (*B*) colon polyp. (Original magnification: *A,* 40×; *B,* 100×.) (*A, inset*) Negative control IgG staining of colon adenocarcinoma. (*C*) High CTGF expression is found in differentiated adenocarcinoma tissue. CTGF is localized distinctly in the apical cytoplasm and membrane of the tumor cells. (*D*) Low CTGF protein expression is noted in a poorly differentiated adenocarcinoma specimen. (*E*) Western blotting of CTGF expression in the tumor part of 24 patients with CRC. The relative fold of protein density to tumor sample 1 is indicated below the graph. *Good prognosis, with survival time greater than the median follow-up time of 58.9 months.

the level of CTGF expression and the clinicopathologic characteristics is summarized in Table 1. No significant relationship was found between the level of CTGF expression and the age of patients, their sex, tumor stage, tumor site, grade of differentiation, preoperative carcinoembryonic antigen level, and invasion depth. Colorectal tumors with low CTGF expression were more often detected as lymph node metastasis ($P = .034$). Furthermore, CTGF expression is associated with disease outcome, that is, patients with low CTGF expression had the shorter survival ($P < .001$) and more frequent recurrence ($P < .001$) compared with patients with CRC with high CTGF expression. In addition, to confirm the data of immunohistochemical staining, we used Western blotting to check the CTGF protein level in the tumor parts from a subset of patients. As shown in Figure 1*E,* of 24 tumor samples analyzed, the correlation between Western blotting and immunohistochemical staining was high and significant (Spearman correlation coefficient $\rho = .7404$, $P = .0001$). Based on the quantity of CTGF protein, the 24 tumor samples were divided into "poor" and "good" prognoses according to their respective survival time. Poor or good prognoses were

Table 1. Clinical and Pathologic Characteristics for High and Low CTGF Expression in CRC

Characteristics	High CTGF	Low CTGF	*P*
No.	66	53	
Age (*y*, mean ± SD)	61.7 ± 13.7	61.8 ± 13.0	.889
Sex			
Male	38	23	.143
Female	28	30	
Tumor site[a]			
Right	14	15	
Left	34	19	.247
Rectum	18	19	
Tumor differentiation			
Poor	4	3	
Moderate	58	40	.158
High	4	9	
Carcinoembryonic antigen level (*ng/mL*)			
≤3	33	25	.854
>3	33	28	
Stage			
I	10	4	
II	24	13	.059
III	27	24	
IV	5	12	
Lymph node			
N0	40	22	
N1	19	16	.034[b]
N2	7	15	
Invasion depth			
T1	1	3	
T2	13	5	.278
T3	50	39	
T4	2	3	
Intratumor Invasion[c]			
Present	21	18	.846
Absent	45	35	
Recurrence			
No	59	28	<.001[b]
Yes	7	25	
Vital status			
Alive	53	17	<.001[b]
Dead	13	36	

[a]Tumor site: right, cecum to splenic flexure; left, splenic flexure to sigmoid colon.
[b]Statistical significance ($P < .05$).
[c]Intratumor invasion (present): if pathologic report showed one of venous, lymphovessel, or perinerial invasions.

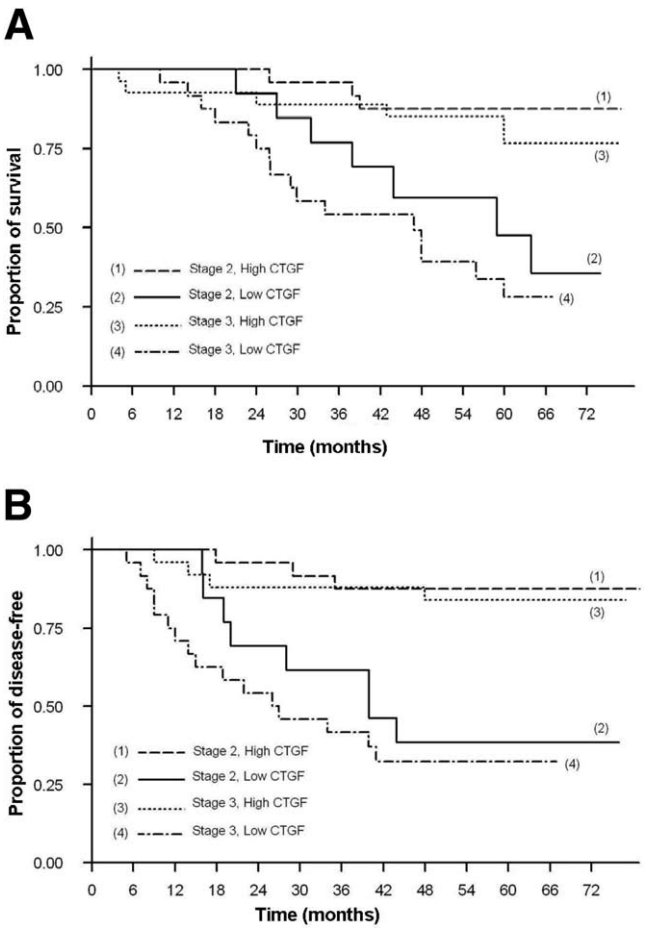

Figure 2. Analysis of survival (Kaplan–Meier) considering patients with stage II and stage III CRC. (*A*) Overall survival and (*B*) disease-free survival of 88 patients according to their tumor CTGF expression.

defined as the survival time less or more than the median survival time (58.9 months). Descriptive statistics showed that CTGF protein levels were significantly higher in the good prognosis group than in the poor prognosis group ($P = .02$). That is, most patients with CRC with a longer survival time had higher CTGF protein levels as compared with patients with a shorter survival time.

When patients were stratified by tumor stage, we found that the patients with stage II and stage III tumors with high CTGF expression had a better survival rate and recurrent-free advantage compared with those with low CTGF expression (Figure 2*A* and *B*). The influence of each clinicopathologic characteristic and the expression pattern of CTGF on patient survival and the disease-free status of patients with stage II and stage III disease were analyzed using the extended Cox regression model, and the results are listed in Table 2. Among the stage II and stage III tumors, patients whose tumor had high CTGF expression also had better disease-free status (hazard ratio, .143; $P < .001$) and better overall survival (hazard ratio, .186; $P < .001$) than those whose tumor had low CTGF expression. The data suggest it is possible that tumor status might increase the risk of recurrence (hazard ratio, 4.12; $P = .016$). CTGF expression was the only significantly independent predictor in this multivariate model analysis, although lymph node status showed a relationship to the disease outcome in the series of patients examined (Table 2). The 5-year survival rate for patients with stage II disease whose tumor had higher CTGF expression was 87.5% compared with those with lower CTGF expression at 47.5% ($P = .005$). In particular, the 5-year survival rate of patients with stage III disease whose tumor had higher CTGF expression was 76.7%, and this contrasted strongly with those having lower CTGF expression at only 28.1% ($P = .004$). Similar results were obtained when the disease-free time was the end point; that is, recurrence occurred earlier in patients whose tumor had lower expression of CTGF. Of the 51 patients with high CTGF expression in stage II and stage III CRC, only 7 (13.7%) developed tumor recurrence (6 distal metastasis and one locoregional). In contrast, 24 of the 37 patients (64.9%) whose tumor had low CTGF expression developed tumor recurrence (20 distal metastases and 4 locoregional). A significant difference in 5-year survival between the patients with (4.3%) and without (94.7%) recurrence was noted ($P < .001$). Taken together, our data strongly suggest that CTGF may act as an independent prognostic factor for patients with CRC, especially for stage II and stage III tumors. Because the death of patients with CRC is largely due to metastasis, CTGF may have a role in modulating the invasiveness and metastasis of CRC.

Invasion Ability of CRC Cell Lines Is Inversely Related to CTGF Expression In Vitro

Because the expression level of CTGF was inversely associated with tumor progression in patients with CRC, we explored whether CTGF affected the invasiveness of human CRC cells. To address this, we initially examined the level of CTGF in 4 human cancer cell lines (HCT116, COLO205, HT-29, and Caco-2) and a mouse cell line (CT26) by RT-PCR and Western blotting assay. As shown in Figure 3*A*, of the cell lines analyzed, HCT116 cells had the lowest RNA and protein levels of CTGF. HT-29 and COLO205 cells exhibited moderate levels of CTGF mRNA and protein. CT26 and Caco-2 cells had the strongest expression level of CTGF. We next tested the in vitro invasive ability of these cell

Table 2. Predictors[a] for Mortality or Recurrence in Stage II or Stage III CRC

Characteristic Category	Label	Overall survival			Disease-free survival		
		Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
Sex							
Male	1	1.39	.7–2.8	.36	1.34	.6–2.7	.42
Female	0						
Age (*y*)							
Older than 65	1	1.66	.5–5.1	.37	1.41	.5–4.3	.54
65 or younger	0						
Tumor site							
Right	0						
Left	1	1.09	.67–1.75	.74	.95	.6–1.5	.84
Rectum	2						
Carcinoembryonic antigen (*ng/mL*)							
>3	1	1.02	.5–2.1	.96	1.10	.5–2.2	.80
≤3	0						
Tumor differentiation							
Well	1						
Moderate	2	1.59	.68–3.75	.29	1.90	.8–4.4	.13
Poor	3						
Lymph node							
N0	0						
N1	1	1.71	1.06–2.77	.03[b]	1.86	1.1–3.0	.01[b]
N2	2						
Tumor status							
T2	2						
T3	3	1.93	.56–6.57	.29	4.12	1.3–13.0	.016[b]
T4	4						
Intratumor Invasion							
Present	1	1.34	.65–2.78	.425	1.87	.8–3.5	.16
Absent	0						
CTGF expression							
High	1	.143	.06–.34	<.001[b]	.186	.09–.41	<.001[b]
Low	0						

CI, confidence interval.
[a]Extended Cox regression model, univariate, with age as the time-varying covariate.
[b]Statistical significance ($P < .05$).

lines using a modified Boyden chamber assay. Figure 3*B* shows that HCT116 cells displayed the highest invasiveness among these cells; in contrast, Caco-2 and CT26 cells showed only weak invasive ability. Collectively, these results showed that invasive ability was inversely correlated with the level of CTGF expression in human CRC cell lines.

Altered Level of CTGF Affects the Invasiveness of Human CRC Cell Lines

To investigate the direct role of CTGF in the invasion and metastasis of human CRC, we transfected sense and antisense CTGF-expressing vectors into HCT116 and CT26 cells, respectively. After G418 selection, CTGF-overexpressing HCT116 (HCT116/CTGF), antisense CTGF-overexpressing CT26 (CT26/AS-CTGF) transfectants, and *Neo* control cells were analyzed for their expression level of CTGF mRNA and protein. Figure 4*A* shows that HCT116/CTGF cells exhibited a 4- to 5-fold increase of CTGF mRNA and protein as compared with HCT116/*Neo* cells. Interestingly, the invasive capacity of HCT116/CTGF cells was dramatically reduced to 20% of that of HCT116/*Neo* cells (Figure 4*B*). Furthermore, the antisense CTGF transfection experiment showed that the endogenous CTGF mRNA and protein levels were effectively diminished in CT26/AS-CTGF cells (Figure 4*A*). In a Boyden chamber assay, the invasive ability of CT26/AS-CTGF cells was increased about 3- to 4-fold over that of CT26/*Neo* cells (Figure 4*B*). This clearly shows that increased CTGF expression level in CRC cells resulted in inhibition of their invasive ability. To rule out the possibility that the effect of CTGF on in vitro cell invasiveness was caused by different proliferation rates among these cell lines, we compared the growth rates of HCT116/CTGF and CT26/AS-CTGF cells with their *Neo* control, respectively (Figure 4*C*). The growth rate of these cells was the same, suggesting that the altered

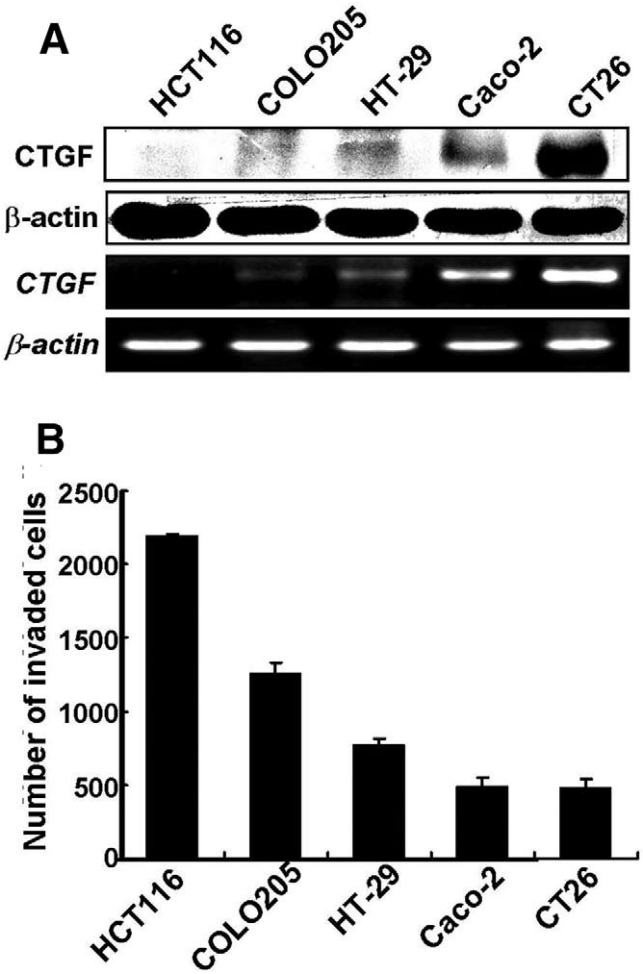

Figure 3. CTGF expression and invasive ability of different colon cancer cell lines. (*A*) Immunoblot analysis of CTGF and β-actin in human colon cancer cell lines. Protein extracts (40 μg/lane) from the indicated cell lines were electrophoresed, transferred, and used for immunodetection of CTGF and β-actin (for an internal loading control) (*upper panel*). mRNA levels of *CTGF* and β-actin were measured semiquantitatively by RT-PCR (*lower panel*). (*B*) In vitro invasion assay performed in human colon cancer cell lines. Approximately 2.5×10^4 cells in 100 μL of complete medium were placed in the upper Boyden chamber with filter inserts (pore size, 8 μm) coated with Matrigel. After 48 hours in culture, cells were fixed in methanol and cells on the underside of the filters were viewed and counted. Each clone was plated in triplicate in each experiment.

invasive ability of those transfectants could not be attributed to their different growth rate.

Because CTGF has been believed to function as a secreted protein, we thus collected conditioned medium from CTGF transfectants (HCT116/*Neo,* HCT116/CTGF, CT26/*Neo,* and CT26/AS-CTGF) after 48-hour culture. Western analysis showed that a substantial level of secreted CTGF protein was detected in 48-hour cultured medium of HCT116/CTGF cells as compared with control cells (Figure 4*D,* upper panel). In contrast, there was a significant reduction of secreted CTGF protein in conditioned medium of CT26/AS-CTGF compared with neo control cells (Figure 4*D,* upper panel). As expected, when NIH3T3 cells treated with the conditioned medium from HCT116/CTGF cells, but not HCT116/*Neo,* showed a significantly increased cell proliferation rate (Figure 4*D,* lower panel). A decreased growth rate of NIH3T3 cells cultured with condition medium from CT26/AS-CTGF was seen when compared with that from CT26/*Neo* cells (Figure 4*D,* lower panel). Moreover, treatment with anti-CTGF neutralizing antibody also notably diminished the proliferation activity of NIH3T3 cells cultured with condition medium of HCT116/CTGF (Figure 4*E*). Taken together, our data suggest that those CTGF transfectants could secrete the functional CTGF protein, which would affect the invasiveness of human CRC cells.

Reduced CTGF Expression in CT26 Cell Line Increased Liver Metastasis In Vivo

To further clarify whether modulation of CTGF expression would affect the in vivo metastasis activity of CRC cells, BALB/c mice were given an intrasplenic/intraportal injection of 5×10^4 CT26/AS-CTGF and CT26/*Neo* cells. Most mice injected with CT26/AS-CTGF cells became moribund within 4 weeks. CT26/AS-CTGF cells formed intrasplenic tumors with the same extent and frequency as compared with CT26/*Neo* cells (Figure 5*A*). However, the number of hepatic metastatic nodules was significantly increased in mice injected with CT26/AS-CTGF as compared with CT26/*Neo* cells (Figure 5*A* and Table 3; $P = .0039$). The liver weight was also remarkably increased for those injected with CT26/AS-CTGF cells but not for those injected with the neo control cells (Table 3; $P = .0008$). Histopathologic examination of the metastatic tumors formed in the livers showed that CT26/AS-CTGF cells had an uneven invasive front, with satellite nodule (circled) in the adjacent liver parenchyma (Figure 5*B,* III). In contrast, CT26/*Neo* cells had a smooth, pushing border (Figure 5*B,* II). The above results showed that CTGF acts as a crucial negative regulator of hepatic metastasis by CRC in mice.

CTGF Inhibits the β-Catenin/Tcf Signaling Pathway in CRC Cells

The β-catenin/Tcf signal pathway has been reported to be directly involved in CRC invasion and metastasis by activating downstream targets (eg, uPAR,[20,21] CD44,[22] MMP-7,[23–25] and fibronectin[26,27]). To investigate whether CTGF inhibits the β-catenin/Tcf signaling pathway, we used HCT116 cells, which have an activated mutation of β-catenin, leading to a consti-

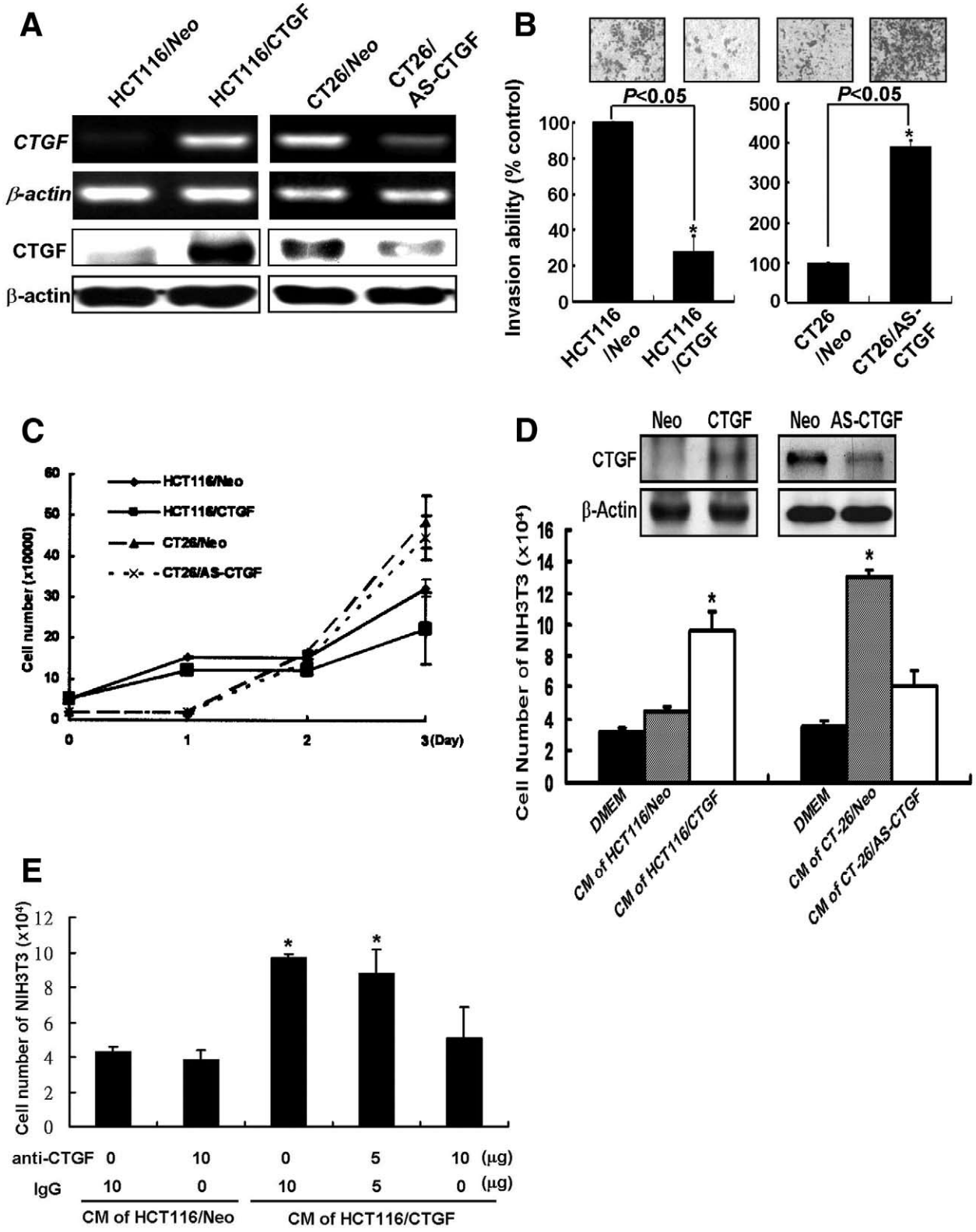

Figure 4. Expression of CTGF and control vector (Neo) in HCT116 cells and CTGF-antisense vector (AS-CTGF) in CT26 cells and the invasion abilities of these transfectants. (*A*) mRNA levels of *CTGF* and β-actin as detected by RT-PCR (*upper panel*). Western blot analysis of total cell lysates probed with antibodies to CTGF and β-actin (*lower panel*). The generation of the stable clones under selection of 800 μg/mL G418. (*B*) Invasion ability of HCT116/Neo and HCT116/CTGF (*left panel*) and CT26/Neo and CT26/AS-CTGF (*right panel*). The micrographs show crystal violet staining of invading cells. (*C*) The growth properties of the vector (Neo)- and CTGF-transfected HCT116 cells and of AS-CTGF–transfected CT26 cells in monolayer culture cell lines. Data are expressed as the mean ± SE. All experiments were performed in triplicate on separate occasions with similar results. (*D*) Effect of condition medium of CTGF transfectant on the growth properties of NIH3T3 cells. (*Upper panel*) Western blot analysis of CTGF expression in condition medium (CM) of HCT116/*Neo,* HCT116/CTGF clones, and CT26/Neo and CT26/CTGF cells. (*Lower panel*) Secreted CTGF increased the proliferation ability of NIH3T3 cells. To compare the proliferation rates of NIH3T3 cells incubated with CM of different transfectants, values were compared with the relative cell numbers of treated Dulbecco's modified Eagle medium alone. Results are expressed as means ± SE of 3 independent experiments. (*E*) Proliferation ability of cotreated CTGF neutralizing antibody and CM of HCT116 transfectants in NIH3T3 cells. HCT116/*Neo* or HCT116/CTGF cells were cultured in confluent condition, and CM was collected after 48 hours. CTGF neutralizing antibody (at 5 and 10 μg/mL) or control IgG was placed with CM in NIH3T3 cells. After 48 hours in culture, the total cells were counted. **P* values of <.05 were considered statistically significant.

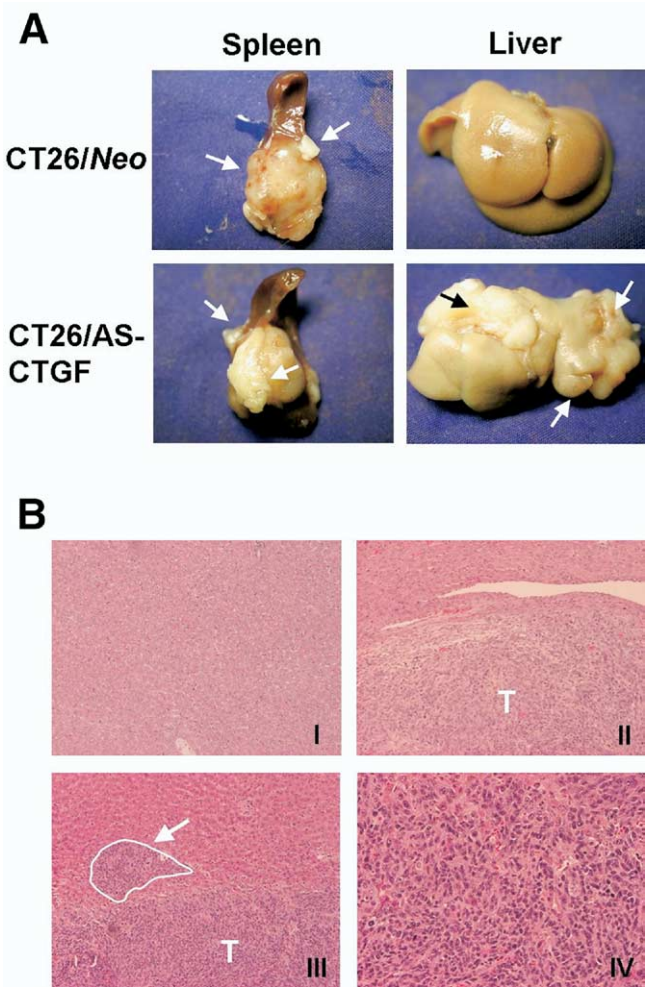

Figure 5. Liver metastasis of CT26/Neo and CT26/AS-CTGF transfectant cells. (*A*) Photographs of spleens and livers from mice that had had their spleen injected with either CT26/Neo or CT26/AS-CTGF cells; the livers and spleens were then harvested when mice were moribund or after 6 weeks. (*B*) Histologic analysis of liver metastasis of CT26/Neo cells (I and II) and CT26/AS-CTGF cells (III and IV) as indicated. Livers were embedded in paraffin, and the paraffin-embedded tissue was sectioned (4 μm thick). The sections were stained with H&E. Metastatic tumors (T) are shown within the liver parenchyma (II and III). The CT26/Neo transfectant shows fewer foci in the liver parenchyma (I), and its metastatic tumor had a smooth margin between the normal hepatocytes (II). The CT26/AS-CTGF tumor shows a satellite nodule (circled) and an unsmooth, invaded border between the liver parenchyma (III). A magnified view of the CT26/AS-CTGF tumor is shown in IV (original magnification 100×).

tutive activation of the pathway. HCT116 cells were transiently transfected with CTGF expression vector and the reporter plasmids TOPflash or FOPflash and pCMV-β-galactosidase to normalize for transfection efficiency. Figure 6*A* (left panel) shows that transfection with various concentrations of CTGF vector dose-dependently inhibited TCF transcriptional activity in HCT116 cells. In HT-29 cells, a similar inhibition of TCF transcription reporter activity by transfection with the CTGF vector was also seen (Figure 6*A,* right panel). Due to the fact that CTGF inhibited the TCF activity, we considered the possibility that CTGF might affect the level or location of β-catenin. Western blot analysis shows that neither the total cellular nor the nuclear fraction levels of β-catenin were altered in HCT116 cells that had been transfected either stably (Figure 6*B*) or transiently (data not shown) with the CTGF expression vector.

To further define whether the downstream effector genes of the β-catenin/Tcf signaling pathway were affected by CTGF, we examined the expression of MMP-7 by RT-PCR and Western blotting. As shown in Figure 6*C,* the level of MMP-7, but not MMP-9, mRNA and protein expression were significantly decreased in HCT116 by stable transfection with CTGF expression vector. In contrast, the level of MMP-7 protein was significantly increased in CT26 cells stably transfected with antisense CTGF (Figure 6*C,* right panel). To confirm the role of MMP-7 in CTGF-modulated CRC cell invasiveness, we further treated CT26/*Neo* and CT26/AS-CTGF cells with MMP-7 neutralizing antibody in the lower chamber. After 48 hours, the invasive ability of CT26/AS-CTGF cells was significantly decreased in a dose-dependent manner after MMP-7 neutralizing antibody treatment (Figure 6*D*). These results indicated that CTGF expression–reduced CRC cell invasion is mediated by interfering with the β-catenin/Tcf signaling pathway and its downstream effector gene MMP-7.

Discussion

In the present study, we defined, for the first time, CTGF as an independent prognostic factor that can successfully predict survival and disease-free survival for patients with CRC with stage II and stage III tumors. The 5-year survival rate for patients with stage II disease whose tumor showed higher CTGF expression was 87.5%, whereas lower CTGF expression resulted in a survival rate of 47.5% ($P = .005$). The 5-year survival rate for patients with stage III disease whose tumor showed higher CTGF expression was 76.7%, whereas lower CTGF expression resulted in a survival rate of only 28.1% ($P = .004$). Western blotting data from a subset of patients also supported that the patients with CRC with longer survival times had higher levels of CTGF protein. In addition, the clinical observations were supported by our experimental research in which a reduction in the CTGF level in human or mouse CRC cells by transfection with CTGF antisense significantly enhanced their invasive and metastatic abilities in vitro and in vivo. Interestingly, the β-catenin/Tcf signaling and its downstream effector gene, MMP-7, were effectively attenuated by CTGF overexpression in human CRC cells.

Table 3. Antimetastatic Effect of Connective Tissue Growth Factor in the Liver Metastasis Model in Mice

	Liver metastasis			
	Nodule count		Liver weight	
Cell line	Median no. (range)	*P* value[a]	Median gram (range)	*P* value[a]
CT26/Neo	0.5 (0–2)	0.0039	1.14 (0.86–1.32)	0.0008
CT26/AS-CTGF	18.5 (6–39)		1.84 (1.36–2.54)	

Note. Each group contained 10 mice. Nodule counts and liver weights were compared between CT26/Neo and CT26/AS-CTGF groups with Mann–Whitney test. All statistical tests were 2-sided.
[a]*P* values of less than 0.05 were considered statistically significant.

Collectively, our current findings provide evidence that CTGF not only potentially acts as a prognostic marker for patients with CRC with stage II and stage III disease but also has a novel function in inhibiting CRC cell invasion and metastasis.

The current prognostic factor in CRC with proven worth, greatest importance, and widest practical clinical use is pathologic staging. In general, the 5-year survival rate of patients with stage I tumors is close to 90%, and this contrasts with around 10% for patients with stage IV tumors. Survival at stages II and III is less predictable than for the early and advanced stages. The range of 5-year survival rates in patients with stage II CRC is about 60%–80% and in patients with stage III CRC is about 30%–60%.[28] About a 20%–30% difference in the 5-year survival rate for the same stage means that there would seem to be certain subgroups of the population of patients with early recurrence, resistance to chemotherapy, and a decreased survival that cannot be predicted by common staging criteria. For these possible "high-risk" patients, adjuvant chemotherapy should be considered. Based on this scenario, several putative molecules have been studied and evaluated for use as possible prognostic markers to identify high-risk patients in the stage II or stage III CRC category. These molecular markers include Ki-67,[29] p53,[29,30] K-ras,[30,31] chromosome 18q loss,[32] c-erbB2,[33] c-myc,[34] MMP,[23–25] and vascular endothelial growth factor.[35] Unlike most molecular markers, CTGF is a secreted protein; this will allow earlier monitoring and/or evaluation of its level using patients' serum. The possible relevance of such a serum marker is shown by the fact that CTGF and its cleaved fragments can be detected in normal human uterine secretory fluid.[36,37]

CTGF is believed to be a multifunctional signaling modulator involved in a wide variety of biologic or pathologic processes, such as angiogenesis, osteogenesis, renal disease, skin disorders, and tumor development.[5–9,17] Of particular interest is the fact that CTGF, in general, is found to be elevated in human tumors, including invasive mammary ductal carcinomas,[38] dermatofibromas, pyogenic granuloma, endothelial cells of angiolipomas, and angioleiomyomas,[39] and in pancreatic tumors.[13] In glioblastoma, CTGF is strongly stained in tumor cells and proliferating endothelial cells, and this has been used to link this with a role for CTGF in angiogenesis.[11] Based on these studies, CTGF seems to be a positive regulator of tumor development and progression. In contrast, a recently increasing body of evidence shows that other CCN family members, whose protein sequences are similar to CTGF, exhibit a negative regulatory effect on tumor development. For example, in neuroblastomas with poor prognostic features, Nov (CCN3) staining is low and moderate within the tumor cells; in tumors with a favorable prognosis, Nov staining is strongly detected in the differentiated ganglion-type cells.[8] A decrease in Nov expression by tumor cells was also observed in human chronic myeloid leukemia[40] and Wilms' tumors.[41] Cyr61 (CCN1) expression was shown to be down-regulated in tumor cells of uterine leiomyomas,[42] rhabdomyosarcomas,[43] prostate carcinomas,[44] and non–small cell lung cancer.[45] In addition, the stable expression of Cyr61 in non–small cell lung cancer cells causes a significant reduction in proliferation rate and tumor growth in mice.[45] Importantly, ectopic overexpression of CTGF in oral squamous cell carcinoma cells induces attenuated cell growth and less tumorigenicity in an animal model.[16] Collectively, these results suggest that the role of CTGF in different types of cancer may vary considerably, depending on the tissue involved. The question of how the cell or tissue context is able to determine the action of the CTGF protein is interesting and deserves further investigation.

The activation of β-catenin/Tcf signaling is crucial in colorectal carcinogenesis,[46,47] because genetic disruption of this signaling should suppress the formation of the CRC.[48] In a search for the target genes trans-activated by the β-catenin/Tcf pathway, many sets of genes have been identified, including cyclin D1,[49] c-myc,[50] vascular endothelial growth factor,[51] MMP-7,[23,24] and so on. When dissecting the functional roles of these genes, the effect of the β-catenin/Tcf signaling pathway should not be limited to the mechanism of colorectal carcinogenesis. In-

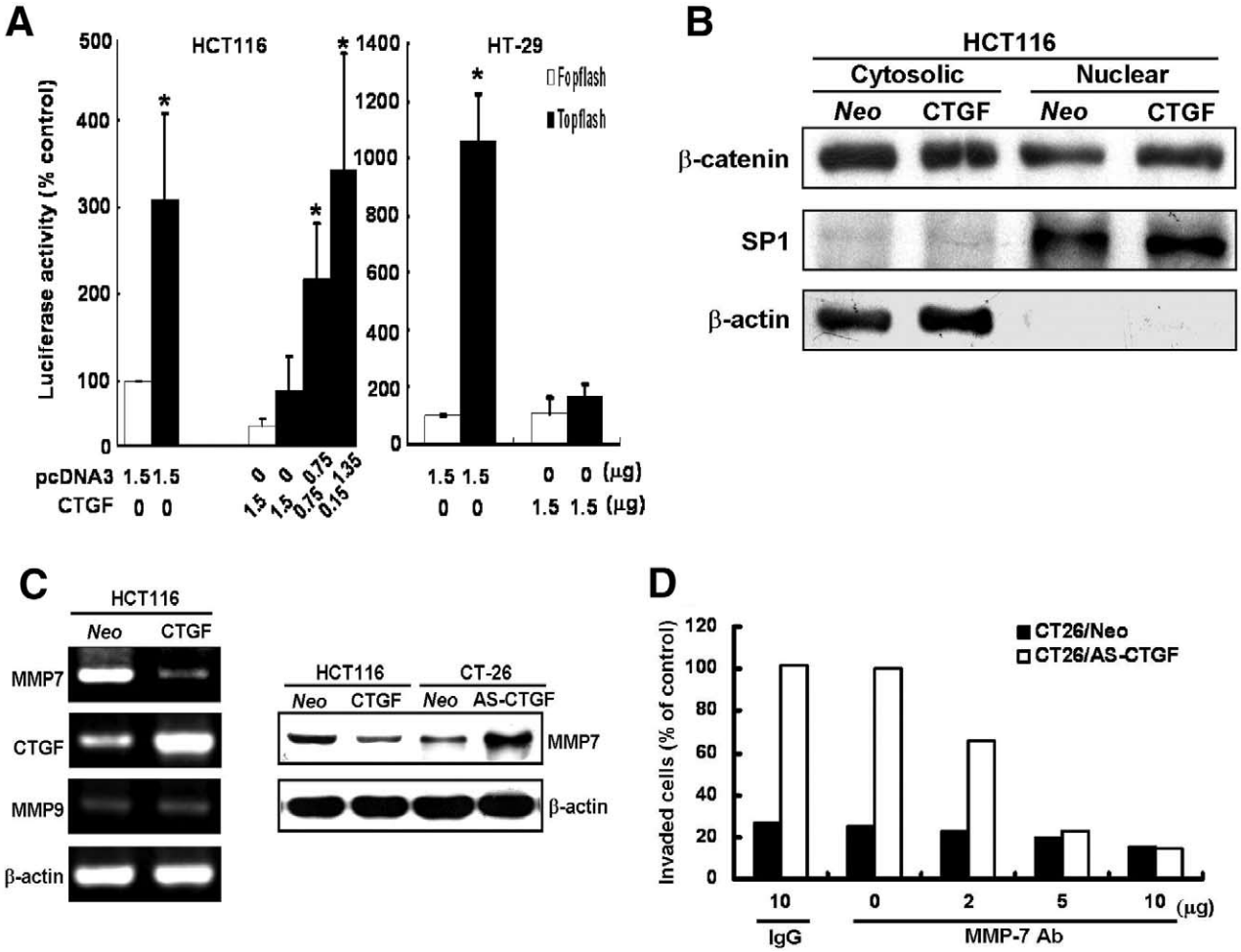

Figure 6. Functional inhibition of the TCF-responsive luciferase reporter gene by CTGF. (*A, left panel*) HCT116 cells were transiently cotransfected with the CTGF vector and one of 2 luciferase reporter plasmids, either with TCF-4 binding sites (TOPflash) or a mutant TCF-binding site (FOPflash). After 48 hours, cells were harvested and processed to determine the luciferase gene-reporter activity. The data points represent the means of triplicate experiments. (*Right panel*) HT-29 cells were transiently cotransfected with CTGF vector and TOPflash or FOPflash as described in Materials and Methods. (*B*) The Western blots show the cytosol and nuclear protein expression of β-catenin, SP1, and β-actin in stable transfected HCT116 cells. SP1 acts as a nuclear protein positive control, and β-actin acts as a cytosol protein positive control. (*C*) RT-PCR analysis of stable transfected CTGF and control vector in HCT116 cells probed with CTGF, MMP-7, MMP-9, and β-actin as described in Materials and Methods (*left panel*). Western blotting assay of stable transfectants of HCT116 and CT26. The total cell lysates probed with antibodies to MMP-7 and β-actin (*right panel*). (*D*) Invasion assay of MMP-7 neutralizing antibody treatment of CT26 transfectants. CT26/*Neo* or CT26/AS-CTGF cells in 100 μL of 1% low-serum medium were placed in the upper Boyden chamber coated with Matrigel, and MMP-7 neutralizing antibody (at 2, 5, and 10 μg/mL) was placed in the lower chamber in the same low-serum medium. After 48 hours in culture, the invasive cells were fixed in methanol and counted. Each clone was plated in triplicate in each experiment. **P* values of $<.05$ were considered statistically significant.

deed, nuclear β-catenin and MMP-7 were found to be coincidently expressed at the invasive front of colorectal tumors, and this has been strongly correlated with a poor prognosis for patients with CRC.[25] Interestingly, our data showed that CTGF overexpression strongly suppresses β-catenin/Tcf activity and MMP-7 mRNA and protein levels in human HCT116 cells (Figure 6*A* and *C*). In contrast, reduced CTGF expression in CT26 cells enhanced MMP-7 protein level. Furthermore, Figure 6*D* showed that anti–MMP-7 neutralizing antibodies actually could abrogate the increased invasiveness of CT26/AS-CTGF. Therefore, we suggest that CTGF acts to inhibit CRC invasion and metastasis and this, at least in part, is mediated by inhibition of β-catenin/Tcf activity and MMP-7 expression.

It has been reported that CTGF can bind with integrins, including $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, and $\alpha_6\beta_1$,[52–55] and low-density lipoprotein receptor.[56] Integrins could modulate the β-catenin level through the integrin-linked kinase and GSK-3β,[57] and it was shown that CTGF binding to low-density lipoprotein receptor could interact with Wnt receptor complex.[58] Previously mentioned is a canonical Wnt pathway in which the cytoplasmic β-catenin protein should translocate into the nucleus. Due to

our previous finding that CTGF failed to alter nuclear or cytoplasmic β-catenin levels, we suggest that other mechanisms or molecules are probably involved in the action of CTGF. In addition, our data describe the inhibition of β-catenin/Tcf activity by CTGF in 2 distinct human CRC cells, HCT116 and HT-29, despite the fact that they have different genetic backgrounds. This implies that CTGF may perhaps induce unknown factor(s) that interact with convergent or critical molecules of the signaling pathway. The accumulating evidence indicates that the signaling/oncogenic activity of β-catenin/Tcf can be trans-repressed by the activation of nuclear receptors such as the retinoic acid receptor,[59,60] the vitamin D receptor,[61] and the androgen receptor.[62] Our preliminary data from microarray analysis show that retinoic acid receptor mRNA was up-regulated in CTGF transfectants (data not shown). It is therefore possible that CTGF expression up-regulates nuclear receptor genes and inhibits β-catenin/Tcf activity by interacting with them. This working hypothesis will need to be tested.

In conclusion, our findings document that on clinical inspection, patients with CRC with decreased CTGF expression have more lymph node metastasis, a shorter recurrence time, and a shorter survival time. Particularly, CTGF seemed to be an independent prognostic factor that will allow the successful differentiation of a high-risk population from the group of patients with stage II and stage III disease. At a mechanistic level, overexpression of CTGF in human CRC cells results in a decrease in the invasive ability. Consistently, reduced CTGF expression significantly enhanced hepatic metastasis of CRC cells in a mouse model. Interestingly, CTGF transfection strongly reduced β-catenin/Tcf signaling and the level of its downstream gene target MMP-7 in human CRC cells. We suggest that CTGF inhibits CRC invasion and metastasis perhaps through inhibition of the β-catenin/Tcf/MMP-7 pathway. Furthermore, the utility of CTGF to inhibit metastasis and invasion suggests that this growth factor may be a relevant candidate for the treatment of patients with CRC.

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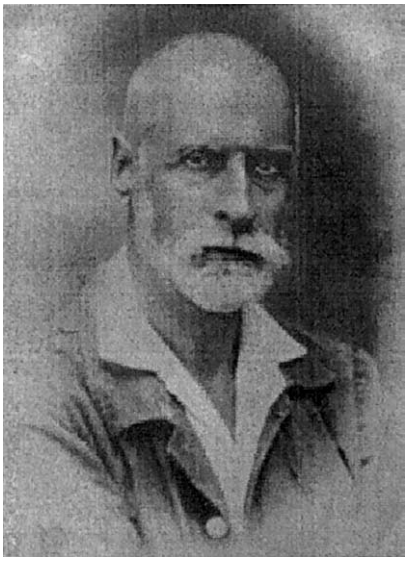
Yersin of Yersinia Infection



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Alexandre Emile John Yersin (1863–1943) was born at Lavaux, Switzerland, in a gunpowder factory where his father was superintendent and the family had lodgings. The father, whose avocation was entomology, died suddenly a few weeks before Alexandre's birth. The widowed mother sought to make a living as headmistress of a finishing school for girls at the nearby town of Morges. Perhaps inspired by the scientific bent of his father, Alexandre enrolled in the medical school of the University of Marburg, but soon chose to continue his studies in Paris where he met Pasteur and became an assistant to the bacteriologist Emile Roux. After qualifying in medicine and becoming a French citizen, he was gripped by wanderlust and ventured to Southeast Asia as a ship's doctor. Entranced by his new, exotic venue, Yersin embarked on a study of plague, then rampant in the Far East. His diligence was rewarded by discovery of the causative bacillus, now known as *Yersinia pestis.* From this an effective vaccine was produced. More familiar to present-day gastroenterologists is *Yersinia enterocolitica,* the cause of acute enteritis and mesenteric adenitis in man and animals. Yersin's illustrious career ended with his death, during World War II, at Nha Trang in Vietnam where candles and incense still burn at his grave.

—Contributed by WILLIAM S. HAUBRICH, M.D.
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