# CLINICAL–ALIMENTARY TRACT

## 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-