Connective Tissue Growth Factor Immunohistochemical Expression Is Associated With Gallbladder Cancer Progression

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• ***Context.***—**Gallbladder cancer (GBC) is an aggressive neoplasia associated with late diagnosis, unsatisfactory treatment, and poor prognosis. Molecular mechanisms involved in GBC pathogenesis remain poorly understood. Connective tissue growth factor (CTGF) is thought to play a role in the pathologic processes and is overexpressed in several human cancers, including GBC. No information is available about CTGF expression in early stages of gallbladder carcinogenesis.**

Objective.—**To evaluate the expression level of CTGF in benign and malignant lesions of gallbladder and its correlation with clinicopathologic features and GBC prognosis.**

Design.—**Connective tissue growth factor protein was examined by immunohistochemistry on tissue microarrays containing tissue samples of chronic cholecystitis (n = 51), dysplasia (n = 15), and GBC (n = 169). The samples were scored according to intensity of staining as low/absent and high CTGF expressers. Statistical analysis was performed using the χ^2 test or Fisher exact probability test with a significance level of $P < .05$. Survival analysis was assessed by the Kaplan-Meier method and the log-rank test.**

Results.—**Connective tissue growth factor expression showed a progressive increase from chronic cholecystitis to dysplasia and then to early and advanced carcinoma. Immunohistochemical expression (score ≥2) was significantly higher in advanced tumors, in comparison with chronic cholecystitis ($P < .001$) and dysplasia ($P = .03$). High levels of CTGF expression correlated with better survival ($P = .04$).**

Conclusions.—**Our results suggest a role for CTGF in GBC progression and a positive association with better prognosis. In addition, they underscore the importance of considering the involvement of inflammation on GBC development.**

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Gallbladder cancer (GBC) is an aggressive neoplasia associated with late diagnosis, unsatisfactory treatment, and poor prognosis.[1] It is characterized by a wide geographic distribution, and Chile is one of the countries with the highest GBC mortality rates in the world.[2] Gallbladder cancer is often discovered incidentally during or after a cholecystectomy, when tumors are unfortunately at an advanced stage. At present, early stage tumors are often curable with a proper resection, whereas advanced GBC requires additional treatment with adjuvant therapy; however, only surgery offers any benefit in terms of survival in GBC.[3–5]

Among the traditional prognostic factors in GBC, the stage of the disease at diagnosis is the most important, followed by histologic grade, depth of wall infiltration, and lymph node metastases.[6] In addition, a large number of molecular alterations with clinical significance have been proposed as potential prognostic markers, including aberrant expression of proteins associated with tumor progression, invasion, and metastases.[7–9] The screening of these alterations in normal epithelium and in benign and malignant gallbladder lesions could help to understand the molecular mechanisms involved in gallbladder carcinogenesis and to identify potential markers for early detection of the neoplastic process.

Connective tissue growth factor (CTGF), also known as CCN2, is a member of the CCN family. All CCN family members are secreted proteins associated with the extracellular matrix, and they are involved in normal processes such as implantation, placentation, embryogenesis, differentiation, and development, as well as in pathologic processes including wound healing, fibrotic disorders, and tumorigenesis.[10] Connective tissue growth factor plays an important role in tumor development and cancer progression, and it is found to be expressed in different types of cancer.[11] The functional implications of CTGF overexpression in the biological behavior of cancer cells depend on the

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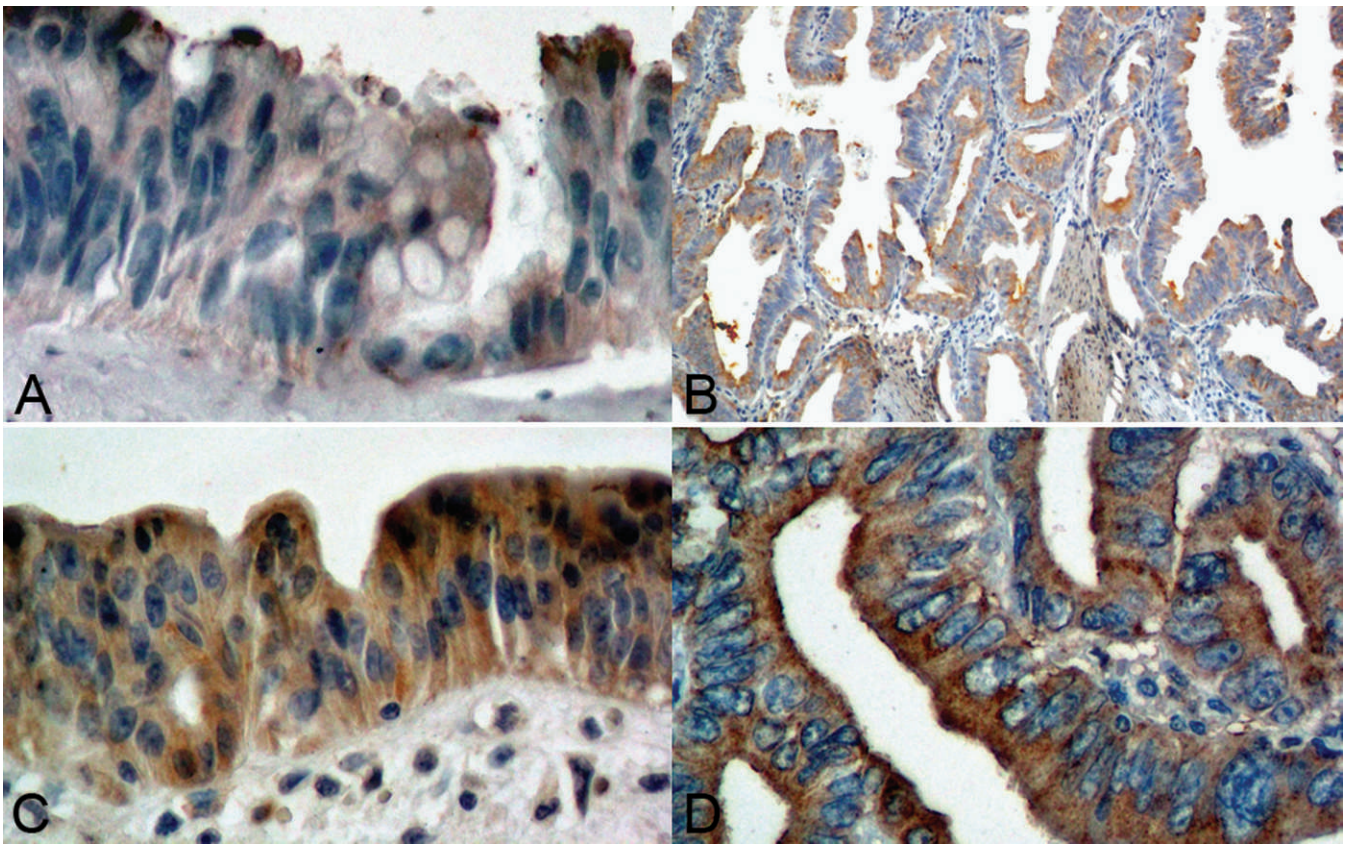


Figure 1. *Immunostaining of connective tissue growth factor in gallbladder tissues. A, Weak staining in early cancer (pT1a). B, Moderate staining in advanced gallbladder cancer (pT2). C, Strong staining in dysplasia. D, Strong staining in advanced gallbladder cancer (pT3) (immunohistochemistry, original magnifications ×100 [B] and ×400 [A, C, D]).*

tumor origin. For example, high CTGF levels have been associated with tumor growth and adverse prognosis in pancreatic cancer[12] and gastric cancer.[13,14] Conversely, CTGF overexpression is associated with metastasis inhibition and is a favorable prognostic marker in colorectal cancers[15] and lung cancers.[16,17]

In GBC, immunohistochemical expression of CTGF in surgical specimens has shown that advanced cancers with high CTGF expression have a favorable prognosis.[18] A better understanding of the role of CTGF in GBC pathogenesis starts by studying the expression of this protein in early stages, including inflammatory and precursor lesions (chronic cholecystitis and dysplasia). Therefore, in the present study we examined by immunohistochemistry the expression of CTGF in benign and malignant gallbladder lesions in order to analyze its possible involvement in malignant progression. Additionally, we evaluated whether CTGF expression is associated with clinicopathologic parameters and prognosis in GBC.

MATERIALS AND METHODS

Patients and Tissue Samples

A total of 235 gallbladder lesions from patients who underwent cholecystectomy between 1987 and 2006 were selected. The formalin-fixed, paraffin-embedded tissues were retrieved from the surgical pathology archives at Hernán Henríquez Aravena Hospital, Temuco, Chile. These samples included 51 chronic cholecystitis samples, 15 dysplasias (10 low-grade and 5 high-grade dysplasias) and 169 adenocarcinomas grouped into 32 early cancers and 137 advanced carcinoma according to T stage (infiltration level): mucosal (pT1a), muscular (pT1b), subserous (pT2) and serous (pT3). According to the TNM staging system for GBC (*AJCC Cancer Staging Manual*, 7th edition),[19] the adenocarcinomas were grouped as stage I (32 cases), stage II (81 cases), stage IIIA and IIIB (24 cases), and stage IVA and IVB (32 cases). The clinicopathologic features were obtained from medical records. Complete postoperative follow-up was available for 121 of 137 patients with advanced GBC. This study was approved by the ethics committee of the Universidad de La Frontera, Temuco, Chile.

Immunohistochemistry

Tissue microarrays were constructed with 2-mm cores of 3 different representative areas of each case. Unstained 4-μm-thick sections were cut from each tissue microarray and then dewaxed in xylene, rehydrated through graded concentrations of ethanol, and placed in an antigen retrieval solution (citrate buffer, pH 6.0) for 15 minutes at 95°C. After cooling for 30 minutes, the tissue sections were quenched with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. The slides were then washed thoroughly with phosphate-buffered saline and stained for 120 minutes at room temperature with goat polyclonal anti-CTGF antibody using a 1:100 dilution (clone L-20; Santa Cruz Biotechnology Inc, Santa Cruz, California). Labeling was detected with the Liquid DAB Substrate-Chromogen System (Dako North America Inc, Carpinteria, California) according to the manufacturer's protocol. Sections were counterstained with hematoxylin, then dehydrated, cleared, and mounted. Negative control was prepared by replacing the primary antibody with phosphate-buffered saline.

Evaluation of CTGF Immunostaining

Connective tissue growth factor is expressed in the cytoplasmic compartment with membranous accentuation. Based on intensity

of labeling, a semiquantitative scale of 0–3 was used to score the reactivity of the samples (0, absent; 1, weak; 2, moderate; 3, strong), using a method that had been validated earlier.[18] Subsequently, the 235 samples were arbitrarily classified as absent/low CTGF expressers (score 0 and 1) or high CTGF expressers (score 2 and 3). The evaluation of the immunohistochemical staining was independently performed by 2 pathologists without knowledge of clinical data.

Statistical Analysis

The analyses were performed using the statistical package SPSS version 17.0 (SPSS Inc, Chicago, Illinois). A comparison of the background data was made between the low-CTGF and the high-CTGF groups. The correlation of CTGF expression with the clinical and pathologic variables was assessed using the χ^2 test or Fisher exact probability test (2-sided). Kaplan-Meier survival curves were plotted for patients with high versus low CTGF expression and compared using a stratified log-rank test. The stratification factor was the infiltration level, because this covariate has been recognized as a strong predictor of survival in patients with advanced GBC.[6,20] The level of significance was set at $P < .05$.

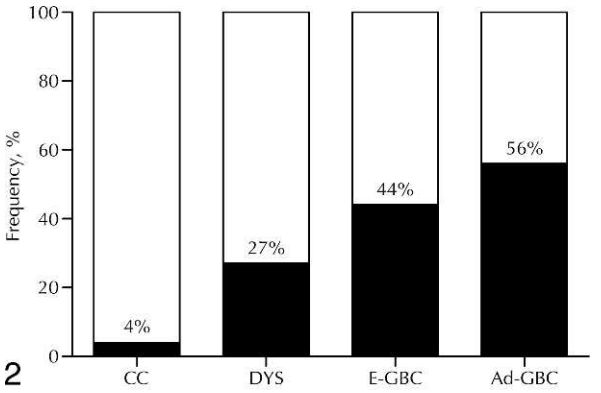
RESULTS

Connective tissue growth factor immunoreactivity showed a distinctive labeling in the cytoplasm and membrane of nonneoplastic and neoplastic epithelial cells. Examples of staining intensity are illustrated in Figure 1, A through D. In only 16 of 235 cases (1 early cancer and 15 advanced carcinomas) did the tumor cells show complete absence of staining; these were classified as CTGF low expressers. Of the 51 chronic cholecystitis analyzed, 49 (96%) had low CTGF expression, whereas only 2 (4%) showed high CTGF levels. In dysplasia, low and high expression levels of CTGF were 73% (11 of 15) and 27% (4 of 15), respectively. According to the degree of dysplasia, a high CTGF expression was observed in 30% (3 of 10) of the cases with low-grade dysplasia and in 20% (1 of 5) of the cases with high-grade dysplasia.

The incidence of high CTGF expression among the adenocarcinomas was 44% (14 of 32) in early cancers, and 56% (76 of 137) in advanced carcinomas (Figure 2). As shown in Table 1, the level of CTGF was significantly higher in gallbladder adenocarcinomas than in either dysplasia tissues ($P = .03$) or chronic cholecystitis ($P < .001$). No statistically significant differences were found in CTGF levels between sequential lesions (chronic cholecystitis to dysplasia; dysplasia to early cancer).

The relationship between CTGF expression and each clinicopathologic factor was analyzed for each gallbladder lesion. Consistent with previous study,[18] no significant correlation was found between the level of CTGF and patients' age, sex, ethnic group, or tumor differentiation (Table 2).

Clinical outcome was analyzed in 121 patients with advanced GBC (excluding 16 patients who died before 30 days postsurgery). The observation time ranged from 1 to 243 months, with a median time of 13 months. The relationship between CTGF expression and patient survival at 5 years postsurgery was examined by Kaplan-Meier analysis. The entire group (n = 121) had an estimated 5-year survival rate of 31% with a median survival of 17.1 months. Patients with absent/low CTGF labeling (n = 50) had a 5-year survival rate of 22% with a median survival of 14.8 months, whereas patients with high CTGF labeling (n = 71) had a 5-year survival rate of 37% with a median survival of 18.1 months. According to a predetermined criterion



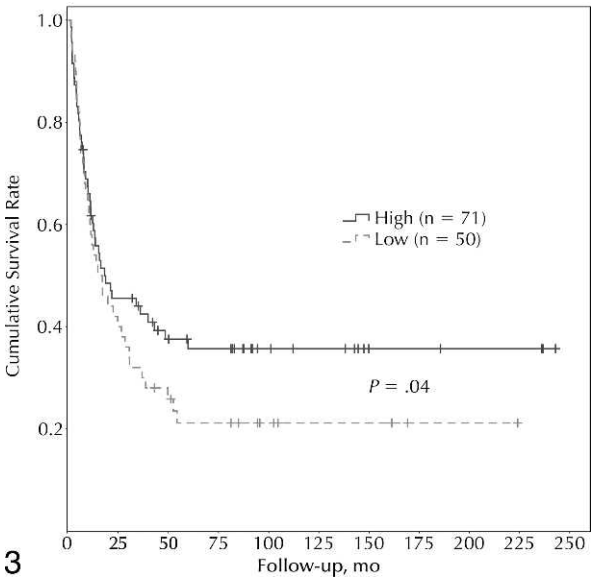


Figure 2. *Frequency distribution for high connective tissue growth factor expression in sequential gallbladder lesions. Ad-GBC, advanced cancer; CC, chronic cholecystitis; DYS, dysplasia; E-GBC, early cancer.*

Figure 3. *Five-year survival curves (Kaplan-Meier) for patients with advanced gallbladder carcinoma. The solid line indicates patients whose tumors express high levels of connective tissue growth factor (CTGF) and the dotted line indicates patients with low CTGF expression (*P *=.04; stratified log-rank test).*

(stratified log-rank test), survival distributions were statistically significant between groups and high CTGF expression was associated with better patient outcomes ($P = .04$) (Figure 3).

Table 1. Connective Tissue Growth Factor (TGF) Expression in Benign and Malignant Lesions of Gallbladder

Tissue Type	Total No.	High CTGF, No. (%)	χ^2	*P*[a]
Advanced GBC	137	76 (56)		
Early GBC	32	14 (44)	1.432	.23
Dysplasia	15	4 (27)	4.500	.03
High-grade	5	1 (20)		
Low-grade	10	3 (30)		
Chronic cholecystitis	51	2 (4)	40.69	<.001

Abbreviation: GBC, gallbladder cancer.

[a] Compared with advanced GBC.

Table 2. Association Between CTGF Expression (Low/High) and Clinicopathologic Parameters												
	Chronic Cholecystitis (n = 51)			Dysplasia (n = 15)			Early Cancer (n = 32)			Advanced Cancer (n = 137)		
	Low	High	*P*	Low	High	*P*	Low	High	*P*	Low	High	*P*
Age			>.99[a]			.60[a]			.31[b]			.43[b]
<60 y	34	2		6	3		11	6		24	25	
≥60 y	15	0		5	1		7	8		37	51	
Sex			.64[a]			.64[a]			.17[a]			.95[b]
Female	39	2		9	3		15	14		54	67	
Male	10	0		2	1		3	0		7	9	
Ethnicity			>.99[a]			. . .			>.99[a]			.22[b]
Hispanic	42	2		11	4		16	13		51	57	
Mapuche	7	0					2	1		10	19	
Tumor differentiation									.96[b]			.31[b]
Well							6	4		14	23	
Moderate							6	5		32	30	
Poor							6	5		15	23	
TNM staging									. . .			.47[b]
I							18	14				
II										38	43	
IIIA										4	12	
IIIB										4	4	
IVA										4	3	
IVB										11	14	

Abbreviation: CTGF, connective tissue growth factor.

[a] Fisher exact test.
[b] Pearson χ^2 test.

The relevance of 5-year survival and other clinicopathologic characteristics were also assessed by Kaplan-Meier analysis, which showed that serosal infiltration (pT3) was significantly associated with poorer survival of patients with advanced GBC ($P < .001$), whereas age, sex, ethnicity, tumor differentiation grade, and TNM staging did not account for poor prognosis (Table 3).

COMMENT

In the present study we evaluated the expression of CTGF in the epithelial component of benign (chronic cholecystitis), premalignant (dysplasia), and malignant gallbladder lesions (early and advanced carcinoma). We found that CTGF is overexpressed with significantly higher frequency in advanced tumors, showing a progressive increase from chronic cholecystitis to dysplasia and then to early and advanced carcinoma. Furthermore, in accordance with the work of Alvarez et al,[18] Kaplan-Meier analysis showed that overexpression of CTGF was significantly associated with better survival of patients with advanced GBC.

Several lines of evidence support a role for CTGF in fibrotic disorders and tumorigenic processes. In fact, it has been documented that CTGF and the other members of the CCN family are aberrantly expressed in cancer and that they are linked with either promotion or inhibition of the pathologic processes.[21,22] This seemingly contradictory role

Table 3. Five-Year Survival Analysis According to Clinicopathologic Factors					
Variable	Cases (n = 121)	Events (n = 83)	5-y Survival Rate, %	Median Survival, mo	*P*
Age					.11
<60 y	47	35	25.5	13.5	
≥60 y	74	48	35.1	22.5	
Sex					.51
Female	109	75	31.2	17.1	
Male	12	8	33.3	9.9	
Ethnicity					.76
Hispanic	96	65	32.3	17.1	
Mapuche	25	18	28.0	13.3	
Tumor differentiation					.25
Well	38	22	42.1	48.4	
Moderate	47	35	25.5	14.8	
Poor	36	26	27.8	12.2	
Infiltration					<.001
Subserous (pT2)	97	61	37.1	22.5	
Serous (pT3)	24	22	8.3	5.9	
TNM Staging					.23
II	71	46	35.2	21.8	
III + IV	50	37	26.0	13.3	
CTGF expression					.045
High	71	44	38.0	18.1	
Low	50	39	22.0	14.8	

Abbreviation: CTGF, connective tissue growth factor.

of CTGF in human malignancies appears to depend on the tissue involved. It has been reported that high CTGF expression is associated with a worse overall survival in gastric cancer[14] and esophageal squamous cell carcinoma,[23] whereas a reduced expression of CTGF correlates with advanced stage of disease, lymph-node metastases, and/or shorter survival in breast cancer,[24] lung cancer,[16,25] intrahepatic cholangiocarcinoma,[26] and GBC.[18] The possible molecular mechanisms involved in CTGF-mediated modulation of tumor cell behavior have been studied in some of these neoplasms. They have been found to be related to the expression of some important cancer progression/suppression-related molecules or pathways, such as TGF-β, HIF-1α, β-catenin/Tcf/MMP-7, and PI3K/AKT.[11]

Collectively, our findings suggest that CTGF is involved in gallbladder carcinogenesis. Our results showed that CTGF is expressed at low levels in chronic cholecystitis, suggesting a role for this protein in the inflammatory process of the gallbladder. Several lines of evidence have demonstrated the participation of CCN proteins in inflammation. Interestingly, CCN expression is regulated by several factors that act as mediators of the inflammatory process (eg, nitric oxide, interleukins, TNF-α, TGF-β) and, conversely, these also participate in the regulation of the expression of cytokines, chemokines, and matrix metalloproteinases.[27]

It is now accepted that several cancers are linked to chronic inflammatory states, and that GBC usually emerges from a background of gallstones and chronic inflammation of the gallbladder mucosa.[28] In the dysplasia-carcinoma sequence the initial lesions on the mucosal epithelium are attributable to inflammation.[29] The inflammatory process could be activated by the irritation caused by gallstones, which creates a propitious condition for the development of a persistent local inflammatory state. The chronically inflamed mucosa undergoes adaptive changes such as metaplasia that could progress to dysplasia, the most widely accepted precursor lesion for GBC.[30] Several cytokines, growth factors, and small molecules, such as TGF-β, TNF-α, and COX2, that are actively involved in the inflammatory response are deregulated in GBC and have been associated with malignant transformation of gallbladder epithelium.[31–35] As mentioned in the literature, CTGF may be differentially regulated by some components of the inflammatory process, contributing to pathogenesis in highly inflamed tissues.[27,36] We found that CTGF levels increase progressively from CC to advanced GBC, and, in a cancer-related inflammation context, it might be possible that complex interactions between crucial inflammatory mediators and CTGF in early stages of gallbladder carcinogenesis—in association with other carcinogenic stimuli—may contribute to the development of sequential histologic changes of the mucosal epithelium favoring the appearance of precursor lesions that ultimately lead to invasive carcinoma.

We found that CTGF protein levels in infiltrating gallbladder carcinoma were significantly higher than in chronic cholecystitis and dysplasia. Furthermore, Kaplan-Meier curves revealed that high CTGF expression was significantly associated with improved survival of patients with advanced GBC (stratified log-rank test, $P = .04$). These results suggest that overexpression of CTGF could interfere with the invasive process in later stages of gallbladder carcinogenesis. As a key downstream modulator of TGF-β signaling, CTGF influences the composition of tumor microenvironment by stimulating the synthesis of extracellular matrix proteins, promoting a desmoplastic reaction and epithelial-mesenchymal transition through autocrine/paracrine signaling.[37] Furthermore, it is noteworthy that CTGF has been found to be expressed either in neoplastic cells and/or in the surrounding stromal cells. In some cancers, such as pancreatic and prostate carcinoma, the expression of CTGF in stromal cells modulates the tumor behavior.[38–40] Advanced GBCs usually show an invasive growth with desmoplastic reaction. It is possible that an enhanced CTGF expression in GBC cells may regulate the stromal composition by means of paracrine action, which could reduce the tumor growth and spread.

In conclusion, our results suggest that CTGF might be playing a dual role in the malignant transformation of the gallbladder. Tumor progression from premalignant to advanced disease would be accompanied by increased levels of CTGF expression with an inflammatory background. This intricate and complex network of inflammatory components may contribute to carcinogenesis by induction of genetic and epigenetic changes, causing alterations in critical pathways and promoting several protumor functions, including enhanced proliferation, resistance to apoptosis, tumor neovascularization, and matrix remodeling. In later stages of gallbladder carcinogenesis, a high expression of CTGF in tumor cells may affect the spectrum of biological responses associated to this protein. The mechanisms involved in this response are unknown, but they are likely related to important modifications in the tumor microenvironment. Functional studies in cell lines and animal models will be necessary to determine whether CTGF acts in an autocrine or paracrine manner on gallbladder tumor initiation and progression.

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