Featured Article

Quantification of Plasma β-Catenin mRNA in Colorectal Cancer and Adenoma Patients

Sze Chuen Cesar Wong,[1] Siu Fong Elena Lo,[1] Moon Tong Cheung,[2] Kai On Enders Ng,[3] Chun Wah Tse,[2] Bo San Paul Lai,[4] King Chung Lee,[1] and Y. M. Dennis Lo[3]

Departments of [1]Pathology and [2]Surgery, Queen Elizabeth Hospital, Hong Kong Special Administrative Region, and Departments of [3]Chemical Pathology and [4]Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong Special Administrative Region, Hong Kong, China

Abstract

Purpose: **Colorectal cancer is an important cause of cancer deaths. Here, we focused our investigation on the β-*catenin* gene which is implicated in colorectal carcinogenesis and tested whether β-*catenin* mRNA is detectable in the plasma of colorectal carcinoma and adenoma patients using quantitative reverse transcriptase-PCR.**

Experimental Design: **Plasma β-catenin mRNA was measured from 58 colorectal carcinoma patients, 49 colorectal adenoma patients, and 43 apparently normal subjects using intron-spanning primers and Taqman probes. Five clinicopathological parameters were studied and correlated with plasma β-*catenin* mRNA concentration. Additionally, 19 colorectal carcinoma patients after tumor removal were also recruited for plasma β-*catenin* mRNA measurement to further demonstrate the clinical usefulness of this test.**

Results: **β-catenin mRNA was detected with median concentrations of 8737 (range: 1480–933,100), 1218 (range: 541–2,254) and 291 (range: 0–1,366) copies/ml plasma in colorectal carcinoma, colorectal adenoma, and apparently normal subjects, respectively. Statistical analysis demonstrated that plasma β-*catenin* mRNA concentration was correlated to tumor stage but not sex, age, lymph node status, and degree in differentiation. Moreover, plasma β-*catenin* mRNA concentration decreased significantly after tumor removal in 16 of 19 (84%) colorectal carcinoma patients.**

Conclusions: **We conclude that plasma β-*catenin* mRNA may potentially serve as a marker for colorectal cancer.**

Introduction

Colorectal cancer is the second leading cause of cancer deaths in Western countries, but detection and treatment of this cancer at an early stage can dramatically improve survival (1) Thus, there has been great interest in developing population-based screening for colorectal cancer to detect asymptomatic colorectal carcinoma, as well as its precursor, colorectal adenoma. Many methods have been evaluated, such as fecal occult blood testing, flexible sigmoidoscopy, screening colonoscopy, virtual colonoscopy, and barium enema, but they have limited sensitivity, specificity, or are invasive procedures (2). Recently, immunostaining for minichromosome maintenance protein 2 in colonocytes and analysis of mutations in DNA extracted from stool have been investigated for diagnosis of colorectal cancer (1, 3).

Assays of tumor or virus-associated DNA and RNA in plasma or serum have been shown to be sensitive and promising techniques for detecting and monitoring various cancers (4–8). Moreover, it is now widely accepted that the pathogenesis of colorectal cancer is caused by sequential multistep accumulation of genetic alterations (9). Among them, the *adenomatous polyposis coli* gene is the most important gatekeeper of colonic epithelial cell proliferation and also responsible for the down-regulation of β-catenin, whose loss of function may lead to the transition of normal colonic mucosa into adenoma and finally colorectal carcinoma (10). β-catenin is a M_r 92,000 protein that is one of the key targets in the Wnt-signaling pathway (11). Several reports indicated that uncontrolled β-catenin expression in Wnt signaling is one of the major pathways that lead to the development of colorectal cancer (10, 12, 13). Immunostaining for β-catenin usually shows abnormal nuclear accumulation in tumor cells, which is a potential prognostic marker in colorectal cancer patients (12). Furthermore, in previous studies, we have noted that: (*a*) colorectal tumors had a randomly distributed pattern of apoptosis (14, 15) and a high microvessel density (16); (*b*) nucleic acid in plasma may come from lysis of circulating cancer cells caused by tumor necrosis, apoptosis, or active release (17); (*c*) hematopoietic cells were reported to be non-adherent in nature (18); and (*d*) our group has studied in β-catenin before with observations that all hematopoietic cells did not express membrane and nucleus β-catenin protein (unpublished data). On the basis of these latter four points, we hypothesize that at least some of the apoptotic cells would release β-*catenin* mRNA into the circulation and that such β-*catenin* mRNA might be detectable in the patients' plasma. Thus, the present study explores the possibility of using quantitative reverse transcriptase-PCR (Q-RT-PCR) in measuring plasma β-*catenin* mRNA in colorectal cancer and adenoma patients. To test whether mRNA other than β-*catenin* is ele-

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Requests for reprints: S. C. Cesar Wong, Department of Pathology, Queen Elizabeth Hospital, Hong Kong Special Administrative Region, Hong Kong, China. Phone: 852-29586813; Fax: 852-29586812; E-mail: cesar01@netvigator.com.

Table 1 Clinicopathological data of studied subjects

Colorectal carcinoma patients ($n = 58$)	
Sex	
Male	39 (68%)
Female	19 (32%)
Age	
Range	30–88 years old
Median	67.5 years old
TNM[a] classification	
Tumor stage	
T1	0 (0%)
T2	15 (26%)
T3	30 (52%)
T4	13 (22%)
Lymph node status	
N0	21 (37%)
N1	24 (41%)
N2	13 (22%)
Degree in differentiation	
Well	0 (0%)
Moderate	44 (76%)
Poor	14 (24%)
Colorectal adenoma patients ($n = 49$)	
Sex	
Male	31 (63%)
Female	18 (37%)
Age	
Range	28–73 years old
Median	53 years old
Degree in dysplasia	
Mild	4 (8%)
Moderate	31 (63%)
Severe	14 (29%)
Apparently normal subjects ($n = 43$)	
Sex	
Male	15 (34%)
Female	28 (66%)
Age	
Range	25–70 years old
Median	34.5 years old

[a] TNM, Tumor-Node-Metastasis.

vated, we also measured the plasma mRNA of a housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Finally, β-*catenin* mRNA level was measured in colorectal cancer patients after tumor removal to further demonstrate the potential clinical relevance of our observations.

Materials and Methods

Plasma Samples. This study was approved by the local institutional ethics committee, and informed consent was obtained from all patients and apparently normal subjects between November 2002 and April 2003. Blood samples from 58 colorectal carcinoma patients before surgical operation and 19 follow-up patients (5–7 days after surgical operation) were collected. Forty-nine patients with suspected colorectal adenoma were also recruited within 20 min after endoscopic removal. The blood samples were sent to the laboratory within 15 min after collection. Gloves were worn during the whole process of treatment. Diagnosis of patients with colorectal carcinoma and adenoma was confirmed by histopathology. Clinical and pathological data, including sex, age, tumor stage, lymph node status, and degree in differentiation were all collected. (Table 1). Forty-three apparently normal subjects were studied as negative controls.

RNA Extraction and Reverse Transcription Reaction. Six milliliters of blood were collected in three EDTA tubes of 2 ml each. They were immediately centrifuged at 3838 × *g* for 8 min at 4°C, followed by careful separation of plasma. They were then stored at −80°C until use. mRNA was extracted from 2 ml of plasma using 1.6 ml of TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD), further purified with an RNeasy column (Qiagen, Inc., Hilden, Germany), according to manufacturers' instructions and dissolved in 15 μl of DEPC-treated water followed by treatment with DNase I (Life Technologies). RT-PCR using Omniscript Reverse Transcriptase Kit (Qiagen) was exploited to generate cDNA for Q-RT-PCR to detect the β-*catenin* and *GAPDH* genes. Each batch of reaction included a positive control from a plasma sample with β-*catenin* mRNA or *GAPDH* mRNA and negative controls without RNA and reverse transcriptase.

Q-RT-PCR Assay. Specific intron-spanning primers and fluorescent probes were designed for β-*catenin* detection (Table 2), whereas those for *GAPDH* were purchased from Applied Biosystems. Q-RT-PCR was set up in a reaction volume of 50 μl using Core Reagents Kit (Applied Biosystems), and 6 μl of cDNA were used for each reaction. Standard protocol of the ABI Prism 7000 Sequence Detector (Applied Biosystems) was used in Q-RT-PCR assay. Calibration curve for β-*catenin* mRNA quantification was prepared by amplifying serial dilutions of a synthetic oligonucleotide (Table 2), with concentrations ranging from 2.5×10^6 to 25 copies/μl. Similarly, the *GAPDH* mRNA calibration curve was prepared by serial dilutions of human control cDNA (Applied Biosystems), with concentrations ranging from 2.6×10^5 to 41 copies/μl. Each batch of amplification included a positive control from β-*catenin* or *GAPDH* calibration oligonucleotide and a negative control without cDNA. Duplicate tests were performed. Finally, the average was calculated for each sample.

Statistics. Statistical analyses (computer software package SPSS v11 for Windows XP; SPSS, Inc.) were carried out using nonparametric Kruskal-Wallis, Spearman correlation, and Wilcoxon matched pairs tests to compare mRNA concentrations among the three groups of subjects and explore the relationship of β-*catenin* mRNA concentrations with sex, age, tumor stage, lymph node status, and degree in differentiation in colorectal cancer patients and between pre and postoperation in colorectal cancer patients, respectively. A $P < 0.05$ was considered significant.

Results

β-*catenin* mRNA was detected in the plasma of all 58 colorectal cancer, 49 colorectal adenoma patients, and 36 (84%) of 43 normal subjects, with median concentrations of 8,737 (range: 1,480–933,100), 1218 (range: 541 to 2,254), and 291 (range: 0–1,366) copies/ml plasma, respectively (Fig. 1*A*). Kruskal-Wallis test showed significant differential quantities among those three groups ($P < 0.0001$). Results were confirmed by another pair of specific intron-spanning primers with a product size of 250 bp visualized in 1.5% agarose gel for 20 colorectal carcinoma and 20 colorectal adenoma plasma samples (Fig. 2).

Table 2 Sequences of primers, probe, and calibration oligonucleotide

Intron-spanning primers	Forward	Exon 2	5′-GCGTGGACAATGGCTACTCA-3′
	Reverse	Exon 3	5′-CCGCTTTTCTGTCTGGTTCC-3′
Dual-labelled fluorescent probe		Exon 3	5′-(FAM[a])TGATTTGATGGAGTTGGACATGGCCA (TAMRA[b])-3′
Oligonucleotide for calibration			5′-ATCCAGCGTGGACAATGGCTACTCAAGCTGA TTTGATGGAGTTGGACATGGCCATGGAACCA GACAGAAAAGCGCTGTT-3′

[a] 6-carboxyfluorescein.
[b] 6-carboxytetramethylrhodamine.

The plasma *GAPDH* mRNA concentration was considered to be a reflection of the total plasma mRNA. All plasma samples from the three groups showed detectable quantities of *GAPDH* mRNA with median concentrations of 1713 (range: 243-4948), 1236 (range: 70–5729), and 1225 (61–6765) copies/ml, respectively (Fig. 1*B*). Kruskal-Wallis test showed no significant difference in the concentrations among those three groups ($P = 0.0712$). The presence of similar quantities of mRNA from a housekeeping gene in all three groups of subjects suggested that plasma β-*catenin* mRNA was selectively elevated in patients with colorectal carcinomas and adenomas. All positive controls had detectable quantities of β-*catenin* and *GAPDH* mRNA, and negative controls did not have any signal in each batch of assay. In addition, Spearman correlation analysis showed that β-*catenin* mRNA concentration in plasma was correlated to tumor stage ($P < 0.05$, $r = 0.778$) but not sex ($P = 0.27$), age ($P = 0.49$), lymph node status ($P = 0.35$), and degree in differentiation ($P = 0.42$) in colorectal cancer patients.

As a further demonstration of the potential clinical relevance of these data, β-*catenin* mRNA concentration was found to decrease significantly ($P < 0.001$; Wilcoxon matched pairs test) in 16 of 19 colorectal cancer patients after tumor removal (Fig. 1*C*).

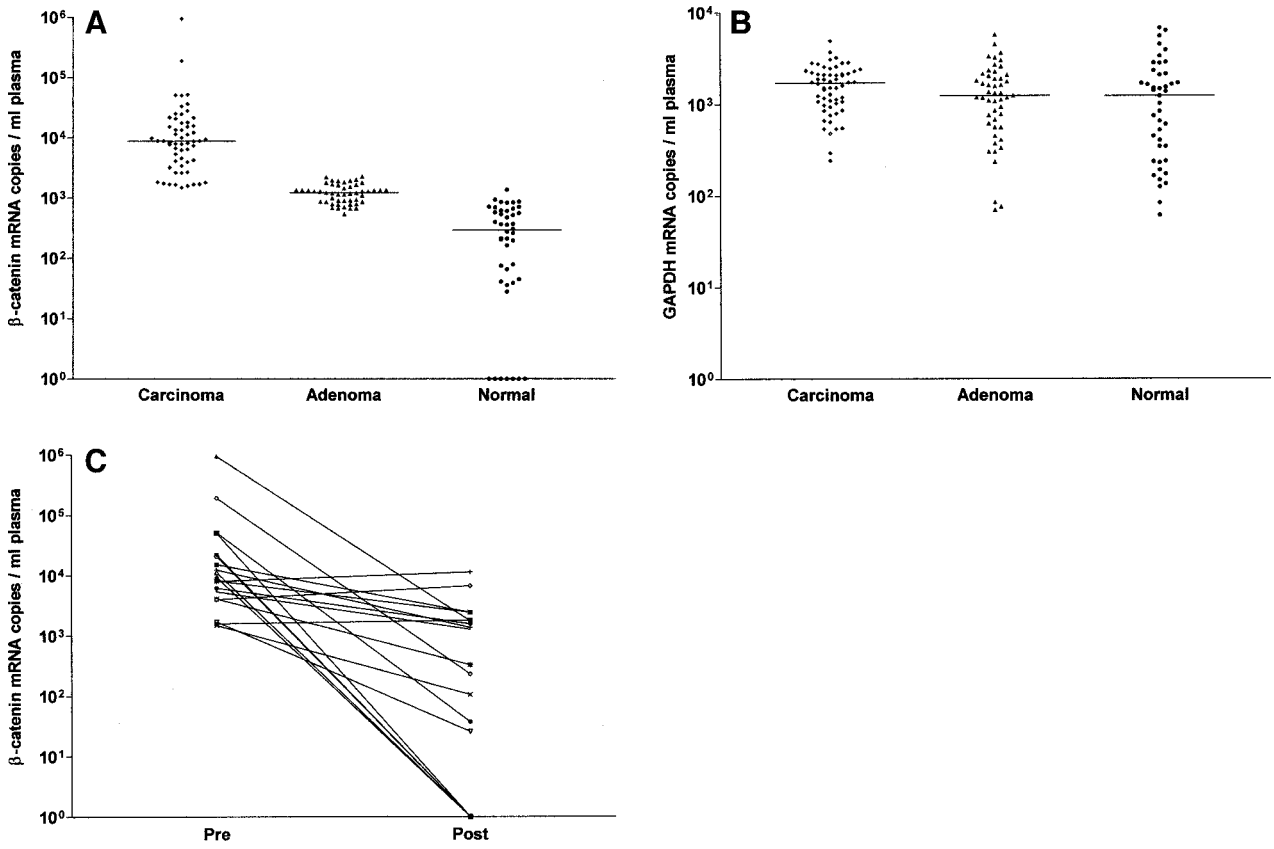

Fig. 1 *A*, β-catenin mRNA in plasma of colorectal carcinoma, adenoma patients, and healthy subjects by quantitative reverse transcriptase-PCR. *Black horizontal line*, the median in each group of subjects. *B*, *glyceraldehyde-3-phosphate dehydrogenase* mRNA in plasma of colorectal carcinoma, adenoma patients, and healthy subjects by quantitative reverse transcriptase-PCR. *Black horizontal line*, the median in each group of subjects. *C*, pre and postoperative β-*catenin* mRNA in 19 colorectal carcinoma patients.

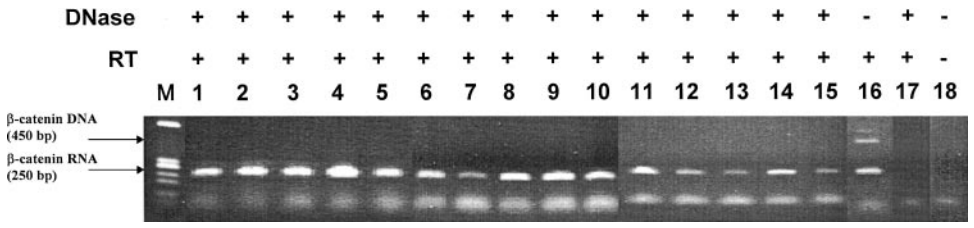

Fig. 2 Detection of plasma β-*catenin* mRNA: (*a*) *Lane 1–10,* β-catenin RNA (250 bp) from 10 colorectal carcinoma patients; (*b*) *Lane 11–15,* β-catenin RNA (250 bp) from 5 colorectal adenoma patients; (*c*) *Lane 16,* β-catenin RNA (250 bp) and DNA (450 bp) from a positive control without DNase; (*d*) *Lane 17,* negative control; (*e*) *Lane 18,* water control; and (*f*) *Lane M,* molecular markers.

Discussion

The finding of a detectable quantity of β-*catenin* mRNA in the plasma of colorectal cancer patients is an exciting development, because it may provide opportunities to establish noninvasive tests for detecting and monitoring this cancer. Our results are promising in the tested cancer subjects, which may be attributable to appropriate selection of mRNA target (β-*catenin*) and the efficiency of our plasma RNA extraction protocol (19). Moreover, this study provides evidence that reference ranges of β-*catenin* mRNA for colorectal carcinoma, adenoma patients, and normal subjects should be established in clinical application.

The measurement of *GAPDH* mRNA can demonstrate at least that there was amplifiable mRNA in a particular plasma sample. Previous studies were mainly based on nonquantitative methods, which may limit the potential clinical usefulness of such systems (6, 7). Our approach of using Q-RT-PCR offered a valuable quantitative dimension to the study and obviated the need for postamplification manipulation, which greatly improved the sensitivity and throughput and reduced the risk of carryover contamination.

One interesting finding from this study is that β-*catenin* mRNA concentration in colorectal cancer patients was only associated to tumor stage but not other parameters, including lymph node status, degree in differentiation, sex, and age. That significant relationship indicates that mRNA from residual tumor cells may get to the circulation, because angiogenesis is a common feature in tumor formation (16, 20), even before the tumor extends through muscularis propria into lymph nodes. This postulation is further supported by no association between β-*catenin* mRNA concentration and lymph node status. A more intensive study is necessary to explore whether plasma β-*catenin* mRNA concentration may become a new prognostic factor, in addition to lymph node status (21), in colorectal cancer patients in the future.

The elevation in plasma β-*catenin* mRNA in patients with colorectal adenoma compared with normal subjects suggests that adenoma may also be detected using this assay. It is possible that the sampling of such adenoma patients after endoscopic removal might have resulted in a lower level than would have been had the blood sampling been taken before adenoma removal. In fact, we managed to obtain five plasma samples from adenoma patients before endoscopy, and the median plasma β-*catenin* concentration was 20–25% higher than when they were obtained after endoscopy (data not shown). The presence of excess plasma β-*catenin* mRNA in adenoma patients implies that it is possible to detect early stage premalignant disease (5).

In addition, the significant reduction in plasma β-*catenin* mRNA level after tumor removal demonstrates not only the potential of using this assay for monitoring the efficacy of treatment (22) but also provides grounds that it may originate from the tumor. Although the decline of plasma β-*catenin* mRNA did not correlate to either tumor stage or nodal status, continuous follow-up of those 19 colorectal cancer patients is essential to assess the potential usefulness of plasma β-*catenin* mRNA measurement in the early detection of tumor recurrence or metastasis, because of three cancer patients who had higher β-*catenin* mRNA after surgical operation, two of them had T4 tumor stage without lymph node metastasis, which indicates that tumor cells may directly invade visceral peritoneum and even other organs.

Overall, the findings of correlation between β-*catenin* mRNA concentration and tumor stage together with elevated β-*catenin* mRNA concentration in colorectal adenoma patients indicate that its level in plasma may reflect the overall tumoral/lesional mass, which is further supported by our postoperation findings.

In conclusion, this study is the first to demonstrate the potential of plasma β-*catenin* mRNA as a marker in colorectal cancer patients. Additional experimentations, such as collection of blood from colorectal adenoma patients before endoscopy and establishment of an optimized schedule for blood collection and storage to prevent mRNA degradation, are necessary before clinical applications can be carried out. Finally, large-scale studies would be needed to investigate whether plasma β-*catenin* mRNA measurement might have a role in population screening for colorectal cancer.

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