Clinical Significance of Telomerase Activity in Peritoneal Disseminated Cells: Gastrointestinal Cancers

Inna L Botchkina,[1] David E Rivadeneira,[2] Kevin Watkins,[2] Martin S Karpeh,[2] and Galina I Botchkina[2]

[1]Department of Physiology, University of California San Francisco, San Francisco, California, USA; and
[2]Department of Surgery/Surgical Oncology, Stony Brook University Medical Center, Stony Brook, New York, USA

Early detection and accurate staging of gastrointestinal (GI) cancers are difficult. The aim of this study was to evaluate whether telomerase activity (TA) in exfoliated/disseminated epithelial cells could be used as a reliable marker for GI cancers. TA was evaluated with the real-time RTQ-TRAP in immunomagnetically sorted peritoneal epithelial cells from 60 patients undergoing surgical treatment. Thirty-two patients were clinically diagnosed with a variety of GI cancers: 1 had premalignant disease, 2 had history of GI cancers, and 25 patients were clinically negative for cancer. Here we report that all types and all cases of gastrointestinal cancers were telomerase positive, thereby demonstrating 100% sensitivity for cancer. Eighteen of 25 nonmalignant cases had undetectable levels of TA, 2 had low, and 5 of 25 expressed high TA levels. Because normal epithelial cells usually have low TA and a lesser tendency to exfoliate compared with cancer cells, it is of great importance to have close follow-up for these patients to exclude possible malignant disease. We conclude that RTQ-TRAP assessment of TA in immunomagnetically sorted peritoneal epithelial cells has 100% sensitivity and 100% negative predictive value for GI cancers, and therefore, can be considered as a valuable tool and useful addition to current standard diagnostic methods. Clinical significance of unusually high telomerase activity in some clinically negative for cancer cases requires further study.
Online address: http://www.molmed.org
doi: 10.2119/2007-00069.Botchkina

INTRODUCTION

Gastrointestinal (GI) cancers, which include cancers of the esophagus, stomach, intestines, colon, rectum, pancreas, liver, and bile duct have the highest incidence of all cancers worldwide, presenting 3 million cases per year and 2.2 million deaths. The generally asymptomatic onset and further development of GI cancers accounts for the frequently advanced stage at time of diagnosis and high mortality rates. Approximately 90% of cancer deaths are due to metastasis, and peritoneal carcinomatosis is the most common mode of the GI cancer relapse that predicts a poor prognosis. There is growing evidence that neoplastic cells shed into biological fluids at an early stage of cancer development, long before any clinical or morphological manifestations (1-2). Further development of GI cancers and invasion of the serosal surfaces also promotes exfoliation of cancer cells into peritoneal cavity (3). Free viable cancer cells have been identified by a large number of investigators in different biological fluids of cancer patients, but not in normal individuals (1-2), and some occult disseminated cells eventually become precursors of metastases, which can arise after many years of homing in secondary organs after curative resection of primary tumor. As we previously reported, an accurate pre- or intraoperative GI cancers staging may have an impact on the survival of GI cancer patients (4). Detection and analysis of free cancer cells in body fluids can provide the unique opportunity for noninvasive/minimally invasive early cancer diagnosis and prognosis. However, although the prognostic significance of gross peritoneal metastasis is obvious and well studied (5-6), that of free malignant cells in the peritoneum of GI cancer patients is still controversial due to two presently existing major obstacles: the very low number of these cells, and until recently, the lack of suitable tools for analysis. Although the presence of free cancer cells itself does not necessarily indicate the inevitable development of metastatic disease, as their fate depends on their malignant potential and crosstalk with the host microenvironment (7), large number of studies have reported that patients with detected free cancer cells are at an increased risk for recurrence and have a considerably worse prognosis (1,5-6,8-12). Therefore, the timely and sensitive detection of free cancer cells at the earliest possible stage is critical to predict relapse before clinical manifestation, be-

***Address correspondence and reprint requests to** GI Botchkina, Department of Surgery/Surgical Oncology, Stony Brook University Medical Center, HSC-18, room 050, Stony Brook, New York, 11794-8191. Fax: 631-444-6348; E-mail: gbotchkina@notes.cc.sunysb.edu*
Submitted July 5, 2007; Accepted for publication October 22, 2007

cause it can potentially improve and optimize the disease management and treatment strategy. However, currently used direct microscopic detection of disseminated cells in body fluids on the basis of routine morphological criteria has very low sensitivity, with positive findings in about 4% to 13% of GI cancer cases (3,13-14). Such a low sensitivity of the peritoneal cytology, in particular, makes positive findings only a determinant of the stage IV GI cancer cases with poorest prognosis, and only 2% of the 5-year survival rate (9,15). In this context, peritoneal cytology obviously can not be used as an indicator of cancer or of the probability of cancer recurrence because neither the presence nor the absence of free cancer cells can be identified accurately. To adequately evaluate the clinical significance of these cells, they should be detected by sensitive and robust methods, such as PCR-based assays, because PCR is the most appropriate tool for detection of submicroscopic alterations. However, at present time, there is no consensus on the type of molecular marker(s) suitable for detection of disseminated tumor cells in a particular type of cancer.

Data accumulated during the last two decades on potential molecular markers for common human cancers suggest that telomerase is probably the most valuable cancer marker despite the multitude of different oncogenic pathways and tumor suppressor mechanisms. Telomerase is a ribonucleoprotein complex with reverse transcriptase activity that catalyzes the elongation of chromosomal telomeric repeats by addition of noncoding hexamers (16), thereby allowing cancer cells to proliferate indefinitely. However, growing evidence suggests that the functional role of telomerase is not limited by the elongation of telomeres, and the acquisition of constitutive telomerase activity is a critical and rather universal step during the malignant transformation of human cells (17- 18), even in rare cancer types with alternative mechanisms of telomere elongation (19). Indeed, telomerase is expressed in a vast majority of cancers, is generally absent in normal adult somatic tissues, and has significantly lower levels in proliferating cells (germ line and stem cells inherently express telomerase activity). In a large number of studies, telomerase has been proven to have high diagnostic and prognostic value, and the highest combination of sensitivity and specificity for cancer compared with recent standard methods of cancer diagnosis, especially at early stages of cancer development (18). In particular, the upregulation of telomerase activity was demonstrated in all types of gastrointestinal cancers, and a large number of recent studies have demonstrated nearly 100% sensitivity for cancer (20-24). However, two major arguments should be considered when telomerase is applied to GI cancers. First, because telomerase is activated in highly proliferating cells, such as GI epithelial lining and activated lymphocytes, as well as in tissue stem cells, its determination in gross surgical specimens of GI normal and cancerous tissues can be misleading. This is because GI tissues have very fast turnover and high number of the tissue stem cells (although in general, TA levels in normal GI tissues usually are significantly lower compared with cancer). Thus, TA was detected in all esophageal cancers and up to 87% of the noncancerous tissues (25-26). A recent study has shown that 97% of biopsy specimens from colorectal carcinoma, 67% of ulcerative colitis, and 27% of normal controls expressed telomerase activity (23). The second argument is that determination of the activity of the telomerase enzyme is prior to the determination of the expression of its different components, because even the expression of the rate-limiting hTERT subunit is necessary, but not sufficient for correct assembly of the functional telomerase ribonucleoprotein complex, which is regulated at multiple levels (19,27). Thus, it was demonstrated that hTERT expression was not related to telomerase activity in both renal and pancreatic carcinoma and normal tissues (28-29). In contrast to the analysis of gross tissue specimens, if cells were enriched by microdissection, TA was detected in 82.2% of esophageal tumors and only in 5% of normal epithelia. In addition, the activity was significantly lower in these cells (30). Also, when telomerase activity was determined in luminal washes, 62% colorectal cancer patients were telomerase-positive, whereas none of normal or ulcerative colitis patients expressed it (23). Similarly, in pancreatic cancer patients, TA in the pancreatic juice was detected in 80% cases and none with chronic pancreatitis (31). Significant difference of TA levels in contrast with the hTERT mRNA expression was also reported in pancreatic juice samples from normal and cancer patients (29). These data clearly support an emerging knowledge that (a) the evaluation of telomerase activity is preferable compared with the analysis of the expression of telomerase components, and (b) more prominent correlation between expression of telomerase and clinicopathological data in exfoliated cells suggests that they better present the malignant status of particular tumor than gross tumor tissue specimens. In addition, analysis of exfoliated cells in body fluids allows noninvasive/minimally invasive sampling and is a technically more convenient way for the enrichment and isolation of meaningful cell pools compared with tissue specimens, providing a basis for accurate molecular characterization.

Therefore, the purpose of this study was to evaluate whether quantitative analysis of telomerase activity in the epithelial pool of exfoliated/disseminated cells in the peritoneal wash can be used as a reliable diagnostic tool and accurate molecular marker for GI cancers.

MATERIALS AND METHODS

Patients and Samples

Patients assigned to gastrointestinal surgery for both malignant and nonmalignant reasons were asked to participate in this study and sign the informed consent that was prior approved by local

ethic committee. At the beginning of each operation, 300 mL sterile saline was introduced into abdominal cavity, aspirated after gentle stirring from the pelvis, right and left upper quadrants of the abdomen, and combined. If clinical specimen was contaminated with blood, the SUPERAse-In inhibitor cocktail (Ambion) was added for all time-consuming steps, including the incubation with Abs and centrifugation. One portion of combined washing fluid was examined cytopathologically on routine basis (no peritoneal cytology was done for normal cases), and another portion (70-100 mL) was subjected to the enrichment and isolation of malignant and normal epithelial cells by immunomagnetic cell sorting (MACS; Miltenyi Biotec, CA, USA) followed by total protein extraction. Washes were centrifuged for 7 min at 1500 rpm and 4°C, rinsed with PBS, incubated with Abs against human epithelial antigen (HEA-125, current name is CD326, or EpCAM) conjugated with paramagnetic microbeads and sorted as recommended by the manufacturer. The pool of epithelial cells was pelleted again using the same parameters of centrifugation and resuspended in 30 mL ice-cold CHAPS lysis buffer (Intergen Co. Atlanta, GA, USA). After incubation on ice for 20 min, lysates were centrifuged at high speed (16,000 *g* for 10 min at 4°C), and the supernatant fluid was removed, promptly aliquoted, snap frozen, and stored at -80°C. The protein concentration was measured using the BCA protein assay (Pierce, IL, USA) or Qubit Quanti-iT Protein Assay (Invitrogen/Molecular Probes).

Real-Time Quantitative (RTQ) TRAP Assay

Telomerase activity was assessed by the real-time RTQ-TRAP assay, in immunomagnetically sorted epithelial cells isolated from peritoneal washings obtained from 50 patients with various gastrointestinal malignant and benign conditions. Real-time PCR amplification of telomeric repeat fragments, which is a measure of telomerase activity, was carried out in a 96-well plate using the Opticon MJ Research instrument and optimized standard SYBR Green protocol as described elsewhere (31). Collected fluorescence signals were analyzed with the Opticon software. The TS and ACX primers (TS: 5′-AATCCGTCGAGCAGA GTT-3′; ACX: 5′-GCGCGGCTTACCCTT ACCCTTACCCTAACC-3′) were synthesized by Invitrogen Life Technologies, CA, USA. Stock solution of each primer (1 μg/mL) was aliquoted and kept at -20°C. Total PCR reaction volume was 20 mL per well, containing 10 mL of 2 × SYBR Green Master Mix (Qiagen, CA, USA), 0.1 μg of each primer, 2 mL protein extract, and 7.8 mL of RNase-free water. The reaction mixture was first incubated at 25°C for 20 min to allow the telomerase presented in the protein extract to elongate the TS primer by adding TTAGGG repeat sequences. The PCR was then started at 95°C for 15 min (hot start) to activate the Ampli*Taq* polymerase, followed by 40-cycle amplification (denaturation at 95°C for 20 s, annealing at 55°C for 30 s, extension at 72°C for 60 s, and plate reading at 60°C for 10 s). All samples were run in duplicates. Serial dilutions of the protein extracts from known number of the reference cancer cells were used in each r-t PCR run as a reference standard and positive control (as a reference material we always use PC-3 cells as a very common cell line that allows comparative analysis of different human cancers and other cell lines between different laboratories). Reaction mixture with 2 mL of CHAPS lysis buffer instead of protein extract (no target) was used as a negative control. The melting curve analysis was performed at the end of each PCR reaction to ensure that there was only one specific product in each sample after PCR amplification.

Flow Cytometry (FACS) Analysis

Cells were analyzed with multiparametric BD FACS*Aria* cell sorter (Becton Dickinson, CA, USA). Prepared as described above cell pellets were labeled with one or several markers conjugated with different fluorophores, such as anti-human EpCAM-FITC (Miltenyi Biotec, CA, USA), CD45-APC (BD Biosciences, San Jose, CA, USA), carcinoembryonic antigen, CD66-PE (BD Pharmingen, San Jose, CA, USA), and multiple isotype controls (BD Biosciences). After 15 min incubation with appropriate dilution of Abs and blocking reagent (Miltenyi Biotec) at 4°C, stained cells were washed, resuspended in 500 μL of 1% paraformaldehyde in MACS buffer and analyzed.

Statistical Analysis

The levels of telomerase activity in patient samples (integrated fluorescence per PCR cycle) were normalized to the levels of activity in the known number of PC-3 cancer cells using a standard curve and Opticon MJ software. Telomerase activity oriented toward detection of GI cancers was compared with the standard diagnostic criteria based on standard clinicopathological parameters. We have evaluated a sensitivity (percent of the telomerase-positive cases among cases with clinically localized different types of GI cancers as a total category), specificity (percent of the telomerase-negative cases among clinically defined nonmalignant cases), positive predictive value, PPV (a probability to have clinically localized GI cancer if telomerase activity is positive), and negative predictive value, NPV (a probability to be clinically free of GI cancer if telomerase activity is negative). Sensitivity, specificity, PPV, NPV, overall percent agreements, and kappa statistics were estimated based on one cut point, which was determined by the limit of sensitivity of the Opticon MJ Research instrument.

RESULTS

FACS Analysis of the Cellular Content of the Peritoneal Washes

To determine major cell types in the peritoneal washes obtained during GI surgery, to evaluate the purity of immunomagnetic cell sorting, and to define potential contributors to the measured levels of telomerase activity, specimens

from normal patients were divided into two portions, one of which was spiked with 700,000 colon cancer HCT116 cells to mimic metastatic cancer conditions. Unsorted, positive, and negative cell populations after EpCAM-MACS sorting of each specimen were analyzed with BD FACS*Aria*. Contaminating red blood cells were lysed with 0.15 M NH_4Cl, 1.0 mM $KHCO_3$, and 0.1 mM EDTA (pH 7.2-7.4) before FACS analysis to get more accurate data. Each cell pellet was labeled with the mixture of antibodies against several cell markers conjugated with different fluorescent dyes, including EpCAM-FITC, CD66-PE and CD45-APC, or only with EpCAM-FITC. The majority of cells in normal peritoneal washes were presented by red and white blood cells, epithelial cells, and cellular debris (Figure 1). A predominant leukocyte population was granulocytes, the vast majority of which were neutrophils. The second largest population was presented by lymphocytes (Figure 1A). Unsorted peritioneal cells from normal individuals usually had relatively low numbers of epithelial cells (in some cases, up to 20%-22%; Figure 1B). The EpCAM immunomagnetic sorting almost completely removed epithelial cells, and negatively selected populations had about 0.3% of EpCAM-immunoreactive cells (D). In the negatively sorted fraction from peritoneal washes spiked with cancer cells (Figure 1C), the proportion of epithelial cells was higher (about 3%-4%; Figure 1E), suggesting that these cells escaped sorting due to high numbers of cells in the separating column. However, accurate purification of the negative fraction of the peritoneal lavage was not an immediate goal of this study, which was focused on the enrichment and purification of the epithelial cells in its positive fraction. Analysis of positive fractions from both normal and spiked specimens (after exclusion of cellular debris; left corners on Figure 1A, 1F, and 1H) has shown high purity of their epithelial populations: 94.5% for spiked specimens (Figure 1G) and 96.8% for normal peritoneal wash (Figure 1I). The majority of epithelial cells in cancer cell-spiked specimens expressed moderate-to-high levels of immunofluorescence (M2 region on Figure 1I) whereas the vast majority of epithelial cells from normal specimens expressed low-to-moderate EpCAM immunoreactivity (M1 region on Figure 1G). These data indicate that epithelial cancer cells have higher affinity to EpCAM immunomagnetic beads and a higher chance to be isolated from peritoneal washes by immunomagnetic sorting compared with normal epithelial cells.

Telomerase Activity (RTQ-TRAP)

The linearity and accuracy of real-time quantitative TRAP assay performed with the Opticon MJ Research instrument were tested using the same samples (total protein extracts of PC-3 cells as a reference material) analyzed in 12 separate experiments in duplicates as described previously (32). These tests have shown high reproducibility of the RTQ-TRAP assay with systemic error 0.121 PCR cycle and $P < 0.001$. The limit of sensitivity of the RTQ-TRAP assay was determined as about 10 cancer cells by analysis of serially diluted protein extracts from known number of PC-3 cells (standard curve). Telomerase activity in clinical samples was automatically calculated based on standard curve (Figure 2) and expressed as a reference standard cell equivalent (CE). Telomerase activity was considered as negative if after normalization to the reference material CE the numbers were less than 10. To evaluate the potential presence of telomerase and/or PCR inhibitors in each clinical sample, we analyzed an original, 1:5, and 1:10 diluted protein extracts. Using this approach, we were able to reach conditions when effects of inhibitors were minimized or abolished, but target was still in amounts sufficient for the real-time PCR amplification. We have determined that aspiration of washing solution from the upper left, upper right, and pelvic abdominal quadrants, and analysis of the combined cellular sediment is critical for obtaining more objective information because levels of TA, as well as the presence of TA and PCR inhibitors in each abdominal region were significantly different. When automatic evaluation of TA was completed, final clinicopathological data were obtained by participating surgeons, and all patients were assigned to the categories presented in Table 1. Our study included total 60 patients, 32 of which were clinically diagnosed with a variety of GI cancers, one with esophageal high-grade displasia, two with history of GI cancers, and 25 patients were clinically negative for cancer by the end of this study. Different levels of telomerase activity were detected in all types and all cases of GI cancers, demonstrating 100% sensitivity for cancer (Table 2). In particular, very high TA levels were detected in all colon cancer patients, and in the majority of pancreatic (86%) and esophageal (75%) cancer patients. Levels of TA in gastric cancer varied from low to very high. Patient with high-grade esophageal displasia and both patients with a history of GI cancers also revealed high levels of telomerase activity. Among nonmalignant by standard clinical evaluation cases, 18 of 25 had undetectable levels of TA, reflecting 72% specificity for GI cancers (Table 3), two had low TA, and five of 25 clinically benign diseases, including appendicitis, one case of cholecystitis, duodenal polyp, lipoma, and splenic hemangioma had very high levels of telomerase activity.

Statistical Analysis

Table 1 represents a summary of all cases with standard pathological evaluation and telomerase activity measurements. One case with high grade esophageal displasia, and two cases with history of malignant diseases were excluded because current diagnosis was indefinite. Diagnostic performance of the telomerase activity was analyzed for base cut-off level of 10 cell equivalent because the limit of sensitivity for the RTQ-TRAP assay was determined as ≥ 10 CE. Diagnostic performance of telomerase activity was summarized in Tables 2 and 3. Because all types and all cases of GI can-

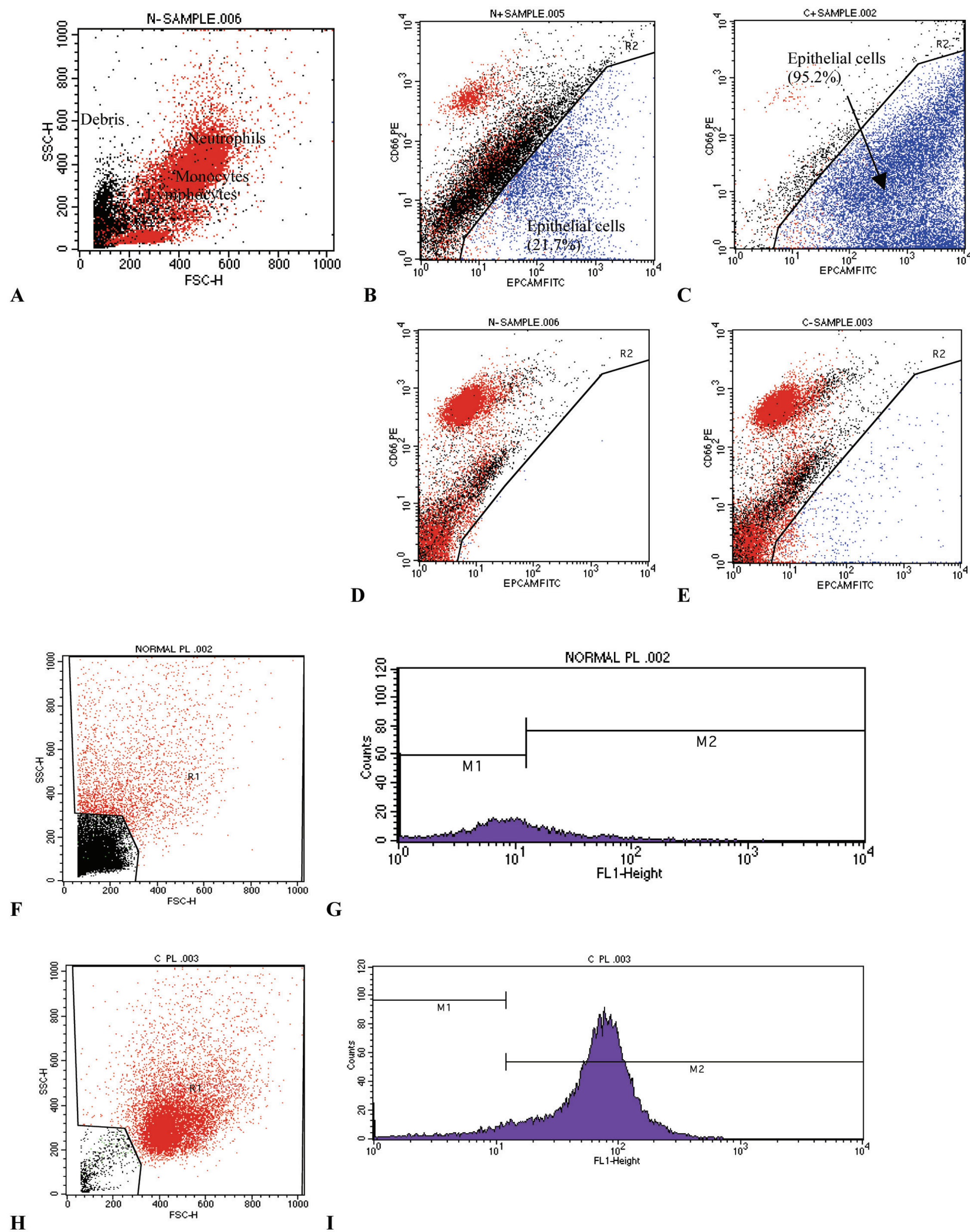

Figure 1. Flow cytometry analysis of peritoneal wash specimens. (A) FACS analysis of normal peritoneal wash showing major cell types (neutrophils, monocytes, and lymphocytes) and cellular debris. (B, C) Unsorted cellular contents of normal (B) and spiked with cancer cells (C) peritoneal washes, labeled with multicolor cocktail of Abs (EpCAM-FITC, CD66-PE, and CD45-APC). (D, E) Negatively sorted fraction from normal (D) and cancer spiked (E) specimens showing very few epithelial cells that escaped MACS sorting. MACS sorted positive fractions of normal (F) and spiked (H) specimens, labeled with EpCAM-FITC Abs, have shown high purity of MACS sorting (96.8% and 94.5%, respectively; cellular debris were excluded). Histograms reflect significantly higher expression of immunofluorescence by the majority of cancer cells (I) compared with normal exfoliated epithelial cells (G). Same number of cells in each pair of specimens were analyzed: 12,000 EpCAM-positive cells (F and H) and 20,000 unsorted and negatively sorted populations (A, B-E).

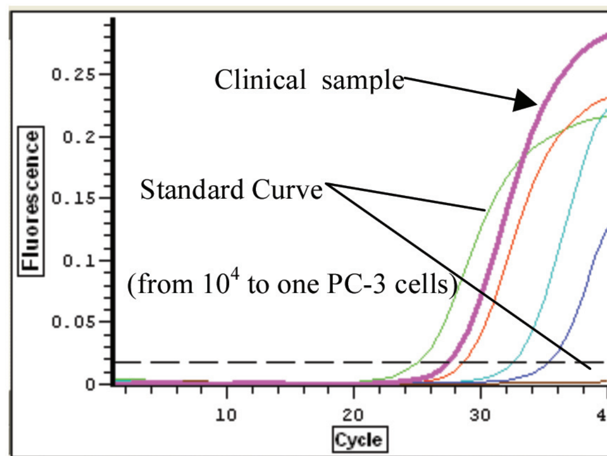

Figure 2. Relative levels of telomerase activity (RTQ-TRAP assay) in disseminated cells isolated and immunomagnetically sorted from the peritoneal washing fluid of gallbladder cancer patient. Thin lines present the standard amplification curves of the telomerase activity products from known number of cancer cells (five serial 1:10 dilutions of 10^4 PC-3 cells; the green curve corresponds to the highest protein concentration from 10^4 PC-3 cells; no amplification was seen from one PC-3 cell: brown curve below the threshold line, Ct). Thick magenta line presents TA in clinical sample that was automatically calculated by the Opticon MJ Research software and was equivalent to 3,395 PC-3 cells. Quantification of the TA levels in clinical specimens in relation to the common cultured cancer cells as a standard reference material allows an accurate comparative analysis of different clinical specimens, different experiments, and data obtained from different laboratories.

cers were telomerase-positive, the sensitivity, or percent of the telomerase-positive cases among cases with clinically localized GI cancers was calculated as 100% (32 of 32). Assay specificity with cut-off level of 10 CE (a determined limit of sensitivity of the RTQ-TRAP assay), or percent of the telomerase-negative cases among nonmalignant cases determined by standard clinical evaluation (which in general has low sensitivity for GI cancers), was 72% (18 of 25). PPV, or a probability to have clinically localized cancer if telomerase activity is positive, was 84.2% (32 clinically localized GI cancers among 38 cases with positive telomerase activity measurements). NPV, or a probability to be clinically free of cancer if telomerate activity is negative, was calculated as 100% because all telomerase-negative cases (18 of 18) were clinically normal. Because peritoneal cytology was not routinely used by all participating surgeons as a standard clinical tool, and because peritoneal cytology has extremely low sensitivity for GI cancers, statistical evaluation of its specificity, PPV and NPV was avoided as misleading. Overall percent agreement and kappa statistics were estimated based on one cut-off point as 85% and 0.744, respectively.

DISCUSSION

The early detection and accurate staging of GI cancers with currently available diagnostic and prognostic tools are difficult. Thus, about 40% of patients predicted to be resectable by computed tomography were found to be not resectable during surgical exploration (10). However, open and laparoscopic surgery, as well as preoperative colonoscopic examination, radiofrequency tumor ablation, and even general anesthesia itself increase dissemination of malignant cells (6,33-35). Moreover, surgical insult triggers adhesion molecule cascades, which facilitate rapid adherence and implantation of disseminated cancer cells to the peritoneum in the presence of multiple growth factors (36). Therefore, although surgical resection often remains the most effective current therapy for patients with GI cancers, surgical exploration needs to be minimized and assisted with less invasive and more sensitive techniques. More importantly, disseminated cancer cells can progress independently from the primary tumor (37), so analysis of micrometastases has high potential for further understanding of carcinogenesis and practical use of these cells for diagnostic and prognostic purposes (38-40). However, existing direct microscopy-based morphological methods for cancer detection, especially detection of low number of disseminated cancer cells in body fluids were proven to have extremely low sensitivity (3,6,13-14). Another problem is that the final conclusion about a malignant status of detected cells entirely depends on the expertise and opinion of the pathologist. In this context, automated real-time PCR-based molecular assays have high potential for early cancer detection due to their high sensitivity. However, the source and the quality of sample are critical for interpretation of molecular data. Thus, sensitive PCR-based methods are often used for analysis of the gross tumor specimens or, more recently, for analysis of the total cellular sediments in body fluids, without enrichment and isolation of cancer cells from contaminating cell types, which can abrogate real target gene expression, or illegitimately express some molecular markers.

The purpose of this study was to evaluate whether telomerase activity in exfoliated/disseminated epithelial cells immunomagnetically isolated from peritoneal washes can be used as a reliable molecular marker for the detection and staging of different GI cancers. In contrast to other studies focused on the role of telomerase in cancer, we evaluated TA levels in a double-blind manner, in a mixed population of patients with malignant, benign GI diseases, and healthy individuals. Quantitatively, by

Table 1. Correlation between levels of telomerase activity and standard clinicopathological data

Patients	*n*	TA (+) (CE)	TA (-) (*n*)	Positive cytology (*n*)	Negative cytology (*n*)	TNM stage	Gender	Age
Colon cancer	5	95	0	-	-	-	f	58
		735	0	0	1	$T_1N_0M_0$	m	69
		427	0	0	1	$T_2N_0M_x$	m	75
		496	0	-	-	$T_3N_0M_0$	f	67
		2930	0	-	-	pT_3 pN_1 pM_x	m	75
Esophageal cancer	8	22	0	0	1	$T_3N_0M_0$	m	70
Esoph Hi-Gr Displ	1	395	0	0	1	$T_3N_1M_0$	f 76	
		423	0	0	1	$T_2N_0M_0$	f	67
		1235	0	0	1	$T_2N_1M_x$	m	53
		595	0	0	1	$pT_3N_{1a}M_x$	m	71
		960	0	0	1	$T_1N_1M_x$	m	56
		70	0	0	1	pT_2 pN_0 M_0	m	77
		405	0	0	1	$pT_2N_0M_x$	m	62
		258	0	-	-	-	m	73
Gastric cancer	7	22	0	0	Atypia	$T_1N_0M_0$	m	81
		795	0	0	1	$T_3N_1M_x$	-	
		40	0	0	ReMesCells	$T_3N_2M_x$	m	77
		25	0	0	1	$pT_3N_3pM_x$	f	70
		20	0	1	0	Stage IV	m	82
		14356	0	1	0	Stage IV	m	65
		47	0	-	-	-	-	
Pancreatic cancer	7	7380	0	-	-	$T_3N_1M_1$	m	56
		784	0	0	1	-	m	86
		410	0	0	Atypia	Stage IV	m	67
		145	0	0	ReMesCells	Stage IV	m	83
		290	0	1	0	Stage IV	f	68
		16	0	0	1	-	m	84
Other GI cancers	5							
Gallbladder		3395	0	0	Atypia	$T_2N_0M_x$	f	75
Duodenal		1215	0	0	1	$T_4N_1M_x$	m	74
Liver		83	0	0	1	-	f	59
Liver		242	0	0	ReMesCells	$T_4N_0M_0$	f	54
Rectal		213	0	0	1	Stage IV	m	47
History of cancer		1196	0	-	-	-	m	67
Esophageal		454	0	-	-	-	f	79
Gastric								
Benign/Normal	25							
Appendicitis		1547	0	-	-	-	m	78
Cholecystitis		1907	0	-	-	-	m	79
Cholecystitis		0	1	-	-	-	m	40
Cholecystitis		0	1	-	-	-	-	
Diverticulosis		0	1	0	1	-	m	47
Diverticulosis		0	1	0	ReMesCells	-	-	
Duodenal polyp		1725	0	0	1	-	m	55
Lipoma		6515	0	0	1	-	f	42
Liver cyst		0	1	-	-	-	m	82
Pancreatic pseudocyst		0	1	-	-	-	m	32
Pancreatic serous cystadenoma		41	0	-	-	-	m	57
Pancreatic mass	5	1	-	-	-	f	71	
Pancreatic mass		50	0	-	-	-	f	51
Spl. hemangioma		9000	0	0	1	-	-	
Normal		0	1	-	-	-	-	

Continued

Table 1. *Continued*

Patients	*n*	TA (+) (CE)	TA (-) (*n*)	Positive cytology (*n*)	Negative cytology (*n*)	TNM stage	Gender	Age
Normal		0	1	-	-	-	-	
Normal		0	1	-	-	-	-	
Normal		0	1	-	-	-	-	
Normal		0	1	-	-	-	-	
Normal		0	1	-	-	-	-	
Normal		0	1	-	-	-	-	
Normal		0	1	-	-	-	-	
Normal		0	1	-	-	-	-	
Normal		0	1	-	-	-	-	
Normal		0	1	-	-	-	-	

Note: Because sensitivity of the RTQ-TRAP was determined as 10 PC-3 cells, relative levels of TA lower then 10 CE were considered as TA-negative.
Abbreviations: ReMesCells, reactive mesenchimal cells; Spl. Hemangioma, splenic hemangioma.

Table 2. Sensitivity of telomerase activity and peritoneal cytology for GI cancers.

Cancer type	Colon *n* = 5	Esophageal *n* = 8	Gastric *n* = 7	Pancreatic *n* = 7	Others *n* = 5	All cancers *n* = 32
Positive telomerase activity	100% (5 of 5)	100% (8 of 8)	100% (7 of 7)	100% (7 of 7)	100% (5 of 5)	100% (32of 32)
Positive peritoneal cytology	0% (0 of 1)	0% (0 of 8)	33.30% (2 of 6)	16.70% (1 of 6)	0% (0 of 5)	10.70% (3 of 28)

the real-time RTQ-TRAP assay in relation to the known number of PC-3 cells as a standard reference material; and in enriched and purified population of exfoliated/disseminated epithelial cells, which might include only normal or reactive mesothelial cells, and carcinoma cells. We analyzed combined serially diluted specimens of the peritoneal washings collected from different abdominal quadrants, because, as we have determined, the levels of the measured by RTQ-TRAP assay TA in each abdominal region were significantly different. This observation is in accordance with a previous study which has demonstrated that the sites of peritoneal lavage collection differ significantly with respect to positivity rates by both RT-PCR and cytology (41). Enzymatic activity of telomerase, as well as the PCR efficiency also highly depends on the presence of inhibitors, therefore RTQ-TRAP requires prompt sample preparation and comparative analysis of diluted samples.

Our data have shown that all analyzed types of gastrointestinal cancers, including those of the colon, duodenum, gallbladder, liver, pancreas, stomach and rectum, as well as all analyzed malignant cases were telomerase positive, thereby demonstrating 100% sensitivity for cancer (Tables 1 and 2). Particularly high levels of TA were detected in all colon cancer patients, and in the majority of pancreatic (86%) and esophageal (75%) cancer patients. Although it is believed that colorectal cancers usually metastasize to the liver via circulation, uniformly high levels of telomerase activity in peritoneal washing of colon cancer patients show that free cancer cells are present in a large numbers in the peritoneal cavity, and most likely it indicates a potential or early existing peritoneal recurrence. This suggestion is in agreement with another study that has demonstrated that patients after curative surgery for colorectal cancers with high levels of TA had significantly lower survival rates, and 64% of cases resulted in death had high TA levels (42). Although we could not evaluate the prognostic value of TA in this study due to the short follow-up period, we can suggest that cases with extremely high levels of TA most likely represent a poor prognosis. Levels of TA in gastric cancer varied from low to very high, although 5 of 7 cases presented metastatic disease. Patient with high-grade esophageal displasia, and both patients with history of

Table 3. Diagnostic performance of telomerase activity for GI cancers.

	Sensitivity	Specificity	PPV	NPV	Kappa
Telomerase Activity	100% (32 of 32)	72% (18 of 25)	81.60% (32 of 38)	100% (18 of 18)	0.744

GI cancers also revealed high TA levels, which most likely reflected an early stage of malignant disease, and its recurrence, respectively.

Although the majority of nonmalignant cases had undetectable levels of TA, reflecting 72% specificity for GI cancers, 2 cases had low TA, and 5 of 16 clinically benign diseases, including appendicitis, 1 case of cholecystitis, duodenal polyp, lipoma, and splenic hemangioma had very high TA levels. As we determined by FACS analysis, EpCAM-MACS sorting practically exclude any significant sample contamination by cells other than epithelial telomerase competent cells (for example, by activated lymphocytes). Also, because normal epithelial cells usually exfoliate after final differentiation when TA is not expressed, and reactive mesothelial cells at some nonmalignant GI pathologies was shown to have relatively low TA (43), we can suggest that unusually high telomerase activity most likely can be attributed to the presence of free cancer cells or severe damage of the epithelial lining. This suggestion is in agreement with a recent study that has demonstrated that some clinically benign conditions, such as bile duct cysts, have cancer in up to 30% of all cases, and increased telomerase activity in these cases was considered as an early event (44). Recent study has shown that approximately one-third of cytology-negative patients were either with peritoneal (35%) or nonperitoneal (27%) recurrence (45).

In general, high measured levels of TA should correlate either with a larger number of exfoliated proliferating epithelial and/or cancer cells, or with higher activity of the telomerase enzyme. According to our observations, the cell pellets after immunomagnetic cell sorting is usually almost invisible, although levels of TA can differ in several orders of magnitude, so we can suggest that major contributor to the measured levels of TA is the enzyme activity rather than the number of exfoliated cells. We suggest that it is rather not practical to set up particular cut-offs for the measured levels of TA because both the number of disseminated telomerase-competent cells in particular clinical specimen and the level of telomerase activation might significantly vary. We also suggest that unusually high levels of TA in patients with some clinically benign diseases (20% in present study) should be alarming and considered as a sign of potential presence of cancer, or severe damage of the serosal surface and appearance of a large number of activated mesothelial cells (46) that require close follow-up and additional clinical tests.

Determination of TA by RTQ-TRAP assay has several advantages, including measurement of TA in relation to the largely available standard reference material (PC-3 cells) that allows comparison of results between different patients, experiments, and laboratories. Sensitivity and specificity of the RTQ-TRAP is evaluated in each particular experiment because serial dilution of the known number of cancer cells are used. False-positive results, including contamination of the sample and the illegitimate amplification of the target gene, are minimized because (1) epithelial cells are highly enriched by MACS, and contaminating cell types (noncancerous epithelial cells) have much a lower rate of exfoliation and lower EpCAM immunoreactivity compared with cancer cells; (2) real-time PCR TRAP assay allows analysis of the melting curves for all obtained PCR products, so illegitimate amplification can be easily detected and suspicious samples can be further analyzed. In addition, automatic data acquisition allows avoid subjective interpretation of the obtained results. In contrast, our study revealed that peritoneal cytology had very low sensitivity for GI cancers (10.7%), which is in agreement with many other reports (*see* Introduction). Such a low sensitivity for cancer makes further evaluation of its diagnostic performance, including specificity and positive and negative predictive values, meaningless, because the lack of positive findings in clinically benign cases does not mean high NPV, but rather reflects the fact that this method in general is not suitable for detection of relatively low number of disseminated cancer cells in biological fluids. Higher sensitivity of the peritoneal cytology in gastric cancer cases in this study is in agreement with previous reports that positive findings usually correlate with the most advanced stage IV disease (9,15).

In conclusion, we report that determination of TA by quantitative real-time PCR RTQ-TRAP assay in epithelial cells isolated from peritoneal washings by immunomagnetic sorting has 100% sensitivity and 100% negative predictive value for GI cancers. In contrast, peritoneal cytology performed as a standard diagnostic test revealed the lack of sensitivity for the majority of GI cancers. To determine a statistically significant correlation between levels of telomerase activity (with different cut-offs) and GI cancers stage, as well as to determine a prognostic value of quantitatively measured TA in exfoliated/disseminated epithelial cells in peritoneal fluid, a larger clinical study is necessary. Also, clinical significance of extremely high levels of TA in particular benign cases needs to be further investigated. Because the number of exfoliated/disseminated cells in body fluids depends not only on tumor size, stage and its biological features, but also on other factors, such as anesthesia, surgical procedures, and sampling techniques, accurate standardization of all procedures is necessary for the evaluation of quantitative correlations.

ACKNOWLEDGMENTS

We thank SBU Sensor CAT for support. We thank Barbara Smith, NP (SBU Medical Center) for irreplaceable help during the performance of this project. We also thank Dr. Henry Thode for his assistance with statistical analysis.

REFERENCES

1. Pantel K, Woelfle U. (2006) Circulating tumor cells as an indicator of invasion. *AACR Educ. Book* 1:116-119.
2. Pantel K, von Knebel Doeberitz M. (2000) Detection and clinical relevance of micrometastatic cancer cells. *Curr. Opin. Oncol*. 12:95-101.
3. Ludeman L, Shepherd NA. (2005). Serosal in-

volvement in gastrointestinal cancer: its assessment and significance. *Histopathiology* 47:123-131.
4. Noguchi Y et al. (2000) Is gastric carcinoma different between Japan and the United States? A comparison of patient survival among three institutions. *Cancer* 89:2237-2246.
5. Kienle P, Koch M. (2002) Are "micrometastases" of the peritoneum equivalent to distant metastases? *Dig. Surg.* 19:453-458.
6. Koppe MJ, Boerman OC, Oyen WJ, Bleichrodt RP. (2006) Peritoneal carcinomatosis of colorectal origin: Incidence and current treatment strategies. *Ann. Surg.* 243: 212-222.
7. Yoneda T, Hiraga T. (2005) Crosstalk between cancer cells and bone microenvironment in bone metastasis. *Biochem. Biophys. Res. Commun.* 328:679-687.
8. Burke EC, Karpeh MS Jr, Conlon KS, Brennan MF. (1998) Peritoneal lavage cytology in gastric cancer: an independent predictor of outcome. *Ann. Surg. Oncol.* 5:411-415.
9. Bando E et al. (1999) Intraoperative lavage for cytological examination in 1297 patients with gastric carcinoma. *Am. J. Surg*.178:256-262.
10. Jimenez RE, Warshaw AL, Fernandez-del Castillo C. (2000) Laparoscopy and peritoneal cytology in the staging of pancreatic cancer. *J. Hepatobilliary Pancreat. Surg.* 7:15-20.
11. Yamamoto S, Akasu T, Fujita S, Moriya Y. (2003) Long-term prognostic value of conventional peritoneal cytology after curative resection for colorectal carcinoma. *Jap. J. Clin. Oncol.* 33:33-37.
12. Cristofanilli M et al. (2004). Circulating Tumor Cells, Disease Progression, and Survival in Metastatic Breast Cancer. *NEJM*, 531:781-791.
13. Nieveen van Dijkum EJ et al. (1998) Cytology of peritoneal lavage performed during staging laparoscopy for gastrointestinal malignancies: is it useful? *Ann. Surg.* 228:728-733.
14. Bentrem D, Wilton A, Mazumdar M, Brennan M, Coit D. (2005) The value of peritoneal cytology as a preoperative predictor in patients with gastric carcinoma undergoing a curative resection. *Ann. Surg. Oncol.* 12:347-353.
15. Kodera Y et al. (1999) Prognostic value of positive findings in patients with gastric carcinoma undergoing a potentially curative resection. *J. Surg. Oncol.* 72:60-65.
16. Blackburn EH. (1990) Telomeres and their synthesis. *Science* 3:489-490.
17. Dong CK, Masutomi K, Hahn WC. (2005) Telomerase: regulation, function and transformation. *Crit. Rev. Oncol. Hematol.* 54:85-93.
18. Hiyama E, Hiyama K. (2002) Clinical utility of telomerase in cancer. *Oncogene* 21:643-649.
19. Stewart SA et al. (2002). Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *PNAS* 99:12606-12611.
20. Boldrini L et al. (2002). Evaluation of telomerase in the development and progression of colon cancer. *Int. J. Mol. Med.* 10:589-592.
21. Domont J et al. (2005). Catalytic subunit of human telomerase reverse transcriptase is an independent predictor of survival in patients undergoing curative resection of hepatic colorectal metastases: a multicenter analysis. *Clin. Oncol*. 23:3086-3093.
22. Sanz-Casla MT et al. (2005). Telomerase activity as a prognostic factor in colorectal cancer. Onkologi 28:553-557.
23. Myung S et al. (2005) Clinical usefulness of telomerase for the detection of colon cancer in ulcerative colitis patients. *J. Gastroenterol. Hepatol.* 20:1578-1583.
24. Wong S, Yu H, and So JB. (2006) Detection of telomerase activity in gastric lavage fluid: a novel method to detect gastric cancer. *J. Surg. Res*. 131:252-255.
25. Kubota M, Yamana H, Sueyoshi S, Fujita H, Shirouzu K. (2002) The significance of telomerase activity in cancer lesions and the noncancerous epitheliumof the esophagus. *Int. J. Clin. Oncol.* 7:32-37.
26. Tominaga T et al. (2002) Telomerase activity and expression of human telomerase catalytic subunit gene in esophageal cancer. *J. Gastroenterol.* 37:488-490.
27. Kyo S, Inoue M. (2002) Complex regulatory mechanisms of telomerase activity in normal and cancer cells: How can we apply them for cancer therapy? *Oncogene* 21:688-697.
28. Rohde V et al. (2000) Expression of the human telomerase reverse transcriptase is not related to telomerase activity in normal and malignant renal tissue. *Clin. Cancer Res.* 6:4803-4809.
29. Ohuchida K et al. (2005) Quantitative assessment of telomerase activity and hTERT mRNA levels in pancreatic juice samples for the diagnosis of pancreatic cancer. Clin Cancer Res 11:2285-2292.
30. Li C, Liang Y, Wu M, Cai W. (2002) Telomerase activity analysis of esophageal carcinoma using microdissection-TRAP assay. *Chin. Med. J.* 115:1405-1408.
31. Mizumoto K, Tanaka M. (2004) Detection of telomerase activity in patients with pancreatic cancer. *Methods Mol. Med*. 103:199-206.
32. Botchkina GI et al. (2005) Noninvasive detection of prostate cancer by quantitative analysis of telomerase activity. *Clin. Cancer Res.* 11:3243-3249.
33. Tomiki Y et al. (2000) Cancer cell exfoliation by preoperative colonoscopic examination. Digest. Endosc. 12:327-330.
34. Koch M et al. (2004) Hematogenous tumor cell dissemination during colonoscopy for colorectal cancer. Surg. Endoscopy 18:587-591.
35. Jaskolka JD et al. (2005) Needle tract seeding after radiofrequency ablation of hepatic tumors. *J. Vasc. Interv. Radiol.* 16:485-491.
36. Alkhamesi NA, Ziprin P, Pfistermuller K, Peck DH, Darzi AW. (2005) ICAM-1 mediated peritoneal carcinomatosis, a target for therapeutic intervention. *Clin. Exp. Metast*. 22:449-459.
37. Schmidt-Kittler O et al. (2003) From latent disseminated cells to overt metastasis: Genetic analysis of systemic breast cancer progression. *Proc. Natl. Acad. Sci. U.S.A.*, 100:7737-7742.
38. Weihrauch MR et al. (2002) Immunomagnetic enrichment and detection of micrometastases in colorectal cancer: correlation with established clinical parameters. *J. Clin. Oncol.* 20:4338-4343.
39. Waguri N et al. (2003) Sensitive and specific detection of circulating cancer cells in patients with hepatocellular carcinoma; detection of human telomerase reverse transcriptase messenger RNA after immunomagnetic separation. *Clin. Cancer Res.* 9:3004-3011.
40. Hess JL, Atha DH, Xu JF, Highsmith WE Jr. (2004) Telomerase activity measurement in magnetically captured epithelial cells: comparison of slab-gel and capillary electrophoresis. Electrophoresis 25:1852-1859.
41. Tokuda K et al. (2003) Clinical significance of CEA-mRNA expression in peritoneal lavage fluid from patients with gastric cancer. *Int. J. Mol. Med.* 11:79-84.
42. Tatsumoto N et al. (2000) High telomerase activity is an independent prognostic indicator of poor outcome in colorectal cancer. *Clin. Cancer Res.* 6:2696-2701.
43. Zendehrokh N, Dejmek A. (2005) Telomere repeat amplification protocol (TRAP) in situ reveals telomerase activity in three cell types: malignant cells, proliferartive mesothelial cells and lymphocytes. *Modern Pathol.* 18:189-196.
44. Soreide K, Soreide JA. (2007) Bile duct cyst as precursor to biliary tract cancer. *Ann. Surg. Oncol.* 14:1200-1211.
45. Mori K et al. (2007) Detection of minimal gastric cancer cells in peritoneal washings by focused microarray analysis with multiple mrkers: clinical implications. *Ann. Surg. Oncol.* 14:1694-1702.
46. Musaers S. (2002) Mesothelial cells: Their structure, function and role in serosal repair. Respirology 7(3):171-191.