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Molecular Detection of Circulating Tumor Cells in the Peripheral Blood of Patients with Colorectal Cancer Using RT-PCR: Significance of the Prediction of Postoperative Metastasis

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Abstract

Background: Approximately 20%–45% of colorectal cancer (CRC) patients ultimately develop local recurrence or metastasis following curative surgical resection. The latter is caused by tumor cells shed from the primary carcinoma prior to or during operation, currently undetected by standard clinical staging. Fortunately, the presence of tumor cells in peripheral blood can be detected by molecular methods and is being regarded increasingly as a clinically relevant prognostic factor.

Materials and Methods: To detect the presence of circulating tumor cells and evaluate their relationship to postoperative metastatic relapse, we simultaneously examined human telomerase reverse transcriptase (hTERT), cytokeratin-19 (CK-19), cytokeratin-20 (CK-20), and carcinoembryonic antigen (CEA) mRNA (messenger RNA) in the peripheral blood of 72 CRC patients and 30 healthy individuals. Using a reverse-transcriptase polymerase chain reaction (RT-PCR), these tumor-related mRNAs were amplified; in addition, analyses were carried out for their correlation with patients' clinicopathologic features, as well as the occurrence of postoperative metastasis.

Results: In RT-PCR analysis of the peripheral blood, 69.4% (50 out of 72), 66.7% (48 out of 72), 52.8% (38 out of 72), and 72.2% (52 out of 72) of CRC patients were positive for hTERT, CK-19, CK-20, and CEA mRNA respectively. All 30 healthy individuals were negative for hTERT and CEA mRNA expression, while 2 were positive for either CK-19 mRNA or CK-20 mRNA expression. The detection of CEA mRNA was significantly correlated with depth of tumor invasion ($P = 0.012$), vessel invasion ($P = 0.035$), TNM stage ($P < 0.0001$), and postoperative metastasis ($P < 0.0001$), while positive hTERT mRNA was correlated with TNM stage ($P = 0.037$) and CK-19 was correlated with depth of tumor invasion ($P = 0.039$) and postoperative metastasis ($P = 0.017$). In addition, multivariate logistic regression showed that only CEA mRNA was an independent and significant predictor of postoperative metastasis ($P = 0.006$). Our findings suggest that CEA mRNA may be a more reliable marker than hTERT, CK-19, and CK-20 for the detection of circulating cancer cells in the peripheral blood of CRC patients.

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Conclusions: Using RT-PCR for the detection of CEA mRNA is feasible and may be a promising tool for early detection of micrometastatic circulating tumor cells in CRC patients. CRC patients expressing positive CEA mRNA in peripheral blood have a significantly higher risk of postoperative metastasis. Nevertheless, confirmation of CEA mRNA as a prognostic predictive factor requires the continuation of patient follow-up.

Colorectal cancer (CRC) is one of the most frequent malignancies and is also the third major cause of cancer-related death in Taiwan, with over 7,000 new cases and 3,000 deaths per year (http://www.doh.gov.tw/statistic/index.htm; accessed in August 2005). Despite recent advances in various diagnostic and therapeutic methodologies, distant metastases remain a major cause of death for CRC patients.[1,2] It has been reported that about 30% of CRC patients who undergo a curative resection nevertheless subsequently develop metastatic disease, suggesting that micrometastasis exists and may play a key role in relapse.[3–5] Even with the recent development of image studies and other diagnostic modalities, it is not always possible to detect metastasis at a very early stage of disease. Use of the reverse-transcriptase polymerase chain reaction (RT-PCR) allows sensitive and reliable detection of a very small number of circulating tumor cells in blood or bone marrow.[5–9] RT-PCR assays exploiting epithelial markers are based on the principle that carcinoma cells detach from the site of the primary tumor and are distributed to hematopoietic or lymphatic tissue.[4,5] This can lead to the appearance of gene transcripts that are not normally expressed in these host tissues.

Several reports have described the use of human telomerase reverse transcriptase (hTERT), cytrokeratin-19 (CK-19), cytrokeratin-20 (CK-20), and carcinoembryonic antigen (CEA) mRNA (messenger RNA) as markers for the detection of occult residual disease for CRC.[8,10–13] However, there have been no previous reports simultaneously analyzing the mRNA molecular markers of hTERT, CK-19, CK-20, and CEA for the detection of circulating tumor cells in the peripheral blood of CRC patients, as well as the prediction of postoperative metastasis when comparing these four mRNA markers. We present here the initial data analysis of the mRNA expression profiles of hTERT, CK-19, CK-20, and CEA in peripheral blood samples from CRC patients, and explore the correlation between the expression of these four molecular markers and a variety of clinicopathological features. Eventually, these mRNA molecular markers could be involved in a potentially noninvasive approach, and used to identify patients at a higher risk of postoperative metastasis.

MATERIALS AND METHODS

Sample Collection

Seventy-two patients undergoing elective surgery for CRC at the Department of Surgery of Kaohsiung Medical University Hospital between April 2002 and November 2003 enrolled in this study. Forty-two were males and 30 were females. The mean age was 64.5 years (range: 39–88). A 4-ml sample of peripheral blood was obtained from each CRC patient during the induction of anesthesia in the operative room. In addition, peripheral blood samples taken from 30 healthy individuals served as controls. Healthy subjects with no history of cancer undergoing hemorrhoidectomy were recruited as non-cancer controls. To prevent contamination of epithelial cells, peripheral blood samples were obtained through a catheter inserted into a peripheral vessel, and the first 5 ml of blood were discarded. Written informed consent was obtained from all subjects and/or guardians for the use of their blood samples. Sample acquisition and subsequent use were also approved by the institutional review board of the Kaohsiung Medical University. Clinical stages and pathological features of primary tumors were defined according to the criteria of the American Joint Commission on Cancer.[14]

Total RNA Isolation and First Strand cDNA Synthesis

Total RNA was extracted from the fresh whole blood of CRC patients by using a QIAamp RNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically on the basis of absorbance at 260 nm. First strand cDNA was synthesized from total RNA by using a RT-PCR kit (Promega, Madison, WI, USA). The reverse transcription was carried out in a reaction mixture consisting of 1 $\times$ Transcription Optimized 5 $\times$ Buffer, 25 μg/ml Oligo(dT)15 Primer, 100 mmol/l PCR Nucleotide Mix, 200 μmol/l MLV Reverse Transcriptase, and 25 μl Recombinant RNasin Ribonuclease Inhibitor. The reaction mixtures with 600 μg of total RNA were incubated at 42°C for 2 hours, heated to 95°C for 5 minutes, and then stored at 4°C until analysis.

Table 1.
List of all primers used with polymerase chain reaction (PCR) amplification conditions

Primer	5′-3′ sequence	PCR conditions	Size of PCR (bp)
hTERT (sense)	AAG TTCCTGCACTGGCTGAT	(94°C /20 s, 60°C /10 s, 74°C /20 s) × 35 cycles	265
hTERT (antisense)	CACGACGTAGTCCATGTTCA		
CK-19 (sense)	ATGAAAGCTGCCTTGGAAGA	(94°C /20 s, 60°C /10 s, 74°C /20 s) × 33 cycles	138
CK-19 (antisense)	TGATTCTGCCGCTCACTATCAG		
CK-20 (sense)	CTGAATAAGGTCTTTGATGACC	(94°C /20 s, 60°C /10 s, 74°C /20 s) × 35 cycles	138
CK-20 (antisense)	ATGCTTGTGTAGGCCATCGA		
CEA (sense)	AACTGGTGTCCCGGATATCA	(94°C /20 s, 60°C /10 s, 74°C /20 s) × 34 cycles	138
CEA (antisense)	ATATTCTTTGCTCCTTGCCA		
GADPH (sense)	CCTCAAGATCATCAGCAATGC	(94°C /20 s, 60°C /10 s, 74°C /20 s) × 35 cycles	165
GADPH (antisense)	GGAAACTGTGGCGTGATGG		

RT-PCR

The target genes for PCR detection included hTERT, CK19, CK20, and CEA. Sequences of the oligonucleotide primers were designed according a PCR primer selection program based on primer3 at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Also, glyceraldehyde-3-phosphate dehydrogenase (GADPH) primers were added as internal controls to correct the differences in total RNA amounts between the CRC patients and healthy individuals. Each RT-PCR reaction mixture contained 1 × PCR buffer (10 mmol/l Tris-HCL, pH 8.3, 50 mmol/l KCL, 2 mmol/l $MgCl_2$), 50 μmol/l dNTP, 0.1 μmol/l sense and antisense primers for target genes, and 0.01 μmol/l sense and antisense primers for GADPH, and 2.5 U Taq DNA polymerase in a total volume of 50 μl. PCR amplification was carried out in a programmable thermal cycler (Primus 25; MWG-BIOTECH, Ebersberg, Germany). The cycle was repeated independently of the results of the PCR cycle number quality control test. PCR products were analyzed in 3% agarose gel. The signals on UV transilluminator for each target gene and GAPDH expression levels were scanned with a computing laser densitometer (Alpha Inotech, San-Leandro, CA, USA) to calculate the relative mRNA density ratio. The sequences of primers, PCR conditions, and sizes of PCR products are listed in Table 1.

Sensitivity of RT-PCR assay

The detection sensitivity of RT-PCR assay for CEA mRNA was evaluated by a dilution test. SW-480 (ATCC, Manassas, VA, USA) colon cancer cells (100, 25, 12, 6 cells) were mixed with 5 ml of peripheral blood obtained from a healthy volunteer.

Follow-up

All the patients were carefully followed up regularly at 3-month intervals until August 2005. At each visit, physical examinations, routine blood work, serum CEA measurement and liver function tests were conducted as appropriate. Chest X-ray and abdominal ultrasonography were performed every 6 months. Computed tomography or magnetic resonance imaging was carried out if indicated. The development of new recurrent or metastatic lesions following operation is defined as a postoperative metastasis. With a median follow-up of 28 months (range: 16–40), the correlation between the postoperative metastasis and the detection of the individual molecular markers was analyzed.

Statistical Analysis

All data were analyzed using the Statistical Package for the Social Sciences Version 10.0 software (SPSS, Chicago, IL, USA). Data were presented as mean ± standard deviation. The two-sided Pearson χ^2 test and the Fisher exact test were used to compare the clinicopathological parameters between mRNA marker-positive patients and mRNA marker-negative patients. To clarify the clinical significance of these mRNA markers as the predictors of postoperative metastasis, multivariate adjustment was performed by the logistic regression analysis. A probability of less than 0.05 was considered to be statistically significant.

RESULTS

The demographic data and clinicopathologic characteristics of all patients are summarized in Table 2. With

Table 2.
Demographic and clinical description of the colorectal cancer patients

	Number of patients
Total cases	72
Age (years)	64.5 ± 11.6
<60	28
≥60	44
Sex	
Male	42
Female	30
Tumor size	
<5 cm	29
≥5 cm	43
Location	
Colon	44
Rectum	28
Differentiation	
Well	9
Moderate	45
Poor	18
Depth of tumor invasion	
T1	3
T2	12
T3	44
T4	13
Lymph node metastasis	
No	33
Yes	39
TNM stage	
I	8
II	22
III	30
IV	12
Vessel involvement	
Absent	36
Present	36
Perineurial involvement	
Absent	42
Present	30
Postoperative metastasis	
Absent	43
Present	29

regard to the histological types of these tumors, 9 were well-differentiated carcinomas, 45 were moderately differentiated carcinomas, and 18 were poorly differentiated carcinomas. Of the 72 CRC patients, 8 were subsequently diagnosed with stage I, 22 with stage II, 30 with stage III, and 12 with stage IV. Twelve patients were shown to have distant metastases, which were confirmed by chest X-ray, ultrasonography, computed tomography or magnetic resonance imaging.

Carcinoembryonic antigen transcript was detected at a concentration as low as 25 colon cancer cells in 5 ml of blood, *i.e.*, five cancer cells per 1 ml of blood. Figure 1 shows the expression patterns of hTERT, CK-19, CK-20, and CEA mRNAs from blood samples of CRC patients and healthy individuals by multiplex RT-PCR assay. In the RT-PCR analysis of peripheral blood, the positive rates of hTERT, CK-19, CK-20, and CEA mRNA in CRC patients were 69.4% (50 out of 72), 66.7% (48 out of 72), 52.8% (38 out of 72), and 72.2% (52 out of 72) respectively (Table 3). However, only 2 out of 30 healthy individuals were found to be positive for these molecular markers: one for CK-19 and the other for CK-20. We found that CEA mRNA expression was the most significant indicator for clinicopathologic characteristics and closely correlated with depth of tumor invasion ($P = 0.012$), vessel invasion ($P = 0.035$), TNM stage ($P < 0.0001$), and postoperative metastasis ($P < 0.0001$). Twenty-eight out of 52 CRC patients with positive CEA mRNA subsequently developed metastatic disease, whereas only 1 of 20 patients with negative CEA mRNA developed metastasis. The presence of CEA mRNA marker was observed in 12.5% (1 out of 8) in stage I patients, 77.3% (17 out of 22) in stage II patients, 73.3% (22 out of 30) in stage III patients, and 100% (12 out of 12) in stage IV patients. Positive hTERT mRNA was correlated with TNM stage ($P = 0.037$), and CK-19 was correlated with depth of tumor invasion ($P = 0.039$) and postoperative metastasis ($P = 0.017$). Table 4 shows that there existed a significant correlation between the expression of CEA mRNA and the occurrence of clinical metastases postoperatively identified by a multivariate analysis ($P = 0.006$), in addition to depth of tumor invasion ($P = 0.033$). The CRC patients with positive CEA mRNA expression had an odds ratio of about 11-fold to develop postoperative metastasis compared with patients without CEA mRNA expression. Therefore, CEA mRNA expression is probably a significant and potential predictor for the prognosis of CRC patients following surgical resection.

DISCUSSION

Mortality from CRC is not due to the primary tumor mass, but is a consequence of distant metastasis. Postoperative recurrence is generally considered to develop from micrometastasis already existing at the time of the operation.[15] Hence the detection of circulating tumor cells in peripheral blood may predict disease recurrence, metastasis, and prognosis. In recent years, there has been an increasing number of reports on the RT-PCR detection of very small numbers of circulating cancer cells in a background of normal cells.[4–13,16–18] The prognostic

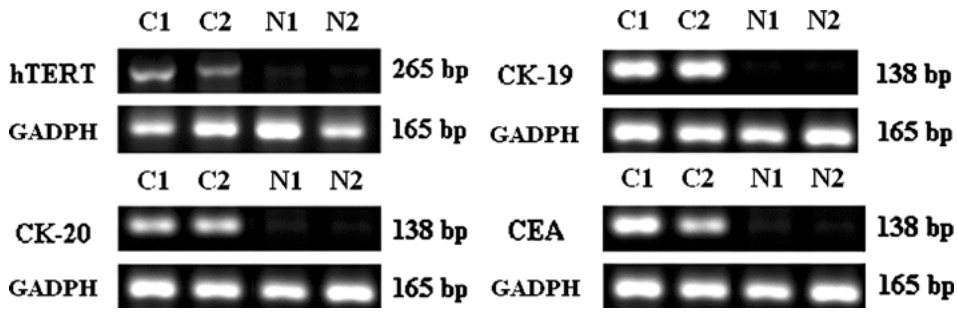

Figure 1. Detection of hTERT, CK-19, CK-20, and CEA mRNAs by reverse transferase-polymerase chain reaction (RT-PCR) in the peripheral blood of colorectal cancer patients. *C* colorectal cancer, *N* healthy individual. GAPDH is an internal control.

Table 3.
Clinicopathological features of colorectal cancer patients with or without mRNA expression in peripheral blood

	hTERT			CK-19			CK-20			CEA		
	+	−	*P*	+	−	*P*	+	−	*P*	+	−	*P*
Number of patients	50	22		48	24		38	34		52	20	
Age (years)												
<60	19	9		16	12		13	15		19	9	
≥60	31	13	0.816	32	12	0.171	25	19	0.389	33	11	0.509
Sex												
Male	30	12		29	13		25	17		28	14	
Female	20	10	0.665	19	11	0.612	13	17	0.175	24	6	0.213
Tumor size												
<5 cm	20	9		21	8		14	15		19	10	
≥5 cm	30	13	0.942	27	16	0.396	24	19	0.530	33	10	0.297
Location												
Colon	30	14	0.771	31	13	0.393	26	18	0.179	34	10	0.230
Rectum	20	8		17	11		12	16		18	10	
Differentiation												
Well	4	5		4	5		4	5		5	4	
Moderate	31	14		30	15		23	22		36	9	
Poor	15	3	0.117	14	4	0.223	11	7	0.669	11	7	0.156
Depth of tumor invasion												
T1	1	2		2	1		0	3		1	2	
T2	7	5		4	8		6	6		5	7	
T3	33	11		34	10		22	22		34	10	
T4	9	4	0.364	8	5	0.039	10	3	0.087	12	1	0.012
Lymph node metastasis												
Absent	21	12		19	14		15	18		21	12	
Present	29	10	0.325	29	10	0.132	23	16	0.252	31	8	0.135
TNM stage												
I	2	6		3	5		1	7		1	7	
II	16	6		14	8		13	9		17	5	
III	23	7		22	8		18	12		22	8	
IV	9	3	0.037	9	3	0.248	6	6	0.101	12	0	<0.0001
Vessel involvement												
Absent	24	12		26	10		18	18		22	14	
Present	26	10	0.609	22	14	0.317	20	16	0.637	30	6	0.035
Perineurial invasion												
Absent	28	14		28	14		22	20		28	14	
Present	22	8	0.545	20	10	1.000	16	14	0.936	24	6	0.213
Metastasis												
Absent	27	16		24	19		21	22		24	19	
Present	23	6	0.136	24	5	0.017	17	12	0.415	28	1	<0.0001

Table 4.
Correlation between postoperative metastasis and molecular markers by reverse transcriptase-PCR (RT-PCR) or clinicopathological features from colorectal cancer (CRC) patients using multivariate logistic regression analysis

Variables	β[a]	SE[b]	*P* value	Odds ratio	95% confidence interval
Depth (T3+T4/T1+T2)	2.385	1.118	0.033	10.858	1.214–97.100
Lymph node metastasis (Presence/absence)	−0.034	1.330	0.980	0.967	0.071–13.088
Stage (III+IV/I+II)	−0.166	1.348	0.902	0.847	0.060–11.898
Vessel involvement (Presence/absence)	0.909	0.671	0.176	2.481	0.666–9.243
Perineurial invasion (Presence/absence)	−0.164	0.633	0.795	0.848	0.245–2.932
hTERT mRNA	0.112	0.746	0.881	1.118	0.259–4.828
CK-19 mRNA	−0.179	0.737	0.809	0.836	0.197–3.549
CK-20 mRNA	−0.224	0.589	0.705	0.800	0.252–2.539
CEA mRNA	2.420	0.873	0.006	11.243	2.033–62.177

β: coefficient; SE: standard error.

and clinical value of this molecular detection method has gained increasing attention. To the best of our knowledge, this is the first comprehensive report of simultaneous analysis including the correlation between hTERT, CK-19, CK-20, and CEA mRNA expression and clinicopathologic features of CRC, and a comparison of the superiority of these markers in predicting postoperative metastasis for CRC patients.

We have demonstrated that detection rates of circulating tumor cells in the peripheral blood of CRC patients using RT-PCR amplification of tumor-specific mRNAs were between 52.8% and 72.2%, with the best sensitivity by CEA mRNA. The incidence of CEA mRNA expression in the peripheral blood of CRC patients in reports varies considerably, ranging from 41% to 69%,[8,11,12,19] but our detection rate seems to be higher than those published in previous research. One possible explanation might be related to our blood sampling during the operation, and that surgical manipulation is considered to enhance the release of tumor cells into the circulation.[15] There were significant differences between patients with and without positive CEA mRNA in the depth of tumor invasion and vessel involvement. Consistent with previous investigations,[13,15,20] the detection rate increased with the stage of the tumor in the present investigation. Especially at stage IV, 9 patients expressed positive hTERT and all 12 expressed positive CEA mRNA. Ito *et al.*[9] have disclosed that RT-PCR amplification of CEA mRNA is an efficient means of detecting circulating cancer cells in the peripheral blood of CRC patients, and that these disseminated tumor cells are associated with high metastatic recurrence. Our observations show that the detection of these circulating tumor cells may be of prognostic value and therefore will have therapeutic implications for CRC patients. Moreover, only positive CEA mRNA correlated directly with the postoperative metastases, and consequently it might represent the most promising molecular marker for surveillance of CRC patients following surgery. Nevertheless, only 53% (28 out of 51) of CRC patients with positive CEA mRNA developed metastasis during the course of this study. One reason may be the relatively short follow-up period. Alternatively, this may be quite reasonable because few carcinoma cells shed into the bloodstream succeed in establishing metastatic disease.[19]

One problem in our results is that they showed that hTERT or cytokeratin mRNA is neither as sensitive as CEA mRNA for the detection of circulating tumor cells, nor as effective as an independent predictor for postoperative surveillance in CRC patients. Lledo *et al.*[10] suggested that detection of hTERT mRNA in peripheral blood might be helpful for differentiation between healthy and CRC patients, but no information about its prognostic significance for CRC patients is available. In addition, the controversial roles of cytokeratin mRNA as molecular markers from previous observations further emphasise the potential importance of CEA mRNA.[21–23] The more multiple molecular markers are used in the detection of circulating tumor cells, the higher the sensitivity of the method becomes.[12,24] Similarly, our detection rate of circulating CRC cells increased to 87.5% (63 out of 72) using these four mRNAs concurrently (data not shown). On the other hand, we detected 2 healthy individuals with positive molecular markers: 1 positive for CK-19 mRNA and 1 positive for CK-20 mRNA. This false-positive in healthy individuals might be attributed to the design of primers, contamination of epithelial cells, the presence of a pseudogene,[22] or the handling of samples.

In conclusion, our study indicates that using CEA mRNA for the detection of circulating tumor cells in

peripheral blood is a rational approach for the surveillance of CRC patients following operation. This analysis can offer a simple, noninvasive, and promising tool for the early detection of micrometastatic tumor cells in CRC patients. However, a further study with long-term follow-up in a larger number of patients is required to confirm the clinical application of this molecular marker.

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