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Persistent Presence of Postoperative Circulating Tumor Cells is a Poor Prognostic Factor for Patients with Stage I–III Colorectal Cancer after Curative Resection

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Aim: To detect pre- and postoperative circulating tumor cells (CTCs) in stage I–III colorectal cancer (CRC) patients undergoing curative resection and so identify a subgroup of patients who are at high risk for relapse.

Methods: Four mRNA molecular markers including human telomerase reverse transcriptase, cytokeratin-19, cytokeratin-20, and carcinoembryonic antigen mRNA were used to detect CTCs in 438 CRC patients underwent curative resection.

Results: Out of 438 patients, 80 CRC patients were classified to preoperative (−)/postoperative (−), 221 patients were preoperative (+)/postoperative (−), while 137 patients were preoperative (+)/postoperative (+). Univariately, postoperative relapse was significantly correlated with depth of invasion ($P = 0.032$), lymph node metastasis ($P < 0.001$), vascular invasion ($P = 0.001$), perineural invasion ($P = 0.013$), and persistent presence of CTCs ($P < 0.001$). Using a multivariate proportional hazards regression analysis, the presence of lymph node metastasis ($P = 0.012$; HR, 7.652; 95% CI: 4.162–14.827), vascular invasion ($P = 0.033$; HR, 4.360; 95% CI: 2.793–10.847), and the persistent presence of CTCs ($P < 0.001$; HR, 29.486; 95% CI: 10.281–87.792) were demonstrated to be independent predictors for postoperative relapse. Combination of these three independent predictors showed that patients with any one positive predictor had a hazard ratio of sevenfold to develop postoperative relapse ($P < 0.001$; HR, 7.064; 95% CI: 4.354–11.464). Furthermore, the

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persistent presence of CTCs was strongly correlated with poorer relapse-free survival rates (all $P < 0.001$).

Conclusion: The promising results of this study suggest that persistent presence of postoperative CTCs may be a crucial prognostic factor adjuvant to conventional tumor markers in CRC patients who have undergone curative resection. Identification of these high-risk patients of persistent CTCs positivity is important and thus could help to define patients for adjuvant therapy with this tumor entity.

Key Words: Circulating tumor cells—Molecular markers—Colorectal cancer—Prognosis—Postoperative surveillance.

Colorectal cancer (CRC) is the third most common cancer and is also the third major cause of cancer-related death in Taiwan, with over 8,000 new cases and 4,000 deaths per year.[1] In addition, this disease has one of the highest rates of increased incidence in Taiwan, gradually approaching Western figures in recent decades. Even with the recent advances in diagnostic and surgical techniques, the outcome remains poor in the cases of advanced disease, and only CRC diagnosed at an early stage is likely to be cured by surgical resection.[2–4] Pathologic prognostic factors of primary tumor invasion, regional lymph node involvement, and the presence or absence of metastasis have been used for many decades as the three major prognostic determinants for CRC patients, and predict the risk of relapse of this disease. Relapses have an important meaning in relation to survival for curative surgical intervention of CRC patients. Unfortunately, despite the curative resection for CRC patients, some patients with apparently localized disease at diagnosis will subsequently develop recurrent or metastatic diseases. It is important to note that as many as 25–40% of patients who undergo curative resection nevertheless subsequently develop metastatic disease, suggesting that undetected micrometastasis exists and may play a key role in relapse.[5–7] One of the major causes is the presence of disseminated tumor cells shed from the primary carcinoma into circulation prior to, during, or after surgery. The fact that the overall survival rate remains poor strongly suggests that the dissemination of these cells occurs early in the disease process and emphasizes the need for finding feasible diagnostic methods with sufficient sensitivity and specificity. Therefore, development of a sensitive, specific and convenient diagnostic method for detecting circulating tumor cells (CTCs) at a very early stage could be used as postoperative surveillance, and ultimately affect future patient prognosis.

The most commonly used technique for the detection of nucleic acid material of disseminated tumor cells is the use of polymerase chain reaction (PCR), reverse-transcriptase PCR (RT-PCR), or real-time quantitative PCR (Q-PCR) assays, which now permit sensitive detection of CTCs in peripheral blood. Accumulated reports have described the detection of CTCs in the peripheral blood of CRC patients, which has important prognostic and therapeutic implications.[8–12] Due to the heterogeneity of gene marker expression in blood and lymph nodes, a multimarker assay is regarded as more reliable and sensitive than a single-marker assay.[13–16] Our recently developed membrane array-based multimarker assay can detect CTCs in the peripheral blood of CRC patients; this is found to be a rational approach for the postoperative surveillance of CRC patients.[17–20] Though many messenger RNA (mRNA) molecular markers have been evaluated as putative prognostic markers in CRC patients, no information about the multimarker assay [human telomerase reverse transcriptase (hTERT), cytokeratin-19 (CK-19), cytokeratin-20 (CK-20), and carcinoembryonic antigen (CEA)] in the detection of CTCs as a prognostic tool for CRC patients undergoing curative resection has been obtained. The aim of this study was to detect both pre- and postoperative CTCs in peripheral blood of stage I–III CRC patients who had undergone curative resection by a panel of molecular markers using a constructed membrane array method, and evaluate whether persistence of CTCs positivity after primary CRC excision was related to clinical outcome.

PATIENTS AND METHODS

Patients and Sample Collection

Included in this prospective study were International Union against Cancer (UICC) stage I–III CRC patients admitted to the Department of Surgery of Kaohsiung Medical University Hospital for elective surgery between January 2002 and December 2005. Patients with other malignant diseases in their medical

history were excluded. Of a total 461 stage I–III CRC patients, 16 were lost to follow-up, 4 had surgical mortality, and 3 with surgical resection margin positive for tumor invasion were excluded. The remaining 438 stage I–III CRC patients (234 males and 204 females; mean age 65.6 ± 13.1 years) with curative resection for the primary lesion were finally enrolled in the present study. Curative resection was defined as any gross residual tumor that did not remain in the surgical bed and in which the surgical resection margin was pathologically negative for tumor invasion. Patients diagnosed as either high-risk stage II or III received adjuvant chemotherapy. Patients with risk factors for relapse (tumor poorly differentiated, tumor perforation, number of lymph nodes examined <12 or lymphatic/vascular invasion) were considered as high-risk stage II cases. Patients were administered three to six 8-week cycles of adjuvant chemotherapy. Each cycle consisted of leucovorin (LV) 100 mg/m^2 administered as a 2-h infusion and given weekly for three to six cycles, and 5-fluorouracil (5-FU) 500 mg/m^2 administered as an intravenous bolus 1 h after the start of LV infusion and repeated weekly for six doses. This cycle was then repeated after a 2-week rest period. CTCs in peripheral blood of these 438 patients were detected using our constructed membrane array method. Postoperative surveillance consisted of medical history, physical examination, and laboratory studies including serum CEA levels every 3 months. Abdominal ultrasonography or computed tomography was performed every 6 months, and chest radiography and total colonoscopy were performed once a year. Patients were followed up at 3-monthly intervals for 2 years and 6-monthly intervals thereafter; median follow-up was 44 months (range 21–66 months). The development of new recurrent or metastatic lesions after operation was defined as a postoperative relapse. The type of postoperative relapse was designated as local recurrence (tumor growth restricted to the anastomosis or the region of primary operation) or distant metastases (distant metastases or diffuse peritoneal seeding).

A 4-ml sample of peripheral blood was obtained from each CRC patient preoperatively (1 day prior to operation) and postoperatively (1 week after operation) for total RNA isolation. 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 each subject and/or guardian. Sample acquisition and subsequent use were also approved by the hospital's institutional review board. Clinical stage and pathological features of primary tumors were defined according to the criteria of the American Joint Commission on Cancer/International Union Against Cancer (AJCC/UICC).[21]

Detection of Serum CEA

Additional 3 ml peripheral blood samples from 438 CRC patients were obtained less than 1 week prior to operation (preoperative) and 4 weeks after operation (postoperative). Serum CEA levels were determined by means of an enzyme immunoassay test kit (DPC Diagnostic Product Co., Los Angeles, CA) with the upper limit of 5 ng/ml defined as normal according to the manufacturers of the kits that were used.

mRNA Isolation and First Strand cDNA Synthesis

Total RNA was extracted from the fresh whole blood of preoperative/postoperative CRC patients using a QIAmp® RNA Blood Mini Kit (QIAGEN Inc., Valencia, CA) 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 Corp., Madison, WI).

Membrane Arrays

The procedure of the membrane array method for the detection of CTC-related mRNA molecular markers was performed according to our recent study.[17,20,22] Patients overexpressing all four molecular markers by membrane-array methods were considered as positive results of CTCs.[20] In our previous investigation, the sensitivity limit of this technique was established at approximately one tumor cell per 10^6 white blood cells (5 cells per 1 ml blood).[17,22]

Statistical Analysis

All data were statistically analyzed using the Statistical Package for the Social Sciences, version 12.0 (SPSS Inc., Chicago, IL). A P value less than 0.05 was considered statistically significant. The univariate analysis of clinicopathologic features and expression of preoperative/postoperative molecular markers between the two groups (relapse group versus non-relapse group) was compared using the chi-square test. Expression of molecular markers was also ana-

lyzed according to types of postoperative relapse, either local recurrence or distant metastasis. Independent prognostic factors for postoperative relapse were determined using a multivariate Cox proportional hazards regression analysis. The combination of lymph node metastasis, vascular invasion, and presence of postoperative CTCs as predictors of postoperative relapse was analyzed using the chi-square test. The relapse-free survival rates of CRC patients were further categorized according to the tumor location. The relapse-free survival rates were calculated by the Kaplan–Meier method, and the differences in survival rates were analyzed by the log-rank test.

RESULTS

Two hundred and thirty-four men (53.4%) and 204 women (46.4%) were recorded. The average age was 65.6 years (range 27–90 years). Two hundred and eighty-two tumors (64.4%) were in the colon and 156 (35.6%) in the rectum. With regard to the histological type of these tumors, 42 (9.6%) were well differentiated, 342 (78.1%) were moderately well differentiated, and 54 (12.3%) were poorly differentiated carcinomas. The clinicopathologic characteristics of these 438 UICC stage I–III CRC patients are listed in Table 1; 66 patients were subsequently diagnosed with stage I CRC, 188 with stage II, and 184 with stage III. Overall, 80 of 438 (18.3%) patients were identified to have no detectable CTCs in neither preoperative nor postoperative peripheral blood; 221 of 438 patients (50.4%) were found to have detectable CTCs in preoperative but not in postoperative peripheral blood; 137 of 438 patients (31.3%) had CTCs in both preoperative and postoperative peripheral blood.

Table 2 shows the distribution of preoperative/postoperative serum carcinoembryonic antigen levels and presence of CTCs according to stage (I–III) of CRC patients. The frequency of abnormal serum preoperative CEA level (≥5 ng/ml) for UICC stage I, II, and III patients was 12.1% (8/66), 46.8% (88/188), and 48.4% (89/184) respectively. Meanwhile, the corresponding values for abnormal serum postoperative CEA levels were 4.5% (3/66), 10.6% (20/188), and 14.7% (27/184) respectively. The frequency of preoperative CTCs positivity for UICC stage I, II, and III patients was 33.3% (22/66), 84.6% (159/188), and 96.2% (177/184) respectively. Simultaneously, the corresponding values for frequency of postoperative CTCs positivity were 6% (4/66), 30.3% (57/188), and 41.3% (76/184), respectively. Our results show that only 60 (13.7%) of 185 patients with abnormal serum preoperative CEA level were not found to convert to normal serum CEA level, whereas 137 (38.3%) of 358 patients with preoperative presence of CTCs were observed to have persistent CTCs positivity despite later curative resection. Distribution of preoperative/postoperative serum CEA levels and presence of CTCs is not related to the tumor location. During the follow-up period, 79 of 282 (28%) colon cancer patients and 51 of 156 (32.7%) rectal cancer patients were identified with postoperative relapse. Of 130 CRC patients with postoperative relapse, 38 patients had local recurrent and 92 patients had distant metastasis diseases.

From the correlation between postoperative relapse and clinicopathologic features or the persistent presence of CTCs positivity of 438 CRC patients using univariate analyses, we found the depth of

TABLE 1. *Clinicopathologic characteristics of 438 colorectal cancer patients undergoing curative resection*

Variables	Number (%)
Gender	
Male/female	234 (53.4)/204 (46.4)
Age (years)	
<65/≥65	192 (43.8)/246 (56.2)
Maximum tumor size (cm)	
<5/≥5	225 (51.4)/213 (48.6)
Tumor location	
Colon/rectum	282 (64.4)/156 (35.6)
Depth of tumor invasion	
T_1+T_2/T_3+T_4	96 (21.9)/342 (78.1)
Lymph node metastases	
No/yes	254 (58.0)/184 (42.0)
UICC stage	
I/II/III	66 (15.1)/188 (42.9)/ 184 (42.0)
Vascular invasion	
No/yes	309 (70.5)/129 (29.5)
Perineural invasion	
No/yes	333 (76.0)/105 (24.0)
Differentiation	
Well/moderately/poorly	42 (9.6)/342 (78.1)/ 54 (12.3)
Type of tumor	
Adenocarcinoma/mucinous	409 (93.4)/29 (6.6)
Presence of CTCs positivity	
Preoperative (−)/postoperative (−)/ Preoperative (+)/postoperative (−)/ Preoperative (+)/postoperative (+)	80 (18.3)/221 (50.4)/ 137 (31.3)
Preoperative colonic obstruction/perforation	
No/yes	413 (94.3)/25 (5.5)
Postoperative relapse	
No/yes	308 (70.3)/130 (29.7)
Patients with postoperative relapse	
UICC stage I/II/III	3 (2.3)/54 (41.5)/73 (56.2)

CTCs, circulating tumor cells; UICC, International Union against Cancer.

TABLE 2. *Distribution of preoperative/postoperative serum carcinoembryonic antigen levels and presence of circulating tumor cells according to various stages colon and rectal cancer patients*

	CEA levels					Presence of CTCs positivity			
	Stage I *N* (%)	Stage II *N* (%)	Stage III *N* (%)	*P* value*		Stage I *N* (%)	Stage II *N* (%)	Stage III *N* (%)	*P* value*
Preoperative					Preoperative				
<5 ng/ml	58 (87.9)	100 (53.2)	95 (51.6)		Negative	44 (66.7)	29 (15.4)	7 (3.8)	
Colon	42 (24.0)	67 (38.3)	66 (37.7)	0.774	Colon	32 (56.1)	20 (35.1)	5 (8.8)	0.941
Rectum	16 (20.5)	33 (42.3)	29 (37.2)		Rectum	12 (52.2)	9 (39.1)	2 (8.7)	
≥5 ng/ml	8 (12.1)	88 (46.8)	89 (48.4)		Positive	22 (33.3)	159 (84.6)	177 (96.2)	
Colon	6 (4.8)	59 (47.2)	60 (48.0)	0.889	Colon	15 (6.0)	109 (43.8)	125 (50.2)	0.909
Rectum	2 (3.3)	29 (48.3)	29 (48.3)		Rectum	7 (6.4)	50 (45.9)	52 (47.7)	
Postoperative					Postoperative				
<5 ng/ml	63 (95.5)	168 (89.4)	157 (85.3)		Negative	62 (94.0)	131 (69.7)	108 (58.7)	
Colon	46 (17.0)	115 (42.6)	109 (40.4)	0.797	Colon	44 (19.4)	98 (43.2)	85 (37.4)	0.518
Rectum	17 (14.4)	53 (44.9)	48 (40.7)		Rectum	18 (24.3)	33 (44.6)	23 (31.1)	
≥5 ng/ml	3 (4.5)	20 (10.6)	27 (14.7)		Positive	4 (6.0)	57 (30.3)	76 (41.3)	
Colon	2 (6.1)	13 (39.4)	18 (54.5)	0.993	Colon	3 (2.9)	43 (41.0)	59 (56.2)	0.954
Rectum	1 (5.9)	7 (41.2)	9 (52.9)		Rectum	1 (3.1)	14 (43.8)	17 (53.1)	

* Results of two-sided Pearson χ^2 test between colon and rectal cancer patients. CTCs, circulating tumor cells.

invasion ($P = 0.032$), the presence of lymph node metastasis ($P < 0.001$), the presence of vascular invasion ($P = 0.001$), the presence of perineural invasion ($P = 0.013$), and the persistent presence of CTCs positivity ($P < 0.001$; Table 3) to show significant correlation. Moreover, no clinicopathologic parameters were significantly different between the colon and rectal cancer patients in the groups with or without postoperative relapse. Using a Cox proportional hazard regression analysis, the presence of lymph node metastasis ($P = 0.012$; HR, 7.652; 95% CI: 4.162–14.827), vascular invasion ($P = 0.033$; HR, 4.360; 95% CI: 2.793–10.847), and the persistent presence of CTCs positivity ($P < 0.001$; HR, 29.486; 95% CI: 10.281–87.792) were demonstrated to be independent predictors for postoperative relapse (Table 4). Moreover, the combination of presence of lymph node metastasis, vascular invasion, and persistent CTCs positivity as high-risk predictors of postoperative relapse is shown in Table 5. CRC patients with one high-risk predictor had a relative risk of 7.064 of developing postoperative relapse compared to those without any high-risk predictor ($P < 0.001$; HR, 7.064; 95% CI: 4.354–11.464). Furthermore, statistically significant difference was observed in terms of relapse-free survival rate between CRC patients with and those without persistent presence of CTCs positivity using the log-rank test, in all patients with CRC, and in colon cancer or rectal cancer (Fig. 1; all $P < 0.001$). Patients with a failed conversion of the preoperative detectable CTCs to the postoperative undetectable CTCs showed the worst relapse-free survival rate when compared with the other two groups ($P < 0.001$).

DISCUSSION

The recent identification of genes overexpressed in CRC, combined with advances in molecular biology, provides the opportunity to establish more sensitive, specific, and cost-effective ways of identifying metastatic disease. Among the current possibilities, one of the most compelling is the development of a highly sensitive molecular diagnostic procedure that permits the detection of tumor cells in different tissues and biologic fluids, especially peripheral blood. Because metastasis is such a key process, there is an enormous effort to identify markers that can detect disseminated tumor cells in the circulation, which will aid early diagnosis. However, the heterogeneity of the expression of tumor genes and the variable performance of these assays has posed major problems for the detection of CTCs. Our membrane array assay was able to simultaneously detect a panel of informative molecular markers for the presence of CTCs in CRC patients undergoing curative resection, with advantages in terms of time saving and cost effectiveness.[20,23] The current investigation has demonstrated that patients identified with persistent presence of CTCs postoperatively using our multimarker membrane array method exhibit higher incidence of postoperative relapse and poorer relapse-free survival rate. Even patients with preoperative CTCs positivity would eventually attain a better prognosis after curative resection when their postoperative CTCs convert to negativity. Because the low frequency (10.4%) of abnormal serum CEA levels could be determined at 4 weeks after operation, the considerably higher frequency (31.3%) of CTCs

TABLE 3. *Correlation between postoperative relapse and clinicopathologic features of colorectal cancer patients using univariate analysis*

	Postoperative relapse (+)			Postoperative relapse (−)			
	Colon (N = 82) (%)	Rectum (N = 48) (%)	P value*	Colon (N = 200) (%)	Rectum (N = 108) (%)	P value*	P value**
Gender							
Male/female	44/38	22/26	0.389	108/92	60/48	0.794	0.469
Age (years)							
<65/≥65	27/55	21/27	0.217	92/108	52/56	0.718	0.058
Maximum size (cm)							
<5/≥5	37/45	25/23	0.443	101/89	52/56	0.470	0.317
Depth of tumor invasion							
T_1+T_2/T_3+T_4	14/68	6/42	0.486	50/150	26/82	0.857	0.032
Lymph node metastases							
No/yes	27/55	14/34	0.656	141/59	72/36	0.487	<0.001
Vascular invasion							
No/yes	67/15	39/9	0.948	129/71	74/34	0.478	0.001
Perineural invasion							
No/yes	70/12	40/8	0.757	143/57	81/27	0.510	0.013
Differentiation							
Well/moderately/poorly	10/58/14	4/37/7	0.740	18/160/22	10/87/11	0.975	0.077
Type of tumor							
Adenocarcinoma/mucinous	74/8	44/4	0.787	189/11	102/6	0.984	0.154
Presence of CTCs positivity							
Preoperative (−)/postoperative (−)/ Preoperative (+)/postoperative (−)/ Preoperative (+)/postoperative (+)	4/24/54	2/12/34	0.842	52/115/33	22/70/16	0.443	<0.001
Preoperative colonic obstruction/perforation							
No/yes	76/6	54/2	0.355	189/11	104/4	0.485	0.245

* Results of two-sided Pearson χ^2 test between colon and rectal cancer patients; ** results of two-sided Pearson χ^2 test of overall colorectal cancer patients between postoperative relapse (+) and (−) groups; CTCs, circulating tumor cells.

TABLE 4. *Correlation between postoperative relapse and clinicopathologic features of colorectal cancer patients using multivariate Cox proportional hazard regression analysis*

Variables	Hazard ratio	95% CI	P value
Lymph node metastases (yes/no)	7.652	4.162–14.827	0.012
Vascular invasion (yes/no)	4.360	2.793–10.847	0.033
Presence of CTCs positivity (yes/no)	29.486	10.281–87.792	<0.001

CI, confidence interval; CTCs, circulating tumor cells.

positivity at 1 week would play a crucial role in the early detection of micrometastasis and CTCs. Recently, our study also revealed that molecular detection of postoperative CTCs is helpful in the early prediction of postoperative relapse in CRC patients with normal perioperative serum CEA levels, with a median lead time of 6 months before the measurement of abnormal CEA levels.[20] Consequently, detecting preoperative/postoperative CTCs seems to be an auxiliary diagnostic tool to conventional serum tumor marker-CEA in the early identification of high-risk stage I–III CRC patients having undergone curative resection.

In our current study, CTCs positivity for UICC stage I, II, and III CRC patients was observed with an incidence of 6%, 30.3%, and 41.3% respectively. The detection incidence of our constructed membrane array methods is almost approaching the incidence of presence of postoperative relapse in stage I, II, and III. The detection incidence of the membrane array in stage I, II, III CRC patients were 6%, 30.3%, and 41.3%. And the incidence of postoperative relapse in stage I, II, and III CRC patients were 2.3%, 28.7%, and 39.7%. Consistent with our findings, Patel et al. have disclosed that 30% of stage I + II and 65% of stage III CRC patients could be identified with CTCs at 1 week after operation, either by CEA or CK-20 mRNA markers.[11] They pointed out that no significant reduction in RT-PCR positivity was detected after apparently complete tumor excision among patients with lymph-node-positive tumors (Dukes' C) in whom there was a high probability of recurrence, compared with the significant reduction detected in node-negative tumors (Dukes' A or B), where the risk of recurrence was lower. Likewise, multivariate analysis indicated that detection of the simultaneous presence of CEA/CK-20 mRNA by RT-PCR or Q-PCR in tumor drainage blood is a potent

TABLE 5. *Combination of the depth, vascular invasion and presence of circulating tumor cells as predictors of postoperative relapse for stage I-III colorectal cancer patients following curative resection*

Lymph node (+) or vascular invasion (+) or presence of CTCs positivity (+)	No. of relapse patients (n = 130)	No. of non-relapse patients (n = 308)	Hazard ratio	95% CI	P value
Any one predictor					
Positive	103	108	7.064	4.354–11.464	<0.001
Negative	27	200			

CI, confidence interval; CTCs, circulating tumor cells.

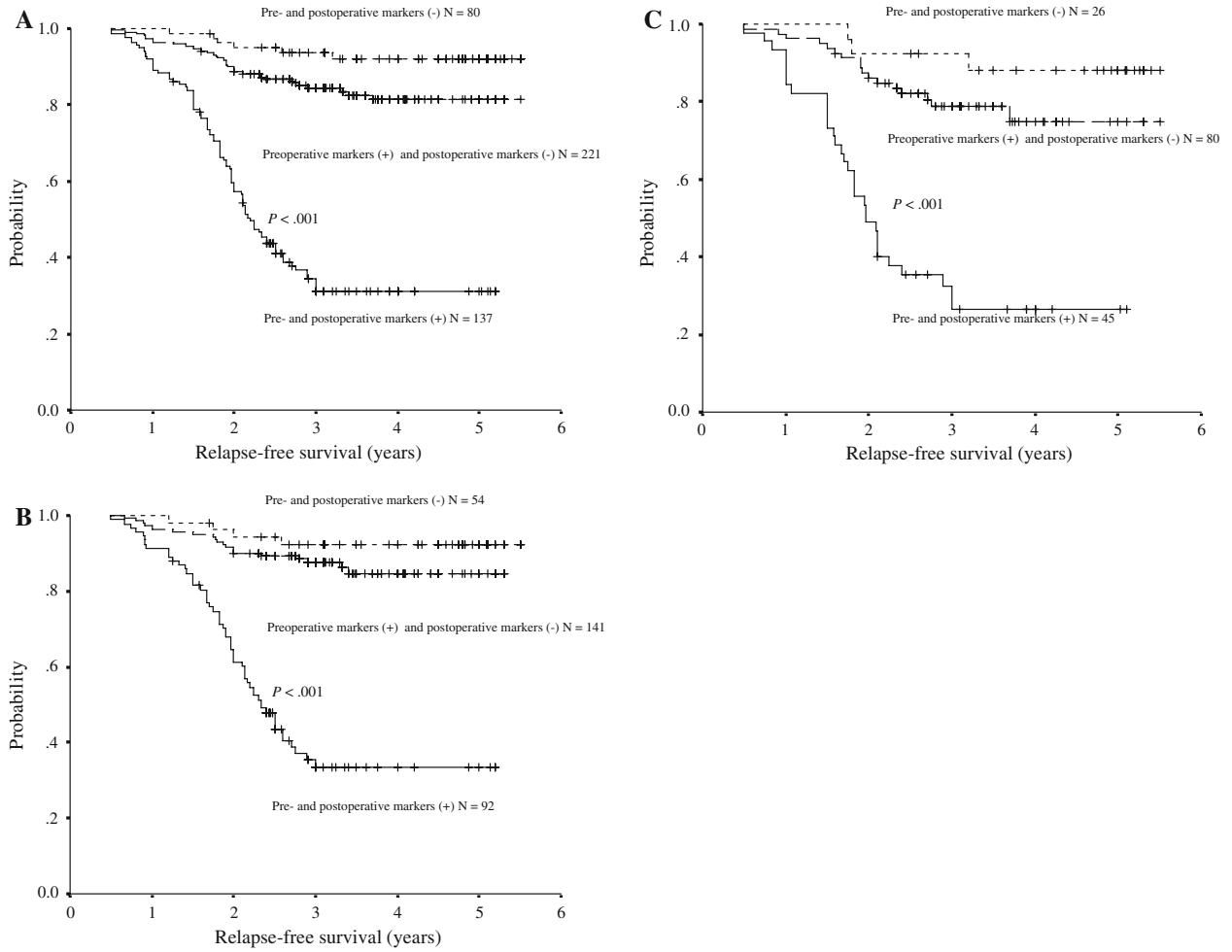

FIG. 1. Relapse-free survival rates of stage I–III colorectal cancer patients analyzed by the Kaplan–Meier method with the differences compared by a log-rank test according to tumor location. (**A**) All 438 stage I–III colorectal cancer patients with a failed conversion of the preoperative detectable CTCs to the postoperative undetectable were found to have the worst overall survival rate while patients with preoperative/postoperative undetectable CTCs were found to have the best relapse-free survival rate among the three groups ($P < 0.001$); (**B**) All 282 stage I–III colon cancer patients with a failed conversion of the preoperative detectable CTCs to the postoperative undetectable CTCs showed the worst relapse-free survival rate when compared with the other two groups ($P < 0.001$); (**C**) All 156 stage I–III rectal cancer patients with a failed conversion of the preoperative detectable CTCs to the postoperative undetectable CTCs showed the worst relapse-free survival rate when compared with the other two groups ($P < 0.001$).

prognostic factor independent of the traditional pathologic parameters.[10,24,25] Furthermore, the disease-free and/or overall survival of patients with CEA/CK-20-positive peripheral blood was significantly shorter than that of marker-gene-negative patients.[10,24,25] Similar results were obtained where detection of CTCs in blood samples of patients with stage II CRC identified patients with poor outcome.[23,26,27]

In contrast, some recently published studies report conflicting results regarding the prognostic value of CTCs.[28,29] A major problem of most of the published studies is that only small, inhomogeneous patient groups with relatively short follow-up periods were evaluated. Moreover, the methods used for CTCs detection also need to be taken into account, as sensitivity and specificity are of major importance and may differ significantly.[20,23,30] Our membrane array assay is unlikely to be 100% accurate in predicting postoperative relapse, suggesting that there is room for the improvement of this method. Recently, Khair et al. proposed that the discrepancies in the published results of many PCR studies discussed in this review are most likely because of one or more of the following factors:[30] (1) the method of blood sample preparation—some studies have measured CTC by using extracellular markers and others intracellular; (2) time lapse between sample collection and processing, as variations in handling are likely to affect the viability and expression of mRNA in particular; and (3) PCR conditions, i.e., single or multiple, number of PCR cycles and whether the PCR is standard, nested or real time. Of three factors, the timing of blood sampling is particularly important in the molecular detection of CTCs. Surgical manipulation probably enhances this release of hematogenic tumor cells into the circulation.[10] Therefore, the detection of CTCs in blood samples taken during surgery is unstable and unreliable. Patel et al. obtained blood samples before and until 3 months after surgery and reported a significant decrease of the CTC-positive patients at 24 h after surgery in Dukes' A/B CRC patients.[11] Analogously, cancer cells experimentally injected into the peripheral vein of mice rapidly decreased with time and were detected 3 days but not 7 days after injection.[31] One recent report also suggests the detection of circulating cancer cells in peripheral blood at 7–10 days after surgery was associated with significantly increased risk of recurrence.[32] However, Allen-Mersh et al. point out that RT-PCR CTCs-positivity within 24 h of primary CRC resection is a strong predictor of CRC recurrence, and may be useful clinically.[33] From the viewpoint of surgical manipulation and clearance of CTCs after operation, it is significant that a direct association was observed between the time when the specimen was obtained and molecular detection of tumor cells.

Combining three independent prognostic markers identified in our patient cohort, including the presence of lymph node metastasis, the presence of vascular invasion and postoperative persistent presence of CTCs positivity, stage I–III CRC patients have a sevenfold risk of developing postoperative relapse compared with those without any high-risk predictor. Concomitant molecular diagnosis of CTCs with a multimarker panel is a justifiable supplementary approach to the current pathological staging system, which may help physicians make appropriate judgments on clinical management and predictive prognosis for stage I–III CRC patients following operation. Additionally, a positive correlation with pathological parameters compatible with more aggressive tumors and CTCs was observed in patients with tumor mRNA present in plasma.[12,34] In the future, these criteria might enable the selection of high-risk patients who would benefit from adjuvant treatment. Hence, therapeutic decision-making models are likely to be further redefined by the inclusion of perioperative changes of such mRNA markers.

In conclusion, the constructed membrane array method for the detection of CTCs has been demonstrated to be complementary to conventional serum CEA level for the surveillance of stage I–III CRC patients. We suggest that CRC patients with persistent positive CTC blood samples should be evaluated for further adjuvant therapeutic strategies after surgery. However, multicenter and long-term clinical follow-up is warranted to address the potential clinical significance.

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