Prognostic Significance of Multiple Molecular Markers for Patients With Stage II Colorectal Cancer Undergoing Curative Resection

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Objective: The aim of this study was to determine whether our constructed high-sensitivity colorimetric membrane-array method could detect circulating tumor cells (CTCs) in the peripheral blood of stage II colorectal cancer (CRC) patients and so identify a subgroup of patients who are at high risk for relapse.

Summary Background Data: Adjuvant chemotherapy is not routinely recommended in patients diagnosed with UICC stage II CRC. However, up to 30% of patients with stage II disease relapse within 5 years of surgery from recurrent or metastatic disease. The identification of reliable prognostic factors for high-risk stage II CRC patients is imperative.

Methods: Membrane-arrays consisting of a panel of mRNA markers that included human telomerase reverse transcription (hTERT), cytokeratin-19 (CK-19), cytokeratin-20 (CK-20), and carcinoembryonic antigen (CEA) mRNA were used to detect CTCs in the peripheral blood of 194 stage II CRC patients who underwent potentially curative (R0) resection between January 2002 and December 2005. Digoxigenin (DIG)-labeled cDNA were amplified by RT-PCR from the peripheral blood samples, which were then hybridized to the membrane-array. All patients were followed up regularly, and their outcomes were investigated completely.

Results: Overall, 53 of 194 (27.3%) stage II patients were detected with the expression of all 4 mRNA markers using the membrane-array method. After a median follow up of 40 months, 56 of 194 (28.9%) developed recurrence/metastases postoperatively. Univariately, postoperative relapse was significantly correlated with the depth of invasion ($P < 0.001$), the presence of vascular invasion ($P < 0.001$), the presence of perineural invasion ($P = 0.048$), the expression of all 4 mRNA markers ($P < 0.001$), and the number of examined lymph nodes ($P = 0.031$). Meanwhile, using a multivariate logistic regression analysis, T4 depth of tumor invasion ($P = 0.013$), the presence of vascular invasion ($P = 0.032$), and the expression of all 4 mRNA markers ($P < 0.001$) were demonstrated to be independent predictors for postoperative relapse. Combination of the depth of tumor invasion, vascular invasion, and all 4 mRNA markers as predictors of postoperative relapse showed that patients with any 1 positive predictor had a hazard ratio of about 27-fold to develop postoperative relapse ($P < 0.001$; 95% CI = 11.42–64.40). The interval between the detection of all 4 positive molecular markers and subsequently developed postoperative relapse ranged from 4 to 10 months (median: 7 months). Furthermore, the expression of all 4 mRNA markers in all stage II CRC patients, or either stage II colon or rectal cancer patients were strongly correlated with poorer relapse-free survival rates by survival analyses (all $P < 0.001$).

Conclusions: The pilot study suggests that the constructed membrane-array method for the detection of CTCs is a potential auxiliary tool to conventional clinicopathological variables for the prediction of postoperative relapse in stage II CRC patients who have undergone curative resection.

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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 8000 new cases and 4000 deaths per year (*http://www.doh.gov.tw/statistic/index.htm*; accessed in January 2007). Adjuvant chemotherapy with 5-fluorouracil (FU)-based therapy has now become an accepted standard of care for patients with International Union Against Cancer (UICC) stage III colon cancer since the early 1990s, and has resulted in a 30% to 40% decrease in relapse and mortality rates versus treatment with surgery alone.[1–3] More recently, the addition of oxaliplatin to 5-FU-based therapy has further improved patient outcomes, thus establishing this combination as a new standard of care.[4,5] Patients with stage II CRC are generally considered to be at low risk for developing postoperative relapse; therefore, patients with CRC in this stage are not recommended to undergo routine

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adjuvant chemotherapy.[3,6–8] However, about 25% to 30% of CRC patients with stage II disease are at high risk for postoperative relapse. Indeed, the clinical outcome of patients with high-risk stage II disease is similar to that of patients with stage III disease.

With regard to stage II CRC, a wide variety of potential clinical and pathologic risk factors for recurrence have been investigated. The most important factors for predicting the risk of recurrence are emergency presentation (bowel perforation or obstruction), poorly differentiated tumor (histologic grade), depth of tumor invasion and adjacent organ involvement (T4), extramural venous invasion, and peritoneal involvement.[9,10] Recently, we have also demonstrated that the depth of invasion, the presence of vascular invasion and number of examined lymph nodes may prominently affect the prognosis of patients with stage II CRC.[11] It is therefore of high importance to define reliable prognostic factors for this patient group to help identify high-risk patients (for tumor relapse) who might benefit from adjuvant therapeutic regimes.[12,13]

With recent developments in molecular technology, the use of polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR), or real-time quantitative-CR (Q-PCR) assays now permit sensitive detection of circulating tumor cells (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.[14–18] 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 surveillance of postoperative CRC patients.[18–21] Though many mRNA (messenger RNA) molecular markers have been evaluated as putative prognostic markers in CRC patients, no information about the multimarker assay [human telomerase reverse transcription (hTERT), cytokeratin-19 (CK-19), cytokeratin-20 (CK-20), and carcinoembryonic antigen (CEA)] in the detection of CTCs as a prognostic tool for stage II CRC patients has ever been obtained. The aim of this study was to analyze stage II CRC patients who have undergone curative resection by a panel of molecular markers using a constructed membrane-array method and evaluate their significance in postoperative surveillance.

PATIENTS AND METHODS

Patients and Samples

Included in this prospective study were 194 stage II 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 disease in their medical history were excluded. Circulating tumor cells in peripheral blood of these 194 patients were detected using our constructed membrane-array method. All 194 patients underwent radical resection for the primary lesion. Radical (R0) resection is defined as any gross residual tumor that did not remain in the surgical bed, and the surgical resection margin is pathologically negative for tumor invasion. 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 40 months (range, 14–62 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 postoperatively (at least 1 week after surgery) for total RNA isolation. No additional blood samples were drawn for the detection of CTCs. 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

TABLE 1. Clinicopathologic Characteristics of 194 Stage II Colorectal Cancer Patients

Variables	No. (%)
Gender	
Male/female	105 (54.1)/89 (45.9)
Age (yr)	
<65/≥65	84 (43.3)/110 (56.7)
Maximum tumor size (cm)	
<5/≥5	100 (51.5)/94 (48.5)
Tumor location	
Colon/rectum	128 (66)/66 (34)
Depth of tumor invasion	
T_3/T_4	185 (95.4)/9 (4.6)
Vascular invasion	
Yes/no	52 (26.8)/142 (73.2)
Perineural invasion	
Yes/no	66 (34)/128 (66)
Histology	
WD/MD/PD	17 (8.8)/157 (80.9)/20 (10.3)
Type of tumor	
Mucinous carcinoma	
Yes/no	10 (5.2)/184 (94.8)
Four molecular markers	
Yes/no	53 (27.3)/141 (72.7)
Number of examined lymph nodes	
<12 nodes/≥12 nodes	119 (61.3)/75 (38.7)
Preoperative colonic obstruction/perforation	
Yes/no	10 (5.2)/184 (94.8)
Adjuvant chemotherapy	
Yes/no	125 (64.4)/69 (35.6)

WD indicates well differentiated; MD, moderately differentiated; PD, poorly differentiated.

TABLE 2. Correlation Between Postoperative Relapse and Clinicopathologic Features of Stage II Colorectal Cancer Patients Using Univariate Analysis

	Postoperative Relapse (+) (N = 56) (%)	Postoperative Relapse (−) (N = 138) (%)	*P*
Gender			
Male/female	28 (50)/28 (50)	77 (55.8)/61 (44.2)	.463
Age (yr)			
<65/≥65	20 (35.7)/36 (64.3)	64 (46.4)/74 (53.6)	.174
Maximum size (cm)			
<5/≥5	27 (48.2)/29 (51.8)	73 (52.9)/65 (47.1)	.554
Tumor location			
Colon/rectum	36 (64.3)/20 (35.7)	92 (66.7)/46 (33.3)	.751
Depth of tumor invasion			
T_3/T_4	48 (85.7)/8 (14.3)	137 (99.3)/1 (0.7)	<.001
Vascular invasion			
Yes/no	34 (60.7)/22 (39.3)	18 (13)/120 (87)	<.001
Perineural invasion			
Yes/no	25 (44.6)/31 (55.4)	41 (29.7)/97 (70.3)	.048
Histology			
WD/MD/PD	3 (5.4)/44 (78.5)/9 (16.1)	14 (10.1)/113 (81.9)/11 (8)	.163
Mucinous carcinoma			
Yes/no	3 (5.4)/53 (94.6)	7 (5.1)/131 (94.9)	.935
Four molecular markers			
Yes/no	45 (80.4)/11 (19.6)	8 (5.8)/130 (94.2)	<.001
Number of examined lymph nodes			
<12/≥12	41 (73.2)/15 (26.8)	78 (56.5)/60 (43.5)	.031
Preoperative colonic obstruction/perforation			
Yes/no	3 (5.4)/53 (94.6)	7 (5.1)/131 (94.9)	.935
Adjuvant chemotherapy			
Yes/no	34 (60.7)/22 (39.3)	91 (65.9)/47 (34.1)	.491

WD indicates well differentiated; MD, moderately differentiated; PD, poorly differentiated.

approved by the hospital's institutional review board. Clinical stage and pathologic features of primary tumors were defined according to the criteria of the American Joint Commission on Cancer/International Union Against Cancer (AJCC/UICC).[22]

mRNA Isolation and First Strand cDNA Synthesis

Total RNA was extracted from the fresh whole blood of CRC patients and healthy volunteers 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 work.[18,23] Patients overexpressing all 4 molecular markers by membrane-array methods were considered as positive results.[18] In our previous investigation, the sensitivity limit of this technique was established at approximately 1 tumor cell per 10^6 white blood cells (5 cells per 1 mL blood).[19]

Statistical Analysis

All data have been statistically analyzed using the Statistical Package for the Social Sciences, version 11.5 (SPSS Inc., Chicago, IL). A *P* value less than 0.05 was considered to be statistically significant. Two-sided Pearson χ^2 test and the Fisher exact test were used to analyze the potential correlation between the expression of molecular markers used in combination and the clinicopathologic features of the study subjects. The multivariate analysis of independent prognostic factors for postoperative relapse was determined using the logistic regression analysis. 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

One hundred five men (54.1%) and 89 women (45.9%) were included in the study. The average age was 64.9 years (range, 28–90 years). With regard to the histologic type of

TABLE 3. Circulating Tumor Cells Used for the Prediction of Postoperative Relapse (Local Recurrence and Distant Metastasis) in 36 Colon and 20 Rectal Cancer Patients

	Local Recurrence			Distant Metastasis		
	Colon (N = 8)	Rectum (N = 8)	*P*	Colon (N = 28)	Rectum (N = 12)	*P*
Four molecular markers						
Positive	6	7	.522	23	9	.605
Negative	2	1	—	5	3	—

TABLE 4. Correlation Between Postoperative Relapse and Clinicopathologic Features of Stage II Colorectal Cancer Patients Using Multivariate Logistic Regression Analysis

Variables	β	SE	*P*	Hazard Ratio	95% CI
Depth (T_4/T_3)	1.406	0.565	.013	4.080	2.348–11.348
Vascular invasion (yes/no)	2.684	0.911	.032	3.541	1.681–13.432
Four molecular markers (yes/no)	3.653	0.520	<.001	38.597	13.931–106.938

β indicates coefficient; SE, standard error; CI, confidence interval.

TABLE 5. Combination of the Depth, Vascular Invasion, and Molecular Markers as Predictors of Postoperative Relapse for Stage II Colorectal Cancer Patients

T4 or Vascular Invasion (+) or Molecular Markers (+)	No. Relapse Patients (n = 56)	No. Nonrelapse Patients (n = 138)	*P*	Hazard Ratio	95% CI
Any one predictor					
Positive	48	25	<.001	27.12	11.421–64.397
Negative	8	113	—	—	—

CI indicates confidence interval.

these tumors, 17 (8.8%) were well-differentiated, 157 (80.9%) were moderately well differentiated, and 20 (10.3%) were poorly differentiated carcinomas. The clinicopathologic characteristics of these 194 stage II patients are listed in Table 1. Overall, 53 of 194 (27.3%) patients were detected with the expression of all 4 mRNA markers using the membrane-array method. During the follow-up period, 36 of 128 (28%) colon cancer patients and 20 of 66 (30%) rectal cancer patients were identified with postoperative relapse. The sensitivity and specificity of the membrane-array method for the prediction of postoperative relapse was 80.4% (45 of 11) and 94.2% (130 of 138), respectively. Eight patients (15%) with positive result of molecular marker expression did not develop postoperative relapse, whereas 11 patients (7.8%) without positive result of molecular marker expression developed postoperative relapse subsequently.

From the correlation between postoperative relapse and clinicopathologic features or molecular markers of stage II CRC patients using univariate analyses, depth of tumor invasion ($P < 0.001$), vascular invasion ($P < 0.001$), perineural invasion ($P = 0.048$), positive molecular markers ($P < 0.001$), and the number of examined lymph nodes ($P = 0.031$) were statistically significant (Table 2). No significant differences existed between the positive molecular markers and the presence of local recurrence or distant metastasis respectively, in either colon or rectal cancer patients (both $P > 0.05$; Table 3).

Using a multivariate logistic regression analysis, the depth of invasion ($P = 0.013$; hazard ratio = 4.080), vascular invasion ($P = 0.032$; hazard ratio = 3.541), and positive molecular markers ($P < 0.001$; hazard ratio = 38.597) were demonstrated to be independent predictors for postoperative relapse (Table 4). Moreover, the combination of depth of tumor invasion, vascular invasion, and 4 positive molecular markers as high-risk predictors of postoperative relapse is shown in Table 5. Stage II CRC patients with 1 high-risk

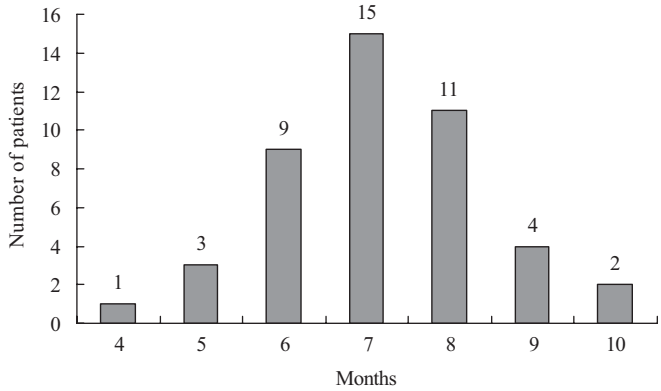

FIGURE 1. The interval between subsequently developed postoperative relapse and the presence of all 4 positive molecular markers in the 45 CRC patients.

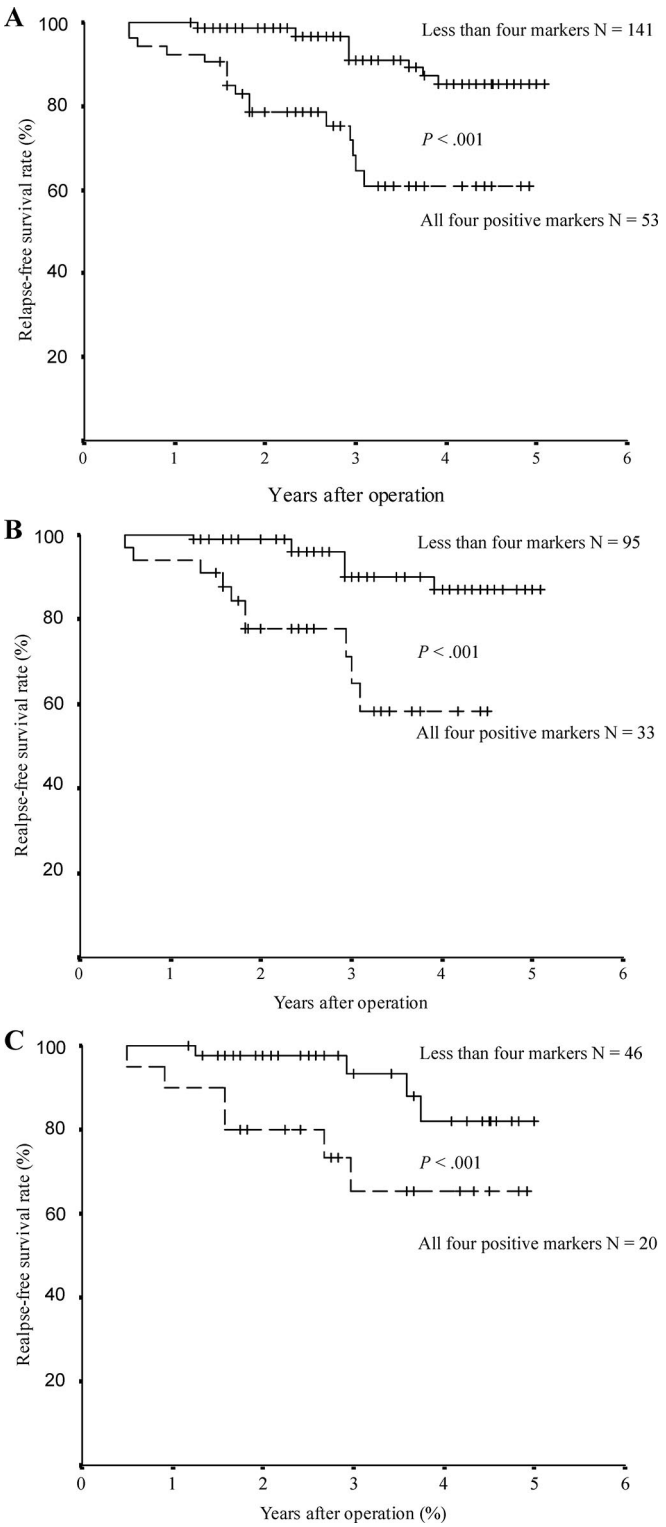

FIGURE 2. Relapse-free survival rates of stage II colorectal cancer patients were analyzed by the Kaplan-Meier method with the differences compared by a log-rank test. A, All 194 stage II colorectal cancer patients with all 4 mRNA markers expression in the peripheral blood showed a significantly poorer survival rate than those with less than 4 positive mRNA maker expression ($P < 0.001$); B, One hundred and twenty-eight stage II colon cancer patients with all 4 mRNA markers expression in the peripheral blood showed a significantly poorer survival rate than those with less than 4 positive mRNA maker expression ($P < 0.001$); C, Sixty-six stage II rectal cancer patients with all 4 mRNA markers expression in the peripheral blood showed a significantly poorer survival rate than those with less than 4 positive mRNA maker expression ($P < 0.001$).

predictor had a relative risk of 27.12 of developing postoperative relapse compared with those without any 1 high-risk predictor ($P < 0.001$). The lead-time between the detection of all 4 positive molecular markers and subsequently developed postoperative relapse ranged from 4 to 10 months (Fig. 1; median: 7 months). Furthermore, statistically significant difference was observed in terms of relapse-free survival rate between CRC patients with expression of all 4 markers and those with less than 4 positive markers using the log-rank test, in all patients with CRC, and in colon cancer or rectal cancer (Fig. 2; all $P < 0.001$).

DISCUSSION

Patients who undergo radical resection of stage II primary CRC are reported to have a 5-year survival rate of around 75%.[24] There is growing evidence that the prognosis of certain stage II CRC patients with unfavorable prognostic factors can be improved by adjuvant chemotherapy.[25,26] Accordingly, there is clearly a need to identify novel predictive factors to guide the identification of stage II CRC patients who are likely to experience relapse. More recently, there has been an attempt to identify novel panels of molecular and biochemical markers that may be used to more precisely define prognosis, and predict benefit of adjuvant treatment in CRC. Several retrospective studies have suggested that a number of molecular markers may now define patients with a higher risk of relapse with both stage II and stage III disease.[27–30] However, none of these are currently in clinical application regarding the decision whether patients with stage II CRC should receive adjuvant chemotherapy.

Detection of micrometastases and CTCs in patients with malignancies undergoing surgery for cure remains a challenge for oncologists, because dissemination of neoplastic cells is the main determinant of distant relapse and cancer-related death. There are numerous publications about conventional RT-PCR or Q-PCR detection of CTCs in CRC patients,[14–17,31,32] but 1 of the limitations is that the methodology could analyze only 1 molecular target at a time. Because of the heterogeneity of tumor-related genes, a multimarker assay is regarded as more reliable and sensitive than a single marker assay.[33–35] Our membrane-array assay was able to simultaneously detect a panel of informative molecular markers for the presence of CTCs in stage II CRC patients, with advantages of time-saving and cost-effectiveness.[18] Consistent with our findings, Koch e al also showed the prognostic significance of tumor cells detected in blood samples of patients with stage II CRC using CK-20 RT-PCR.[36] Similarly, Lloyd et al have disclosed that for a subgroup of patients with stage I and II CRC, detection of

marker-positive cells by immunobead RT-PCR in peritoneal lavage fluid taken during laparotomy was a significant risk factor for reduced survival after curative resection.[37] This risk factor was independent of the established prognostic factors of tumor stage and site of primary tumor and may be useful in determining those patients who would benefit from adjuvant chemotherapy.[37]

Conversely, some recently published studies report conflicting results regarding the prognostic value of CTCs.[38,39] A major problem of most of the published studies is that only small, inhomogeneous patient groups with 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.[18,36] A false positive rate of 15% and a false negative rate of 7.8% for the prediction of postoperative relapse using our membrane-array assay suggest that there is room for the improvement of this method. In fact, using microarray technology and gene-expression profiling to identify more specific markers of risk of relapse in stage II patients might improve the accuracy of molecular detection methods.[40]

Despite curative resection, 28.9% of Stage II CRC patients ultimately developed postoperative relapse in our observation. Our constructed membrane-array method could detect CTCs in 80% of these stage II CRC patients with postoperative relapse. This method is helpful for the prediction of both local recurrence and distant metastasis in either colon or rectal cancer patients postoperatively. Multivariate analysis revealed 3 independent prognostic markers in our patient cohort, including T4 depth of tumor invasion, the presence of vascular invasion and all 4 molecular markers. Likewise, Koch et al confirmed that tumor cell detection in blood, T-category and number of removed lymph nodes to be independent prognostic factors for survival rates of stage II CRC patients.[36] Overall, stage II CRC patients with 1 high-risk predictor, T4 or positive vascular invasion or all 4 molecular markers, have a 27-fold risk of developing postoperative relapse compared with those without any 1 high-risk predictor. Concomitant molecular diagnosis of CTCs with a multimarker panel is a justifiable supplementary approach to the current pathologic staging system, which may help physicians make appropriate judgments on clinical management and predictive prognosis for stage II CRC patients. Hence, therapeutic decision-making models are likely to be further redefined by the inclusion of such molecular markers.

Finally, this current investigation has demonstrated that our membrane-array methods could identify stage II CRC patients at high risk of relapse at an earlier stage, with a median lead-time (the time between the presence of molecular markers and the onset of clinically detectable recurrence) of 7 months. In practice, 7 months is adequate for the consideration of new therapeutic strategies to possibly cure these patients. Incidentally, the lead-time advantage of routine serum CEA measurement for surveillance of CRC patients is only 4 months.[41] Therefore, it is an approximate 3-month benefit for the earlier prediction of postoperative relapse when comparing our membrane-array method and serum CEA measurement. Moreover, relapse-free survival rates of stage II colon or rectal cancer patients during a median follow up of 40 months are significantly lower in those patients with 4 molecular markers. Consequently, to determinate whether the introduction of adjuvant chemotherapy for stage II patients with positive CTCs is advantageous and efficacious would be an imperative issue for future investigation.

In conclusion, the constructed membrane-array method for the detection of CTCs has been demonstrated to be complementary to the surveillance of stage II CRC patients. The highly sensitive and high-throughput assay is a promising tool for early detection of postoperative relapse, with a median lead-time of 7 months before the development of postoperative relapse. However, large scale and long-term clinical studies follow up is warranted, to confirm the clinical significance of membrane-arrays as decision-making models for adjuvant chemotherapy.

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