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# Circulating tumor cells as a surrogate marker for determining clinical outcome to mFOLFOX chemotherapy in patients with stage III colon cancer

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**Background:** This study was aimed to detect post-chemotherapeutic circulating tumour cells (CTCs) in stage III colon cancer patients and identify those who were at high risk of relapse.

**Methods:** We used human telomerase reverse transcriptase, cytokeratin-19, cytokeratin-20, and carcinoembryonic antigen (CEA) as the biomarkers to detect CTCs in 90 stage III colon cancer patients undergoing curative resection followed by mFOLFOX chemotherapy.

**Results:** Post-chemotherapeutic relapse occurred in 30 (33.3%) patients. By univariate analysis and multivariate proportional hazards regression analysis, perineural invasion (hazard ratio (HR): 2.752; 95% confidence interval (CI): 1.026–7.381), high post-chemotherapeutic serum CEA levels (HR: 2.895; 95% CI: 1.143–7.333) and persistent presence of post-chemotherapeutic CTCs (HR: 6.273; 95% CI: 2.442–16.117) were independent predictors of post-chemotherapeutic relapse. In addition, the persistent

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presence of post-chemotherapeutic CTCs strongly correlated with reduced disease-free survival and overall survival. Accuracy of detecting relapse in post-chemotherapeutic stage III colon cancer patients by analysing the persistent presence of post-chemotherapeutic CTCs was higher than that by post-chemotherapeutic CEA levels (odds ratio: 50.091 vs 5.211).

**Conclusion:** The persistent presence of post-chemotherapeutic CTCs is a potential powerful surrogate marker for determining clinical outcome in stage III colon cancer patients receiving adjuvant mFOLFOX chemotherapy.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females worldwide, with over 1.2 million new cancer cases and 608 700 deaths estimated to have occurred in 2008 (Jemal *et al*, 2011). Curative surgery remains the mainstay of therapy for CRC, but half of the patients receiving curative surgery alone will ultimately relapse and die of metastatic disease (Obrand and Gordon, 1997). Although there have been significant improvements in the treatment of advanced CRC using a multidisciplinary approach, individuals with postoperative relapse or metastatic disease still have poor prognosis (Gallagher and Kemeny, 2010).

In recent years, much progress has been achieved in the adjuvant chemotherapy of patients with colon cancer. Oxaliplatin, a third-generation alkylating platinum derivative that inhibits DNA synthesis, appears to be one of the most effective chemotherapeutic agents for metastatic CRC (de Gramont *et al*, 2000). To determine if oxaliplatin combined with 5-fluorouracil (5-FU) and leucovorin (LV) can also show improved efficacy in adjuvant settings, Andre *et al* (2004) conducted the MOSAIC (the Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer) study, a phase III clinical trial, and found that adding oxaliplatin to a traditional regimen of 5-FU/LV (FOLFOX4) significantly improved disease-free survival (DFS) in stage II and III colon cancer patients compared with the same regimen of 5-FU and LV. Because of the results of the MOSAIC trial, the US Food and Drug Administration approved the FOLFOX4 regimen for postoperative adjuvant chemotherapy in patients with stage III colon cancer in November 2004.

In the extended period of MOSAIC trial follow-up, the use of oxaliplatin-based adjuvant chemotherapy after curative surgery also improved DFS and overall survival (OS) in patients with stage III colon cancer (Andre *et al*, 2009). These results were strikingly similar to a parallel clinical trial, NSABP C-07 (the National Surgical Adjuvant Breast and Bowel Project, C-07) in the United States (Kuebler *et al*, 2007). However, there was a constant rate, about 30%, of recurrence in colon cancer patients receiving the postoperative FOLFOX4 adjuvant regimen in these studies (Kuebler *et al*, 2007; Andre *et al*, 2009). Relapse in stage III CRC patients receiving postoperative adjuvant chemotherapy is attributed mainly to reduced response to the chemotherapeutic regimen. Therefore, it would be valuable to identify potential metastases earlier or chemotherapy-resistant patients who would benefit from other therapeutic attempts.

For many decades, efforts have made to detect recurrent tumours early to ensure adequate and effective treatment and improve the patient's prognosis (Rodriguez-Moranta *et al*, 2006). The identification of specific colon tumour-associated molecular markers and the development of a robustly accurate assay method for effective disease monitoring would significantly improve the early diagnosis of recurrence and guide more effective treatment (Nannini *et al*, 2009). Undetected micrometastatic tumour cells with reduced response to chemotherapeutic regimens contribute to the failure of primary curative surgery with subsequent adjuvant chemotherapy in advanced CRC patients (Steinert *et al*, 2008). Therefore, the detection of tumour-shed cells in the bloodstream is very important for early identification of postoperative and/or

adjuvant chemotherapeutic CRC patients requiring further optimal therapy (Rahbari *et al*, 2010).

Circulating tumour cells (CTCs) were first discovered in the blood of a cancer patient (post-mortem) by Ashworth (1869). More recently, with refined techniques and advances in molecular biology, the identification of CTCs via nucleic acid-based methodologies and PCR has developed into a useful tool for the detection of occult metastases (Allan and Keeney, 2010). Our recent investigations have demonstrated that the persistent presence of postoperative CTCs is a poor prognostic factor for patients with CRC after curative resection (Uen *et al*, 2008; Lu *et al*, 2011).

Our current study examined the prognostic significance of post-chemotherapeutic CTCs in multiple blood samples for the detection of relapse in stage III colon cancer patients who have undergone curative surgery and mFOLFOX adjuvant chemotherapy in order to help define recurrent or metastatic lesions for further therapeutic planning.

## MATERIALS AND METHODS

**Patients.** Between August 2007 and July 2010, a total of 108 American Joint Commission on Cancer/International Union Against Cancer (AJCC/UICC) (Edge *et al*, 2010) stage III colon cancer patients who received curative surgery and modified FOLFOX (mFOLFOX) adjuvant chemotherapy at the Kaohsiung Medical University Hospital were retrospectively analysed. Of 108 patients, 5 patients with other malignancies, 13 patients with <8 cycles of mFOLFOX administration or followed-up for <1 year, and cases of surgical mortality or surgical resection margins positive for tumour invasion were excluded. All of the finally enrolled 90 patients completed 12 cycles of mFOLFOX adjuvant chemotherapy. Curative surgery was defined as surgical bed free of any gross residual tumour and surgical resection margins pathologically negative for tumour invasion. To decrease the false-negative rate of CTCs in predicting post-operative relapse, multiple peripheral blood samples were obtained for cDNA analysed. Therefore, a total of 90 sets of cDNA from 1 week and 4 weeks after completion of adjuvant chemotherapy were entered into this study. Post-chemotherapeutic relapse was defined as the development of new postoperative and post-chemotherapeutic recurrent or metastatic lesions. Postoperative surveillance consisted of medical history, physical examination, and laboratory studies, including serum carcinoembryonic antigen (CEA) levels every 2–3 months. Abdominal ultrasonography or computed tomography was performed every 6 months, and chest radiography and total colonoscopy were performed once a year. An elevated CEA is defined as over 50% increase in CEA level compared with previous CEA level on two successive determinations of CEA value. Furthermore, a significantly elevated CEA is defined as two consecutively elevated CEA levels at an interval of 2–3 months' regular check (Tsai *et al*, 2008). If a significantly elevated CEA value was found in 4–6 months, abdominal or chest-computed tomography was done before the annual check-up. Patients were followed-up at 3-month intervals for 2 years and 6-month intervals

thereafter. We followed these enrolled patients intensively until September 2012; the median follow-up time was 36 months (range, 18–61 months). Of the 90 patients, 30 (33.3%) developed post-chemotherapeutic relapse during the follow-up period. The postoperative adjuvant chemotherapy with mFOLFOX regimen was as follows: on day 1, oxaliplatin 85 mg m<sup>-2</sup> and leucovorin 200 mg m<sup>-2</sup> were administered, followed by a 24-h continuous infusion of 5-FU at a dose of 2000 mg m<sup>-2</sup> for 44–46 h. The cycles were repeated every 2 weeks for half a year.

Circulating tumour cells in peripheral blood of these 90 patients were detected using our previously constructed membrane-array method (Wang *et al*, 2006; Yeh *et al*, 2006). Briefly, a 4-ml sample of peripheral blood was obtained from each colon cancer patient at 1 and 4 weeks after chemotherapy, respectively, for total RNA isolation. To prevent contamination by epithelial cells, peripheral blood samples were obtained through a catheter inserted into a peripheral vessel, and the first 5 ml of blood were discarded. Sample acquisition and subsequent use were approved by the hospital's institutional review board. Pre-operative staging methods included chest radiography, abdominal ultrasound, bone scan, and abdominal-computed tomography. Clinical stage and pathological features of primary tumours were defined according to the criteria of the AJCC/UICC (Edge *et al*, 2010).

**Detection of serum CEA.** Additional 3-ml peripheral blood samples from the 90 colon cancer patients were obtained at 1 week and 4 weeks after chemotherapy, respectively. Serum CEA levels were determined by means of an enzyme immunoassay test kit (DPC Diagnostic Product Co., Los Angeles, CA, USA) with the upper limit of 5 ng ml<sup>-1</sup> defined as normal according to the manufacturers of the kits used.

**mRNA Isolation and first strand cDNA synthesis.** Total RNA was extracted from the fresh whole blood of post-chemotherapeutic colon cancer patients using a QIAamp RNA Blood Mini Kit (Qiagen Inc., 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 synthesised from total RNA by using a RT-PCR kit (Promega Corp., Madison, WI, USA).

**Membrane-arrays.** The procedure of the membrane-array method for the detection of CTCs-related mRNA molecular markers was performed according to our previous study (Wang *et al*, 2006; Yeh *et al*, 2006). Four mRNA molecular markers including human telomerase reverse transcriptase, cytokeratin-19, cytokeratin-20, and CEA mRNA were used as the biomarkers to detect CTCs according to our previous studies (Lu *et al*, 2011). Patients overexpressing all four molecular markers by membrane-array method in peripheral blood samples obtained post-chemotherapeutically (at both 1 and 4 weeks after chemotherapy) were considered to have persistent presence of CTCs. In our previous investigation, the sensitivity limit of this technique was established at approximately 1 tumour cell per 10<sup>6</sup> white blood cells (5 cells per 1 ml blood) (Wang *et al*, 2006; Yeh *et al*, 2006).

**Statistical analysis.** All data were statistically analysed using the Statistical Package for the Social Sciences, version 14.0 (SPSS Inc., Chicago, IL, USA). A *P*-value <0.05 was considered statistically significant. Receiver-operating characteristics curve analyses were carried out to determine the sensitivity and specificity for each membrane-array mRNA marker. The cutoff values for each mRNA marker were set at points representing the highest accuracy of analysis (minimal false-negative and false-positive results). The difference between data obtained by membrane array and real-time quantitative (Q)-PCR was calculated by using linear regression and Pearson's correlation. The univariate analysis of clinicopathological features and persistent presence of CTCs between the two groups (relapse group vs non-relapse group) was compared using the

**Table 1.** Clinicopathological features of 90 stage III colon cancer patients undergoing mFOLFOX chemotherapy

| Variables   | Number (%)                 |
|---|----------------------------|
| <b>Gender</b>   |                            |
| Male/female   | 51 (56.7)/39 (43.3)        |
| Age (years) (mean ± s.d.)   | 63.1 ± 12.9                |
| <b>Maximum tumour size (cm)</b>   |                            |
| <5/≥5   | 54 (60.0)/36 (40.0)        |
| <b>Depth of invasion</b>  |                            |
| T <sub>1</sub> +T <sub>2</sub> /T <sub>3</sub> +T <sub>4</sub>  | 19 (21.1)/71 (78.9)        |
| <b>Lymph node metastasis</b>  |                            |
| N1/N2   | 62 (68.9)/28 (31.1)        |
| <b>Vascular invasion</b>  |                            |
| Yes/no  | 47 (52.2)/43 (47.8)        |
| <b>Perineural invasion</b>  |                            |
| Yes/no  | 52 (57.8)/38 (42.2)        |
| <b>Histology</b>  |                            |
| WD/MD/PD  | 8 (8.9)/67(74.4)/15 (16.7) |
| <b>Post-chemotherapeutic CEA (ng ml<sup>-1</sup>)</b>   |                            |
| <5/≥5   | 73 (81.1)/17 (18.9)        |
| <b>Post-chemotherapeutic CTCs</b>   |                            |
| No/yes  | 69 (76.7)/21 (23.3)        |
| Abbreviations: CEA = carcinoembryonic antigen; CTCs = circulating tumour cells; MD = moderately differentiated; PD = poorly differentiated; WD = well differentiated. |                            |

$\chi^2$ -test. Independent prognostic factors for post-chemotherapeutic relapse were determined using a multivariate Cox proportional hazards regression analysis. Disease-free survival was defined as the time elapsed between primary surgery and recurrence of colon cancer. Overall survival was defined as the time elapsed between primary surgery and death from any cause. The DFS rates and OS rates were calculated by the Kaplan–Meier method, and the differences in survival rates were analysed by the log-rank test.

## RESULTS

The average age of the patients was 63.1 years (range, 32–80 years; Table 1). Forty-one colon tumours (45.6%) were right-sided and 49 (54.4%) left-sided. By histological type, 8 (8.9%) of the tumours were well differentiated, 67 (74.4%) were moderately differentiated, and 15 (16.7%) were poorly differentiated carcinomas. With regard to clinicopathological features, 47 (52.2%) patients had vascular invasion, and 52 (57.8%) patients were found to have perineural invasion (PNI). High serum CEA levels (≥5 ng ml<sup>-1</sup>) were observed in 17 (18.9%) of the postoperative adjuvant chemotherapeutic colon cancer patients, and persistent post-chemotherapeutic CTCs were detected in 21 (23.3%) of the 90 patients.

Table 2 reported the correlation between post-chemotherapeutic relapse and the clinicopathological features of the 90 study patients receiving the mFOLFOX regimen. On univariate analysis, depth of invasion (*P* = 0.018), vascular invasion (*P* = 0.037), PNI

**Table 2.** Correlation between post-chemotherapeutic relapse and clinicopathological features by univariate analysis

|  | Non-relapse (N = 60) (%) | Relapse (N = 30) (%) | P-value <sup>a</sup> |
|--|--------------------------|----------------------|----------------------|
| <b>Gender</b>  |                          |                      |                      |
| Male/female  | 35 (68.6)/25 (64.1)      | 16 (31.4)/14 (35.9)  | 0.652                |
| <b>Age (years)</b>   |                          |                      |                      |
| < 65/≥ 65  | 30(60.0)/30 (75.0)       | 20 (40.0)/10 (25.0)  | 0.134                |
| <b>Maximum size (cm)</b>   |                          |                      |                      |
| < 5/≥ 5  | 39 (72.2)/21 (58.3)      | 15 (27.8)/15 (41.7)  | 0.171                |
| <b>Depth of invasion</b>   |                          |                      |                      |
| T <sub>1</sub> + T <sub>2</sub> /T <sub>3</sub> + T <sub>4</sub> | 17 (89.5)/43 (60.6)      | 2 (10.5)/28 (39.4)   | 0.018                |
| <b>Lymph node metastasis</b>                                     |                          |                      |                      |
| N1/N2  | 40 (64.5)/20 (71.4)      | 22 (35.5)/8 (28.6)   | 0.520                |
| <b>Vascular invasion</b>   |                          |                      |                      |
| Yes/no   | 24 (55.8)/36 (76.6)      | 19 (44.2)/11(23.4)   | 0.037                |
| <b>Perineural invasion</b>                                       |                          |                      |                      |
| Yes/no   | 19 (50.0)/41 (78.8)      | 19 (50.0)/11 (21.2)  | 0.004                |
| <b>Histology</b>   |                          |                      |                      |
| WD + MD/PD   | 52 (69.3)/8 (53.3)       | 23 (30.7)/7 (46.7)   | 0.230                |
| <b>Post-chemotherapeutic CEA (ng ml<sup>-1</sup>)</b>            |                          |                      |                      |
| < 5/≥ 5  | 54 (74.0)/6 (35.3)       | 19 (26.0)/11 (64.7)  | 0.002                |
| <b>Post-chemotherapeutic CTCs</b>                                |                          |                      |                      |
| No/yes   | 58 (84.1)/2 (9.5)        | 11 (15.9)/19 (90.5)  | <0.001               |

Abbreviations: CEA = carcinoembryonic antigen; CTCs = circulating tumour cells; MD = moderately differentiated; PD = poorly differentiated; WD = well differentiated.  
<sup>a</sup>χ<sup>2</sup>-Test.

**Table 3.** Correlation between post-chemotherapeutic relapse and clinicopathological features by multivariate Cox proportional hazards regression analysis

| Variables   | Coefficient | s.e.  | P-value <sup>a</sup> | Hazard ratio <sup>b</sup> |
|---|-------------|-------|----------------------|---------------------------|
| Depth of invasion (T <sub>3</sub> + 4/T <sub>1</sub> + 2) | 1.158       | 0.798 | 0.147                | 3.184 (0.666–15.214)      |
| Vascular invasion (yes/no)                                | 0.244       | 0.510 | 0.632                | 0.783 (0.288–2.129)       |
| Perineural invasion (yes/no)                              | 1.012       | 0.503 | 0.044                | 2.752 (1.026–7.381)       |
| Post-chemotherapeutic CEA (≥ 5/ < 5)                      | 1.063       | 0.474 | 0.025                | 2.895 (1.143–7.333)       |
| Post-chemotherapeutic CTCs (yes/no)                       | 1.836       | 0.481 | <0.001               | 6.273 (2.442–16.117)      |

Abbreviations: CEA = carcinoembryonic antigen; CTCs = circulating tumour cells.  
<sup>a</sup>Multivariate Cox proportional hazards regression analysis.  
<sup>b</sup>Values in parentheses are 95% confidence intervals.

**Table 4.** Accuracy of carcinoembryonic antigen vs circulating tumour cells in peripheral blood for prediction of post-chemotherapeutic relapse

| Methods                             | Relapse (N = 30) | Non-relapse (N = 60) | P-value | Odds ratio (95% CI)     |
|-------------------------------------|------------------|----------------------|---------|-------------------------|
| <b>Post-chemotherapeutic CEA*</b>   |                  |                      |         |                         |
| ≥ 5 ng ml <sup>-1</sup>             | 11               | 6                    | 0.002   | 5.211 (1.694–16.029)    |
| < 5 ng ml <sup>-1</sup>             | 19               | 54                   |         | 1                       |
| <b>Post-chemotherapeutic CTCs**</b> |                  |                      |         |                         |
| Positive                            | 19               | 2                    | <0.001  | 50.091 (10.182–246.427) |
| Negative                            | 11               | 58                   |         | 1                       |

Abbreviations: CI = confidence interval; CEA = carcinoembryonic antigen; CTCs = circulating tumour cells. \*Sensitivity: 64.71% (48.4–81.0%); specificity: 74.0% (59.0–84.0%); positive predictive value: 36.7% (20.2–53.1%); negative predictive value: 90.0% (79.8–100.2%); and accuracy: 72.2% (56.9–87.5%) for CEA. \*\*Sensitivity: 90.5% (80.5–100.5%); specificity: 80.6% (71.6–96.5%); positive predictive value: 63.3% (46.9–79.8%); negative predictive value: 96.7% (90.5–102.8%); and accuracy: 85.6% (73.6–97.5%) for CTCs.

(*P* = 0.004), high post-chemotherapeutic serum CEA level (*P* = 0.002), and the persistent presence of post-chemotherapeutic CTCs (*P* < 0.001) were significant in the relapse of stage III colon cancer patients receiving postoperative adjuvant mFOLFOX chemotherapy. When multivariate Cox proportional hazards regression analysis was used, the presence of PNI (*P* = 0.044; hazard ratio (HR): 2.752, 95% confidence interval (CI): 1.026–7.381), high post-chemotherapeutic serum CEA level (*P* = 0.025; HR: 2.895, 95% CI: 1.143–7.333) and the persistent presence of post-chemotherapeutic CTCs (*P* < 0.001; HR: 6.273, 95% CI: 2.442–16.117) independently predicted post-chemotherapeutic relapse in these patients (Table 3). The prognostic accuracy of CTCs at both 1st and 4th week is superior to either 1st week or 4th week (85.6% vs 80% or 82.2%) for predicting relapse of stage III colon cancer patients following adjuvant chemotherapy (Supplementary Table 1).

Table 4 showed the accuracy of CEA vs CTCs for the prediction of post-chemotherapeutic relapse in stage III colon cancer patients receiving adjuvant mFOLFOX chemotherapy. Among the patients with high post-chemotherapeutic CEA levels, 11 of 17 patients developed recurrence (*P* = 0.002; odds ratio: 5.211, 95% CI: 1.694–16.029). However, 19 of 21 patients with the persistent

presence of post-chemotherapeutic CTCs developed recurrence during the follow-up period (*P* < 0.001; odds ratio: 50.091, 95% CI: 10.182–246.427). Thus, the detection of CTCs was statistically more powerful than CEA in the prediction of relapse in stage III colon cancer patients receiving postoperative mFOLFOX chemotherapy.

The correlation between post-chemotherapeutic OS and clinicopathological features of our studied patients by univariate and multivariate Cox proportional hazard regression analysis was shown in Table 5. It demonstrated the presence of vascular invasion (*P* = 0.017), PNI (*P* = 0.029), and post-chemotherapeutic CTCs (*P* = 0.009) are significant predictors of OS for stage III colon cancer patients following adjuvant mFOLFOX chemotherapy.

The median disease-free times for positive CTCs patients and negative CTCs patients were 11 and 32 months, respectively. The median OS times for persistent positive CTCs patients and negative CTCs patients were 27 and 38 months, respectively. Log-rank test showed that both DFS (*P* < 0.001) and OS (*P* < 0.001) in patients with persistent positive peripheral blood CTCs were significantly lower than in patients without CTCs (Figure 1).

Table 5. Univariate and multivariate Cox proportional hazard regression analysis of clinicopathological features on overall survival

| Parameters   | Number | Univariate analysis   |         | Multivariate analysis |         |
|--|--------|-----------------------|---------|-----------------------|---------|
|  |        | Hazard ratio (95% CI) | P-value | Hazard ratio (95% CI) | P-value |
| Age ( $\geq 65$ / $< 65$ )years                                    | 40/50  | 1.02 (0.40–2.60)      | 0.960   | 3.09 (0.92–10.40)     | 0.069   |
| Sex (male/female)  | 51/39  | 0.75 (0.30–1.89)      | 0.543   | 0.35 (0.12–1.03)      | 0.057   |
| Tumour size ( $\geq 5$ / $< 5$ )cm                                 | 36/54  | 1.53 (0.61–3.85)      | 0.371   | 0.73 (0.20–2.64)      | 0.725   |
| Depth (T3 + T4/T1 + T2)  | 71/19  | 5.45 (0.72–41.09)     | 0.100   | 2.25 (0.26–19.73)     | 0.465   |
| Lymph node metastasis (N2/N1)                                      | 28/62  | 0.77 (0.27–2.15)      | 0.614   | 0.42 (0.90–1.91)      | 0.415   |
| Vascular invasion (yes/no)   | 43/47  | 2.63 (0.98–7.03)      | 0.055   | 2.56 (0.66–9.88)      | 0.017   |
| Perineurial invasion (yes/no)                                      | 38/52  | 3.17 (1.19–8.45)      | 0.021   | 4.13 (1.15–14.77)     | 0.029   |
| Histology (PD/MD + WD)   | 15/75  | 2.06 (0.73–5.77)      | 0.017   | 3.75 (0.80–17.72)     | 0.095   |
| Post-chemotherapeutic CEA ( $\geq 5$ / $< 5$ ) ng ml <sup>-1</sup> | 17/73  | 2.33 (0.87–6.22)      | 0.093   | 1.94 (0.48–7.81)      | 0.350   |
| Post-chemotherapeutic CTCs (yes/no)                                | 21/69  | 9.48 (3.54–25.39)     | <0.001  | 5.37 (1.52–19.04)     | 0.009   |

Abbreviations: CI = confidence interval; CEA = carcinoembryonic antigen; CTCs = circulating tumour cells; MD = moderately differentiated; PD = poorly differentiated; WD = well differentiated.

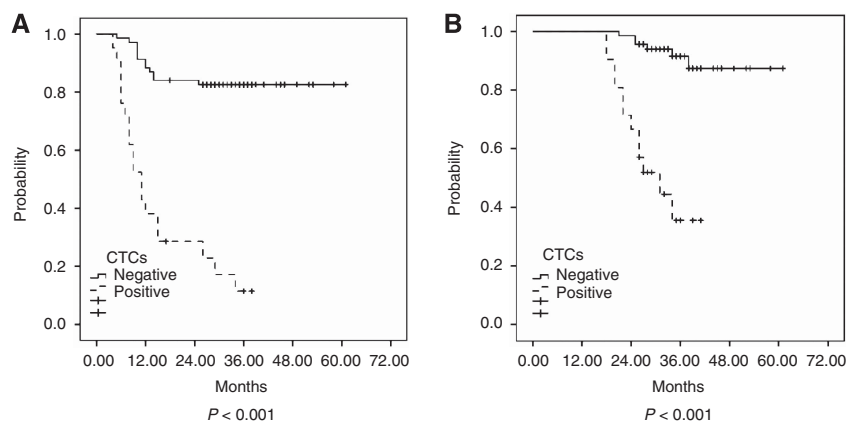


Figure 1. (A) Cumulative disease-free survival (DFS) of 90 stage III colon cancer patients undergoing mFOLFOX chemotherapy according to the post-chemotherapeutic presence of circulating tumour cells (CTCs). Colon cancer patients with positive CTCs in peripheral blood showed a significantly reduced DFS than those without CTCs in the peripheral blood ( $P < 0.001$ ); (B) Cumulative OS of 90 stage III colon cancer patients undergoing mFOLFOX chemotherapy according to the post-chemotherapeutic presence of CTCs. Colon cancer patients with persistent post-chemotherapeutic CTCs in peripheral blood showed a significantly reduced OS than those without CTCs in the peripheral blood ( $P < 0.001$ ).

## DISCUSSION

There is a recurrence rate of  $\sim 30\%$  in stage III colon cancer patients undergoing curative surgery and subsequent adjuvant chemotherapy, which are the current standard and so-called best recommendation for this subgroup of patients (Jonker *et al*, 2011). The recurrence rate was 33.6% during the 5-year MOSAIC follow-up period in stage III colon cancer patients receiving a post-operative FOLFOX4 regimen (Andre *et al*, 2009) and 27.8% during 4 years of the NSABP C-07 study in stage II and III colon cancer patients using the same adjuvant regimen (Kuebler *et al*, 2007). These findings are consistent with the 33.3% of recurrence rate in our present study. This phenomenon suggests that undetected micro-metastases resistant to adjuvant FOLFOX4 chemotherapy are present and have a decisive role in subsequent relapse. Tumour relapse after curative resection of CRC and adjuvant chemotherapy is attributed to tumour cell dissemination and resistance to the adjuvant chemotherapy. Heterogeneous tumour behaviours and individual patient responses to chemotherapeutic agents lead to variable outcomes. Although CEA is a widely used tumour marker

for the follow-up of CRC patients, its lack of sensitivity remains unsolved. In addition, Sorbye and Dahl (2003) have reported a transient CEA surge (15%; 4/27) in metastatic CRC patients receiving oxaliplatin-based chemotherapy, despite a good objective response among these patients. This inappropriate elevation of CEA level could incorrectly guide the CRC patient's therapeutic protocol, and make disease progression possible. Consequently, the 2006 update of The American Society of Clinical Oncology recommendations stated that caution should be used when interpreting a rising CEA level during the first 4–6 weeks of a new therapy, as spurious early rises may occur especially after oxaliplatin (Locker *et al*, 2006). These data suggest that there is still room to develop a more effective marker than CEA for monitoring the response of metastatic CRC patients to systemic therapy.

CTCs are thought to be shed from the primary tumour into the bloodstream before or during operation (Yamaguchi *et al*, 2000). Accumulated reports have documented the presence of CTCs in pre- or post-operative CRC patients, which would probably lead to postoperative relapse among these patients (Yamaguchi *et al*, 2000; Uen *et al*, 2008; Peach *et al*, 2010; Lu *et al*, 2011). The current study has confirmed that stage III colon cancer patients identified with

persistent post-chemotherapeutic CTCs by a multi-marker membrane array method exhibit reduced DFS and OS rates. In a recent investigation in Japan, a total of 64 metastatic CRC patients received oxaliplatin-based chemotherapy (Matsusaka *et al*, 2011). Patients with  $\geq 3$  CTCs at baseline and at 2 and 8–12 weeks after initiation of chemotherapy had shorter median progression-free survival (8.5, 7.3, and 1.9 months, respectively) than those with  $< 3$  CTCs (9.7, 10.4, and 9.1 months, respectively). Patients with  $\geq 3$  CTCs at 2 and 8–12 weeks after initiation of chemotherapy had a shorter median OS (10.2 and 4.1 months, respectively) than those with  $< 3$  CTCs (29.1 and 29.1 months, respectively). The investigators concluded that  $< 3$  persistent CTCs at 2 weeks after initiating oxaliplatin-based chemotherapy in metastatic CRC patients was a strong indicator that the current therapy is effective, whereas three or more CTCs indicated that any benefits were likely to be short-term only (Matsusaka *et al*, 2011). Therefore, persistent presence of post-chemotherapeutic CTCs appears to be valuable in the identification of chemotherapy-resistant patients, who could benefit from shifting treatment programme and/or further investigational approaches.

In our study of stage III colon cancer patients receiving adjuvant mFOLFOX chemotherapy, both elevated post-chemotherapeutic CEA level and persistent positive CTCs had significant roles in predicting relapse by either univariate or multivariate analysis. Pre- and post-therapeutic elevated serum CEA levels in CRC patients have previously been shown to predict deeper local invasion of tumours, higher risk of occult metastases, or higher rates of post-therapeutic relapse (McCall *et al*, 1994; Wiratkapun *et al*, 2001). Our previous work further indicated that molecular detection of postoperative CTCs is helpful in the earlier prediction of postoperative relapse in CRC patients with normal perioperative serum CEA levels, with a median lead time of 6 months before detection of elevated CEA values (Wang *et al*, 2007). The present study shows that persistent post-chemotherapeutic CTCs is also more powerful than elevated post-chemotherapeutic CEA levels in predicting relapse in stage III colon cancer patients undergoing curative surgery and subsequent adjuvant mFOLFOX chemotherapy (odds ratio: 50.091 *vs* 5.211). Therefore, the persistent presence of post-chemotherapeutic CTCs in stage III colon cancer patients might bring more therapeutic considerations and options, such as prolonged duration of adjuvant chemotherapy or changing therapeutic agents.

Perineural invasion is a distinct pathological entity, yet less commonly observed than lymphovascular invasion in CRC patients (Washington, 2008). Report of Poeschl *et al* (2010) concluded that PNI in postoperative specimens of CRC patient is significantly associated with several histopathological variables indicating aggressive tumour behaviour, such as lymphatic invasion, venous invasion, tumour budding, infiltrative tumour growth pattern, and incomplete tumour-free resection margin. In fact, 5-year DFS rate for patients with positive PNI is significantly worse than negative-PNI patients (11% *vs* 68%, respectively) (Poeschl *et al*, 2010). In addition, Zorzos *et al*, 2003 found that PNI in colon cancer patients was significantly related to the over-expression of P-glycoprotein, which was regarded as a multidrug resistant protein. That study partially explains the significant role of PNI in CRC patients resistant to systemic chemotherapy. Consistent with these investigations, our present study also reveals that PNI is a significant independent predictor of post-chemotherapeutic relapse.

In summary, our study shows that in addition to the assessment of PNI and post-chemotherapeutic CEA levels, the persistent presence of post-chemotherapeutic CTCs may be a potentially valuable tool in predicting relapse and survival rate in stage III colon cancer patients undergoing curative surgery and adjuvant mFOLFOX chemotherapy. However, further validation studies are mandatory to apply CTCs as prognostic factors or therapeutic

strategies in the development process of a new biomarker for stage III colon cancer patients following adjuvant mFOLFOX chemotherapy.

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## CONFLICT OF INTEREST

The authors declared no conflict of interest.

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