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ORIGINAL ARTICLE

Enhanced prognostic value of combined circulating tumor cells and serum carcinoembryonic antigen in patients with colorectal cancer

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Abstract

Background: Circulating tumor cells (CTCs) have been investigated as a potential biomarker for predicting prognosis and monitoring therapeutic responses in colorectal cancer (CRC). However, the sensitivity of CTCs detection is low, thus limiting the clinical utility of CTCs. We aim to examine the clinicopathological parameters that improve prognosis prediction for CRC using CTCs as a biomarker.

Methods: We enumerated CTCs in 186 CRC patients and associated the number of CTCs with the clinicopathological features and overall survival (OS) using a univariate and multivariate Cox regression model and Kaplan–Meier survival analysis.

Results: The presence of CTCs from 186 CRC patients was significantly associated with stage, preoperational carcinoembryonic antigen (CEA), and CA19-9 levels. Using Kaplan–Meier survival and Cox regression analysis, patients with five or more CTCs exhibited significantly worse OS compared to patients with fewer than five CTCs. The combination of CTCs with tumor marker CEA has a better OS prediction than individual CTCs or CEA and serves as a more effective prediction model in patients with CRC.

Conclusion: We identified that patients with more than five CTCs exhibited significantly worse OS. Additionally, patients with the normal level of CEA, but who also had more than five CTCs trended towards a worse OS.

Keywords: Carcinoembryonic antigen; Circulating tumor cell; Colorectal cancer; Mesenteric vein blood; Overall survival; Prognosis

1. INTRODUCTION

Colorectal cancer (CRC) ranks third among newly diagnosed cancers and cancer-related deaths worldwide.[1] More than 130 000 new CRC cases are diagnosed annually in the United States with an additional 15 000 new cases annually in Taiwan.[1,2] The high mortality of CRC patients is tightly linked to late diagnosis, undetected recurrence, resistance to therapies, and distant metastases.[3] The understanding, prevention, and treatment of CRC progression and metastasis are of paramount clinical importance for clinicians. Early detection of tumor recurrence and metastases could guide treatment strategies and improve patient outcome. The tumor marker carcinoembryonic antigen (CEA) found in peripheral blood, is widely used as an indicator of disease progression or recurrence after resection. CEA is recommended as a reliable tumor marker for CRC patients by the National Comprehensive Cancer Network and the American Society of Clinical Oncology.[4] Serum CEA level is an important prognostic factor, it is also an indicator of therapeutic efficacy and recurrence in CRC patients.[5,6] However, it is not as useful as a predictor of prognosis, because the expression of CEA can be influenced by several factors, including liver status, chronic inflammation, and chemotherapy, thereby limiting the precision in recurrence prognosis, especially in advanced-stage cancers. Low specificity, weak sensitivity, and poor accurate rates were often displayed.[7–9] There remains an unmet clinical need for

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reliable biomarkers and simple tests for disease detection, prognosis, and therapeutic response monitoring.

Metastasis is a multistep process involving the dissemination of tumor cells from the primary tumor.[10] Cancer cells that undergo intravasation result in circulating tumor cells (CTCs) in the bloodstream or lymphatic system and have the potential for metastatic tumor formation at distant sites.[11,12] Several studies have shown that the number of CTCs correlated with progression-free survival and overall survival (OS) in clinical stage IV metastatic CRC patients.[13,14] These studies were primarily conducted with stage IV patients where distant metastasis had already occurred,[15–17] providing limited insight into whether CTC count predisposes the development of metastasis.[18] CTC detection has been studied for early cancer diagnosis and prognosis prediction in CRC patients[19–22] as well as for response to therapy and for monitoring by sequential blood analysis.[23,24]

However, the detection and isolation of CTCs are difficult due to their scarcity and heterogeneity.[25,26] The high cost and low sensitivity of CTC detection has limited the use of CTC as an effective biomarker in CRC diagnostics. Label-free approaches of cell isolation, such as by size, typically suffer from low recovery, clogging of filters, complicated integration of external force fields, low cell purity, and loss of smaller rare cells limiting their broad utility.[27] Antibody-based methods to isolate rare cells based on the expression of surface marker proteins typically use immunomagnetic isolation by magnetic fields using antibodies immobilized to magnetic beads;[28] microfluidics approaches with antibodies immobilized on a microfluidic chip;[29] or fluorescence-activated methods where rare cells are detected and sorted based on laser-induced fluorescence of fluorophore-labeled antibodies.[30] New technologies and tools enabled the automation of CTC isolation, antibody labeling, and fluorescence imaging, allowing for consistent measurement over the whole study period. Given the highly heterogeneous nature of CRC, a single tumor marker may not be able to represent an accurate diagnostic standard with sufficient sensitivity or specificity in all cases. Recent studies have indicated that combining multiple tumor markers may improve the accuracy of diagnostic and prognostic evaluations.[31–37] In this study we demonstrate the potential enhanced prognostic value of combining CTC enumeration with CEA measurements in CRC patients during the early follow-up period after the first clinical treatment.

Table 1

Demographic and clinicopathological characteristics of CRC patients with their associated mean number of circulating tumor cell

	No. of cases	Mean no. of CTCs ± SD	*p*
Age			
≥67	93	6.5 ± 18.9	0.3319
<67	93	3.1 ± 10.2	
Gender			
Male	103	5.6 ± 17.8	>0.9999
Female	83	3.8 ± 11.4	
Location			
Right	51	2.1 ± 5.2	0.1136
Left	85	4.3 ± 12.6	
Rectal	50	7.1 ± 16.8	
Tumor size (cm)			
≥5	27	3.6 ± 12.3	0.0786
2–5	109	38 ± 138.7	
<2	50	8.1 ± 21.2	
Tumor stage			
T1	26	0.5 ± 1.8	0.1724
T2	28	2.7 ± 8.6	
T3	87	5.4 ± 17.9	
T4	45	7.5 ± 16.8	
Node Status			
N0	110	4 ± 11.7	<0.001***
N1	49	2.5 ± 7.5	
N2	27	12.1 ± 30.2	
Distant metastasis			
M0	163	3.3 ± 10.5	<0.001***
M1	23	15.2 ± 32	
Differentiation			
Poor	5	1.8 ± 1.9	0.1825
Moderate	177	5.0 ± 15.6	
Well	4	1 ± 1.4	
CEA (5 ng/ml)			
>5	62	7.5 ± 21.2	0.0182*
≤5	124	3.4 ± 11.1	
CA19-9 (U/ml)			
>37	32	9.3 ± 26.5	<0.001***
≤37	154	3.8 ± 11.8	

CEA = carcinoembryonic antigen; CTC = circulating tumor cells.
*$p < 0.05$.
***$p < 0.001$.

2. METHODS

2.1. Patients and sample collection

A total of 186 patients who underwent curative surgical resection at Taipei Veterans General Hospital (VGHTPE) between October 2016 to July 2019 were enrolled in this study. The study was approved by the institutional review board of VGHTPE and all patients signed the informed consent forms (VGHIRB number: 2016-07-005CC). The median follow-up time for patients was 702 days (range 13–1253 days). A minimum of 8 mL of mesenteric vein blood was collected in dipotassium ethylenediaminetetraacetic acid (K_2EDTA) anticoagulant tubes for CTC enumeration. CEA was routinely examined before operation. Table 1 shows the demographic and clinicopathological characteristics of the patients before surgery.

2.2. CTC enumeration

The MiSelect R system (MiCareo, Taiwan) was used to quantify CTCs in 8 mL blood samples drawn from patients. Blood samples were collected in 10 mL K_2EDTA blood collection tubes, stored at room temperature, and processed within 24 hours of the collection according to the manufacturer's instructions. Briefly, whole blood was incubated with the anti-Epithelial cell adhesion molecule (EpCAM) antibody for 20 minutes at room temperature. After incubation, the sample was washed with ISOTON II buffer to remove the unbound antibody and then loaded on the MiSelect R system. The MiSelect R system includes an optical detection system, a microfluidic active cell sorting scheme, and an on-chip filter for cell labeling and fluorescence imaging enumeration. Upon detection of fluorescently labeled cells in the whole blood, CTCs were diverted to a channel that led to an on-chip filter, where they were fixed, permeabilized, and labeled with confirmation antibodies before fluorescence imaging. The staining reagent contained PE-anti-EpCAM, allophycocyanin-anti-panCK, fluorescein isothiocyanate-anti-CD45, and 4',6-diamidino-2-phenylindole (DAPI) dilactate. The anti-panCK antibody targeted cytokeratins.[4–6,11,13,16,21,22] CTCs are defined as cells with a DAPI-positive nucleus, positive membrane staining for EpCAM, cytoplasmic staining for cytokeratins, and the absence of CD45 expression.

2.3. Statistical analysis

Comparative analysis of two categorical variables was performed using a chi-square test. Comparative analysis of the

two independent groups where the data are continuous was performed using the Mann-Whitney U test. The OS analysis was performed using the Kaplan–Meier method and comparing survival curves with the log-rank test using GraphPad Prism Program. The Cox regression model of IBM SPSS software was used for univariate and multivariate analysis of prognosis factors. All tests were two-sided and considered significant at $p < 0.05$. Not all stratified groups have yet reached statistical significance, due to the population size and completed follow-up time period.

3. RESULTS

3.1. CTC enumeration in patients with CRC

The isolated cells were labeled with additional antibodies and imaged using the automated instrument. CTCs were defined as nucleated cells that were positive for EpCAM and cytokeratin, but negative for CD45 (Fig. 1).

The mesenteric vein blood from 186 CRC patients who underwent curative surgical resection was analyzed for the presence of CTCs. The number of CTCs was significantly associated with CEA, CA19-9, and both N and M staging (Table 1). The number of CTCs found in patients at different stages of CRC is presented in Fig. 2. CTCs were found in 69 (37%) of all CRC patients. Of the 23 metastatic patients, 17 (74% of metastatic total) had CTCs, whereas only 32% of nonmetastatic patients had CTCs. The mean number of CTCs found in all of the nonmetastatic patients was 3.3, whereas metastatic patients had a mean of 15.2 CTCs with a strong statistical significance of $p < 0.0001$.

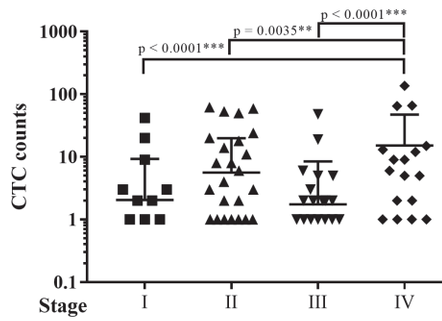
3.2. High number of CTCs correlates with poor prognosis

Patients who had more than one CTC were statistically more likely to have a shorter OS than patients with no CTCs (Fig. 3A). To improve the sensitivity and specificity of using CTCs as a prognostic indicator, we plotted all of the cases in a receiver operating characteristic (ROC) curve with different CTC count thresholds (Fig. 3B). A CTC count threshold of 4.5 resulted in an area under the curve (AUC) of 0.79, a sensitivity of 61.5%, and a specificity of 86.7%. Patients with a CTC count ≥5 had a significantly shorter OS compared to those with a CTC count <5 (Fig. 3C). After 36 months, 12.9% of patients with <5 CTCs were still alive compared to only 3.2% of patients still living who had ≥5 CTCs.

Patients with more than five CTCs had a statistically strong association with the clinicopathological characteristics (Table 2). In total 48% of patients with stage IV CRC had five or more CTCs compared with just 12% of stage I, II, and III patients. A strong positive association was also found the individual T, N, and M staging and higher CEA levels associated with patients with five or more CTCs. No association was found between patients with more than five CTCs and gender, tumor location, differentiation, and CA19-9.

3.3. Patient overall survival prediction based on CEA level and CTC count

Patients with a CEA level higher than 5 ng/mL had a shorter OS trend ($p = 0.0389$) and an increased risk of death trend, with a hazard ratio (HR) of 3.055 compared to patients with a low level of CEA (HR, 95% confidence interval (CI), 0.9726–9.596; $p = 0.0389$) (Fig. 4A and Table 3). To evaluate the combination of CEA level and CTC count as a predictor for OS, patients were divided into four groups based on CEA level and CTC count. Patients with a CEA level >5 showed a sharply



Stage	I	II	III	IV
No. of Patients	41	63	59	23
Patients with CTCs	10	24	18	17
% of Patients with CTCs	24.4	38.1	30.5	73.9
Mean No. of CTCs	2.0	5.6	1.7	15.2
Range of CTCs	0-32	0-42	0-62	0-136

Fig. 2 The number of CTCs found in patients at different stages of CRC. The Y axis uses a log scale to show the range of CTCs. The *p*-value shows the statistical significance between the stage IV patients and all of the other stages. Patients with no detected CTCs are not shown. CRC = colorectal cancer; CTC = circulating tumor cells.

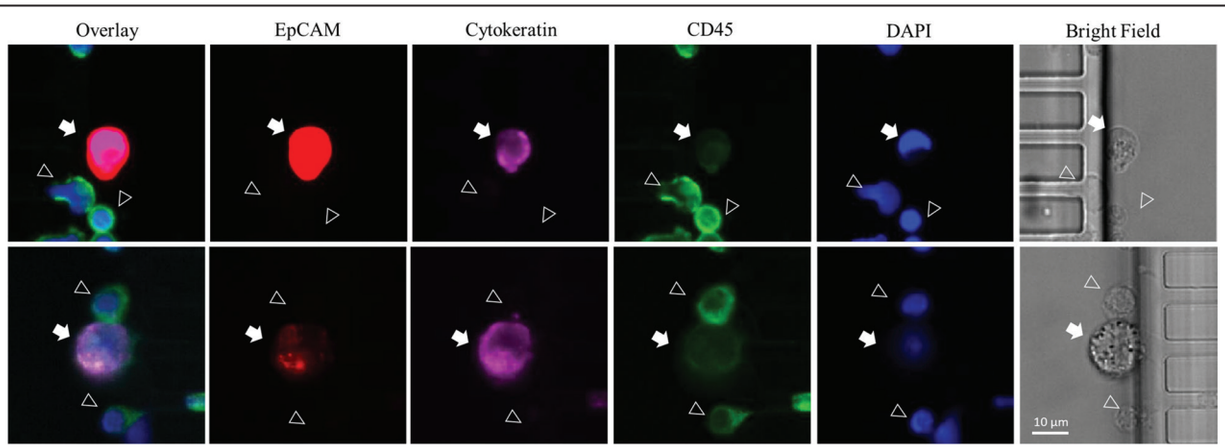


Fig. 1 Blood is incubated with fluorescently tagged indicated antibodies and loaded on the MiSelect R System. Isolated CTCs (marked with a white arrow) from a CRC patient are analyzed to determine biomarker expression using the automatic immunofluorescence imaging of the MiSelect R system where it is processed with eDAR sorting in a microfluidic chip. CD45-positive white blood cells are indicated with a triangle. CEA = carcinoembryonic antigen; CRC = colorectal cancer; CTC = circulating tumor cells; eDAR = ensemble-decision aliquot ranking; EpCAM = epithelial cell adhesion molecule; TNM = tumor-node-metastasis.

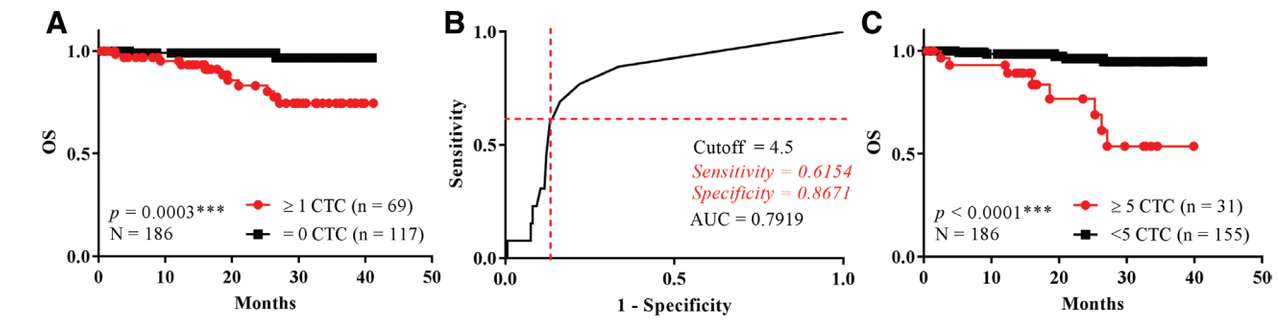


Fig. 3 Kaplan–Meier survival curves of overall-survival at all cancer stages of patients, stratified with CTC. (A) Kaplan–Meier overall survival curve for patients with one or more CTC. (B) The ROC curve showing that five CTCs was the optimal threshold to use for prognostic analysis. (C) Kaplan–Meier overall survival curve for patients with five or more CTCs. CTC = circulating tumor cells.ROC = receiver operating characteristic.

Table 2

Demographic and clinicopathological characteristics of patient cohorts segregated by patients with 5 or more CTCs

	NO. of case (%)	NO. of case ≥5 CTC (%)	NO. of case <5 CTC (%)	
	N = 186	N = 31	N =155	*p*
Age	67 (range 38–90)			
Gender				
Male	103 (55)	19 (18.4)	84 (81.6)	0.5544
Female	83 (45)	12 (14.5)	71 (85.5)	
Location				
Right colon	51 (27)	7 (13.7)	44 (86.3)	0.6977
Left colon	85 (46)	14 (16.5)	71 (83.5)	
Rectal	50 (27)	10 (20.0)	40 (80.0)	
TNM Stage				
I	41 (22)	3 (7.3)	38 (92.7)	<0.001***
II	63 (34)	12 (19.0)	51 (81.0)	
III	59 (32)	5 (8.5)	54 (91.5)	
IV	23 (12)	11 (47.8)	12 (52.2)	
Tumor stage				
T1-T2	54 (29)	3 (5.6)	51 (94.4)	0.0088**
T3-T4	132 (71)	28 (21.2)	104 (78.8)	
Node status				
N0	110 (59)	15 (13.6)	95 (86.4)	0.0423*
N1	49 (26)	7 (14.3)	42 (85.7)	
N2	27 (15)	9 (33.3)	18 (66.7)	
Distant metastasis				
M0	163 (88)	20 (12.3)	143 (87.7)	<0.001***
M1	23 (12)	11 (47.8)	12 (52.2)	
Differentiation				
Poor	5 (3)	1 (20.0)	4 (80.0)	0.6537
Moderate	177 (95)	30 (16.9)	147 (83.1)	
Well	4 (2)	0 (0.0)	4 (100.0)	
CEA (5 ng/ml)				
>5	62 (33)	16 (25.8)	46 (74.2)	0.0223*
≤5	124 (67)	15 (12.1)	109 (87.9)	
CA19-9 (U/ml)				
>37	32 (17)	9 (28.1)	23 (71.9)	0.0688
≤37	154 (83)	22 (14.3)	132 (85.7)	

CEA = carcinoembryonic antigen; CTC = circulating tumor cells; eDAR = ensemble-decision aliquot ranking; TNM = tumor-node-metastasis.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

different OS based on the CTC count. A CTC count ≥5 indicated a shorter OS (median OS = 27.1 months; HR = 9.008; 95% CI, 1.817–44.65; $p = 0.0011$) compared to those with a CTC count <5, where greater than 95.7% of patients were still alive at 40 months (Fig. 4B and Table 3). In addition, patients with a low CEA level showed the same split in OS based on the CTC count. Patients with low CEA and a CTC count ≥5 had a significantly shorter OS and increased risk (HR = 9.831; 95% CI, 0.28–357.7; $p = 0.0019$) compared to patients with a low level of CEA and CTCs (Fig. 4C and Table 3).

Univariate analysis results, including age, gender, tumor stage, node status, distant metastasis, CEA, CA19-9, and CTC were analyzed for the prediction of OS. CTC was identified as a predictor of poor survival. Multivariate analysis indicated that CTC, in addition to gender, distant metastasis, CA19-9, was a predictor of poor OS, as determined by multiple logistic regression analysis after adjusting for age, gender, tumor stage, node status, distant metastasis, CEA, CA19-9 ($p = 0.029$, Table 4).

4. DISCUSSION

Previous studies have indicated that CTCs are rarely detected in the peripheral venous blood of patients.[28,38] The rarity of CTCs in the peripheral venous blood of patients with nonmetastatic adenocarcinoma greatly limits its use as a predictor for metastasis. Our previous studies and other studies showed that CTCs can be detected at a higher rate and in higher numbers in tumor mesenteric blood than in peripheral venous blood in CRC patients.[39–41] CTCs have been detected in the blood of colorectal, breast, prostate, lung, and other cancer patients,[42–45] and have been shown to correlate with progression-free survival and OS in metastatic CRC using CellSearch.[13] Previous studies have reported that 22% of stage II and III patients and 41% of stage IV patients have >1 CTC per 7.5 mL of blood, as measured by CellSearch.[46,47] Particularly, the early stage of CRC and the precancer polyps also have CTC detected by the newly developed system.[19] In our study, 34.4% of stage II and III patients and 73.9% of stage IV patients demonstrated at least 1 CTCs/8.0 mL blood. CTCs detection still has many limitations. The cutoff value of 1–6 CTCs was analyzed using Cox regression analysis. The cutoff value of five CTCs had the most statistical significance for OS detection. Aggressive tumor cells lose epithelial markers EpCAM due to epithelial-mesenchymal transition and will not be detected in the blood.[48,49] CEA levels are an important prognostic factor and indicator of therapeutic effect and recurrence in patients with CRC.[5,6] CEA did not effectively detect treatable recurrences at an early stage, and a clinically relevant effect on patient mortality remains to be proven.[50] In the log-rank and Cox regression analysis of predictors, the CEA level was not a predictor of poor OS (Tables 3,4). However, a single tumor marker such as the serum CEA level in CRC has limited sensitivity and specificity, therefore it is necessary to select and combine a variety of markers to improve

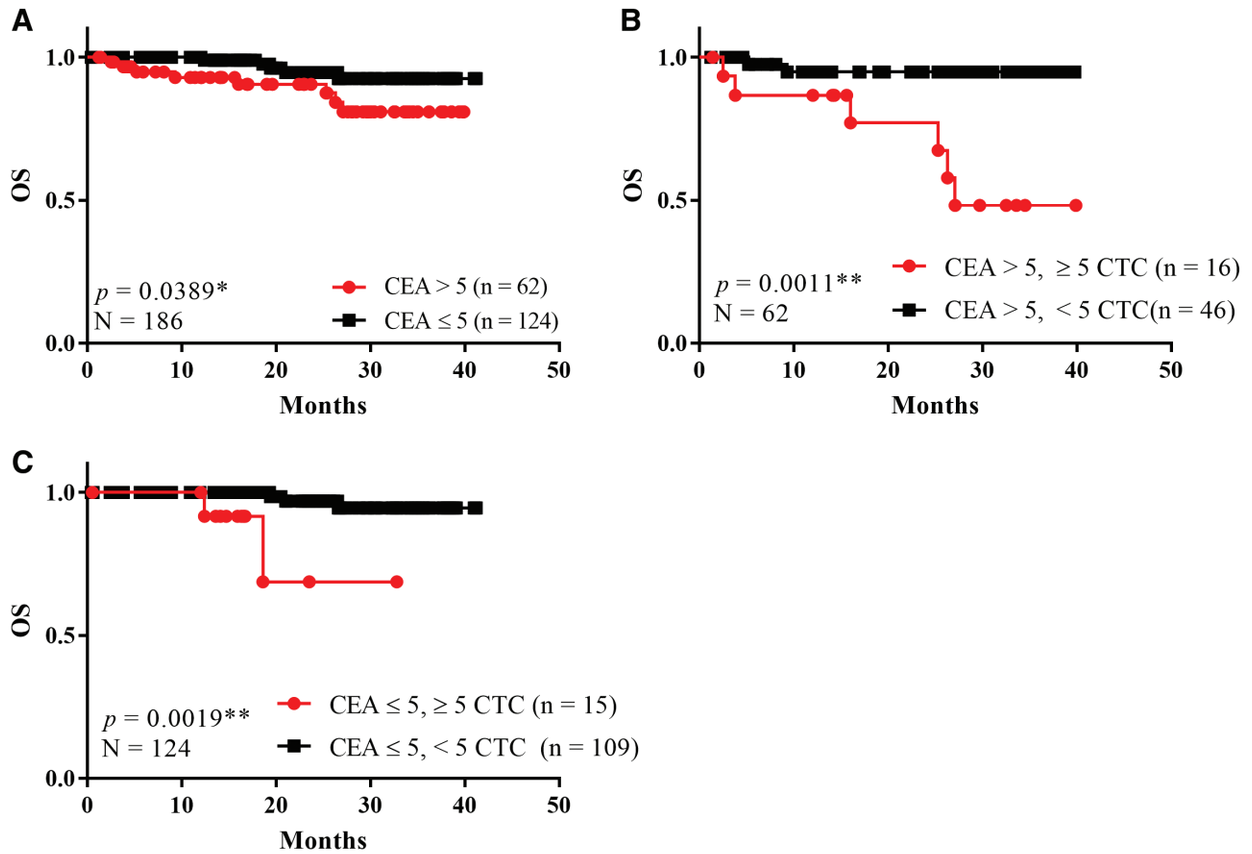


Fig. 4 Patients were stratified based on their CTC count and CEA level. (A) Kaplan–Meier OS curve for patients with high CEA vs low CEA. (B) OS curves for all patients with high CEA, segmented by CTC count. (C) OS curves for all patients with low CEA, segmented by CTC count. CEA = carcinoembryonic antigen; CTC = circulating tumor cells; OS = overall survival.

Table 3

Overall survival and hazard ratio of patient cohorts segregated by CEA level and CTC count

OS	HR	95% CI	*p*
≥5 CTC, CEA >5 ≥5 CTC, CEA ≤5	1.73	0.4103–7.292	0.4826
<5 CTC, CEA >5 <5 CTC, CEA ≤5	1.592	0.233–10.87	0.6074
≥5 CTC, CEA >5 <5 CTC, CEA >5	9.008	1.817–44.65	0.0011**
≥5 CTC, CEA ≤5 <5 CTC, CEA ≤5	9.831	0.2702–357.7	0.0019**
CEA >5 CEA ≤5	3.055	0.9726–9.596	0.0389*
≥5 CTC <5 CTC	10.03	2.069–48.57	<0.001***
≥1 CTC CTC=0	9.624	3.109–29.79	0.0003***

CEA = carcinoembryonic antigen; CTC = circulating tumor cells.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$

prognostic value.[31–37] In this study, we prospectively investigated CTC counts as a prognostic marker in 186 patients with non-metastatic and metastatic CRC. Previous studies have assessed the relationship between CTCs and tumor markers in solid tumors.[51–53] Additionally, the combination of CTC count and conventional tumor markers such as CEA, has been suggested to enhance prognostic power and clinical prediction in lung cancer patients.[54,55] Here, we emphasize the improvement of prognosis prediction using the combination of CTC counts and CEA level in CRC. A recent study revealed the diagnostic value of CTC enumerations and found that improved accuracies of AUC in predicting metastatic CRC samples were obtained by analyzing the combination of CEA and CTCs, rather than by CEA alone.[56]

A strong association existed between patients with CTCs and cancer stage as well as the individual T, N, and M staging. High CEA levels are also correlated in patients with five or more CTCs. No association was found between five or more CTCs and gender, tumor location, or histological differentiation. This finding was expected and strengthens the understanding of CTCs as indicative of disease progression. As expected, both CTCs and CEA demonstrated clinical utility for predicting OS (Table 2). A high CEA level had an HR of 3 compared to a low CEA level, which while statistically significant is only of marginal clinical utility as a prognostic indicator on an individual basis. Moreover, the prediction power of CTCs ($p < 0.0001$) was greater than the prediction power of the CEA tumor marker for OS ($p = 0.0389$). The prediction power of combined CTC count and CEA level ($p = 0.0011$ and 0.0019) was also greater than the prediction power of CEA level alone ($p = 0.0389$). In addition, a high CA19-9 level has a significant higher CTC than a low CA19-9 ($p \leq 0.001$, Table 1), but further log-rank analysis of CA19-9 and CTC shows no significant static difference in OS

Table 4

Univariate and multivariate analysis of OS predictors by Cox regression model

	Univariate cox regression			Multivariate cox regression		
	HR	95% CI	*p*	HR	95% CI	*p*
Age (≧65 vs <65)	0.966	0.323–2.890	0.951	4.135	0.903–18.932	0.067
Gender (male vs female)	0.352	0.108–1.143	0.082	0.019	0.002–0.212	0.001**
Tumor stage (T3-4 vs T1-2)	0.204	0.026–1.568	0.127	0.219	0.014–3.307	0.273
Node status (N1-2 vs N0)	8.267	1.832–37.314	0.006**	5.113	0.724–36.124	0.102
Distant metastasis (M1 vsM0)	21.377	6.561–69.653	<0.001***	17.713	2.479–126.588	0.004**
CEA (>5 vs ≦5)	3.088	1.009–9.447	0.048*	0.050	0.006–0.451	0.008**
CA19-9 (>37 vs ≦37)	9.632	3.146–29.490	<0.001***	28.039	3.726–210.980	0.001**
CTC (≧5 vs <5)	10.498	3.400–32.408	<0.001***	7.436	1.224–45.177	0.029*

CEA = carcinoembryonic antigen; CI = confidence interval; CTC = circulating tumor cells HR = hazard ratio.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

prediction (Supplementary Table 1 http://links.lww.com/JCMA/A188).

In the subgroup analysis, the patient population with low CEA levels, but high CTCs had a large HR of 9.8. The large difference in OS in this group suggests that CEA level combined with CTC count may provide important clinical utility. It may be recommended to use the combined CTC count with CEA level analysis to modify patient monitoring schedules and protocols to increase the probability of detecting recurrence at an earlier and more actionable stage. In summary, we demonstrate the improved prognostic power of combining CTC enumeration with CEA measurements in CRC patients. The detection of more than five CTCs predicted a worse OS and was particularly relevant for patients who had a low level of CEA.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://links.lww.com/JCMA/A188.

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