

Mesenchymal and Phosphatase of Regenerating Liver-3 Status in Circulating Tumor Cells May Serve as a Crucial Prognostic Marker for Assessing Relapse or Metastasis in Postoperative Patients With Colorectal Cancer

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INTRODUCTION: Circulating tumor cells (CTCs) and phosphatase of regenerating liver-3 (PRL-3) have been considered to be significant prognostic indicators in metastatic colorectal cancer (CRC). This study discusses the prognostic significance of mesenchymal CTCs with PRL-3 (M+ PRL-3+ CTCs) in postoperative patients with CRC.

METHODS: We detected CTC subtypes (including epithelial CTCs, biphenotypic epithelial/mesenchymal CTCs, and mesenchymal CTCs) and PRL-3 in CTCs from the peripheral blood samples of 156 patients. Receiver operating characteristic curve analysis, Kaplan-Meier analysis, and Cox proportional hazards regression analysis were performed to identify the prognostic value of mesenchymal CTCs with PRL-3+. Immunohistochemistry was used to detect the expression of PRL-3 in tumor tissues from some of the patients to explore the connection between CTCs and tissues.

RESULTS: All CTCs were positive in all samples, both mesenchymal CTCs and PRL-3-positive cells. The count of mesenchymal and PRL-3+ CTCs was significantly associated with recurrence, and the optimal cutoff value was 2 (area under the curve = 0.690, $P < 0.001$). In addition, these patients had a significantly shorter median disease-free survival than those who did not fulfill the criteria (8.5 vs 24 months, $P < 0.001$) according to multivariable and multinomial logistic regression. Immunohistochemistry was applied to explore the associations between PRL-3 expression and significant prognostic risk factors, including recurrence ($R = 0.566$; $P < 0.001$), and M+ PRL-3+ status in CTCs ($R = 0.452$; $P = 0.001$).

DISCUSSION: The status of M+ PRL-3+ in CTCs may serve as a crucial prognostic marker for assessing clinical outcomes in CRC.

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INTRODUCTION

The most common metastatic target organ of colorectal cancer (CRC) is the liver. Approximately 15%–20% of patients with CRC were found to have liver metastasis at the first diagnosis, and 60% of the remaining patients also had liver metastasis or tumor recurrence during subsequent treatment, which is the main cause of poor efficacy and death from CRC (1). Circulating tumor cells (CTCs) derived from the primary tumor and metastases play an important role in the process of metastasis, the removal of the basement membrane, and of invasion by mobilizing the matrix into blood vessels (2). In addition, tumor cells must make some changes to withstand hemodynamic and fluid shear forces when entering new complete microenvironments, such as the circulatory system

and remote organs (3,4). Overexpression of phosphatase of regenerating liver-3 (PRL-3) and epithelial-mesenchymal transition (EMT) are considered to be the 2 most important changes, associated with the migration, invasion, and metastasis of CRC (5–12). A previous study claimed that mesenchymal markers are highly enriched in CTCs, whereas both mesenchymal and epithelial markers are barely expressed in primary tumors (13). Furthermore, the association between mesenchymal CTCs and the prognosis of metastatic breast cancer and the predictive value of total CTC count are restricted by the heterogeneity of CTCs (13). Hence, the situation in which the mesenchymal markers are overexpressed in CTCs, which occurs in the EMT process, needs further research. Although some researchers have studied the effect

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Table 1. The clinicopathological variables of 156 advanced CRC patients

Variables	All patients (n = 156)	%
Age, mean (range)	60 (24–81)	
Male sex, primary tumor	93	59.6
Colon	113	72.4
Rectum	43	27.6
T status		
T1	6	3.8
T2	9	5.8
T3	27	17.3
T4	114	73.1
N status		
N0	62	39.7
N+	94	60.3
Initial stage of disease (AJCC)		
I	11	7.1
II	47	30.1
III	67	42.9
IV	31	19.9
CEA ^a	4.3 (0.4–5,595)	
CTCs		
Positive	156	100
PRL-3		
Positive	156	100
Subtype of CTCs		
M+ CTCs	103	64.4
E+ CTCs	129	80.6
E+/M+ CTCs	140	87.5
M+ PRL-3+ CTCs		
Positive	78	50
Negative	78	50
E+ PRL-3+ CTCs		
Positive	99	63.5
Negative	57	36.5
E+/M+PRL-3+ CTCs		
Positive	133	85.5
Negative	23	14.5
Chemotherapy		
CapeOX	97	62.1
FOLFIRI	6	3.9
mFOLFOX6	14	9.0
No	39	25
Bevacizumab treatment		
Yes	8	5.1

Table 1. (continued)

Variables	All patients (n = 156)	%
DFS (mo)		
<24	65	41.7
≥24	91	58.3
Time of metastasis		
Synchronous	31	19.9
Metachronous	34	21.8
Metastatic site		
Liver	51	32.7
Lung	7	4.5
Other site	7	4.5
In the rows “disease-free survival and “CTCs,” the value is the number of patients. AJCC, American Joint Committee on Cancer; CEA, carcinoembryonic antigen; CTC, circulating tumor cell; PRL-3, phosphatase of regenerating liver-3. ^a The CEA value is the median (range) in µg/L.		

of mesenchymal CTCs on the prognosis of breast cancer and determined an optimal cutoff value of 10.7%, the specificity is still not high and the area under the curve (AUC) was only 0.581 (14). For this purpose, we focused on PRL-3 in exploring the connection between mesenchymal CTCs and the recurrence of CRC considering the role of PRL-3 in CRC.

In this study, we used the CanPatrol CTC enrichment technique and PRL-3 antibody probe to derive and characterize CTCs and divided CTCs into 3 subpopulations, namely, epithelial CTCs with PRL-3 (E+ PRL-3+ CTCs), biphenotypic epithelial/mesenchymal CTCs with PRL-3 (E+/M+ PRL-3+ CTCs), and mesenchymal CTCs with PRL-3 (M+ PRL-3+ CTCs) (15). Therefore, we investigated the prognosis of CTCs with respect to EMT markers and PRL-3 status in 156 patients with CRC by using the previously described technique.

The purpose of the study was to evaluate the prognostic value of M+ PRL-3+ CTC detection before surgery through peripheral blood collected from patients. In addition, we also investigated the association between the distribution of M+ PRL-3+ CTCs and patient clinicopathological characteristics.

METHODS

Patients

From October 2016 to September 2018, a cohort 156 patients from 248 patients with CRC at Sun Yat-sen Memorial Hospital, Guangdong Province, China, was enrolled in the study and prospectively included after informed consent was obtained. The inclusion criteria were as follows: (i) sporadic primary CRC; (ii) definitive pathological diagnosis of CRC based on the World Health Organization criteria; (iii) complete available information, including age, sex, tumor location, tumor differentiation, depth of invasion, lymphatic invasion, distant metastasis, TNM stage, CTC counts (each subtype), carcinoembryonic antigen (CEA) level, and PRL-3 expression level during the follow-up; (iv) did not receive any previous treatment including gastrointestinal surgery, radiotherapy, and

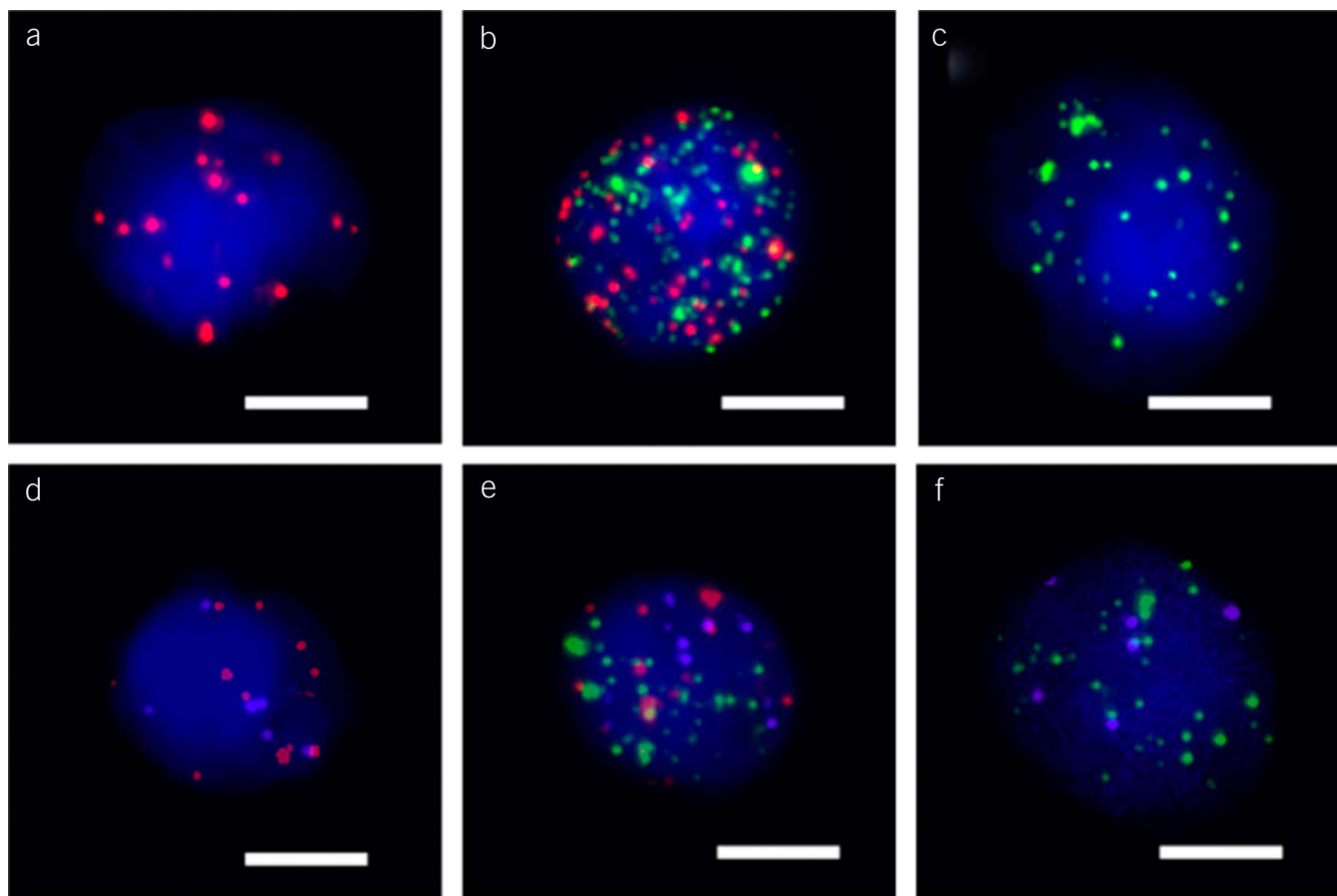


Figure 1. (a–c) CTC subpopulations classified by categorical markers (a: epithelial CTCs, b: epithelial/mesenchymal CTCs, and c: mesenchymal CTCs). Red dots: epithelial biomarker expression. Green dots: mesenchymal biomarker expression. (d–f) PRL-3 expression status of CTC subtypes based on probe (d: epithelial CTCs, e: epithelial/mesenchymal CTCs, and f: mesenchymal CTCs). Purple dots: PRL-3 expression (bars = 5 μ m). CTC, circulating tumor cell; PRL-3, phosphatase of regenerating liver-3.

chemotherapy before the operation; and (v) chemotherapy regimens for patients with CRC were performed according to National Comprehensive Cancer Network clinical practice guidelines of colon cancer (16). In detail, for the stage II and stage III patients, CapeOx and mFOLFOX6 regimen are recommended in priority by the guidelines. As for the stage IV patients, fluorouracil combined with oxaliplatin and irinotecan are used to improve the prognosis. For patients with multiple metastases, bevacizumab and cetuximab are added to the chemotherapy regimen. The exclusion criteria were as follows: (i) previous or present primary malignant tumor of other tissues; (ii) hereditary diseases such as hereditary nonpolyposis CRC or familial adenomatous polyposis; and (iii) patients with serious life-threatening diseases. The clinical staging and clinicopathological classifications were determined according to the American Joint Committee on Cancer staging system. Stage I, II, and III patients underwent radical resection and lymphadenectomy for CRC. Stage IV patients underwent palliative resection and resection of metastatic liver lesions. For patients with stage IV disease, we considered new metastases to be relapses. The clinicopathological characteristics of the enrolled patients are detailed in Table 1. All paraffin-embedded and fresh tissues used in this study were obtained with the consent of each patient and with institutional research ethics committee approval.

Disease-free survival (DFS) was defined as the interval from the time of the collection of peripheral blood to detect CTCs until disease progression during or after chemotherapy. Recurrence or metastasis of the tumor was assessed by computed tomography (CT) scanning or MRI every 8–12 weeks during the chemotherapy course. After chemotherapy, we followed up the patients by telephone to determine their health status. The strategies of postoperative re-examination were based on National Comprehensive Cancer Network guidelines (16). Every patient after operation or chemotherapy returned to hospital every 3–6 months during 2 years to check themselves with chest/abdominal/pelvic CT and detection of CEA. All patients were followed up until progression or October 31, 2019.

CTC detection and characterization

Peripheral blood samples (approximately 5 mL) were collected from CRC patients' preoperation and were used for detection by the CellSearch System (Veridex, LLC, Warren, NJ) which is approved by the Food and Drug Administration. The epithelial marker probes we used included EpCAM, CK8, CK18, and CK19. The mesenchymal marker probes we used included vimentin, Twist, and CD45. The experimental steps have been described in detail elsewhere (17). In brief, an EDTA anticoagulant blood collection vessel was used to collect blood samples from patients,

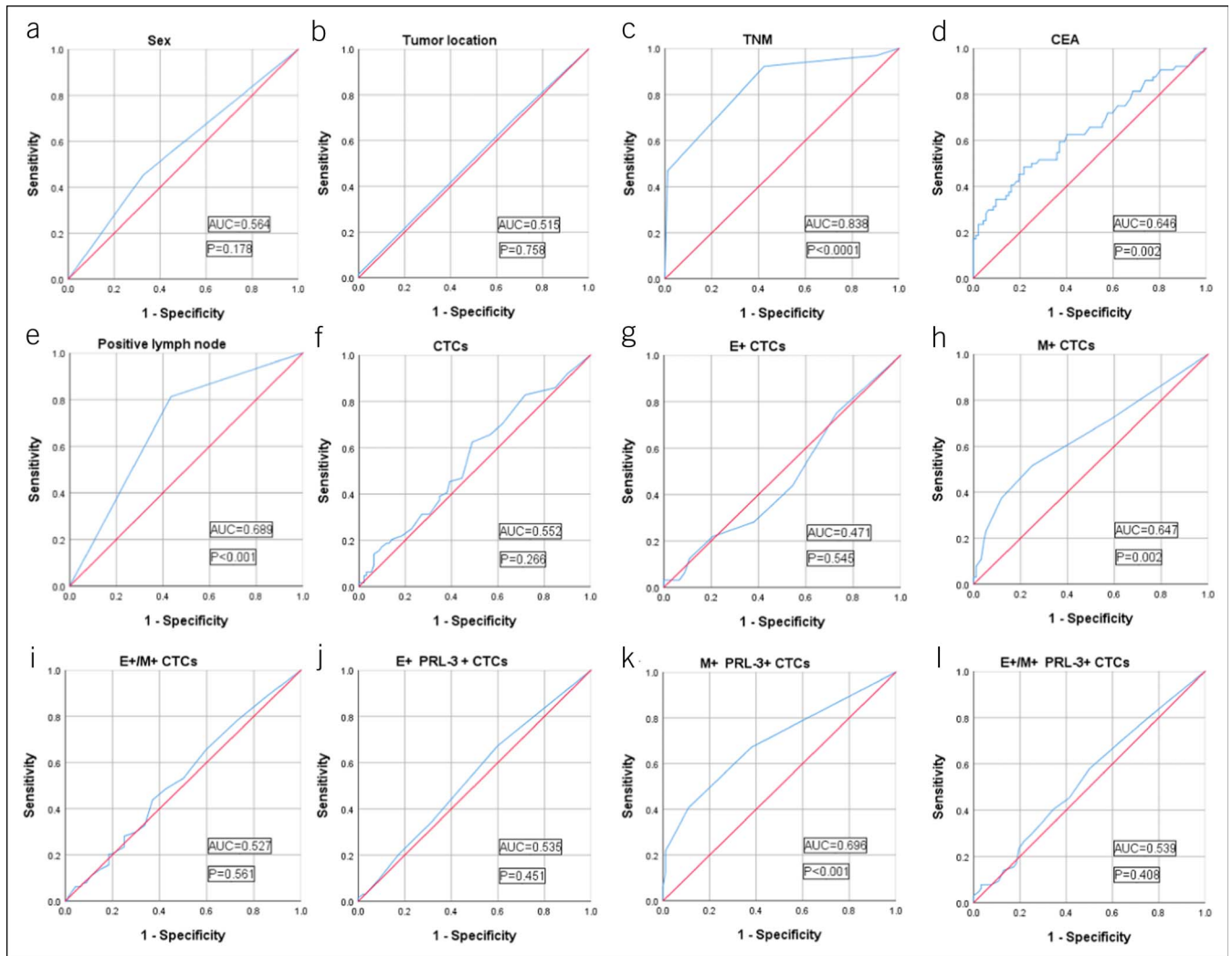


Figure 2. ROC curves were created to determine indicators of DFS. The sensitivity and specificity of each factor are plotted, and the AUCs are indicated. AUC, areas under the curve; DFS, disease-free survival; ROC, receiver operating characteristic.

which were mixed upside-down, and 15 mL of erythrocyte lysate was added to mix them (the formula of the lysis buffer solution was 154-mM NH_4Cl , 10-mM KHCO_3 , and 0.1-mM EDTA). The erythrocytes were lysed at room temperature without shaking for 30 minutes; the blood samples were centrifuged at 500g for 5 minutes to remove the supernatant. The cell precipitate was resuspended in phosphate buffer solution (PBS). The remaining cells were precipitated for 8 minutes with formaldehyde at a final concentration of 4%. The fixed cells were transferred to the filter tube (containing the filter membrane), and the cells were filtered with the filter membrane using a vacuum filter pump. After filtration, the cell membrane samples were fixed at room temperature for 1 hour with 4% formaldehyde. The fixed membrane samples were washed with PBS 3 times and placed in 24-well plates. Protease K (0.1 mg/mL) was added for treatment and left at room temperature for 1 hour to increase membrane permeability. The cells were washed 3 times with PBS, and then, the specific capture probe was added. The reaction was hybridized at 40°C for 3 hours. The unbound probe was washed with 1,000 μL of eluent 3 times (formula: 0.1 \times SSC [Sigma, St. Louis]). Then, 100 μL of preamplification fluid (formula: 30% horse serum [Sigma], 1.5%

sodium dodecyl sulfate [Sigma], and 3-mM Tris-HCl [pH 8.0]) and 0.5-fmol preamplification probe were added and incubated at 40°C for 30 minutes for signal amplification probe reaction. The membrane was cooled, eluted 3 times with 1,000 μL of eluent (0.1 \times sodium citrate buffer [SSC]), and then incubated with 100 μL of amplification solution (30% horse serum, 1.5% sodium dodecyl sulfate, and 3-mM Tris-HCl [pH 8.0]) with 1-fmol preamplification probe at 40°C for 30 minutes. The color developing probe carrying the fluorescent dye Alexa Fluor 647 was added and incubated at 40°C for 30 minutes. Then, 0.1 \times SSC was used for elution, and the nuclei were stained with 4', 6-diamidino-2-phenylindole for 5 minutes. The samples were observed under a 100-fold oil microscope using an automated fluorescence scanning microscope. The CTCs were classified into epithelial CTCs, interstitial CTCs, and mixed CTCs according to epithelial markers, interstitial markers, or both, respectively. Representative images of each CTC population are shown in Figure 1a,b,c.

Detection of PRL-3 expression in each CTC

The expression level of PRL-3 was determined by a PRL-3 antibody probe. Then, it was labeled with a purple fluorescent dye.

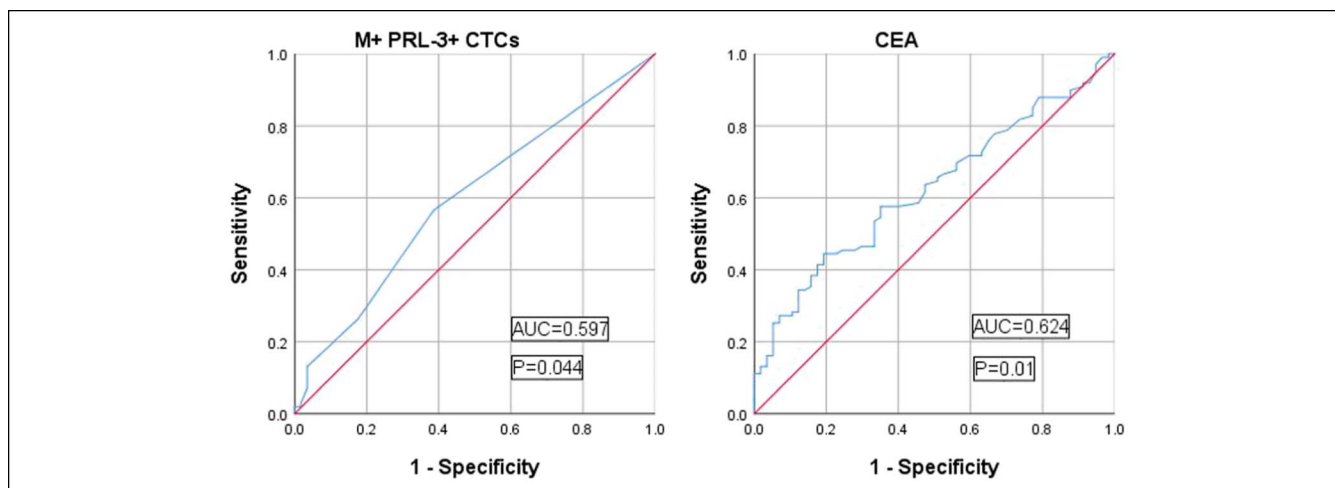


Figure 3. Receiver operating characteristic curves were used to estimate the sensitivity and specificity of each factor, and the AUCs are indicated. AUC, area under the curve.

Furthermore, the PRL-3 expression level of each CTC was classified by 4°, including high-level expression, middle-level expression, low-level expression, and nonexpression. In brief, we divided the levels into 2 groups, 1 for positive and 1 for negative. Images for the PRL-3 status of each CTC subpopulation are shown in Figure 1d,e,f.

Statistical analysis

The statistical analyses and graphics were performed using SPSS 25.0 software (IBM, Armonk, NY), GraphPad Prism 7 software (GraphPad Software, La Jolla, CA), and R 3.6.1 (The R Foundation for Statistical Computing, Lanzhou Province, China). Receiver operating characteristic (ROC) curve analysis was applied to confirm the specific indicators related to TNM stage and DFS by using the closest-to-(0, 1)- criterion and to determine the optimal cutoff value for PRL-3 expression in CTCs (18). The AUC values were calculated. DFS among different prognostic categories was calculated using the Kaplan-Meier method, and significant differences between the survival curves were compared by using the log-rank test. According to the results of all variables in the univariate analyses, only variables with a P value < 0.05 were included in the multivariate Cox regression model to evaluate the influence of independent factors on DFS. All P values were 2-sided, and $P < 0.05$ was deemed to be statistically significant.

Based on the above results, a nomogram for the probability of recurrence was constructed, and its performance was assessed by discrimination and calibration (19). The discriminative quality of the model was determined by the ROC curve, ranging from 0.5 (little discrimination) to 1 (excellent discrimination) (18). The calibration of the model was assessed by a visual calibration plot comparing the predicted and actual probabilities. Furthermore, bootstrap validation was used to assess the predictive accuracy of the nomogram.

Immunohistochemistry

Immunohistochemistry (IHC) was used to examine PRL-3 expression in 41 human CRC specimens from a total of 156 samples. To exclude the high risk of recurrence in stage IV, the samples used were all stage I–III. Two independent observers blinded to the

histopathological features assessed the results and then scored the degree of immunostaining. The scores were based on the proportion of positively stained tumor cells (graded as: 0 [$<5\%$ positive], 1 [6%–25% positive], 2 [26%–50% positive], or 3 [$>50\%$ positive]) and staining intensity (categorized as 0 [no staining], 1 [light yellow], 2 [yellow brown], or 3 [brown]). The staining scores were obtained by multiplying the scores for the staining intensity and for the proportion of positive cells (scored as 0, 1, 2, 3, 4, 6, or 9). In each section, 5 different fields of view were randomly selected and counted, and then, the arithmetic mean was calculated. A staining index score ≥ 4 indicated tumors with high positive PRL-3 expression, and a score of <4 indicated low or no PRL-3 expression. ROC curve analysis was used using variables including PRL-3 expression and patient recurrence to determine the optimal cutoff values of the scores (Figure 6).

RESULTS

Characteristics of the patients

As presented in Table 1, 156 advanced CRC patients, 21–81 years of age, were ultimately included in the study. Generally, CTCs were detected in all samples, and 156 patients (100%) were PRL-3–positive. Regarding the CTC subtypes, the proportion of each subtype was 64.4% M+ CTCs, 80.6% E+ CTCs, and 87.5% E+/M+ CTCs; when PRL-3 was detected cooperatively, the positive rate decreased to 50%, 63.5%, and 85.5%, respectively. Regarding prognosis, 65 patients (41.7%) relapsed.

Selection of DFS indicators and cutoff score

All the samples were positive for CTCs, with a detection rate of 100%. Each sample had a different proportion of CTC subtypes. In total, there were 3 subtypes of CTCs, including M+ CTCs, E+ CTCs, and E+/M+ CTCs, and the expression rate of PRL-3 varied from CTC to CTC. As a result, ROC curve analysis was used to identify the prognostic factors. The results are shown in Figure 2.

The number of M+ CTCs and the expression of PRL-3 were considered to be associated with prognosis, especially as joint indicators ($P < 0.001$). This joint indicator (M+ PRL-3+ CTCs) was used to estimate the prognosis of the patients. To identify an optimal cutoff value for the joint indicator, ROC curve analysis

Table 2. Risk factors for recurrence using Kaplan-Meier curves (univariate analysis) of patients (n = 156)

Variables	No. of patients	Mean DFS (mo)	Log-rank test (P)
Age, yr			0.312
≥60	76	23.7	
<60	80	22.8	
Sex			0.172
Male	97	23.8	
Female	59	21.9	
Primary tumor			0.696
Right	48	23.6	
Left	108	23.2	
T status			0.554
T1	6	26.5	
T2	9	24.8	
T3	28	24.6	
T4	113	22.7	
N status			<0.001
Positive	92	29.0	
Negative	64	18.9	
TNM			<0.001
I–II	57	32.0	
III–IV	99	18.2	
CEA, ng/mL			<0.001
>9.05	51	18.4	
<9.05	105	25.3	
CTCs			0.110
≥6.5	85	22.1	
<6.5	71	24.6	
M+ CTCs			<0.001
≥3	35	16.0	
<3	121	25.0	
E+ CTCs			0.956
≥2	78	23.6	
<2	78	22.9	
M+/E+ CTCs			0.367
≥6	62	22.4	
<6	94	23.6	
M+ PRL-3+ CTCs			<0.001
≥2	36	14.7	
<2	120	25.5	
E+ PRL-3+ CTCs			0.456
≥1	98	23.0	
<1	58	23.8	

Table 2. (continued)

Variables	No. of patients	Mean DFS (mo)	Log-rank test (P)
M+/E+ PRL-3+ CTCs			0.349
≥3	83	23.8	
<3	73	22.7	

CTC, circulating tumor cell; DFS, disease-free survival; PRL-3, phosphatase of regenerating liver-3.

was used to determine the cutoff score in various patterns. The ROC curves for each clinicopathological characteristic clearly show the dot on the curve closest to (0.0, 1.0), which maximizes both sensitivity and specificity for the outcome. The cutoff values of the indicators including some clinical factors described to be related with DFS were as follows: TNM stage II, CEA (≥ 9.05 $\mu\text{g/L}$, or < 9.05 $\mu\text{g/L}$), number of CTCs (≥ 6.5 , or < 6.5), lymph node positive rate (positive), number of M+ PRL-3+ CTCs (≥ 2 , or < 2), and number of M+ CTCs (≥ 3 , or < 3).

The expression rate of M+ PRL-3+ CTCs predicts TNM stage (early or late).

In this study, the expression rate of M+ PRL-3+ was detected before the operation. These data were used to assess pathological TNM (pTNM) staging, which was well associated with both ($P = 0.044$) (Figure 3). Stages I and II were reduced to the early stage, and stages III and IV were considered the late stage. Furthermore, the CEA index was also well associated with pTNM ($P = 0.01$) (Figure 3). The cutoff values were as follows: number of M+ PRL-3+ CTCs (> 0.5 , or < 0.5) and CEA (≥ 9.05 $\mu\text{g/L}$, or < 9.05 $\mu\text{g/L}$). Therefore, we may predict the pTNM before operation by detecting the CEA index and the expression rate of M+ PRL-3+.

Relapse/progression

In the study, 41.7% of all patients experienced relapse or progression during the follow-up time. At the end of follow-up, 65 of 156 advanced CRC patients were diagnosed with recurrence. The cumulative relapse rates were 16.0%, 34.0%, and 40.0% at 6, 12, and 18 months, respectively. At a median follow-up time of 8.5 months, 50% of the patients whose M+ PRL-3 CTC counts were over 2 experienced progression. Twenty-one of the 36 patients were diagnosed with relapse. Of these patients, 3 were stage II, 9 were stage III, and the others were stage IV. Of the stage IV patients, 5 of 9 had multiple systemic metastases after surgery or during chemotherapy.

The results of the univariate analysis of recurrence are displayed in Table 2. The expression of M+ and PRL-3+, CEA index, positive lymph node status, and TNM stage were considered to be significantly associated with disease-free survival in the follow-up patients ($P < 0.001$). The presence of 2 or more M+ and PRL-3+ CTCs showed a stronger correlation with disease-free survival (Figure 4), and referring to M+ CTCs alone, more than 3M+ CTCs were also related to DFS ($P < 0.001$). However, the counts of the other subtypes of CTCs combined with PRL-3 were not associated with relapse or progression in the patients.

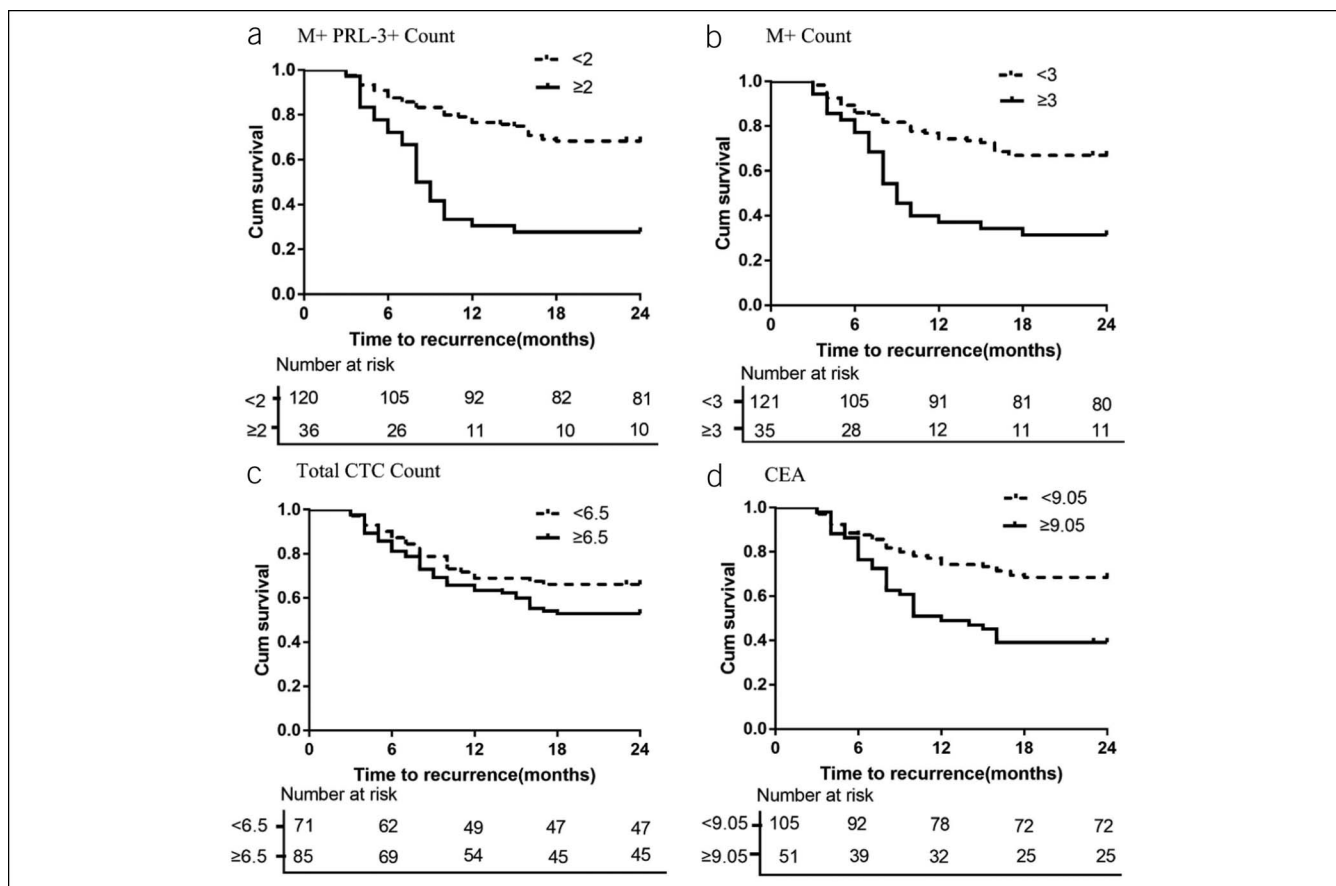


Figure 4. Kaplan-Meier curves of univariate analysis data (log-rank test). (a) DFS curves of patients with M+ and PRL-3+ CTC counts. (b) DFS curves of patients with M+ CTC counts. (c) DFS curves of patients with total CTC counts. (d) DFS curves of patients with CEA ≥9.05 ng/mL. CEA, carcinoembryonic antigen; CTC, circulating tumor cell; DFS, disease-free survival; PRL-3, phosphatase of regenerating liver-3.

Independent risk factors for relapse/progression

The meaningful risk indicators for progression were used for multivariate analysis (Table 3). Patients with 2 or more M+ and PRL-3+ CTCs had a 3.6 times higher risk of relapse than those with less than 2 CTCs ($P < 0.001$). By contrast, the number of CTCs, CEA index, lymph node positivity, and number of M+ CTCs were not identified as independent risk factors for relapse,

although they were considered significantly correlated with DFS in the univariate analysis and ROC curve analysis. At the same time, TNM stage was highly related to recurrence with an increased risk (hazard ratio: 22.21, 95% confidence interval: 6.366–77.479), which is the consensus in the world.

A nomogram model was established to assess the risk of recurrence of patients based on the above analyses of certain

Table 3. Independent risk factors for recurrence using the multivariate Cox regression model

Variable	Level	Hazard ratio	95% CI	P ^a
Age	≥60/<60	1.293	0.756–2.213	0.348
Location	Right/left	1.258	0.693–2.283	0.451
No. of CTCs	>6.5/<6.5	0.947	0.521–1.721	0.859
CEA (μg/L)	>9.05/<9.05	1.433	0.830–2.476	0.197
LN ^a positivity	Yes/No	0.505	0.228–1.116	0.091
No. of M+ PRL-3+	≥2/<2	3.608	1.930–6.742	<0.001
TNM	Early/late	22.210	6.366–77.479	<0.001
No. of M+	≥3/<3	1.387	0.739–2.604	0.309

CI, confidence interval; CEA, carcinoembryonic antigen; CTC, circulating tumor cell; PRL-3, phosphatase of regenerating liver-3.

^aLN, lymph node.

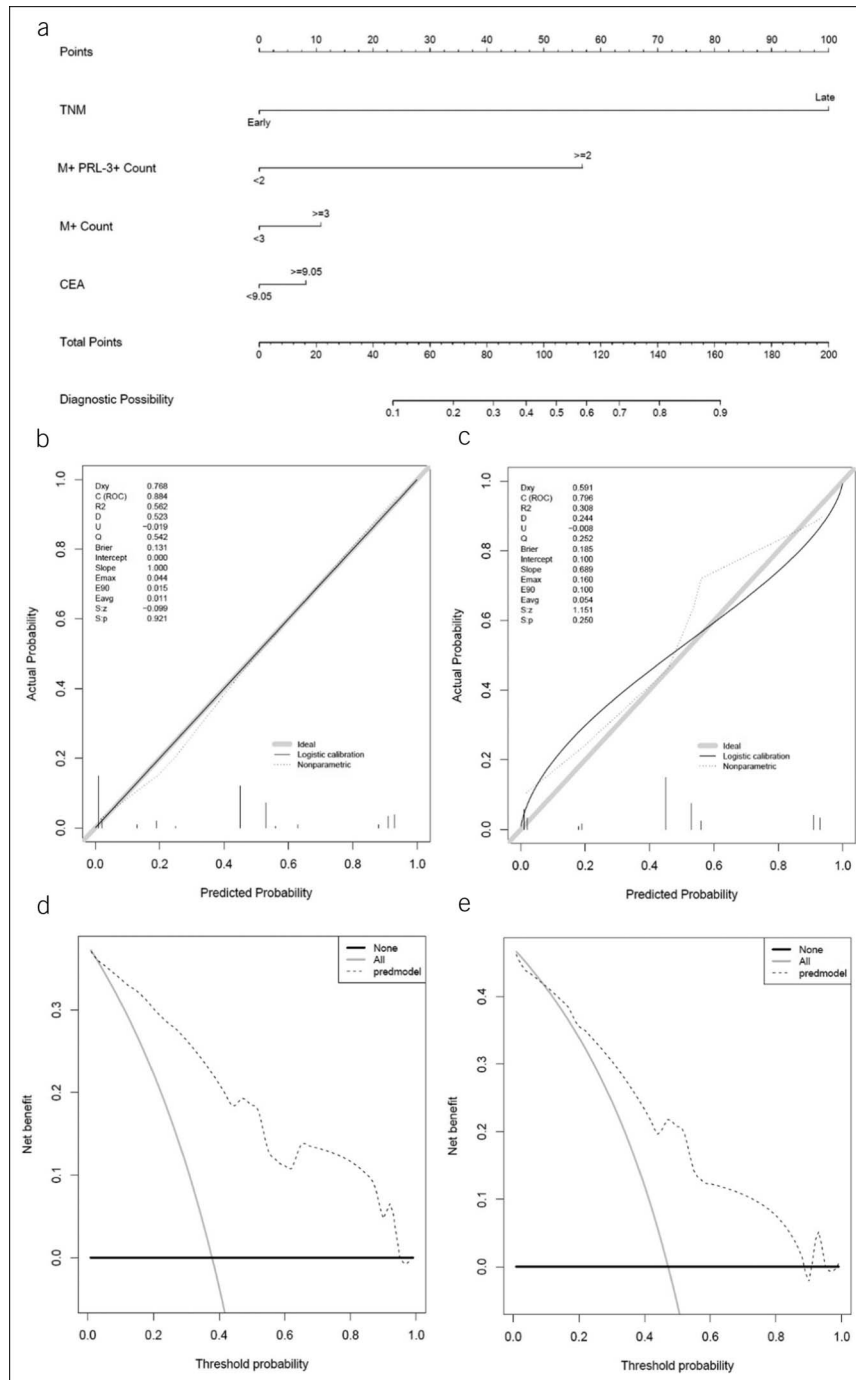


Figure 5. (a) A nomogram predicting the risk of recurrence for patients with the expression of M+ and PRL-3+ in CTCs. The value of each variable is given a score on the point scale axis. A total score could be easily calculated by adding each single score and by projecting the total score to the lower total point scale to estimate the probability of recurrence. The calibration curves for the nomogram (b and c). The x axis represents the nomogram-predicted probability, and the y axis represents the actual probability of recurrence. (a) For the modeling group and (b) for the validation group. A perfect prediction would correspond to the 45° ideal line. The black solid line represents the entire cohort ($n = 156$), and the black dotted line is bias-corrected by bootstrapping ($b = 1,000$ repetitions), indicating observed nomogram performance. (d and e) Decision curve analysis for the nomogram for the modeling group (d) and validation group (e). The y axis measures the net benefit. Horizontal axis: the threshold probability at a range of 0.0 to 1.0. The dotted line represents the nomogram. The real line represents the assumption that all patients have recurrence. The thin black line represents the assumption that no patients have recurrence. The net benefit was calculated by subtracting the proportion of all patients who are false positive from the proportion who are true positive, weighting by the relative harm of forgoing treatment compared with the negative consequences of an unnecessary treatment. CTC, circulating tumor cell; PRL-3, phosphatase of regenerating liver-3.

Table 4. Clinicopathological features and PRL-3 expression of some patients (n = 41) with colorectal cancer

Variables	No. of cases (%)
Age (yr)	
≥60	22 (53.7)
<60	19 (46.3)
Male sex, primary tumor	
Left	26 (63.4)
Right	30 (73.2)
T status	
T1	11 (26.8)
T2	0 (0)
T3	1 (2.4)
T4	11 (26.8)
N status	
N0	29 (0.8)
N+	16 (39)
Initial stage of disease (AJCC)	
I	25 (61)
II	1 (2.4)
III	15 (36.6)
CEA (ng/mL)	25 (61)
≥9.05	15 (36.6)
<9.05	26 (63.4)
CTCs	
Positive	41 (100)
PRL-3 of tissue	
Positive	41 (100)
Strong	19 (46.3)
Weak	22 (53.7)
Subtype of CTCs	
M+ CTCs	26 (63.4)
E+ CTCs	30 (73.2)
E+/M+ CTCs	34 (82.9)
M+ PRL-3+ CTCs	
Positive	22 (53.7)
Negative	19 (46.3)
E+ PRL-3+ CTCs	
Positive	26 (63.4)
Negative	15 (36.6)
E+/M+ PRL-3+ CTCs	
Positive	33 (80.5)
Negative	8 (19.5)
PRL-3+ CTCs	
Positive	41 (100)

Table 4. (continued)

Variables	No. of cases (%)
Disease-free survival (mo)	
<24	14 (34.1)
≥24	27 (65.9)
AJCC, American Joint Committee on Cancer; CTC, circulating tumor cell; PRL-3, phosphatase of regenerating liver-3.	

clinicopathological characteristics (TNM stage, count of M+ CTCs, count of M+ PRL-3 CTCs, and CEA index) (Figure 5). A total of 156 patients were enrolled to construct and validate the nomogram model. The patients were randomly divided into a modeling group and a validation group at a ratio of 2:1 (20–22). The nomogram had outstanding discrimination, with an AUC of 0.884. The calibration curves of the modeling group for the probability of disease-free survival showed optimal agreement between the probability predicted by the nomogram and the actual probability.

IHC

IHC was applied to 41 paraffin-embedded, samples collected from clinical cancer samples, which included 15 and 25 cases of TNM stage II and III, respectively, to investigate the clinical relevance of PRL-3 expression and cancer progression. There was strong positive expression of PRL-3 in 19 (46.3%) CRC specimens, whereas there was no or little detectable staining in the remaining 22 (53.7%; Table 4) clinical samples. PRL-3 was originally localized in the tumor cell membrane (Figure 6). Spearman correlation analysis confirmed the strong association of PRL-3 expression and significant prognostic risk factors, including recurrence ($R = 0.566$; $P < 0.001$), CEA ($R = 0.300$; $P = 0.057$), deep stromal invasion ($R = 0.271$; $P = 0.043$), TNM stage ($R = 0.272$; $P = 0.085$), M+ CTCs ($R = 0.383$; $P = 0.014$), and M+ PRL-3+ CTCs ($R = 0.452$; $P = 0.001$) (Table 5).

DISCUSSION

CTCs have been suggested to be reflective factors of the aggressiveness of solid tumors including breast cancer, liver cancer, prostate cancer, and CRC, by participating in tumor differentiation, invasion, and metastasis. Previous studies used the CellSearch System to detect the number of CTCs in peripheral blood, but CTCs were mainly identified by tumor epithelial cell expression of EpCAM, the completeness of an intact nucleus, and the lack of CD45 (17,23–27). Nonetheless, the above approach is unable to detect CTCs undergoing EMT. Hence, we used categorical markers to isolate the subtypes of CTCs. To date, several studies have examined the number of M+ CTCs from peripheral blood in association with poor clinical outcomes in liver cancer and breast cancer (14,28). However, the significance of M+ status in CTCs of CRC and its effects on the prognosis of CRC is still unclear.

To the best of our knowledge, PRL-3 is an important gene associated with the metastasis of CRC, and the proteins transcribed by this gene are located on cell membranes. Previous studies have shown that PRL-3 enables tumor cells to promote invasion, migration, and metastasis through EMT (18,29). Our previous

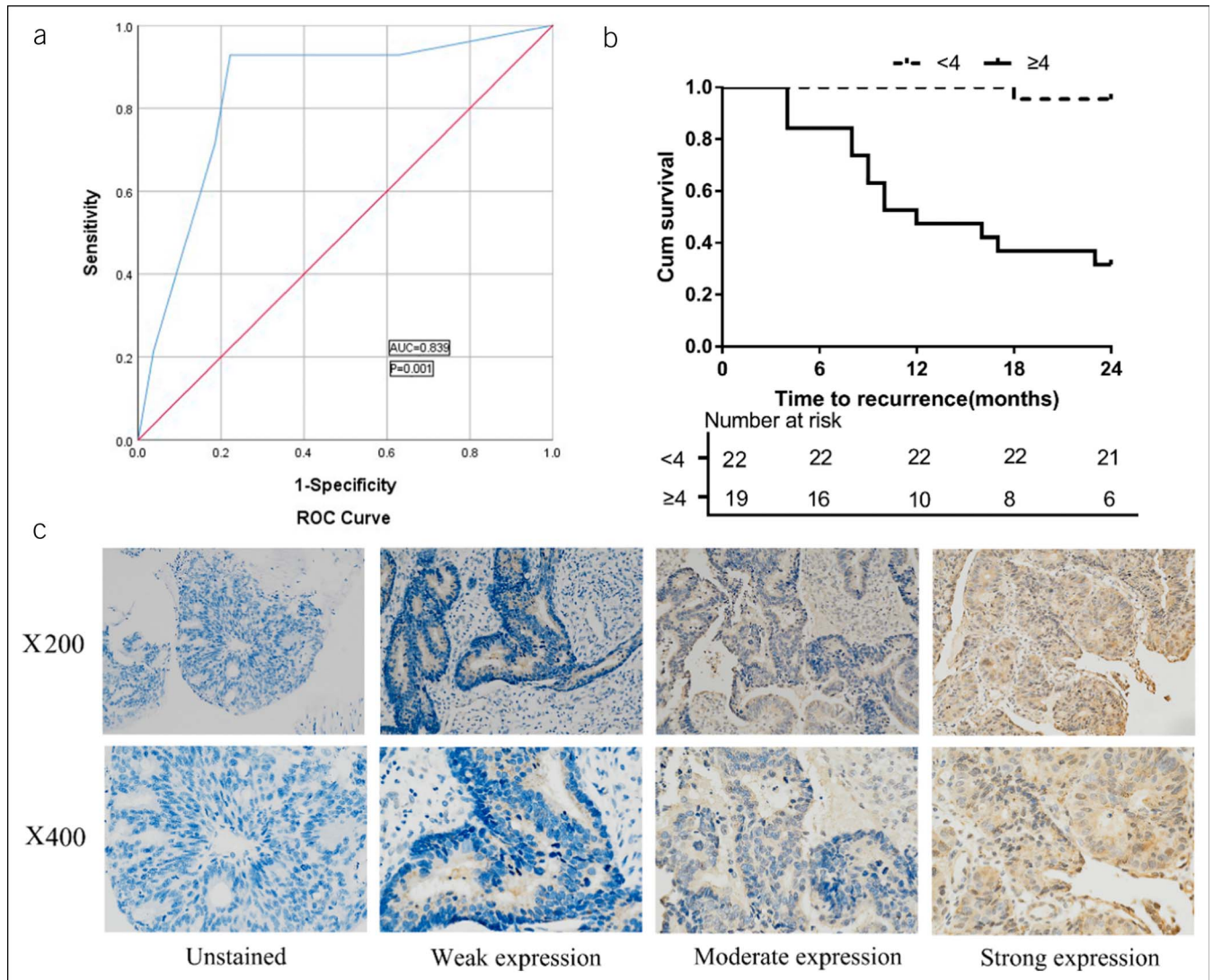


Figure 6. (a) ROC curve analysis to determine the optimal cutoff values for IHC staining index scores; 4 was defined as the cutoff point. Accordingly, scores ≥ 4 were judged as high PRL-3 expression and scores < 4 were categorized as low PRL-3 expression; Kaplan-Meier curves of univariate analysis data (log-rank test). (b) DFS curves of patients with PRL-3 expression in tumor tissues. ($P < 0.001$); Spearman analysis of the correlations of PRL-3 expression in tumor tissues and clinicopathological features. (c) IHC assay of PRL-3 expression in colorectal cancer tissues. Original magnification $\times 200$ or $\times 400$. Positive PRL-3 staining was observed mainly in colorectal cancer cell membranes and early endosomes. From left to right: unstained colorectal cancer tissue; representative images of weak PRL-3 staining in colorectal cancer tissues; representative images of moderate PRL-3 staining in colorectal cancer tissues; and representative images of strong PRL-3 staining in colorectal cancer tissues. DFS, disease-free survival; IHC, immunohistochemistry; PRL-3, phosphatase of regenerating liver-3; ROC, receiver operating characteristic.

research revealed that KCNN-4 channels participate in PRL-3-induced EMT through the c calcium/CaM-kinase II/GSK-3 beta pathway (19). Therefore, the mechanism by which PRL-3 induces EMT in cancer cells was clarified before. However, there are no or few well-known prognostic parameters in clinical practice. Based on this point, we combined M+ and PRL-3+ expression on the cell membranes of CTCs as a joint indicator to predict recurrence rates, which may potentially be an independent prognostic factor in CRC. Thus, our data were used to investigate the prognostic significance of this indicator in patients with CRC.

In the current study, we collected clinical data and detected the number of M+ and PRL-3+ CTCs from the peripheral blood (5 mL) of 156 patients before surgery. ROC curve analysis was applied to integrate various factors, including subtypes of CTCs, PRL-3, and

some fundamental indexes considered to be related to prognosis. We found that TNM stage, CEA index, M+ CTCs, M+ PRL-3+ CTCs, and lymph node status were associated with disease-free survival ($P < 0.05$). More importantly, the 2 largest areas under the curve were 0.838 and 0.696, reaching the boundary value of the evaluation and screening index. For predicting the 2-year DFS rate, the optimal cutoff values of these crucial indicators were evaluated separately and were applied in Kaplan-Meier analysis and Cox proportional hazards regression. The results showed that TNM stage and M+ PRL-3 CTCs were strong independent risk factors for relapse in the total population. Patients whose M+ PRL-3+ CTC count was over 2 had a significantly shorter median DFS than those who did not fulfill the criteria (8.5 vs 24 months, $P < 0.001$). From the above results, the study also showed that the count of M+ PRL-

Table 5. Spearman analysis of the correlation of PRL-3 expression in tissue and PRL-3 expression in CTCs

Variable	The status of PRL-3 expression	
	Spearman correlation	P value
Deep stromal invasion (T)	0.271	0.043
M+ PRL-3+ in CTCs	0.452	0.001
M+ in CTCs	0.383	0.014
TNM stage	0.272	0.085
CEA	0.300	0.057
Positive lymph node	0.276	0.045
Recurrence	0.566	<0.001

CEA, carcinoembryonic antigen; CTC, circulating tumor cell; PRL-3, phosphatase of regenerating liver-3.

3+ CTCs may be associated with TNM stage, which presents a positive correlation. When CRC cells progress until they break through the serous membrane or even metastasize, CTCs are more likely to have undergone EMT and express PRL-3. Therefore, this indicator may be an important aspect of distant metastases. A previous study investigated the prognostic value of dynamic CTC detection based on EMT markers in patients with breast cancer, and a proportion of M+ CTCs surpassing 10.7% was significantly associated with prognosis (14). Former metastatic breast cancer studies have supported that the proportion and count of M+ CTCs may be more appropriate for assessing outcomes than total CTC count (13,30,31). Consistent with the above studies, the count of M+ CTCs was correlated with poor clinical outcomes in patients with CRC. However, surprisingly, there were no significant values in the Cox proportional hazards regression for M+ CTCs, whereas there was significance in the Kaplan-Meier analysis. Overall, M+ PRL-3 CTCs have the potential to serve as a biomarker for assessing patients with a more aggressive form of disease. We speculate that mesenchymal phenotype and PRL-3 are both located in the cell membrane and may participate in EMT, promoting tumor cell differentiation and metastasis. A large cohort of patients with CRCs should be enrolled in future studies.

Some interesting findings were also revealed in that the number of M+ PRL-3+ CTCs and the CEA index detected preoperatively can predict postoperative pathological stages, as shown in Figure 3, with corresponding AUCs of 0.597 ($P = 0.044$) and 0.624 ($P = 0.01$), respectively. Currently, the TNM staging system is classified by the eighth edition of the American Joint Committee on Cancer guidelines as clinical TNM (cTNM) and pTNM. After the preliminary diagnosis of CRC, a clinical stage is mainly obtained according to the results of various auxiliary examinations including CT and nuclear magnetic resonance. cTNM can contribute to clinical decisions making before surgery, whereas its defect is the lack of information on the lymph node status. Moreover, M+ PRL-3 CTCs can assist in improving cTNM staging by simple detection in the peripheral blood.

The CEA index is currently most widely used in clinical diagnostics and in the evaluation of curative effects and the recurrence of patients with CRC; the strength of its recommendation is grade A, as indicated by Atkins et al. (32). Several studies revealed that CEA was an independent prognostic factor and, importantly, predicted prognosis in patients with stage II disease (33–36). Furthermore, compared with other available diagnostic

methods, sustained high CEA levels or the continuous elevation of CEA seems to be the most sensitive to detect early recurrence, especially for liver metastases (36,37). However, the clinical practical value of CEA is still controversial for certain benign diseases (38). Meanwhile, the numerical changes in CEA do not specifically indicate CRC; moreover, increased CEA levels have also been observed in patients with gastric carcinoma, esophageal SCC, and other cancers. In addition, the optimal cutoff level of CEA is still approximately disputed partially because of the different disease stages of patient groups (36). Therefore, it seems that CEA is not a complete indicator for predicting prognosis in CRC.

In our study, a nomogram was constructed based on 4 factors that were predictive of DFS: TNM stage, count of M+ CTCs (M), count of M+ PRL-3 CTCs (MP), and CEA. In our nomogram, TNM stage was the greatest contributor to the risk of DFS, followed by the count of M+ PRL-3+ CTCs (MP), the count of M+ CTCs (M), and CEA. Apparently, the proportion of MP is significantly larger than that of M, which is similar to CEA. In this case, we suspected that the joint indicator may have a stronger effect than any single indicator. However, the limitation of the nomogram is the lack of external validation, which warrants further investigation.

Several studies have indicated that PRL-3 expression is significantly correlated with lymph node and liver metastases in CRC. In addition, the high expression of the PRL-3 gene suggested that the possibility of organ metastasis and postoperative survival time were increased (39,40). The IHC analysis also demonstrated that PRL-3 expression was strongly associated with recurrence ($R = 0.566$, $P < 0.001$) as well as the following well-known prognostic parameters: CEA ($R = 0.300$; $P = 0.057$), deep stromal invasion ($R = 0.271$; $P = 0.043$), and TNM stage ($R = 0.272$; $P = 0.085$), providing evidence that PRL-3 plays an important role in CRC development. Interestingly, in our study, the status of M+ PRL-3+ in CTCs also correlated with PRL-3 expression in tumor tissue ($R = 0.452$; $P = 0.001$). Consequently, the status of M+ PRL-3+ in CTCs may serve as a biomarker for assessing patients with an aggressive form of the disease. Overall, our results indicate the status of M+ PRL-3+ in CTCs as a crucial contributing factor in tumor progression.

In summary, we hypothesize that the expression of M+ PRL-3+ in CTCs may serve as a crucial prognostic marker for predicting TNM stage before surgery and assessing clinical outcomes in CRC, which could aid surgeons in determining more appropriate therapeutic strategies through preoperative peripheral blood tests.

CONFLICTS OF INTEREST

Guarantor of the article: Zhonghua Chu, MD.

Specific author contributions: PengWei Su, Wei Lai, MD, and Lu Liu, MD, contributed equally to this work. Z.C.: designed the study. P.S.: collated the data, analyzed the data, and wrote the manuscript. W.L.: participated in the data analysis. L.L. and Y.Z.: contributed to drafting the manuscript. H.X. and Q.L. participated in the immunohistochemistry assays of. All authors have read and approved the final submitted manuscript.

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Potential competing interests: None to report

Study Highlights

WHAT IS KNOWN

- ✓ Colorectal cancer (CRC) incidence has increased significantly in recent years and a large proportion of patients die from metastases.
- ✓ As a liquid biopsy technique, circulating tumor cells (CTCs) have been widely used in breast cancer. However, the prognostic value of CTCs in CRC remains unknown.
- ✓ High expression of phosphatase of regenerating liver-3 (PRL-3) in tissue is strongly associated with poor prognosis of CRC.

WHAT IS NEW HERE

- ✓ We combined phenotypes of peripheral blood circulating tumor cells and PRL-3 as a new prognostic factor in CRC.
- ✓ When the number of mesenchymal CTCs with PRL-3 (M+ PRL-3+ CTC) in colorectal patient is equal or greater than 2, the risk of recurrence is 3.6 times than that of negative patient.
- ✓ The prediction performance of M+ PRL-3+ CTC is better than that of M+ CTC and carcinoembryonic antigen.
- ✓ The number of M+ PRL-3+ CTC in colorectal patient is positively correlated with the expression of PRL-3 in cancer tissues.

TRANSLATIONAL IMPACT

- ✓ The number of M+ PRL-3+ CTC has important clinical value in predicting the recurrence of colorectal cancer patients.

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