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A retrospective cohort study of clinical value of *PRL-3* in stage III human colorectal cancer

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Abstract

The aim of this study was to investigate the expression of phosphatase of regenerating live-3 (*PRL-3*) in human stage III colorectal cancer (CRC) and to evaluate its correlation with metachronous liver metastasis (MLM) and prognosis.

The retrospective cohort study included 116 stage III CRC primary tumors and 60 normal colorectal tissues. *PRL-3* expression was measured by immunohistochemistry. We investigated the correlation of *PRL-3* with clinicopathologic features by the chi-square test. The association of *PRL-3* expression with MLM was assessed by binary logistic regression. Overall survival (OS) and disease-free survival (DFS) between patients with positive *PRL-3* expression and those with negative *PRL-3* expression were compared by the Kaplan–Meier method and Cox proportional hazards regression model.

We found that 32.8% of stage III CRC primary tumors were *PRL-3* positive, and 15.0% of normal colorectal epithelia showed high *PRL-3* expression ($P=.012$). Seventeen tumors (47.2%) among 36 cases that developed MLM were *PRL-3* positive, and only 21 tumors (26.3%) in the 80 cases that did not develop MLM had positive *PRL-3* expression ($P=.026$). *PRL-3* expression was associated with MLM ($P=.028$). Patients with positive expression of *PRL-3* showed a significantly shorter OS (40.32 ± 3.97 vs 53.96 ± 2.77 months, $P=.009$) and DFS (34.97 ± 4.30 vs 44.48 ± 2.89 months, $P=.036$). A multivariate analysis indicated that *PRL-3* expression was an independent unfavorable prognostic factor for OS ($P=.007$).

Our study suggested that high *PRL-3* expression is an independent risk factor for MLM and poor prognosis. *PRL-3* is expected to be a promising biomarker for predicting the incidence of MLM and prognosis in patients with stage III CRC.

Abbreviations: CEA = carcinoembryogenic antigen, CRC = colorectal cancer, DFS = disease-free survival, MLM = metachronous liver metastasis, OS = overall survival, PRL = phosphatase of regenerating live.

Keywords: metachronous liver metastases, *PRL*-3, prognosis, stage III CRC

1. Introduction

Over the last few decades, colorectal cancer (CRC) incidence and mortality have increased dramatically in many regions.[1–4] In 2018, nearly 2.0 million newly diagnosed CRC cases and more than 0.8 million related deaths were expected to occur worldwide.[5] Of the CRC patients, hepatic metastases are present in 15% to 25% of patients at the time of diagnosis, and

another 25% to 50% of patients develop liver metastases within 3 years following resection of the primary tumor; eventually, up to 50% of these patients die of liver metastases.[6] When liver metastases are curatively resected, the 5-year survival is 60%.[7] However, only 10% to 25% of patients with colorectal liver metastases have a possibility of liver resection.[8] To improve the prognosis of CRC, it is important to select high-risk patients who have suffered metachronous metastases, especially liver metastases, and subsequently treat them with postoperative adjuvant therapy. These patients should be followed-up closely to detect and resect early metastases.[9,10] Therefore, more reliable biological markers are required for early diagnosis, prognosis and follow-up.

In various signal transduction pathways, protein tyrosine phosphatases act as key regulatory enzymes. The phosphatase of regenerating live (*PRL*) represents a novel subfamily of protein tyrosine phosphatases, which comprises 3 members (*PRL-1*, *PRL-2*, and *PRL-3*) sharing a high degree (>75%) of amino acid sequence identity.[11] Many studies have shown that *PRLs* are involved in regulating cell proliferation, oncogenic transformation, migration and metastasis.[12–14] *PRL-1* was originally identified as an immediate early gene with its expression induced in regenerating liver.[15] Overexpression of *PRL-1* and *PRL-2* is associated with tumor cell migration and invasion.[16,17] The *PRL-3* gene, also known as PTP4A3, is located on chromosome 8q24–3. Saha et al[18] reported that *PRL-3* overexpression is detected in all liver metastases and acts as the first link with the metastasis of human cancer. Increasing evidence has further demonstrated that *PRL-3* is associated with tumor proliferation

and invasion, particularly transfer in different types of cancer.[19–24] Using in situ hybridization, Kato et al studied 177 primary colorectal tumors and reported that a significantly higher proportion of the primary tumors from patients with these metastases (liver, 84.4%; lung, 88.9%) had elevated *PRL-3* expression than those without (liver, 35.9%; lung, 42.3%).[25] The *PRL-3* expression levels in primary colorectal tumors contribute to the prediction of liver or lung metastasis development. Similarly, Kim et al found that *PRL-3* over-expression in primary tumors significantly correlates with the development of liver metastases.[26]

We hypothesized that the levels of *PRL-3* expression are elevated in patients with CRC primary tumors that developed metachronously. Hence, *PRL-3* may act as a useful molecular marker for predicting the incidence of metachronous liver metastasis. In the present study, we investigated the expression of *PRL-3* in stage III CRC primary tumors and in normal colorectal epithelia. We also analyzed its relationship with other clinico-pathologic factors and survival, and we assessed whether *PRL-3* can be applied as a prognostic indicator for CRC metachronous liver metastases.

2. Materials and methods

2.1. Patients and tissue specimens

The retrospective cohort study was comprised of patients with completely resected stage III CRC who underwent surgery at the Affiliated Ganzhou Hospital of Nanchang University between January 2014 and December 2017. Patients who received chemotherapy or radiation therapy before surgery were excluded from this study. All patients received postoperative chemotherapy and were followed up at regular intervals of 6 months after surgery. The overall survival (OS) time was calculated from the date of surgery until the date of last visit or death and the disease-free survival (DFS) time from the date of resection until relapse. Metachronous liver metastasis refers to liver metastases that appeared more than 6 months after resection of the primary tumor. The data collected were entered into the registry database.

End points were local recurrence for DFS and cancer-related death or last follow-up for OS.

Tissues of primary tumors and adjacent normal colorectal mucosa epithelia (at least 3 cm distant from the tumor edge) were fixed in formalin, routinely processed and embedded in paraffin. Tumor stage was classified according to TNM staging.

Informed consent was obtained from all the patients, and the Medical Ethics Committee of Ganzhou People's Hospital approved the collection of case data for this clinical retrospective study.

2.2. Immunohistochemical analysis

For immunohistochemical studies, 5-μm sections were cut from paraffin blocks and incubated at 50 to 60°C overnight. The paraffin sections were then dewaxed with xylene and rehydrated through a graded alcohol series. After treatment with 3% hydrogen peroxide solution, sections were incubated for 10 minutes at room temperature to block endogenous peroxidase activity. The sections were blocked with 1% bovine serum albumin for 20 minutes and subjected to a 10-minutes microwave pretreatment in 0.01 M citrate buffer. Sections were then incubated with PRL-3 mono-clonal antibody 318 (Santa Cruz Biotechnology, USA) at 4°C overnight in a humidified chamber followed by incubation with a

second antibody from Supervision™ (Changdao Biotechnology, Shanghai, China) for 30 minutes at room temperature. For each step, sections were washed twice for 5 minutes with phosphate-buffered saline. The reaction product was visualized with diaminobenzidine (DAB-Kit Changdao Biotechnology, Shanghai, China) for 5 minutes at room temperature, and sections were counterstained with hematoxylin.

For the negative control, the primary antibody was omitted from the reaction sequence. The positive sections of liver metastasis from colon cancer were used as positive controls. Cytoplasm and cytoplasmic membrane were evaluated through staining. Tissue samples were estimated in a consecutive analysis to ensure maximal internal consistency. The analysis was assessed according to both the percentage of positive cells and the intensity of the cytoplasmic staining in ten randomly chosen microscopic fields. Assays were scored as negative if <10% of tumor cells were stained and positive if ≥10% of tumor cells were stained. The staining intensity was classified using the following scale: no staining or staining observed in <10% of tumor cells, 0; weak staining detected in ≥10% of tumor cells, 1+; and moderate or strong complete staining observed in ≥10% of tumor cells, 2+ or 3+. A score of 0 or 1+ was considered negative, whereas a score of 2+ or 3+ was considered positive. Two experienced pathologists without any knowledge of the clinical data evaluated the immunoreactivity of *PRL-3*.

2.3. Statistical analysis

All statistical analyses were performed with the IBM SPSS statistical software package 24.0 (SPSS, Inc., Chicago, IL). The Chi-Squared test was used to determine the statistical significance of the rate difference and investigate the association between *PRL-3* expression and clinicopathologic characteristics. Survival curves were estimated using the Kaplan–Meier method and compared with the log-rank test. The association between the incidence of metachronous liver metastasis and clinicopathologic indicators was evaluated by binary logistic regression. We also evaluated the association between overall survival and clinicopathologic parameters by Cox univariate and multivariate proportional hazard models. $P < .05$ was considered statistically significant.

3. Results

3.1. General data of patients and tissue specimens

A total of 116 patients with completely resected stage III CRC who underwent surgery at the Affiliated Ganzhou Hospital of Nanchang University between January 2014 and December 2017. The median follow-up time was 42.0 (range of 6–72) months. There were 69 men and 47 women with an average age of 59.0 (range of 25–87) years. Among the 116 patients, 36 patients eventually developed metachronous liver metastasis, and 80 patients did not develop metachronous liver metastasis.

Tissues of 116 CRC primary tumors and 60 adjacent normal colorectal mucosa epithelia were obtained from the Department of Pathology at the Affiliated Ganzhou Hospital of Nanchang University.

3.2. PRL-3 protein expression and its association with clinicopathological parameters

We investigated the expression of *PRL-3* in 176 colorectal samples by immunohistochemistry, and the frequency of *PRL-3*

PRL-3 expression in primary CRC and adjacent normal tissues.

Tissue type	Number	PRL-3 staining		Positive rate (%)	x^2	P value
		negative	positive			
CRC	116	78	38	32.8	6.372	.012
Normal colorectal tissue	60	51	9	15.0		

CRC = colorectal cancer, PRL-3 = phosphatase of regenerating live-3.

expression is listed in Table 1. As shown in Figure 1, *PRL-3* immunostaining was mainly localized in the cytoplasm of normal or tumor epithelial cells. In 60 cases of normal colorectal tissues, 9 cases (15.0%) were *PRL-3* positive, the *PRL-3* in the paired tumor specimens of the 9 cases were also positive. Among the 116 primary tumor specimens, 38 (32.8%) tumors had positive *PRL-3* expression. Therefore, *PRL-3* expression was significantly higher in stage III CRC primary tumor tissues than in normal colorectal tissues (P=.012). We investigated the association between *PRL-3* expression and clinical characteristics in 116 stage III CRC primary tumors (Table 2). High expression of *PRL-3* was correlated closely with the depth of invasion (P=.029) and metachronous liver metastases (P=.026). There was no relationship of *PRL-3* expression with other factors, such as age, tumor location and size, tumor differentiation or preoperative carcinoembryogenic antigen (CEA).

3.3. A high level of PRL-3 expression in colorectal primary
3.3.1. Tumors is associated with MLM.
In 116 cases of stage III CRC primary tumor tissues, 36 cases eventually developed MLM,

and 80 cases did not develop MLM. Seventeen tumors (47.2%) among the 36 cases that developed MLM were *PRL-3* positive, and in the 80 cases that did not develop MLM only 21 tumors (26.3%) had positive *PRL-3* expression. There was a significant difference between the 2 groups (P=.026; Table 2). We analyzed *PRL-3* expression and several clinicopathologic factors in MLM with the help of binary logistic regression. *PRL-3* expression and preoperative CEA were found to be predictors of distant MLM in univariate analysis (P=.028 and .006, respectively; Table 3). High levels of *PRL-3* and preoperative serum CEA were 2 independent predictors of metastasis (P=.041 and .007, respectively; Table 3). Tumors were then classified according to positive or negative *PRL-3* expression, and the former showed a 2.583-fold average increase in MLM (P=.041, Table 3).

3.4. High expression of PRL-3 in primary stage III CRC correlates with worse survival

The Kaplan–Meier survival curves are shown in Figure 2. Patients with positive expression of *PRL-3* showed a significantly shorter OS [40.32±3.97 months (95% CI, 32.53–48.10) vs 53.96±2.77
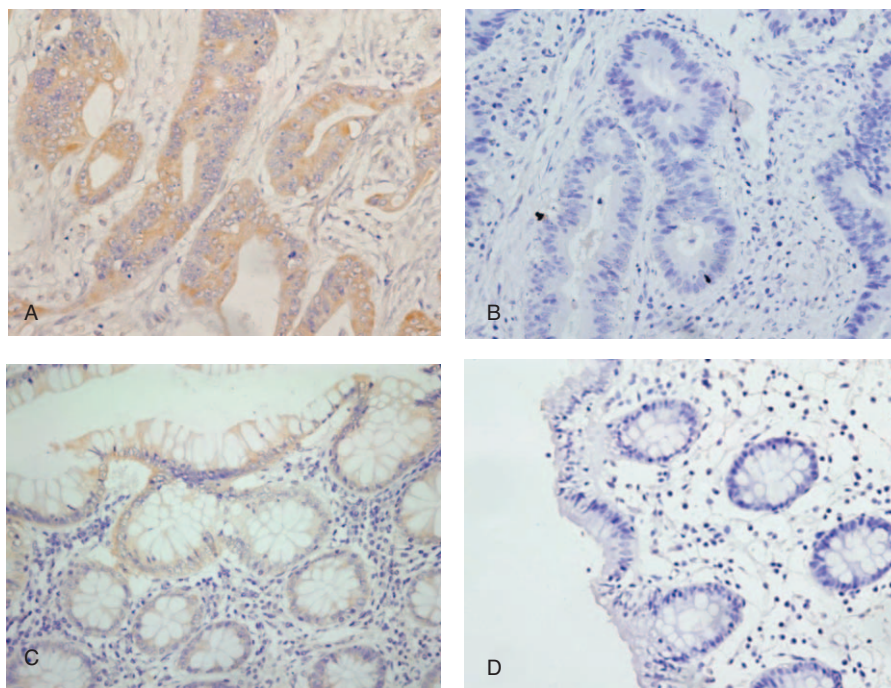
Figure 1. Expression of *PRL-3* protein in human Dukes' C CRC tissues. (A). Positive staining of *PRL-3* in primary tumors (magnification 400×). (B). Negative staining of *PRL-3* in primary tumors (magnification 400×). (C). Positive staining of *PRL-3* in normal mucosal epithelia (magnification 400×). (D). Negative staining of *PRL-3* in normal mucosal epithelia (magnification 400×). CRC = colorectal cancer, PRL = phosphatase of regenerating live.

Table 2

Correlation between *PRL-3* staining and clinicopathologic factors.

| Variable | Number | PRL-3 staining | | Positive rate (%) | x^2 | P value |
		Negative	Positive			
Age						
≤64 yr	78	53	25	32.1	0.054	.816
>64 yr	38	25	13	34.2		
Gender						
Male	69	51	18	26.1	3.441	.064
Female	47	27	20	42.6		
Location						
Colon	43	27	16	37.2	0.614	.433
Rectum	73	51	22	30.1		
Tumor size						
≤5 cm	74	47	27	36.5	0.363	.547
>5 cm	42	29	13	31.0		
Tumor differentiation						
Well/moderate	90	63	27	30.0	0.201	.654
Poor	26	17	9	34.6		
Depth						
Serosa negative	30	25	5	16.7	4.757	.029
Serosa positive	86	53	33	38.4		
Preoperative CEA (ng/ml)						
≤10	85	57	28	32.9	0.066	.798
>10	31	20	11	35.5		
Metachronous metastases						
Absent	80	59	21	26.3	4.957	.026
Present	36	19	17	47.2		

CEA = carcinoembryogenic antigen, *PRL-3* = phosphatase of regenerating live-3.

months (95% CI, 48.53–59.38), respectively; log-rank test, $P = .009$; Fig. 2A] and DFS [34.97 ± 4.30 months (95% CI, 26.54–43.40) vs 44.48 ± 2.89 months (95% CI, 38.80–50.16), respectively; log-rank test, $P = .036$; Fig. 2B]. To elucidate the prognostic factors for stage III CRC patients, we analyzed *PRL-3* expression and several clinicopathologic factors on OS in Cox regression. A univariate analysis showed that there was no significant association between OS and other clinicopathologic factors, such as age, sex, tumor location, tumor size or depth of invasion. Tumor differentiation, preoperative CEA and PRL-3 expression were related to OS ($P = .006$, .000 and .012, respectively; Table 4). A multivariate analysis of these 3 variables indicated that *PRL-3* expression, tumor differentiation and preoperative CEA could independently affect OS ($P = .007$, .009 and .000, respectively; Table 4).

4. Discussion

As a phosphatase, *PRL-3* has a catalytic active signature motif (C104S).[27,28] Stable expression of wild-type active *PRL-3* dramatically enhances cell migration and vice versa with the *PRL-3* (C104S) mutant.[29] These results indicate that the ability of *PRL-3* to promote cell migration depends on its phosphatase activity. Recently, many studies have shown that high expression of *PRL-3* is associated with invasion, metastasis and poor prognosis in colorectal cancer and gastric cancer.[9,30–33] In the present study, we analyzed the expression of *PRL-3* by immunohistochemistry in stage III CRC primary tumors and normal mucosal epithelia. *PRL-3* positive expression was detectable in 15.0% (9/60) of normal colorectal tissues, while 32.8% (38/116) of CRC primary tumor tissues showed positive expression of *PRL-3*. Our immunohistochemical examination

Table 3

Influential factors for metachronous liver metastases in binary logistic regression.

| Parameter | Univariate | | | | Multivariate | | | |
| | P | HR | 95% CI | | P | HR | 95% CI | |
			Lower	Upper			Lower	Upper
Age (≤64 yr vs >64 yr)	.606	1.243	0.543	2.847				
Gender(Male vs Female)	.325	1.491	0.673	3.304				
Location(Colon vs Rectum)	.785	0.894	0.397	2.009				
Tumor size(≤5 cm vs >5 cm)	.488	0.729	0.299	1.779				
Tumor differentiation(Well/moderate vs Poor)	.359	0.621	0.224	1.718				
Depth(Serosa negative vs Serosa positive)	.293	1.672	0.642	4.352				
Preoperative CEA(≤10 ng/ml vs >10 ng/ml)	.006	3.824	1.476	9.903	0.007	3.793	1.436	10.022
PRL-3 expression(negative vs positive)	.028	2.514	1.105	5.721	0.041	2.583	1.038	6.432

CEA = carcinoembryogenic antigen, CI = confidence interval, HR = hazard ratio, *PRL-3* = phosphatase of regenerating live-3.
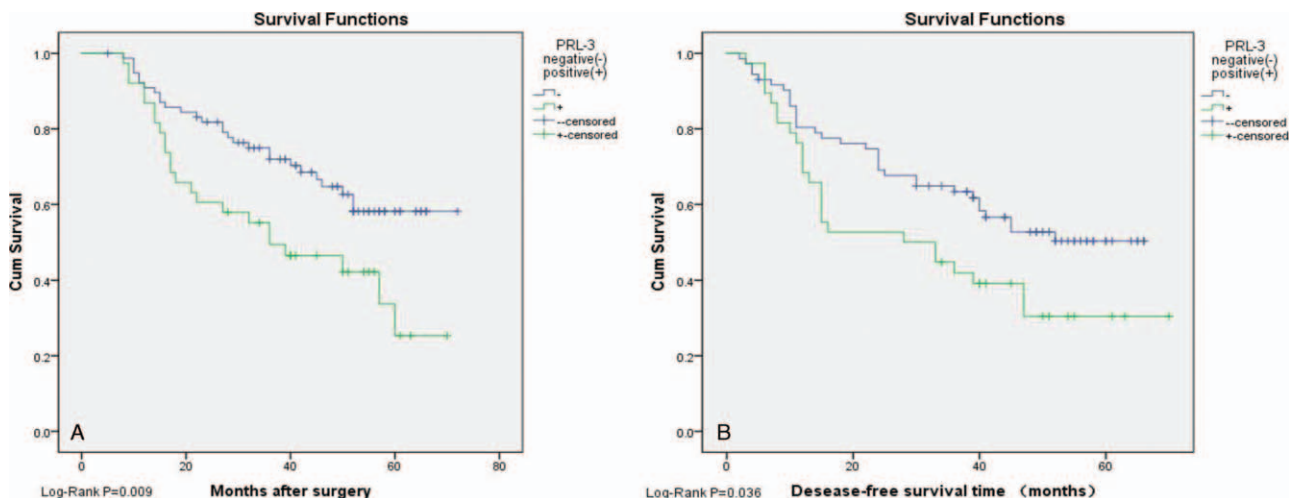
Figure 2. Kaplan–Meier estimates for OS and DFS time according to *PRL-3* expression in Dukes' C CRC primary tumors. (A). OS time stratified by *PRL-3* expression, log-rank test, *P* = .009, (B). DFS time stratified by *PRL*-3 expression, log-rank test, *P* = .036. The results indicated that *PRL-3*-positive tumors are associated with a worse prognosis than *PRL-3*-negative tumors for patients with colorectal cancer. CRC = colorectal cancer, DFS = disease-free survival, OS = overall survival, PRL = phosphatase of regenerating live.

revealed that *PRL-3* expression was significantly higher in CRC primary tumors than in normal colorectal tissues (*P* = .012). Similarly, Tamagawa et al measured the expression level of *PRL-3* mRNA in primary CRC and in the normal adjacent mucosa from 202 patients using quantitative real-time reverse-transcriptase polymerase chain reaction (PCR) and showed that the expression of *PRL-3* is higher in cancer tissues than in the adjacent normal mucosa.[30] Hatate et al showed high *PRL-3* expression in 60% (18/30) of patients with stage III primary colorectal tumors,[19] and Nakayama et al reported that 27.4% of 109 primary colorectal cancer patients are *PRL-3* positive.[9]*PRL-3* expression has also been detected in other human tumors. According to Vandsemb et al, the expression of *PRL-3* in 116 human prostate cancers and 40 normal prostate tissues demonstrated that *PRL-3* expression is significantly higher in prostate cancer than in normal prostate tissues (*P* < .001).[21]

We investigated the relationship between *PRL-3* expression in CRC primary tumors and clinicopathological factors. Among the factors, depth of invasion (*P* = .029) and MLM (*P* = .026) were associated with high *PRL-3* expression in the primary tumors. High *PRL-3* expression was more frequently detected in the

primary CRC tumors of invasive serosa, suggesting that *PRL-3* might play an important role in the invasion of primary CRC. Hatate et al observed high expression of *PRL-3* in primary CRC tissue and reported that it has a close association with depth of invasion (*P* = .0002).[19] In a meta-analysis, Hu et al showed an association between *PRL-3* overexpression and its clinical outcome, and they revealed that *PRL-3* overexpression is significantly associated with the depth of invasion (OR = 2.03; 95% CI = 1.38–2.98; *P* = .001) and vascular invasion.[33]

A portion of CRC patients eventually die from metachronous metastasis, especially liver metastasis. In stage III CRC, approximately 70% of patients develop distant metastases, mainly confined to the liver, during the course of 2 or 3 years following resection of their primary tumor.[34] Therefore, searching for new prognostic indicators that identify patients at high risk of MLM is extremely important. We analyzed the relationship between *PRL-3* expression in CRC primary tumors and MLM. Our results indicated that *PRL-3* is a promising biomarker for predicting stage III CRC patients who are at an increased risk of MLM. In 80 patients with stage III CRC, Mollevi et al reported that 28 out of 38 primary tumors (73.7%)

Table 4

Prognostic factors for overall survival in Cox proportional hazards model.

Parameter	Univariate				Multivariate			
			95% CI				95% CI	
	P	HR	Lower	Upper	*P*	HR	Lower	Upper
Age(≤64 yr vs >64 yr)	.502	1.218	0.685	2.165				
Gender(Male vs Female)	.073	1.654	0.955	2.865				
Location(Colon vs Rectum)	.559	0.847	0.485	1.479				
Tumor size(≤5 cm vs >5 cm)	.955	0.982	0.527	1.831				
Tumor differentiation(Well/moderate vs Poor)	.006	2.316	1.276	4.203	0.009	2.445	1.247	4.791
Depth(Serosa negative vs Serosa positive)	.111	1.755	0.879	3.505				
Preoperative CEA(≤10 ng/ml vs >10ng/ml)	.000	3.627	1.932	6.810	0.000	3.644	1.921	6.913
PRL-3 expression(negative vs positive)	.012	2.040	1.173	3.548	0.007	2.336	1.256	4.345

CEA = carcinoembryogenic antigen, CI = confidence interval, HR = hazard ratio, *PRL*-3 = phosphatase of regenerating live-3.

displayed high *PRL-3* expression and eventually developed metachronous liver or lung metastases but that only 9 out of 42 patients (21.4%) showed low *PRL-3* expression ($P = .000$). These results indicate that a high level of *PRL-3* is the only independent predictor of metastasis ($P = .0001$).[24] Kato et al demonstrated that postoperative development of occult liver and/or lung metastasis appeared in 14 of 104 cases (13.5%). Metastasis-free survival analysis showed that patients with high *PRL-3* expression had a greater risk for metachronous metastasis than those with low *PRL-3* expression ($P < .0001$).[25] However, Hatate et al held the opposite view as they believed that PRL-3 expression has no value in predicting MLM in stage III CRC patients. These researchers examined the relationship between *PRL-3* expression and MLM in 30 stage III CRC patients, and they found MLM in 16 cases (53%) during a follow-up course. The *PRL-3* expression patterns were subdivided into intense/weak/none to faint immunostaining of *PRL-3* in the absence and presence of MLM in 9/2/3 cases and 9/4/3 cases, respectively. These results showed no significant difference between the 2 groups.[19] The reason why they did not expect a good potential of PRL-3 expression predicting MLM may have been based on a small sample size of stage III patients (n = 30) (Supplemental Digital Contents, http://links.lww.com/MD2/A103).

Our survival curve analysis showed that the OS and DFS times of patients with *PRL-3*-positive tumors were shorter than those of patients with *PRL-3*-negative tumors. Our results indicated that *PRL-3* expression is closely associated with OS and demonstrated that *PRL-3* positive expression in primary CRC tumors is a significant independent risk factor for OS ($P = .007$). The present study suggested that *PRL-3* might serve as a novel prognostic factor in stage III CRC, which may help to predict an adverse disease outcome. Mollevi et al reported that high *PRL-3* expression is associated with poor OS and is the only independent predictor of survival in their selected cases of 80 stage III colorectal carcinomas.[24] Similar results indicating *PRL-3* as a valuable prognostic marker in colorectal carcinoma, gastric cancer, prostate cancer and breast cancer have been observed.[21,30,32,35] Thus, high expression of *PRL-3* in primary tumors likely implies an advanced grade of disease.

In recent years, *PRL-3* has been assessed as a potential therapeutic target for tumors. Knockdown of endogenous *PRL-3* by siRNA or *PRL-3* inhibitors, such as farnesyltransferase inhibitors, suppresses metastatic properties.[36–38] Although little is known about *PRL-3* action, elucidation of the molecular mechanisms of *PRL-3* may provide an attractive therapeutic target for colorectal cancer control.

Our study had several limitations. First, our study involved a single center and a relatively small number of patients, which may lead to admission rate bias. Second, this was a retrospective study performed using electronic medical records that may have the potential for information bias. Third, some parameters, such as body mass index and smoking, were not available in the electronic medical records. Therefore, a multicenter, prospective randomized controlled study is required to evaluate the metastatic and prognostic value of *PRL-3* in stage III human colorectal cancer.

In conclusion, our study suggested that *PRL-3* was overexpressed in stage III CRC primary tumors compared with normal colorectal tissues. Additionally, colorectal primary tumors eventually developed MLM, which showed high *PRL-3* expression. High *PRL-3* expression was indicated as an independent risk factor for MLM and poor prognosis. *PRL-3* is expected to be a promising biomarker for predicting the incidence of MLM and prognosis in patients with stage III CRC. Histochemical detection of *PRL-3* expression after curative surgery for primary colorectal cancer may contribute to identifying which patients are at increased risk of MLM to impose postoperative adjuvant therapy and close follow-up.

Author contributions

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