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Original Article/Liver

Retinoblastoma binding protein 4 up-regulation is correlated with hepatic metastasis and poor prognosis in colon cancer patients

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ABSTRACT

Background: Retinoblastoma binding protein 4 (RBBP4) plays an essential role in the development of multiple cancers. However, its relationship with prognosis in colon cancer and colon cancer hepatic metastasis has not been elucidated. The aim of this study was to explore the relationship between RBBP4 expression and prognosis of colon cancer patients and to evaluate RBBP4 as a new prognostic marker in these patients.

Methods: Eighty colon cancer patients underwent surgical resection of the colon were enrolled. Among them, forty colon cancer patients suffered with hepatic metastasis. The colon cancer tissues, para-colon cancer tissues, and hepatic metastatic cancer tissues were collected from the pathological department for further analysis. The expression of RBBP4 proteins was examined by immunohistochemistry and correlated with clinicopathological parameters. The Cancer Genome Atlas (TCGA) database was used to validate the expression and explore its relationship with clinical characteristics.

Results: RBBP4 was up-regulated in the colon cancer tissues compared with the para-colon cancer tissues. The analysis of TCGA database verified the upregulation of RBBP4 in the colon cancer tissues and RBBP4 overexpression was correlated with nerve invasion and poor outcomes of chemotherapy. Moreover, the positive rate of RBBP4 expression in 40 colon cancer patients with hepatic metastasis was higher in the hepatic metastatic cancer tissues (39/40, 97.5%) than in the colon cancer tissues (26/40, 65.0%). Our clinicopathological analysis showed that RBBP4 expression was significantly correlated with vascular invasion, hepatic metastasis, and lymph node involvement (all $P < 0.05$). Additionally, the survival analysis demonstrated that RBBP4 over-expression was correlated with poor prognosis.

Conclusions: RBBP4 was upregulated in the colon cancer. RBBP4 may be a novel predictor for poor prognosis of colon cancer and colon cancer hepatic metastasis.

Introduction

Colon cancer is one of the most common leading causes of cancer-related deaths worldwide. Hepatic metastasis is the main reason of colon cancer caused death, and 5-year overall survival remains poor (only 25%–40%). According to the WHO mortality database, almost half of colon cancer patients were companied with hepatic metastasis [1,2]. Despite the progression of treatment strategies, including systemic chemotherapy and targeted therapies, hepatectomy is still the most effective and potentially curative method for colon cancer patients with hepatic metastasis, which has the best tumor response rates and overall survival [3,4]. The documentations show that many colon cancer patients with liver metastasis who underwent surgery eventually died, which indicated that surgery alone is not enough in many colon cancer patients with hepatic metastasis [5–7]. Therefore, it is essential to investigate the biological mechanism of liver metastasis of colon cancer and find the new therapeutic strategies. Early detection and treatment of colon cancer hepatic metastasis is useful for prolonging patient's survival. There is an urgent need to identify molecules that are correlated with the metastasis of colon cancer to the liver and to explore the potential therapeutic targets for these patients.

Retinoblastoma binding protein 4 (RBBP4), initially defined for its capability to bind to a putative functional domain of retinoblastoma protein Rb [8,9], is a member of several histone deacetylase (HDAC) complexes involved in chromatin remodeling, such as the nucleosome remodeling and deacetylation (NuRD) complex [10], the Sin3 complex [11], and the chromatin assembly factor 1 (CAF-1) complex [12]. RBBP4 has been reported to be

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involved in the development of several cancers. RBBP4 is strongly overexpressed in human thyroid cancer, and is a target of nuclear factor-kappa B activity in thyroid cancer [13]. When RBBP4 is transfected into breast cancer cells, it regulates cellular morphology and cytoskeletal organization by increasing K-Ras activity and signaling through MAPK [14]. However, only one study showed that RBBP4 may be a biomarker of colorectal cancer [15]. The present study aimed to explore the relationship between RBBP4 expression and clinicopathological features of colon cancer, and to evaluate whether RBBP4 is a new prognostic marker in colon cancer patients with or without hepatic metastasis.

Methods

A total of 80 colon cancer patients underwent surgical resection in the Department of Colon & Rectal Surgery, the First Affiliated Hospital, Zhejiang University School of Medicine from January 1, 2009 to December 31, 2014 were enrolled. The study protocol was approved by the Institutional Ethics Committee of the hospital.

Patients were enrolled, if: (1) age greater than or equal to 18 years; (2) For colon cancer patients with hepatic metastasis, the synchronous metastasis confined to the liver as assessed by preoperative radiological imaging assessment and confirmed by surgery at the time of initial diagnosis; (3) For colon cancer patients without hepatic metastasis, the primary colon tumor was verified by preoperative radiological imaging assessment, confined to colon without adjacent and distant tissue invasion, and confirmed by surgery at the time of initial diagnosis; (4) No other chronic diseases, including severe diabetes, hypertension, coronary artery disease, and chronic pulmonary disease.

The colon cancer tissues, para-colon cancer tissues and hepatic metastatic cancer tissues were collected from the pathological department for further analysis. Sections were reviewed by two experienced pathologists. The locations of tumors and distant metastases were determined by computerized tomography (CT), magnetic resonance imaging (MRI), and colonoscopy. All the patients were followed up every three months with a total follow-up of 60 months.

Immunohistochemistry

The selected specimen of colon cancer, para-colon cancer and hepatic metastatic cancer tissues were fixed in 10% neutral-buffered formalin and embedded with paraffin. After being cut, dewaxed and rehydrated, 5 μm sections were subjected to antigen retrieval. The endogenous peroxidase activity was blocked with H_2O_2, and the tissue sections were incubated with the primary antibody (1:100 diluted) against RBBP4 overnight at 4 °C. The streptavidin-biotin peroxidase complex method was used (Lab Vision, Fremont, CA, USA). The prepared slides were examined by two experienced pathologists, and were analyzed and photographed using a BX-60 Olympus microscope attached to a digital camera (Olympus, Tokyo, Japan). Sections stained positively were incubated in each batch, while the negative controls were prepared by replacing the primary antibody with preimmune serum.

The RBBP4 expression was evaluated by staining intensity and the percentage of positive staining cells using the Image-Proplus (IPP) 6.0 software (MEDIA CYBERNETICS Inc., Maryland, USA). Staining intensity was divided into four grades according to the IPP scores: 0 = no staining of cancer cells, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. The percentage of positive cells was scored as follows: 0 = 0%, 1 = 1%–25%, 2 = 26%–50%, and 3 = >50%. The formula was adopted for determining RBBP4 staining positivity: overall score = positive percentage score × intensity score. A score of 0 was defined as "0", 1–2 as "1", 3–6 as "2", and 7–9 as "3". Tumor samples with level 0 or 1 were defined as negative for expression, whereas level 2 or 3 defined as positive [12].

Western blot

The colon cancer tissues and para-colon cancer tissues were lysed in RIPA lysis buffer with 1 mmol/L PMSF (Beyotime, Shanghai, China) for 1 h in ice. Sample proteins were quantified using a Pierce BCA protein assay (Thermo Scientific, Waltham, MA, USA). Equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membranes, and probed with the antibody of interest. The primary antibody RBBP4 and GAPDH (Abcam, Cambridge, MA, USA) concentration was 1:1000, and the corresponding secondary antibody (Abcam) concentration was 1:2000.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Total RNA (0.5 μg) was reversed to cDNA. SYBR Green PCR Master mix (Takara, Dalian, China) was used to determine the mRNA level of RBBP4. All reactions were performed in triplicate. The relative expression of RBBP4 was respectively normalized to the internal reference GAPDH. Data were analyzed using the $2^{\Delta\Delta Ct}$ method. The primers used in the study were as follows: GAPDH forward primer: 5′-ATG GGG AAG GTG AAG GTC G-3′, GAPDH reverse primer: GGG GTC ATT GAT GGC AAC AAT A; RBBP4 forward primer: GCT ATG GGC TTT CTT GGA, RBBP4 reverse primer: CAC AGG CAG ATG GTA TGG.

The cancer genome atlas analysis

The cancer genome atlas (TCGA) database (http://gdc-portal.nci.nih.gov/) were used to validate the expression of RBBP4 in colon cancer. TCGA-COAD cohort consisted of 471 primary colon cancers and 41 normal samples was downloaded and analyzed.

Statistical analysis

The statistical package SPSS version 10.00 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Comparisons between three groups were analyzed by Kruskal-Wallis test. Chi-square test or Fisher's exact test was used to analyze the correlation between RBBP4 expression and various clinicopathological variables. Survival curves were drawn with the Kaplan-Meier method and compared with log-rank test. A P value of < 0.05 was considered statistically significant.

Results

Patient characteristics

Among the 80 colon cancer patients, 51 patients were male (63.7%) and 29 (36.3%) were female. The median age of the patients was 58.6 years (range 38–72 years). Of them, 40 patients received colonic resection only, and 40 received colonic and hepatic resections because of hepatic metastasis. All the patients did not receive radiotherapy, chemotherapy or molecularly targeted therapy before the surgical resection. Forty-one patients (51.3%) had tumor size more than 5 cm in colon. Tumors were located in the ascending colon in 37 patients (46.3%), in the transverse in 22 (27.5%), and in the descending colon in 21 (26.2%). Tumor was well and moderately differentiated in 36 patients (45.0%), and poorly differentiated in 44 patients (55.0%). The vascular invasion was found in 19 patients (23.8%), lymph node involvement in

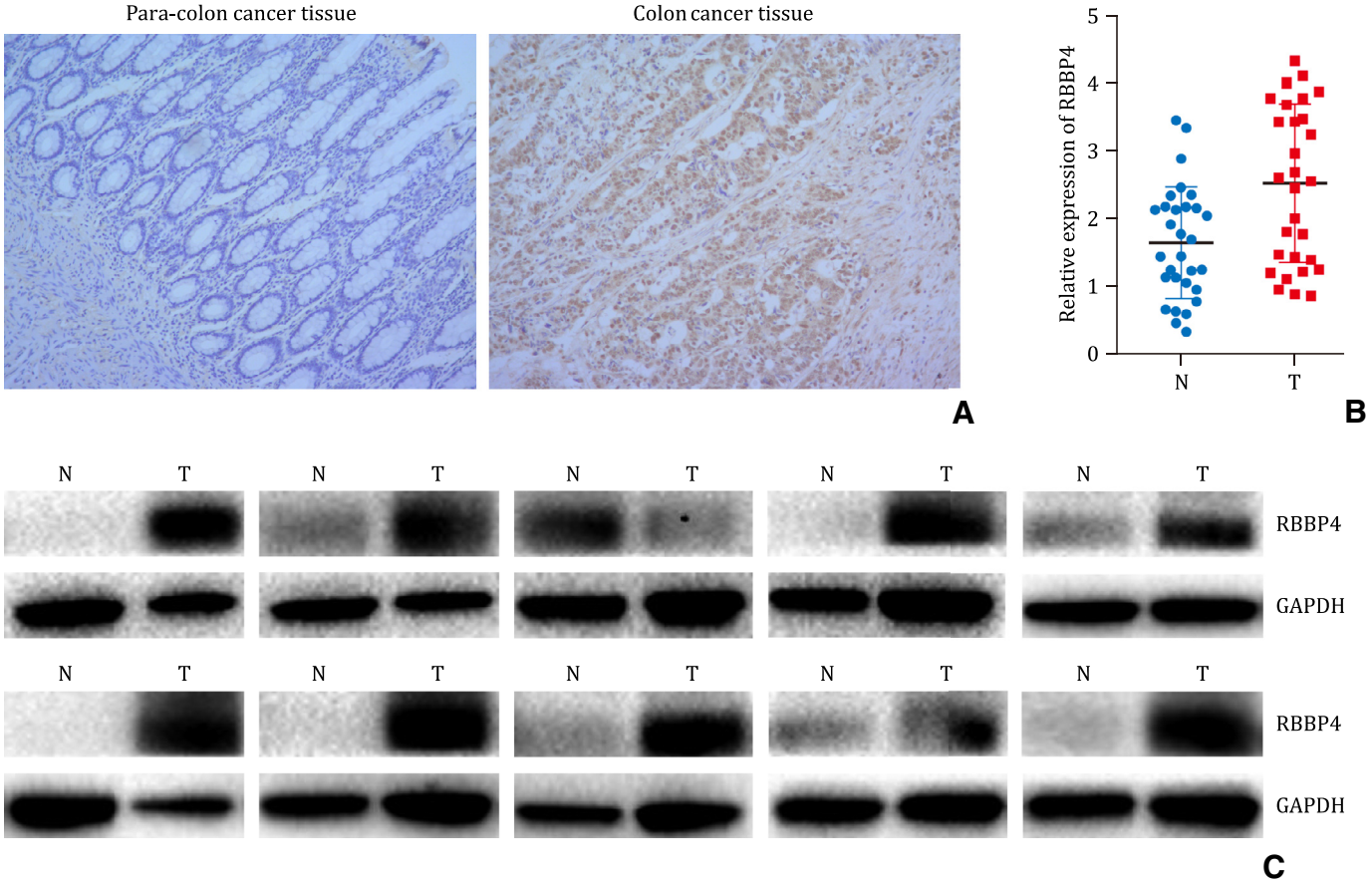

Fig. 1. RBBP4 was up-regulated in the colon cancer tissues. **A**: Negative and positive staining of RBBP4 in the colon cancer tissues and para-colon cancer tissues by immunohistochemistry (original magnification × 100). RBBP4 was located in the cell nucleus; **B**: The expression of RBBP4 in the colon cancer tissues (T) and paired para-colon cancer tissues (N) performed by qRT-PCR; **C**: The expression of RBBP4 in the colon cancer tissues (T) and paired para-colon cancer tissues (N) performed by Western blot.

44 patients (55.0%), and hepatic metastasis in 40 patients (50.0%). Thirty-eight patients (47.5%) had serum CEA level >5 ng/mL. The median follow-up of the patients was 34 months (range 12–60 months).

RBBP4 protein overexpression in colon cancer

We first evaluated the expression of RBBP4 in 80 colon cancer tissues, para-colon cancer tissues, and 40 hepatic metastatic cancer tissues using the immunohistochemical staining. Among these samples, RBBP4 was positive in 43 colon cancer tissues (43/80, 53.70%), but only positive in 11 para-colon cancer tissues (11/80, 13.75%), and the expression of RBBP4 was upregulated in the colon cancer tissues compared with the para-colon cancer tissues (Fig. 1A).

Then, we randomly selected 30 paired colon cancer tissues and para-colon cancer tissues from the 80 colon cancer patients to perform qRT-PCR, and 10 paired colon cancer tissues and para-colon cancer tissues to perform Western blot. The qRT-PCR and Western blot results verified the upregulation of RBBP4 in the colon cancer tissues (Fig. 1B and C). Above all, in the 40 colon cancer patients with hepatic metastasis, 39 (39/40, 97.5%) hepatic metastatic cancer tissues was positive for RBBP4, but only 26 (26/40, 65.0%) paired colon cancer tissues was positive for RBBP4 (Table 1). However, in the 26 double positive patients, we did not find the expression difference of RBBP4 between hepatic metastatic cancer tissues and paired colon cancer tissues (Fig. 2).

In addition, we analyzed the data of colon cancer patients from TCGA database. Our results indicated that RBBP4 was upregulated

Table 1
RBBP4 expression positive samples.

Samples	RBBP4 positive	RBBP4 negative	*P* value*
Colon cancer tissues ($n=80$)	43 (53.8%)	37 (46.2%)	
Para-colon cancer tissues ($n=80$)	11 (13.8%)	69 (86.2%)	<**0.01***
Hepatic metastatic cancer tissues ($n=40$)	39 (97.5%)	1 (2.5%)	
Colon cancer tissues of patients with hepatic metastasis ($n=40$)	26 (65.0%)	14 (35.0%)	<**0.01**#

* *P* value was calculated by Chi-squared test.
# *P* value was calculated by Fisher's Exact test.

in colon cancer tissues (Fig. 3A), and up-regulated RBBP4 levels were correlated with poor chemotherapy result (Fig. 3B) and nerve invasion (Fig. 3C). These results suggested a vital role of RBBP4 in colon cancer. And the ratio of positive expression of RBBP4 in the hepatic metastatic cancer tissues was significantly more than that in the paired colon cancer tissues, which suggested that RBBP4 might play an important role in predicting the colon cancer liver metastasis.

RBBP4 is associated with clinicopathological parameters

Table 2 showed the distribution of RBBP4 expression level in 80 primary colon cancer patients and the relationship between RBBP4 expression level and clinicopathological characteristics, including age, sex, vascular invasion, primary colon tumor

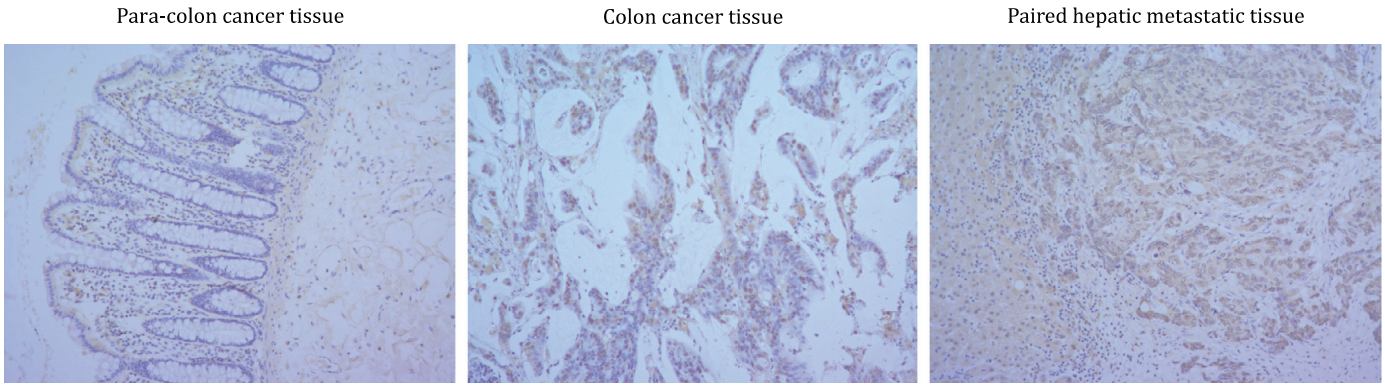

Fig. 2. RBBP4 was positive expression in the hepatic metastasis cancer tissues. The expression of RBBP4 is significantly higher in the hepatic metastasis cancer tissues than that of in the para-colon cancer tissues, but no difference between the colon cancer tissues and paired hepatic metastatic cancer tissues (original magnification × 100).

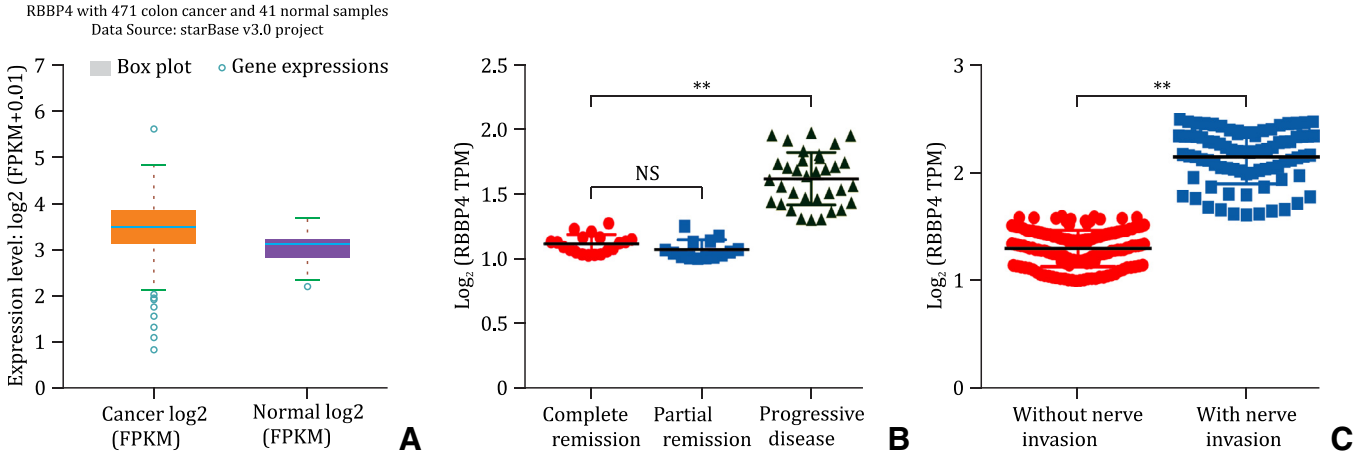

Fig. 3. The bioinformatic analysis of data from TCGA database. **A**: Bioinformatics analysis of RBBP4 expression in TCGA COAD (colon cancer) database; **B**: The relationship between RBBP4 expression and chemotherapy results. **: $P < 0.01$, vs complete remission; **C**: The relationship between RBBP4 expression and nerve invasion. **: $P < 0.01$, vs without nerve invasion. FPKM: fragments Kilobase of exon model per millon mapped reads; TCGA: The Cancer Genome Atlas; RBBP4: retinoblastoma binding protein 4.

location, tumor size, histological differentiation, TNM stage, hepatic metastasis, lymph node involvement, and serum CEA level. Our results showed that overexpression of RBBP4 was correlated with vascular invasion (32.6% vs. 13.5%, $P = 0.046$), hepatic metastasis (72.1% vs. 24.3%, $P < 0.001$), and lymph node involvement (67.4% vs. 40.5%, $P = 0.016$).

Positive-RBBP4 is associated with poor survival in colon cancer patients

The overall survival curves for colon cancer patients with hepatic metastasis subdivided on the basis of RBBP4 expression are shown in Fig. 4. Patients with RBBP4-positive expression had poorer prognosis than those patients with RBBP4-negative expression (log-rank test, $P < 0.001$).

Discussion

According to the WHO mortality database, colon cancer is still one of the most frequent malignant tumor worldwide [16]. The most common distal metastatic target organ of colon cancer is the liver, and almost 50% of patients suffered hepatic metastases (15%–25% appeared with colon cancer at the same time and 20% appeared after the colon cancer) [17,18]. Despite recent progression in diagnosis, and therapeutic strategies, the 5-year overall survival

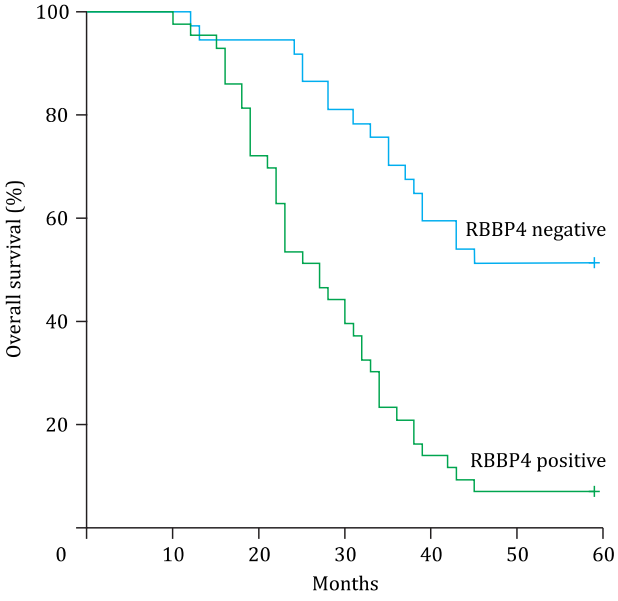

Fig. 4. Kaplan-Meier survival curves of 80 colon cancer patients, grouped by RBBP4 expression. The survival rate for patients with in the RBBP4 negative group ($n = 37$) was significantly higher than that for patients in the RBBP4 positive group ($n = 43$, log-rank test, $P < 0.001$).

Table 2
Clinicopathological characteristics of colon cancer patients with hepatic metastasis ($n = 80$).

Characteristics	Colon cancer tissue		*P* value
	RBBP4 positive($n = 43$)	RBBP4 negative($n = 37$)	
Age			
< 60 yr	21 (48.8%)	19 (51.4%)	0.827
≥ 60 yr	22 (51.2%)	18 (48.6%)	
Sex			
Male	26 (60.5%)	25 (67.6%)	0.510
Female	17 (39.5%)	12 (32.4%)	
Vascular invasion			
Yes	14 (32.6%)	5 (13.5%)	**0.046**
No	29 (67.4%)	32 (86.5%)	
Tumor location			
Ascending colon	21 (48.8%)	16 (43.2%)	0.795
Transverse colon	12 (27.9%)	10 (27.0%)	
Descending colon	10 (23.3%)	11 (29.7%)	
Tumor size			
≤5 cm	21 (48.8%)	18 (48.6%)	0.987
>5 cm	22 (51.2%)	19 (51.4%)	
Tumor differentiation			
Well and moderately	21 (48.8%)	15 (40.5%)	0.457
Poorly	22 (51.2%)	22 (59.5%)	
Hepatic metastasis			
Yes	26 (65.0%)	14 (35.0%)	**<0.01**
No	12 (27.9%)	28 (75.7%)	
Lymph node involvement			
Yes	29 (67.4%)	15 (40.5%)	**0.016**
No	14 (32.6%)	22 (59.5%)	
Serum CEA level			
>5 ng/mL	22 (51.2%)	16 (43.2%)	0.479
≤5 ng/mL	21 (48.8%)	21 (56.8%)	

of colon cancer patients with hepatic metastasis is still very low. Hepatic metastasis is a very pressing problem for the treatment of colon cancer, developing predictive markers for screening patients with hepatic metastasis in high risk groups and prognostic markers for recurrence after hepatectomy is valuable. Although some predictive markers have been reported by researchers, single marker is not enough [19,20]. Hence, it is necessary to explore more specific hepatic metastasis-related or prognosis-related markers to establish the predictive model which contributed to the early detection of hepatic metastasis or prognostic judgment.

Although RBBP4 was reported to be overexpressed in colon carcinoma [14], the clinical relevance of RBBP4 in colon cancer prognosis and hepatic metastasis remains unknown. We therefore focused on exploring the predictive role of RBBP4 in prognosis and hepatic metastasis in colon cancer patients. We examined the RBBP4 expression in 80 colon cancer patients with or without liver metastasis, and the patients was followed up. Our result revealed that the RBBP4 was frequently positive expressed in hepatic metastatic cancer tissues and predicted poor overall survival in colon cancer. These data further indicate that RBBP4 may have a unique role in liver metastasis of colon cancer and deserves further functional study.

In summary, this is the first study showing the expression of RBBP4 in colon cancer and revealing that RBBP4 up-regulation is significantly associated with poor prognosis in colon cancer patients. Our results suggest that RBBP4 may be a new predictor of poor prognosis and liver metastasis in colon cancer patients, and may be a promising candidate for targeted therapy for liver metastasis of colon cancer.

Contributors

LYD proposed the study. LYD and LZ performed the research, wrote the first draft, and collected and analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. ZSS is the guarantor.

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Ethical approval

This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (No. 2013-0022).

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References

[1] Hashim D, Boffetta P, La Vecchia C, Rota M, Bertuccio P, Malvezzi M, et al. The global decrease in cancer mortality: trends and disparities. Ann Oncol 2016;27:926–933.
[2] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69–90.
[3] Shawki S, Ashburn J, Signs SA, Huang E. Colon cancer: inflammation-associated cancer. Surg Oncol Clin N Am 2018;27:269–287.
[4] Primrose JN. Surgery for colorectal liver metastases. Br J Cancer 2010;102:1313–1318.
[5] Paschos KA, Majeed AW, Bird NC. Natural history of hepatic metastases from colorectal cancer–pathobiological pathways with clinical significance. World J Gastroenterol 2014;20:3719–3737.
[6] Truant S, Séquier C, Leteurtre E, Boleslawski E, Elamrani M, Huet G, et al. Tumour biology of colorectal liver metastasis is a more important factor in survival than surgical margin clearance in the era of modern chemotherapy regimens. HPB (Oxford) 2015;17:176–184.
[7] Lan H, Jin K, Gan M, Wen S, Bi T, Zhou S, et al. APOBEC3G expression is correlated with poor prognosis in colon carcinoma patients with hepatic metastasis. Int J Clin Exp Med 2014;7:665–672.
[8] Qian YW, Wang YC, Hollingsworth RE Jr, Jones D, Ling N, Lee EY. A retinoblastoma-binding protein related to a negative regulator of Ras in yeast. Nature 1993;364:648–652.

[9] Parthun MR, Widom J, Gottschling DE. The major cytoplasmic histone acetyltransferase in yeast: links to chromatin replication and histone metabolism. Cell 1996;87:85–94.
[10] Millard CJ, Varma N, Saleh A, Morris K, Watson PJ, Bottrill AR, et al. The structure of the core NuRD repression complex provides insights into its interaction with chromatin. Elife 2016;5:e13941.
[11] Zhang Y, Iratni R, Erdjument-Bromage H, Tempst P, Reinberg D. Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. Cell 1997;89:357–364.
[12] Tyler JK, Bulger M, Kamakaka RT, Kobayashi R, Kadonaga JT. The p55 subunit of drosophila chromatin assembly factor 1 is homologous to a histone deacetylase-associated protein. Mol Cell Biol 1996;16:6149–6159.
[13] Pacifico F, Paolillo M, Chiappetta G, Crescenzi E, Arena S, Scaloni A, et al. RbAp48 is a target of nuclear factor-kappaB activity in thyroid cancer. J Clin Endocrinol Metab 2007;92:1458–1466.
[14] Scuto A, Zhang H, Zhao H, Rivera M, Yeatman TJ, Jove R, et al. RbAp48 regulates cytoskeletal organization and morphology by increasing K-Ras activity and signaling through mitogen-activated protein kinase. Cancer Res 2007;67:10317–10324.
[15] Alvarez-Chaver P, Rodríguez-Piñeiro AM, Rodríguez-Berrocal FJ, García-Lorenzo A, Páez de la Cadena M, Martínez-Zorzano VS. Selection of putative colorectal cancer markers by applying PCA on the soluble proteome of tumors: NDK A as a promising candidate. J Proteomics 2011;74:874–886.
[16] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394–424.
[17] McMillan DC, McArdle CS. Epidemiology of colorectal liver metastases. Surg Oncol 2007;16:3–5.
[18] Manfredi S, Lepage C, Hatem C, Coatmeur O, Faivre J, Bouvier AM. Epidemiology and management of liver metastases from colorectal cancer. Ann Surg 2006;244:254–259.
[19] Shen Y, Zhang F, Lan H, Chen K, Zhang Q, Xie G, et al. FRZB up-regulation is correlated with hepatic metastasis and poor prognosis in colon carcinoma patients with hepatic metastasis. Int J Clin Exp Pathol 2015;8:4083–4090.
[20] Huang J, Hu W, Lin X, Wang X, Jin K. FRZB up-regulated in hepatocellular carcinoma bone metastasis. Int J Clin Exp Pathol 2015;8:13353–13359.