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Original Article

FRZB up-regulation is correlated with hepatic metastasis and poor prognosis in colon carcinoma patients with hepatic metastasis

Yanping Shen[1*], Fang Zhang[2*], Huanrong Lan[3,4*], Ke Chen[1], Qi Zhang[1], Guoming Xie[1], Lisong Teng[5], Ketao Jin[3,4]

[1]Department of Cancer Chemotherapy and Radiotherapy, Yinzhou Hospital Affiliated to Medical School of Ningbo University, Ningbo 315040, Zhejiang Province, P. R. China; [2]Department of Gynecology and Obstetrics, Yinzhou Hospital Affiliated to Medical School of Ningbo University, Ningbo 315040, Zhejiang Province, P. R. China; [3]Department of Gastrointestinal Surgery, Shaoxing People's Hospital, Shaoxing Hospital of Zhejiang University, Shaoxing 312000, Zhejiang Province, P. R. China; [4]Department of Gastrointestinal Surgery, Taizhou Hospital, Wenzhou Medical University, Linhai 317000, Zhejiang Province, P.R. China; [5]Department of Surgical Oncology, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, Zhejiang Province, P.R. China. [*]Equal contributors.

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Abstract: Frizzled-related protein (FRZB) was up-regulated in hepatic metastasis samples compared with primary colon cancer samples in our previous work. However, the clinical relevance of FRZB in colon cancer hepatic metastasis remains uncertain. The aim of this study was to assess the prognostic value of FRZB in patients with colon carcinoma hepatic metastasis after hepatic resection. FRZB expression was evaluated by immunohistochemistry in formalin-fixed paraffin embedded (FFPE) primary colon carcinoma and paired hepatic metastasis tissues from 136 patients with liver metastasis from colon carcinoma that underwent hepatic resection. The relation between FRZB expression and clinicopathologic factors and long-term prognosis in these 136 patients was retrospectively examined. The prognostic significance of negative or positive FRZB exspression in colon carcinoma hepatic metastasis was assessed using Kaplan-Meier survival analysis and log-rank tests. Positive expression of FRZB was correlated with liver metastasis of colon cancer. Univariate analysis indicated significantly worse overall survival (OS) for patients with a positive FRZB expression in colon carcinoma hepatic metastasis than for patients with a negative FRZB expression. Multivariate analysis showed positive-FRZB in colon carcinoma hepatic metastasis to be an independent prognostic factor for OS after hepatic resection (P = 0.001). Positive expression of FRZB was statistically significantly associated with poor prognosis of patients with colon carcinoma hepatic metastasis. FRZB could be a novel predictor for poor prognosis of patients with colon carcinoma hepatic metastasis after hepatic resection.

Keywords: Colon carcinoma, hepatic metastasis, frizzled-related protein (FRZB), prognosis

Introduction

Colon cancer is one of the most common leading causes of cancer-related deaths worldwide [1]. The primary cause of death in patients with colon cancer is hepatic metastasis, and 5-year overall survival is only 25% to 40% [1]. Hepatic metastasis is the most common form of distant spread of primary colon cancer. It is estimated that approximately 50% of patients with colon cancer develop hepatic metastases synchronously or metachronously, and in advanced disease the mortality of colon cancer is principally attributable to the development of hepatic metastases [1].

Despite the improvements of systemic chemotherapy in terms of both tumor response rates and overall survival benefit, hepatic resection is the most effective and potentially curative therapy for patients with hepatic metastasis from colon cancer [2]. However, many colon cancer patients with hepatic metastasis undergoing surgery ultimately die of their disease, which means that surgery is not sufficient in a many patients [3, 4]. The development of effective

chemotherapy regimens, such as 5-fluorouracil (5-FU) with leucovorin (LV) plus oxaliplatin or irinotecan, and the addition of molecular targeted agents, such as anti-vascular endothelial growth factor (VEGF)-based and anti-epidermal growth factor receptor (EGFR)-based antibodies to the existing cytotoxic chemotherapy has improved colon cancer patients with hepatic metastasis response rates [5-8]. However, the overall survival benefits remain modest despite exceedingly high financial costs. It is important to uncover the biological mechanisms underlying hepatic metastasis of colon cancer and accelerate the development of new treatment strategies. Early treatment targeting colon cancer hepatic metastatic might be important for improving patient survival. Therefore, there is an urgent need to identify molecules that are correlated with colon cancer hepatic metastasis and facilitate the metastasis of colon cancer to the liver, which would be potential therapeutic targets for treating patients with colon cancer and hepatic metastases.

Frizzled-related protein (FRZB), a member of the secreted frizzled related protein (sFRP) family, also known as sFRP3, plays an important role in embryonic development. FRZB is one of the Wnt signaling pathway regulators. FRZB contains a 24-amino acid putative transmembrane segment [9], a cysteine-rich domain (CRD) which is similar to the putative Wnt-binding region of the frizzled family of transmembrane receptors, a netrin-like domain (NTR) which is homologous with tissue inhibitors of metalloproteases (TIMPs) [10]. Polymorphisms in the FRZB gene have been associated with osteoarthritis [11] and are considered one of the osteoblast regulatory genes. FRZB affects the cartilage integrity as well as cortical bone thickness and density. The mechanism of this protection can be partly attributed to FRZB suppression of the expression of WNT/β-catenin target genes, including genes for MMP3 and cyclooxygenase 2 (COX2) [12]. Further study demonstrated that FRZB may bind and inhibit MMP3 proteinase activity through its NTR domain [12]. FRZB was seen to be acting as an oncogene in metastatic renal cancer [13]. Tis-suemicroarray analysis showed that the level of FRZB protein was low in primary renal cancer tissues but high in metastatic renal cancer tissues. Functional analysis showed increased cell growth, invasion, and tube formation, and decreased numbers of apoptotic cells in the FRZB-transfected renal cancer cell line A498. The reverse trend was seen in metastatic cells (ACHN and Hs891.T) when FRZB expression was silenced by using a knock-down approach [13].

Our previous work using cDNA microarray analysis found that FRZB was up-regulated in hepatic metastasis samples compared with primary colon cancer samples [14]. However, the clinical relevance of FRZB in colon cancer hepatic metastasis remains uncertain. In this study, we evaluated the relationship between FRZB expression and clinicopathologic features, and assessed the utility of FRZB as a new prognostic marker in patients with colon carcinoma hepatic metastasis after hepatic resection.

Materials and methods

Patients and tumor tissue samples

Formalin-fixed paraffin embedded (FFPE) primary colon carcinoma and paired hepatic metastasis tissue samples were obtained from 136 patients undergoing surgical resection of primary colon carcinoma and liver metastasis at the Department of Surgical Oncology, the First Affiliated Hospital, Zhejiang University School of Medicine and Department of Gastrointestinal Surgery, Taizhou Hospital, Wenzhou Medical University from January 1999 to December 2006. None of 136 patients had received chemotherapy, radiotherapy or molecularly targeted therapy before resection. After resection, patients were followed up every three months. Sections were reviewed by two experienced pathologists to verify the histologic assessment. Prior informed consent was obtained and the study protocol was approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine and Taizhou Hospital, Wenzhou Medical University. The locations of tumors and distant metastases were determined by colonoscopy, computerized tomography (CT), and magnetic resonance imaging (MRI). Patients were enrolled into this study if synchronous metastasis confined to the liver as assessed by preoperative radiological imaging assessment and confirmed by surgery at the time of initial diagnosis. All patients were classified as stage IV (TXNXM1) according to UICC staging of resected specimens.

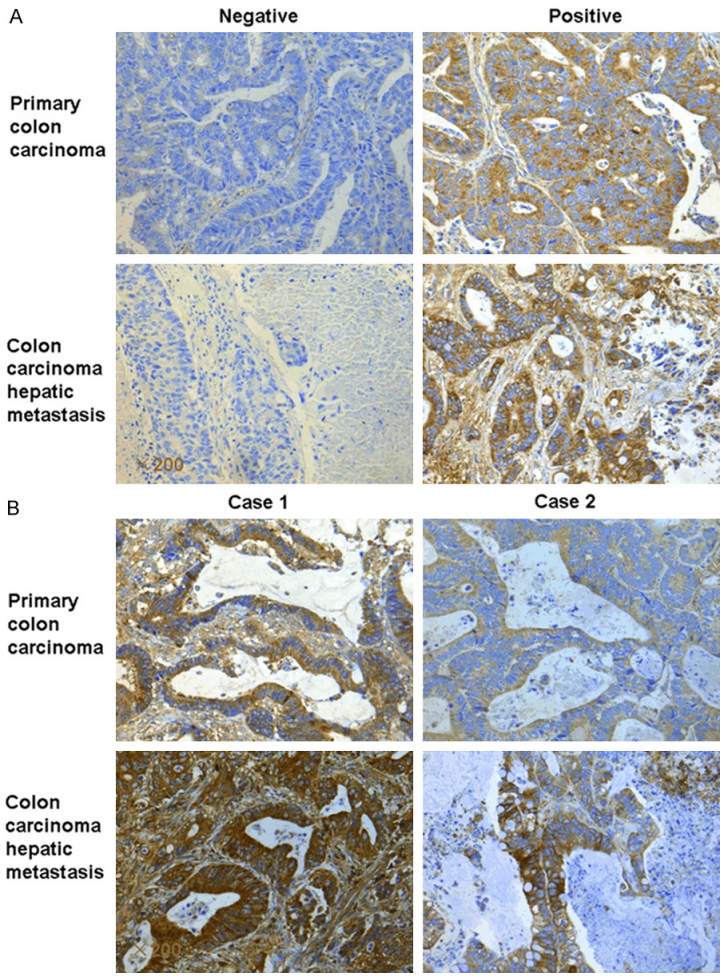

Figure 1. Expression of FRZB in human primary colon cancer and colon cancer heptic metastasis tissues. A: Negative and positive staining of FRZB in primary colon cancer tissues and heptic metastatic tissues (original magnification × 200). B: Representative immunohistochemical staining of FRZB in primary colon cancer tissues and paired heptic metastatic tissues. The expression of FRZB is significantly greater in the colon cancer hepatic metastatsis tissues than in the primary colon cancer tissues (original magnification × 200).

Immunohistochemistry

Five micromolar FFPE sections were cut, dewaxed, rehydrated, and subjected to antigen retrieval. After blocking endogenous peroxidase activity, the sections were incubated with the primary antibody against FRZB (Abcam, Cambridge, MA) (1:200) (overnight at 4 at 4°C). Immunohistochemistry was performed using the streptavidin-biotin peroxidase complex method (Lab Vision, Fremont, CA). The slides were examined and pictures were taken using an Olympus BX60 (Olympus, Japan). Sections known to stain positively were incubated in each batch and negative controls were also prepared by replacing the primary antibody with preimmune sera.

Expression analysis of FRZB in tumor tissue was performed by comparing staining intensity and the percentage of immunoreactive cells. Staining intensity was arbitrarily scored on a scale of four grades: 0 (no staining of cancer cells), 1 (weak staining), 2 (moderate staining), and 3 (strong staining), and the percentage of positive cells was scored as follows: 0 (0%), 1 (1% to 25%), 2 (26% to 50%), and 3 (> 50%). FRZB staining positivity was determined using the following formula: overall score = positive percentage score × intensity score. A score of 0 was defined as "0", > 0 to ≤ 2 as "1", > 2 to ≤ 6 as "2", and > 6 to ≤ 9 as "3". In the end, tumor samples rated as level 0 or 1 were defined as negative for expression, whereas samples rated as level 2 or 3 were defined as positive.

Follow-up

Patient follow-up consisted of physical examination, assesment of serum carcinoembryonic antigen (CEA) levels, and thoracoabdominal computed tomographic scan every 3 months for the first 5 years, then annually thereafter. The patients were followed up until death or until the date of last follow-up. Follow-up was finished on De-cember 31, 2014. The median follow-up was 61 months (range, 8-108 months).

Statistical analysis

Data are expressed as a mean ± SEM. Clinicopathologic parameters were analyzed using the two-tailed chi-square test, and the two-tailed *t* test was used to evaluate association between FRZB expression and clinicopathologic parameters. Overall survival (OS) curves for positive- and negative-FRZB patients were estimated with the Kaplan-Meier method, and the survival functions were compared by the log rank test. Univariate and multivariate analyses

Table 1. Clinicopathological characteristics in relation to FRZB expression in patients with primary colon carcinoma and hepatic metastasis (n = 136)

FRZB expression in primary colon cancer				
Factor		Positive	Negative	*P* value
Patients (n/%)		95/69.9	41/30.1	
Age (years; mean ± SEM)		64.9 ± 7.8	65.7 ± 7.6	0.889
Gender	Female (n)	20	9	0.893
	Male (n)	75	32	
Primary colon tumor location	Ascending colon (n)	16	7	0.651
	Transverse colon (n)	4	2	
	Descending colon (n)	75	32	
Primary colon tumor size	< 5 cm (n)	58	26	0.721
	≥ 5 cm (n)	37	15	
Primary colon tumor histology grade	Low grade (n)	46	17	0.793
	High grade (n)	49	24	
Primary colon tumor differentiation	Well (n)	16	8	0.612
	Moderate (n)	69	30	
	Poor (n)	10	3	
Lymph node involvement	Yes (n)	71	19	0.000
	No (n)	24	22	
Vascular invasion	Yes (n)	36	12	0.321
	No (n)	59	29	
Serum CEA level	> 6 ng/mL (n)	71	11	0.000
	≤ 6 ng/mL (n)	24	30	
HBV infection	Positive (n)	11	7	0.832
	Negative (n)	84	34	
HCV infection	Positive (n)	3	2	0.832
	Negative (n)	92	39	
FRZB expression in colon cancer hepatic metastasis				
Factor		Positive	Negative	*P* value
Patients (n/%)		89/65.4	47/34.6	
Age (years; mean ± SEM)		66.3 ± 9.2	64.7 ± 9.8	0.953
Gender	Female (n)	18	9	0.877
	Male (n)	71	38	
Hepatic metastatic tumor number	Single (n)	74	41	0.843
	Multiple (n)	15	6	
Hepatic metastatic tumor size	< 5 cm (n)	54	29	0.833
	≥ 5 cm (n)	35	18	
Hepatic metastatic histology grade	Low grade (n)	43	17	0.792
	High grade (n)	46	30	

CEA, carcinoembryonic antigen. HBV, hepatitis C virus infection. HCV, hepatitis C virus infection.

were based on the Cox proportional hazards regression model. Factors that significantly influenced overall survival were used in the Cox proportional regression model for multivariate analysis. All *P*-values were considered statistically significant when the associated probability was less than 0.05.

Results

FRZB up-regulated in colon carcinoma hepatic metastatic tissue

To determine whether the observed up-regulation of FRZB gene is a common feature of colon

Table 2. Univariate and multivariate analysis for synchronous hepatic metastasis

Variables	Univariate analysis	Multivariate analysis		
	P value	HR	95% CI	*P* value
FRZB expression (Positive vs. negative)	< 0.001	2.42	1.23-3.87	0.016
Serum CEA level (> 6 ng/mL vs. ≤ 6 ng/mL)	< 0.001	4.53	1.33-27.36	< 0.001
Lymph node involvement (Yes vs. no)	< 0.001	3.26	1.54-8.37	< 0.001
Vascular invasion (Yes vs. no)	0.062	7.43	1.24-54.2	0.091

HR, hazards ratio. CI, confidence interval. CEA, carcinoembryonic antigen. HBV, hepatitis C virus infection. HCV, hepatitis C virus infection.

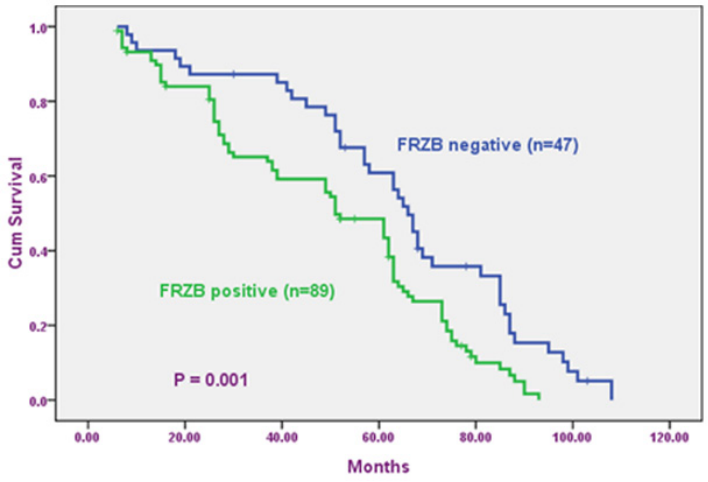

Figure 2. Kaplan-Meier survival curves of patients with metastatic colon carcinoma undergoing liver resection with curative intent, grouped by FRZB expression in metastatic tumor tissues. The survival rate for patients with hepatic metastasis from colon cancer in the FRZB-negative expression group (n = 47) was significantly higher than that for patients in the FRZB-positive expression group (n = 89, log-rank, *P* = 0.001).

cancer liver metastasis [14], we evaluated the expression of FRZB in 136 primary colon cancer samples and paired liver metastatic samples using the method of immunohistochemical staining. We found that among these 136 paired samples, FRZB was positive in 95 primary colon tumors (69.9%) and in 89 paired hepatic metastases (65.4%). The expression of FRZB in the hepatic metastases was noticeably higher than that in the paired primary colon cancer tissues (**Figure 1**). These results suggested that FRZB might play a key role in the colorectal cancer liver metastasis.

Positive expression of FRZB is associated with clinicopathological parameters

Table 1 showed the distribution of FRZB expression level in 136 primary colon cancer tissues and paired hepatic metastases and the relationship between FRZB expression level and clinicopathologic characteristics, including age, gender, primary colon tumor location, serum CEA level, tumor size, histological grade and differentiation, vascular invasion and lymph node involvement. Univariate and multivariate analysis showed that lymph node involvement, serum CEA level, and positive-FRZB expression were independently predictive factors for synchronous hepatic metastasis (**Table 2**).

Positive-FRZB is associated with poor survival in colon cancer patients with synchronous hepatic metastasis

The OS curves for colon cancer patients with hepatic metastasis subdivided on the basis of FRZB expression are shown in **Figure 2**. Positive-FRZB expression was associated with poor prognosis in patients with synchronous hepatic metastasis (log-rank test, *P* = 0.001).

Table 3 lists the relationship between the clinicopathologic variables and overall survival after hepatic resection. Univariate analysis showed that FRZB-positive patients had a significantly poorer prognosis than FRZB-negative colon cancer patients with synchronous hepatic metastasis (*P* < 0.001; **Table 3**). Multivariate analysis showed that lymph node involvement, serum CEA level, and positive-FRZB expression were independent and significant predictors in overall survival (**Table 4**).

Discussion

Colon cancer remains the most common malignant disease worldwide [1]. The liver is the most common target for metastasis in colon

Table 3. Univariate analysis of overall survival after hepatic resection

Factor		Overall survival	
		Patients (n)	*P* value
FRZB expression in colon carcinoma hepatic metastasis	Positive	89	< 0.001
	Negative	47	
Serum CEA level	> 6 ng/mL	83	< 0.001
	≤ 6 ng/mL	53	
Lymph node involvement	Yes	90	< 0.001
	No	46	

CEA, carcinoembryonic antigen.

Table 4. Multivariate analysis of overall survival after hepatic resection

Factor	HR (95% CI)	*P* value
FRZB expression in colon carcinoma hepatic metastasis (Positive)	2.552 (1.861-3.634)	< 0.001
Serum CEA level (> 6 ng/mL)	4.527 (2.002-16.670)	0.0022
Lymph node involvement (Yes)	4.377 (1.936-11.822)	0.0035

HR, hazards ratio. CI, confidence interval. CEA, carcinoembryonic antigen.

cancer patients: it is estimated that approximately 50% of patients develop hepatic metastases (15% to 25% synchronous metastases and 20% metachronous metastases) [15]. Despite recent advances in diagnostic and therapeutic modalities, the prognosis of colon cancer patients with hepatic metastasis remains poor. Hepatic metastasis is a crucial issue for the treatment of colon cancer and it would be invaluable to develop predictive markers for screening high risk groups of patients for hepatic metastasis and prognostic markers for recurrence following hepatic resection. Although numerous studies have reported prognostic factors for recurrence and survival following hepatectomy and predicive factors for hepatic metastasis, the current knowledge remains incomplete. Therefore, identification of the specific tumor metastasis-associated genes or proteins responsible for colon cancer metastasis to the liver would be beneficial to a large proportion of the colon cancer patient population.

FRZB, the first member of the sFRP family, was isolated as a chondrogenic factor in developing cartilage [9]. FRZB binds to both Wnt-8 and Wnt-1 and acts as a functional inhibitor of Wnt-8 activity [16, 17]. It was demonstrated that FRZB was seen to be acting as an oncogene in metastatic renal cancer [13]. Hirata, et al. found that FRZB protein was up-regulated in metastatic renal cancer tissues compared with primary renal cancer tissues [13]. However, FRZB is also involved in malignant tumor generation and progression. Numerous studies strongly suggest a tumor suppressor role of FRZB. Deregulation of FRZB is found in bone-originated malignant diseases. Expression of FRZB was also found to be related to bone involvement at diagnosis in myeloma plasma cells [18]. Loss of FRZB expression was commonly found in osteogenic sarcoma tissues [19]. Expression of FRZB suppresses epithelial original prostate cancer cell in vivo growth and progression [20]. FRZB can function as a melanoma migration and invasion suppressor by interfering with Wnt5a signaling [21]. FRZB decreases growth and invasiveness of fibrosarcoma cells and this inhibition is associated with downregulation of c-Met expression and inhibited Met-mediated signaling [22]. FRZB suppressed gastric cancer cell proliferation and modulated the balance between proliferation and differentiation in gastric cancer [23]. Knockdown of FRZB in gastric cancer cells increased cell growth and migration/invasion [24]. FRZB knockdown may upregulate the Wnt/β-catenin pathway and promote aggressiveness in gastric cancer [24].

The aim of this study was to evaluate the prognostic value of FRZB in patients with colon carcinoma hepatic metastasis after hepatic resection. cDNA microarray analysis showed that FRZB was up-regulated in liver metastasis sam-

ples compared with primary colon cancer samples [14]. Here, we evaluated the FRZB expression in primary colon carcinoma and paired hepatic metastasis tissues from 136 patients with liver metastasis from colon carcinoma that underwent hepatic resection, which had clinical follow-up records. The result demonstrated that the positive expression of FRZB was correlated with liver metastasis ($P < 0.05$) and was coupled with shorter overall survival. These data, for the first time, imply that FRZB has distinct roles in colon cancer liver metastasis and is worthy of further investigation.

In summary, this is the first study showing the expression of FRZB in the hepatic metastases of colon cancer and revealing that FRZB upregulation is significantly associated with hepatic metastasis in patients with colon cancer. Our results also showed that positive expression of FRZB was statistically significantly associated with poor prognosis of patients with colon carcinoma hepatic metastasis. Our results indicated FRZB can be a novel predictor for poor prognosis of patients with colon carcinoma hepatic metastasis after hepatic resection and FRZB might be a promising candidate for targeted therapy of colon cancer hepatic metastasis.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ketao Jin, Department of Gastrointestinal Surgery, Shao-xing People's Hospital, Shaoxing Hospital of Zhejiang University, 568 Zhongxing North Road, Shaoxing 312000, Zhejiang Province, P. R. China. E-mail: jinketao2001@zju.edu.cn

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