

Zinc finger protein 185 is a liver metastasis-associated factor in colon cancer patients

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Abstract. LIM domain proteins are involved in several fundamental biological processes, including cell lineage specification, cytoskeleton organization and organ development. Zinc finger protein 185 (ZNF185) is one of the LIM domain proteins considered to be involved in the regulation of cellular differentiation and/or proliferation. However, the detailed functions and properties of ZNF185 in the multi-step process of cancer biology have not yet been elucidated. In this study, we analyzed the association between ZNF185 and the clinicopathological characteristics of colon cancer, such as patient age and gender, histological type, lymphatic and venous involvement, T and N status, liver metastasis and stage. ZNF185 protein expression was immunohistochemically analyzed and ZNF185 was detected in the cancer cells of 78 of the 87 colon cancer patients. The correlation between ZNF185 and histological type was significant ($P=0.010$, G-test). ZNF185 expression was also significantly correlated with liver metastasis ($P=0.030$, G-test). A multivariate analysis using the Cox proportional hazards model was performed among cause-specific survival rate, ZNF185 expression and clinicopathological characteristics. Histological type, liver metastasis and ZNF185 expression were found to be independent prognostic indicators ($P=0.028$, $P<0.0001$ and $P=0.036$,

respectively). Therefore, ZNF185 expression was found to be an independent indicator of liver metastasis and prognosis in patients with colon cancer.

Introduction

Colorectal cancer is among the three leading causes of cancer-related mortality worldwide. Approximately 50% of patients with colon cancer, the predominant type of colorectal cancer, develop liver metastasis, which is considered to be the main cause of death from advanced-stage colon cancer (1). Therefore, it is crucial to elucidate the biological mechanisms underlying liver metastasis of colon cancer and accelerate the development of new treatment strategies.

The liver is the most common site for metastasis from colon and pancreatic cancer (2). Hepatectomy is a potentially curative treatment option for liver metastasis from colon cancer; however, liver metastasis from pancreatic cancer is not considered an indication for surgical treatment (3). Similarities or differences in the biology of liver metastasis between colon and pancreatic cancer remain to be elucidated. We previously established and investigated the highly liver-metastatic human colorectal cancer cell sublines SW48LM2 and LM-BxPC-3, through the serial intrasplenic transfer of hepatic tumor foci formed by parental SW48 colon cancer cells and BxPC-3 pancreatic cancer cells in NOD/Shi-*scid*/IL-2R γ^{null} mice (4-6). We then performed a quantitative proteome analysis utilizing these established cell lines by our original method (7). The comparison of cellular protein abundance between a pair of 'highly liver-metastatic' cells and its parental cells revealed a series of metastasis-related proteins. In order to identify more universal metastasis-related proteins, we subsequently selected 11 proteins commonly detected among two pairs, i.e., the BxPC-3 and the SW48 subtype pairs (unpublished data). These proteins are expected to be good biomarker candidates

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and/or plausible causal factors for cancer metastasis. Zinc finger protein 185 (ZNF185) is one of these 11 proteins thus selected.

ZNF185 belongs to the family of LIM domain proteins and contains one LIM zinc-binding domain at the COOH-terminus and an actin-targeting domain (ATD) at the NH₂-terminus. The LIM domain is a cysteine- and histidine-rich double zinc-finger motif named after the three homeodomain proteins: Lin-1, Isl-1 and Mec-3 (8-10). The LIM domain is present in a wide range of proteins whose functions include a number of fundamental biological processes, such as cell lineage specification, cytoskeleton organization and organ development (11). Whereas the Zinc finger motif contains the typical DNA binding structures, there is little evidence to support the observation that LIM domains may directly bind DNA (12). LIM domain proteins were found to be distributed in the cytoplasm or nucleus of cells and perform regulatory functions through protein:protein interactions rather than direct interactions with DNA (13,14). ZNF185 is located on chromosome Xq28 and is expressed in the kidney, prostate, pancreas, blood, placenta and ovary, but not in the liver (15). ZNF185 may be involved in regulating cellular differentiation and/or proliferation (16,17). Certain LIM domain-containing proteins were previously shown to be involved in carcinogenic processes (18-27). In basic research on prostate cancer, craniocervical squamous cell carcinoma and non-small-cell lung cancer, the underexpression of ZNF185 mRNA in tumoral tissue was compared to that in matched normal tissue (16,28,29). However, the detailed properties and functions of ZNF185 in the multistep process of tumor invasion have not been investigated in detail (12). The clinical behavior of ZNF185 also remains unknown in relation to the prognosis or treatment of various cancers.

In this study, we investigated the expression level of ZNF185 using immunohistochemistry in 87 cases of colon cancer obtained by complete surgical resection. We also discussed the association between prognosis and the clinical significance of ZNF185 expression.

Materials and methods

Patients. A total of 87 colon cancer specimens were obtained from the surgical specimens of patients with informed consent between April, 2002 and May, 2005. This study has been approved by the Institutional Research Review Board of Tokai University. The tissues were immediately fixed in 40% formaldehyde. The surgical specimens were also processed for routine histopathological analysis.

The patient sample included 48 men and 39 women, with a mean age of 68.30±9.26 years. Well-differentiated adenocarcinomas were found in 60 patients, moderately differentiated adenocarcinomas in 21, poorly differentiated adenocarcinomas in 2 and mucinous adenocarcinomas in 4 patients. The tumors were clinically staged according to the Union for International Cancer Control TNM system. The tumor status was T1 in 5 patients, T2 in 11, T3 in 55 and T4 in 16 patients. A total of 46 patients had lymph node metastasis (N1) and 18 patients had distant metastasis (M1). Lymphatic and venous involvement was found in 74 and 46 patients, respectively. A total of 19 patients had liver metastasis, including 10 synchronous liver metastasis patients. The pathological stages were as follows:

stage I, 9 patients; stage II, 30 patients; stage III, 30 patients; and stage IV, 18 patients.

Immunohistochemical (IHC) analysis. Formalin-fixed, paraffin-embedded tissue sections of the tumor samples were analyzed. The paraffin-embedded sections were deparaffinized and stained using the streptavidin-biotin-peroxidase complex method. Rabbit antibodies specific to ZNF185, activated RNA polymerase II transcriptional coactivator p15 (SUB1), β -N-acetylhexosaminidase A (HEXA), general transcription initiation factor IIF α subunit (GTF2F1), actinin α 4 (ACTN4) and interleukin enhancer-binding factor 3 (ILF3) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Rabbit anti-glucosidase 2 subunit β (Glucos) and clathrin heavy chain (CLTC) antibodies were purchased from Abcam[®] (Cambridge, UK). Briefly, the sections were incubated in 0.3% H₂O₂ in methanol, washed in phosphate-buffered saline (PBS) and non-specific protein binding was blocked with normal rabbit serum (Nichirei, Tokyo, Japan). The sections were then incubated overnight in a humid chamber at 4°C, with affinity purified antibodies diluted in PBS, as recommended by the manufacturers. Following three PBS washes, the sections were incubated with peroxidase-labeled polymer conjugated rabbit anti-goat antibody (Histofine Simple Stain Max PO; Nichirei). The amplified immune products were visualized using a 3,3'-diaminobenzidine tetrahydrochloride reaction.

Statistical analysis. Statistical comparisons of data sets were performed by non-parametric analysis using the Mann-Whitney U test. The G-test (likelihood ratio Chi-square test) was applied for comparisons between group frequencies. On multivariate analyses of the cause-specific survival rate, the Cox proportional hazards model was used. Data are presented as means \pm standard deviation. The analyses were performed using JMP version 8 software (SAS Institute Inc., Cary, NC, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of ZNF185 as a liver metastasis-associated factor. We selected 8 proteins for the IHC staining experiment among the 11 liver metastasis-associated proteins identified and selected by quantitative proteome studies (unpublished data). The expression of each protein in surgically resected specimens from colon cancer was evaluated by IHC staining. Statistical analyses were performed between expression of the ZNF185, SUB1 and HEXA proteins and liver metastasis in 87 colon cancer cases. A significant correlation was only observed for ZNF185 expression, whereas the correlations were not significant for the expression of SUB1 and HEXA (P=0.030, G-test) (Table I). Specific expression of the GTF2F1, ACTN4, ILF3, CLTC and Glucos proteins could not be detected using standard IHC procedures.

ZNF185 expression and clinicopathological characteristics. ZNF185 expression was observed in 78 of the 87 colon cancer cases (Fig. 1). A significant difference was observed between histological type and ZNF185 expression (P=0.010,

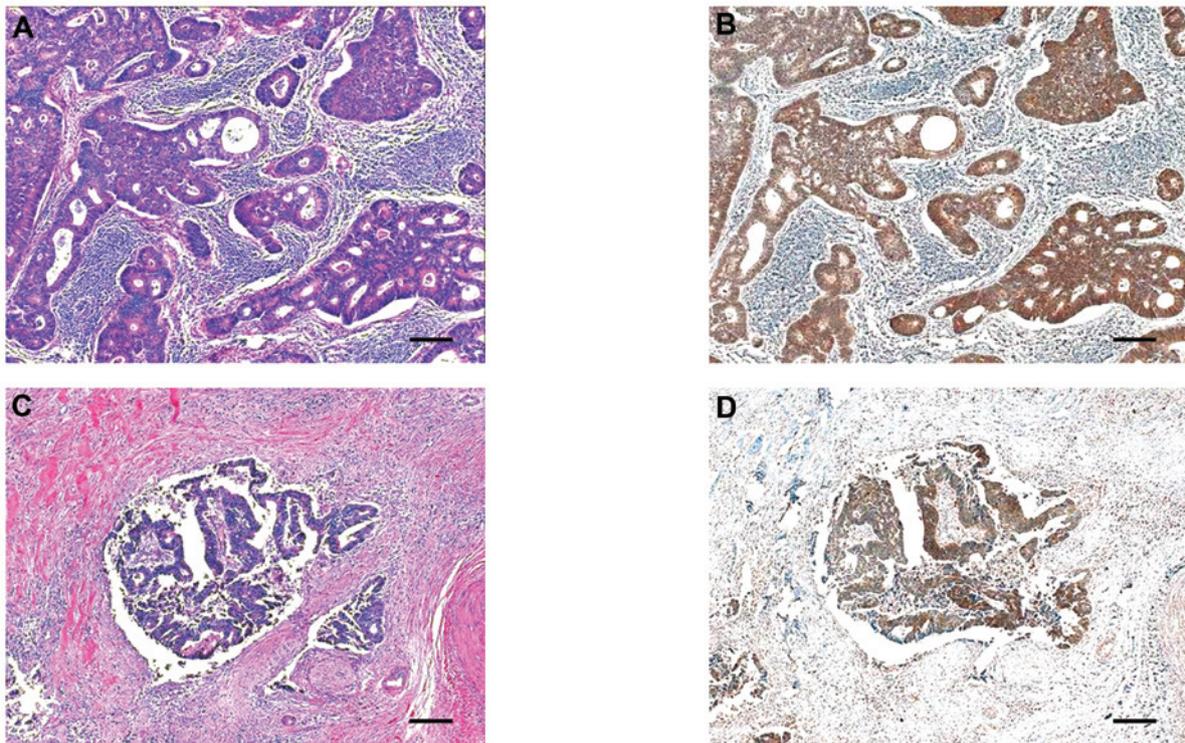


Figure 1. Zinc finger protein 185 (ZNF185) expression in human colon cancer. Colon cancer cells (A and C) exhibit strongly positive cytoplasmic staining for ZNF185 (B and D). Bar, 100 μ m.

Table I. Expression of candidate molecules in liver metastasis from colon cancer.

Candidate molecules (n)	Total liver metastasis		P-value
	Positive (19)	Negative (68)	
ZNF185			
Positive (78)	19	59	0.030 ^a
Negative (9)	0	9	
HEXA			
Positive (69)	14	55	0.347
Negative (18)	5	13	
SUB1			
Positive (84)	19	65	0.473
Negative (3)	0	3	

G-test; ^aP<0.05. ZNF185, zinc finger protein 185.

G-test). Other clinicopathological correlations, including synchronous liver metastasis, were not significant (Table II). The mean age of ZNF185-positive and -negative patients was 67.67 \pm 9.07 and 73.78 \pm 9.61 years, respectively (P=0.071, Mann-Whitney U test).

Correlations between prognosis and ZNF185 expression in colon cancer. We analyzed the correlations among cause-specific survival rate, ZNF185 expression and clinicopathological characteristics, such as patient age and gender, histological type, lymphatic and venous involvement, T and

N status, synchronous liver metastasis and stage, using the Cox proportional hazards model. The multivariate analyses identified histological type, synchronous liver metastasis and ZNF185 expression as independent prognostic indicators (P=0.029, P<0.0001 and P=0.020, respectively) (Table III).

Discussion

In this study, we identified ZNF185 as a significant liver metastasis-associated factor in colon cancer. To the best of our knowledge, this is the first study to investigate the association between ZNF185 and the clinical characteristics of cancer. ZNF185 expression in colon cancer was found to be an indicator of liver metastasis, as well as an independent prognostic indicator. The histological type and synchronous liver metastasis were found to significantly affect the prognosis of colon cancer patients.

ZNF185 belongs to the LIM domain protein family containing two zinc-finger motifs in the C-terminus, classified as group 3 (12,30). ZNF185 is located on chromosome Xq28 and is expressed in the kidney, prostate, pancreas, blood, placenta and ovary, but not in the liver (15). The complete ZNF185 gene was originally cloned from normal human prostate tissue by Zhang *et al* (17). The expression and localization of ZNF185 in prostate cancer cells and fibroblasts revealed that, in addition to F-actin stress fibers, ZNF185 localized to several other cytoskeleton-related areas, including focal adhesions and filopodia/lamellipodia. ZNF185 was also shown to contain an ATD in the N-terminal region and binds to F-actin directly through the ATD, but not the LIM domains (17). Thus, ZNF185 interacts with F-actin and focal adhesion components. Further studies, focused on identifying proteins interacting with the other domains of

Table II. ZNF185 expression in colon cancer.

Clinicopathological characteristics (n)	ZNF185 expression		P-value
	(+)	(-)	
Gender			
Male (48)	44	4	0.496
Female (39)	34	5	
Histological type			
Well differentiated adenocarcinoma (60)	56	4	0.010 ^a
Moderately differentiated adenocarcinoma (21)	18	3	
Poorly differentiated adenocarcinoma (2)	0	2	
Mucinous adenocarcinoma (4)	4	0	
T status			
T1 (5)	5	0	0.599
T2 (11)	9	2	
T3 (55)	50	5	
T4 (16)	14	2	
N status			
N0 (41)	35	6	0.213
N1 (46)	43	3	
M status			
M0 (69)	61	8	0.424
M1 (18)	17	1	
Lymphatic involvement			
Positive (74)	67	7	0.538
Negative (13)	11	2	
Venous involvement			
Positive (46)	41	5	0.835
Negative (41)	37	4	
Synchronous liver metastasis			
Positive (10)	10	0	0.127
Negative (77)	68	9	
Stage			
I (9)	7	2	0.502
II (30)	26	4	
III (30)	28	2	
IV (18)	17	1	

G-test; ^aP<0.05. ZNF185, zinc finger protein 185.

ZNF185, may help clarify the mechanism underlying its diverse subcellular localization and function (12). The LIM domains are generally cysteine- and histidine-rich domains, 50-60 amino acids in size, sharing double characteristic zinc finger motifs. A diverse group of proteins containing LIM domains has been identified, which displays various functions, including gene regulation, cell fate determination, tumoral formation and cytoskeleton organization. LIM domain proteins were previously shown to be distributed in the nucleus as well as the cytoplasm and exert their functions through interactions with various

Table III. Multivariate analyses using the Cox proportional hazards model.

Variable	Strata	P-value
Age (years)	68.30±9.26	0.108
Gender	Male, female	0.967
Histological type (adenocarcinoma)	Well, moderate, poorly differentiated, mucinous	0.029 ^a
T status	T1, T2, T3, T4	0.087
N status	N0, N1	0.268
Synchronous liver metastasis	Positive, negative	<0.0001 ^b
Lymphatic involvement	Positive, negative	0.216
Venous involvement	Positive, negative	0.319
ZNF185 expression	Positive, negative	0.020 ^a

^aP<0.05 and ^bP<0.001. ZNF185, zinc finger protein 185.

protein partners (12). Certain LIM domain proteins are known to play a role in the carcinogenic processes. Epithelial protein lost in neoplasm and testin were found to be downregulated in various cancer cell lines (18,19), whereas LIM domain-only protein 4 is considered to be a negative regulator of breast cancer susceptibility gene 1 and promotes breast tumorigenesis (21,23). LIM and SH3 protein 1 was also identified as a promoter of breast cancer, ovarian cancer and hepatocellular carcinoma and is suggested to be the transcriptional target of p53 (25-27).

ZNF185 gene expression was only shown to be downregulated in matched normal tissues from prostate cancer, craniocervical squamous cell carcinoma and non-small-cell lung cancer (16,17,28,29). Vanaja *et al* (16) reported that the gene expression levels in high-grade (Gleason score 9) prostate cancer cells were suppressed more compared to intermediate grade (Gleason score 6) prostate cancer cells (16). Thus, the dysregulation of ZNF185 gene expression appears to be a frequent event in several cancer types, which suggests its potential role in cancer development. However, there are currently no published reports on ZNF185 in colon cancer. ZNF185 expression was significantly high in well-differentiated adenocarcinoma. In this study, we demonstrated that ZNF185 is a liver metastasis-associated factor, as well as an independent prognosis-deteriorating factor in colon cancer.

Adjuvant chemotherapy is commonly performed to reduce the risk of recurrence and improve the prognosis in patients with colon cancer. According to the National Comprehensive Cancer Network guidelines 2012, all patients with stage III disease should undergo adjuvant chemotherapy. However, stage II patients should also undergo adjuvant chemotherapy when they have high-risk factors, such as T4 lesions, lymphovascular involvement, or poorly differentiated histology (31-33). Liver metastasis is one of the most critical events in the clinical treatment of advanced colon cancer (34). Due to the recent development of clinical studies, certain patients with advanced colon cancer and liver metastasis may become operable. However, the chemotherapeutic regimens for metastatic colon cancer have also improved (35). In our results, ZNF185

indicated liver metastasis with a sensitivity of 100% (19/19) and a specificity of 13% (9/68). These high-sensitivity and low-specificity properties are appropriate for a screening test. There is a possibility that adjuvant chemotherapy may be omitted in ZNF185-negative patients. Therefore, ZNF185 may represent a potential prognostic biomarker of colon cancer.

Cancer cell invasion is a multistep process that includes cell attachment, proteolysis of matrix components and cell migration. Hematogenous liver metastasis, in particular, occurs as a consequence of a well-characterized set of sequential events. The detailed properties and functions of ZNF185 in cancerous invasion have not been fully elucidated. We investigated the clinical significance of ZNF185 in the prognosis and treatment of patients with various types of cancer. The results of the present study, which investigated the behavior of ZNF185 in cancerous invasion, may contribute to the development of novel treatment strategies for advanced colon cancer.

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