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Loss of Fbxw7 expression is a predictor of recurrence in colorectal liver metastasis.

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ABSTRACT:

Background/Purpose

Fbxw7 is a tumor suppressor through ubiquitination and degradation of multiple oncoproteins. Loss of Fbxw7 is frequently observed in various human cancers. In this study, we examined the role of Fbxw7 expression in colorectal liver metastasis (CRLM) and its mechanism.

Methods

Fifty-six patients with CRLM who undergo curative resection were enrolled. Fbxw7 in tumor tissue was determined by immunohistochemistry. Patients were divided into two groups, the Fbxw7 high and low group. Clinicopathological factors including miR-223 expression were compared between the high (n=32) and low Fbxw7 group (n=24).

Results

Fbxw7 expression in tumor tissues was significantly lower than that in normal tissues. The disease-free survival in the low Fbxw7 group was significantly worse than that in the high Fbxw7 group, and 3years disease-free survival of the low and the high Fbxw7 group were 12.5% and 47.0%, respectively ($p=0.023$). On multivariate analysis, loss of Fbxw7 was detected as one of the independent risk factor for recurrence of CRLM (Hazard ratio: 2.390, $p=0.017$). Likewise, Fbxw7 expression inversely correlated to miR-223 expression ($p=0.017$).

Conclusion

Loss of Fbxw7 in tumor tissues could be a reliable predictor of recurrence after hepatectomy in patients with CRLM, and miR223 might be a possible regulator of Fbxw7.

INTRODUCTION:

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females worldwide [1]. Liver is the most common site of metastasis from CRC [2]. Several clinicopathological features have been identified as prognostic factors in colorectal liver metastasis (CRLM) [3]. Recently, although nomogram has been reported for an index of the recurrence prediction after hepatic resection for CRLM from Japanese Society of Hepato-Biliary-Pancreatic Surgery (JHBPS) [4], the promising molecular target for malignant behavior has still been elucidated.

F-box and WD repeat domain-contain 7 (Fbxw7) is a member of the F-box protein family. Fbxw7 serves as a substrate adaptor for the SKP1-CUL1-F-box protein (SCF) ubiquitin ligase complex. SCF ubiquitin ligase contributes to ubiquitin-dependent degradation of proteins with important role in the cell cycle, growth, and differentiation [5]. Fbxw7 acts as a tumor suppressor by targeting several oncogenic regulators such as cyclin E, c-Myc, Notch, Mcl-1, c-Jun, and mTOR [6]. Loss of Fbxw7 in tumor tissues correlates with a poor prognosis in several cancers [7, 8]. Recently, microRNAs (miRs) attracted attention as a mechanism for Fbxw7 regulation. miR-27a and miR-223 have been reported to be direct regulators of Fbxw7 in various types of carcinomas [9]. We also previously reported that the responder cases with rectal cancer for preoperative chemoradiotherapy indicated higher miR-223 compared with the non-responders [10]. Recently, increasing evidence suggests that miRNA-223 may also play an essential part in both hematological malignancies and solid tumors. As yet, the function of miR-223 in cancer has not been fully characterized and understood [11].

To our knowledge, the correlation between Fbxw7 expression and prognostic effect for CRLM has never been reported. Also, its possible mechanism has been elucidated including its relationships to miR-223 in CRLM. Therefore, the aim of this study was to investigate the relationships between Fbxw7 expression and clinicopathological factors after curative hepatic resection (R0) and the role of miR-223 as a Fbxw7 possible regulator for CRLM patients.

METHODS:

Patients

Fifty-six patients with CRLM, who underwent curative hepatic resection (R0) in our institute from 2001 to 2009, were included in this study. The clinicopathological characteristics, including age, gender and pathology, were available for all patients. We excluded the patients who received non-curative surgical treatment (R1, R2) in not only primary tumor but CRLM, and had remote organ metastasis. Among all patients, there were 39 males and 17 females with a mean age of 63.9 years, ranging from 36 to 92 years in age. Synchronous and metachronous CRLM were observed in 32 and 24 patients, respectively. Regarding the primary region, 32 patients had colon cancer and 24 patients had rectal cancer. The mean follow-up period of those 56 patients was 55.0 months (range: 4.4–115.4 months).

Regarding the classifications for extent of liver metastasis, H-stage classification and Grade classification were defined according to the Japanese Classification of Colorectal Carcinoma Second English Edition [12]. H-stage was based on number and largest size of liver metastasis. H1 was less than 4 number with diameter ≤ 5 cm. H2 was more than 5 number or diameter > 5 cm. H3 was more than 5 metastases with diameter > 5 cm. Grade classification was based on H-stage, mesenteric lymph node metastases (pN0/1: ≤ 3 lesions, pN2: ≥ 4 lesions), and extrahepatic metastases (EM0: absence of extrahepatic metastasis, EM1: presence of extrahepatic metastases). Grade A was H1 and pN0/1, Grade B was H2 and pN0/1, or H1 and pN2. Grade C was H3 with any pN, or any H-stage and any pN with EM1.

Of 24 cases with metachronous metastasis, there were 8 cases who underwent adjuvant chemotherapy after primary tumor resection and two of whom had CRLM during the period of adjuvant chemotherapy. Also, there were 37 cases who underwent adjuvant chemotherapy after hepatectomy for CRLM. The mean of time interval between primary tumor resection and liver metastasis in metachronous cases was 25.4 months (range: 3.4-132.2 months).

This study was authorized in advance by the institutional review board of the University of Tokushima Graduate School (approved number; 2395).

Quantitative real-time reverse transcription-PCR (qRT-PCR)

The messenger RNA (mRNA) expression levels of Fbxw7 in tumor and non-tumor liver tissues from 44 patients were evaluated by qRT-PCR. The following assay (assay identification number) were used: Fbxw7 (Hs00217794_ml) and TaqMan human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) endogeneous control

(4326317E, Applied Biosystems) as the housekeeping gene. The mRNA expression levels were calculated as a ratio to that of GAPDH.

The miR-223 expression levels in tumor and non-tumor liver tissues from 47 patients were evaluated by qRT-PCR. The following assay were used: has-miR-223 (2295) and RNU6B (001093, Applied Biosystems) as the housekeeping gene. The miR-223 expression levels were calculated as a ratio to that of U6 small nuclear RNA (RNU6B).

Immunohistochemistry.

Immunohistochemical staining was performed in surgical sections from 56 patients according to previously described methods [7]. We used a rabbit polyclonal anti-Fbxw7 antibody (1:100, ab84873; Abcam, Cambridge, UK) as a primary antibody. Tissue sections were overlaid with 2nd antibody (Dako Real EnVision Kit/HRP, DAKO, Glostrup, Denmark). Peroxidase labeling was developed by incubating in 3,3'-deaminobenzidine tetrahydrochloride. Finally, nuclear counterstaining was completed using Meyer's hematoxylin solution. Then, sections were dehydrated in alcohol and xylene and enclosed in synthetic resin.

To evaluate Fbxw7 expression, the staining score was semiquantitatively calculated by the sum of the percentage score of cells stained at a given staining intensity (0, 0-25%; 1, 26-50%; 2, 51-75%; 3, 76-100%) and the staining intensity score (0, none; 1, weak; 2, moderate; 3, intense) (Fig. 1A-D). And those with a final score of more than 3 were considered high for Fbxw7 expression [7]. All immunohistochemical evaluation was performed by the pathologist who was given no information about individual CRLM.

Statistical analysis.

All statistical analyses were performed using JMP 11 statistical software (SAS Campus Drive, Cary, NC, USA). All results are expressed as the mean \pm standard error. The Welch's t test and Mann-Whitney U-test were used to compare continuous variables. The chi-squared test was applied for categorical data. Survival curves were calculated using the Kaplan-Meier method and compared using the log-rank test. All factors found to be significant by univariate analysis were included in the Cox's proportional hazards model of multivariate analysis to identify independent factors influencing survival. A value of $p < 0.05$ was considered statistically significant.

RESULTS:

The correlation between Fbxw7 expression and clinicopathological characteristics

Fbxw7 mRNA expression in tumor tissues was significantly lower than that of non-tumor tissues ($p<0.001$) (Fig. 2A). Moreover, Fbxw7 protein expression in tumor tissues was significantly lower than that of non-tumor tissues ($p<0.0001$) (Fig. 2B, C). The patients were divided into two groups: the high Fbxw7 ($n=32$) and the low ($n=24$) group in tumor tissues.

Table 1 shows the comparison of clinicopathological characteristics according to Fbxw7 expression. There were no significant differences in the tumor factors, other than high carcinoembryonic antigen (CEA) level in the low Fbxw7 group ($p=0.008$).

Regarding the regimen content and chemo-induction rate of adjuvant chemotherapy after hepatectomy, there was no significant difference between the two groups.

There was no significant difference in overall survival (OS) between Fbxw7 high and low expression group. However, the disease-free survival (DFS) in the Fbxw7 low expression group was significantly worse than that in the high expression group, and 3years disease-free survival rate were 12.5% and 47.0%, respectively ($p=0.023$) (Fig. 3).

Risk factors for tumor recurrence

In the univariate analysis, metastatic grade B and C ($p=0.032$), synchronous liver metastasis ($p<0.001$), nomogram preoperative score ≥ 11 ($p=0.039$), and Fbxw7 low expression ($p=0.023$) were determined to be significant risk factors for tumor recurrence (Table 2). In the multivariable analysis, synchronous liver metastasis and Fbxw7 low expression were found to be the independent risk factors for tumor recurrence (Table 3).

In respect to the recurrence pattern according to Fbxw7 expression in tumor tissues, there were no significant difference between Fbxw7 high and low expression group (Table 4).

miR-223 expression and the relationship to Fbxw7 expression

miR-223 expression in tumor tissues was significantly higher than that in non-tumor tissues ($p=0.048$) (Fig. 4A). Also, miR-223 expression in tumor tissues inversely correlated to the Fbxw7 expression ($p=0.017$) (Fig. 4B). We divided the patients into two groups: the high miR-223 group ($n=37$) and the low group ($n=10$) in tumor tissues according to miR-223 relative expression level (cut-off value: 1.3). The disease-free survival rate in the miR-223 high group was tended to be worse than that in

the low group ($p=0.124$) (Fig. 4C).

DISCUSSION:

Fbxw7 is an important regulator of cell division and differentiation. Fbxw7 acts as tumor suppressor by ubiquitin-dependent degradation of several oncoproteins such as cyclin E, c-Myc, Notch, Mcl-1, c-Jun, and mTOR. The decline of Fbxw7 in tumor tissues has been observed to invite a poor prognosis in several types of the malignancy. In this study, we examined Fbxw7 expression in surgical specimen from patients with CRLM after curative hepatic resection. Consistent with previous reports, Fbxw7 expression levels were significantly decreased in tumor tissues than that in normal liver tissues. In clinicopathological analyses, we found that patients with low Fbxw7 expression in tumor tissues had significantly poorer disease-free survival. Furthermore, on the multivariate analysis, Fbxw7 was extracted as a more powerful independent recurrence defined factor compared to metastatic grade, synchronous metastasis and high preoperative score of nomogram. This suggests that loss of Fbxw7 in tumor tissues may be an important biomarker to predict postoperative recurrence in CRLM.

Regarding the relationship between Fbxw7 and miRNAs, in the previous report, miR-27a was identified to inhibit the expression of FBW7. miR-27a was found to be overexpressed and inversely associated with FBW7 expression in leukemia [13]. Also, it was reported that miR-223 is associated with Fbxw7 expression. Kurashige et al. reported that inverse relationships between miR-223 and Fbxw7 in esophageal squamous cell carcinoma [9]. Li et al. also reported that Fbxw7 protein levels were inversely correlated with miR-223 expression in gastric tumor tissues [14]. Consistent with these findings, in the present study, we demonstrated that miR-223 expression was significantly higher in the low Fbxw7 group. Consequently, it considered the regulation of miR-223/Fbxw7 pathway, which miR-223 suppressed Fbxw7 expression. Moreover, patients with miR-223 high expression in tumor tissues tended that disease-free survival was worse. Regarding malignant behavior of miR-223, in the previous report, it was demonstrated that overexpression of miR-223 decreased Fbxw7 mRNA level, and enhanced c-Myc and c-Jun proteins expression in oesophageal squamous carcinoma cell lines [8]. Additionally, the mechanism was reported that miR-223 could negatively regulate the expression of Fbxw7 by directly targeting 3' untranslated region (UTR) of Fbxw7 mRNA in gastric cancer cell lines, and overexpression of miR-223 induced tumor growth in vivo [14]. In another report, in T-cell acute lymphoblastic leukemia cell lines, it was observed the inverse correlation of miR-223 and Fbxw7 expression and that both Notch and NF- κ B being able to activate cooperatively the transcriptional activity of miR-223 promoter [15]. Consistent with those studies, our findings suggested the involvement of miR-223/Fbxw7 pathway to poor prognosis in CRLM, and strongly

suggested that miR-223/Fbxw7 pathway was an important target in CRLM. However, on the contrary, downregulation of miR-223 has been observed as worse prognostic factor in several types of malignancy, including osteosarcoma [16], hepatocellular carcinoma [17], esophageal carcinoma [18], and acute myeloid leukemia [19]. These previous reports demonstrated that miR-223 may have a side of the tumor suppressor. Taken together, while the regulatory mechanism of miR-223 has not been still elucidated, it could be a useful biomarker in various cancers.

In addition, we have a discrepancy that there was no significant difference in overall survival, while disease-free survival was poor in Fbxw7 low expression groups. For one of the reasons, it may be the influence on the chemotherapy sensitivity via Fbxw7 expression. Previous studies have examined the relationship between Fbxw7 expression and chemosensitivity. It was reported that the low Fbxw7 expression produced the resistance to chemotherapy such as taxol in non-small cell lung cancer [20] and doxorubicin in hepatocellular carcinoma [21]. On the other hands, another report revealed that Fbxw7 low expression group in cholangiocarcinoma patients certainly has poor prognosis, but interestingly they have high chemo-sensitivity [22]. Additionally, it was demonstrated that Fbxw7 depletion leads to the high sensitivity to imatinib in leukemia-initiating cells through c-Myc accumulation [23]. Thus, Fbxw7 is considered to have different role with respect to chemotherapy depending on cancer types and anti-cancer agents. In this study, although we were not able to prove the relationships between Fbxw7 and adjuvant chemotherapy, and it is the examination task for the future, but Fbxw7 may be expected as a clinical application to estimate the efficacy of chemosensitivity.

Together, the present and previous findings supported the importance of conducting further investigations to determine the role of Fbxw7 in cancer for the biomarker for tumor recurrence and its mechanism. In conclusion, Fbxw7 low expression in tumor tissues was an independent risk factor for recurrence in CRLM after curative hepatic resection. miR223/Fbxw7 pathway might regulate the malignant behavior and be a molecular target for CRLM treatment.

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Table 1. Comparison of clinicopathological features between Fbxw7 high and low expression in tumor tissues of colorectal liver metastasis.

Factors		Fbxw7 expression		p-value
		High (n=32)	Low (n=24)	
<u>Metastatic tumor status</u>				
Age	years	65.3 ± 4.3	62.0 ± 5.0	0.296
Gender	Male / Female	24 / 8	15 / 9	0.315
CEA	<100 / ≥100	30 / 2	16 / 8	0.008
CA19-9	<100 / ≥100	24 / 8	19 / 5	0.713
Tumor maximum diameter	<5cm / ≥5cm	24 / 8	15 / 9	0.432
Tumor number	<4 / ≥4	18 / 14	15 / 9	0.637
H Stage	H1 / H2, H3	19 / 14	13 / 11	0.696
Grade	A / B, C	14 / 16	9 / 14	0.582
Metastasis period	synchronous / metachronous	17 / 15	15 / 9	0.482
Adjuvant chemotherapy	yes / no	21 / 10	16 / 5	0.506
Fluoropyrimidine	+ / -	19 / 1	16 / 0	0.273
Oxaliplatin	+ / -	10 / 10	9 / 7	0.708
Irinotecan	+ / -	6 / 14	5 / 11	0.935
Bevacizumab	+ / -	1 / 19	3 / 13	0.188
<u>Primary tumor status</u>				
T	2, 3 / 4	24 / 5	21 / 2	0.361
Location	Colon / Rectum	19 / 13	13 / 11	0.696
Grading (G1, 2, 3)	1 / 2, 3	7 / 22	5 / 16	0.978
Lymph node metastasis	Positive / Negative	17 / 13	17 / 5	0.117
Vascular invasion	Positive / Negative	22 / 7	15 / 5	0.945
Adjuvant chemotherapy	yes / no	4 / 8	4 / 3	0.310

CEA, carcinoembryo antigen; CA19-9, carbohydrate antigen 19-9

Table 2. Risk factor analysis for recurrence after hepatic resection of colorectal liver metastasis: univariate analysis.

Factors		3-year survival (%)	<i>p</i> -value
Metastatic tumor status			
Age	<65 years / ≥65 years	25.0 / 41.2	0.429
Gender	Male / Female	32.6 / 29.4	0.890
Tumor maximum diameter	<5cm / ≥5cm	31.6 / 33.8	0.545
Tumor number	<4 / ≥4	36.3 / 24.1	0.103
H Stage	H1 / H2, H3	34.3 / 27.7	0.135
Grade	A / B, C	36.3 / 20.9	0.032
Metastasis period	Synchronous / metachronous	16.9 / 50.0	0.001
Adjuvant chemotherapy	yes / no	27.8 / 43.6	0.535
Nomogram preoperative score	<11 / ≥11	39.4 / 19.4	0.039

Fbxw7 expression	High / Low	47.0 / 12.5	0.023
Primary tumor status			
T	2, 3 / 4	85.3 / 56.5	0.486
Location	Colon / Rectum	33.5 / 26.1	0.534
Differentiation	Well / Other	45.4 / 25.6	0.126
Grading (G1, 2, 3)	1 / 2, 3	45.4 / 25.6	0.126
Vascular invasion	Positive / Negative	30.0 / 29.0	0.596
Adjuvant chemotherapy	yes / no	50.0 / 54.5	0.820

Table 3. Risk factor analysis for recurrence after hepatic resection for colorectal liver metastasis: multivariate analysis.

Factors		Hazard Ratio	95% CI	p-value
Grade	B, C	1.437	0.674 – 3.123	0.348
Metastasis period	Synchronous	2.145	1.061 – 4.557	0.033
Nomogram preoperative score	≥ 11	1.563	0.700 – 3.411	0.270
Fbxw7 expression	Low	2.390	1.169 – 4.964	0.017

CI, confidence interval.

Table 4. Recurrent pattern after curative hepatectomy in colorectal liver metastasis according to Fbxw7 expression.

Recurrent site	Fbxw7 expression		<i>p</i> -value
	High (n=16)	Low (n=21)	
Liver	12 (75.0%)	19 (90.4%)	0.206
Lung	6 (37.5%)	7 (33.3%)	0.793
Lymph node	4 (25.0%)	2 (9.5%)	0.206
Bone	0 (0%)	1 (4.7%)	0.283
Focal	0 (0%)	2 (9.5%)	0.125

FIGURE LEGENDS:

Figure 1. Immunohistochemical staining of Fbxw7 in colorectal liver metastasis (CRLM).

- (A) Fbxw7 none expression in tumor tissue of CRLM patients.
- (B) Fbxw7 weak expression in tumor tissue of CRLM patients.
- (C) Fbxw7 moderate expression in tumor tissue of CRLM patients.
- (D) Fbxw7 intense expression in tumor tissue of CRLM patients.

Figure 2. Correlation of Fbxw7 expression with that in tumor and non-tumor tissues of colorectal liver metastasis (CRLM).

- (A) Fbxw7 mRNA relative expression by quantitative real-time reverse transcription PCR.
- (B) The representative picture of tumor infiltrating part in CRLM patients.
- (C) Fbxw7 protein expression by immunohistochemical staining score. Fbxw7 expression levels in tumor tissues were significant lower than those in non-tumor tissues of CRLM patients ($p < 0.0001$).

Figure 3. Kaplan–Meier analysis of (A) overall survival and (B) disease-free survival in colorectal liver metastasis patients with high and low Fbxw7 expression.

Figure 4 miR-223 expression in colorectal liver metastasis (CRLM).

- (A) Comparison of miR-223 expression in the tumor and non-tumor tissues of CRLM patients. miR-223 expression in tumor tissues was significantly higher than those in non-tumor tissues.
- (B) Correlation between Fbxw7 expression and miR-223 expression in the tumor tissues of CRLM patients. miR-223 expression in Fbxw7 low expression group was significantly higher than those in Fbxw7 high expression group.
- (C) Disease-free survival rate according to miR-223 expression in tumor tissues of CRLM patients. DFS in miR-223 high expression group was tended to be worse than those in miR-223 low expression group.

Figure 1

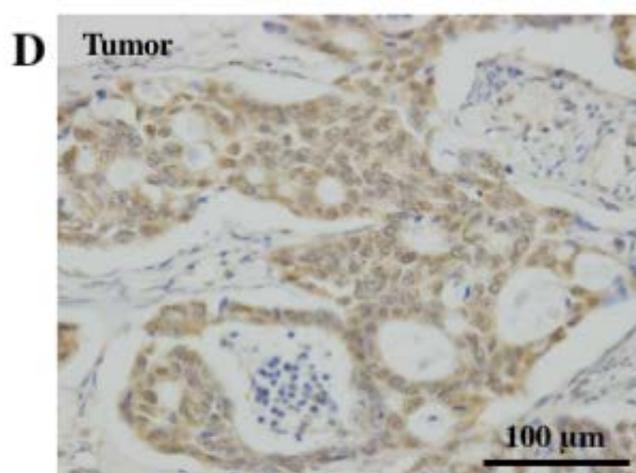
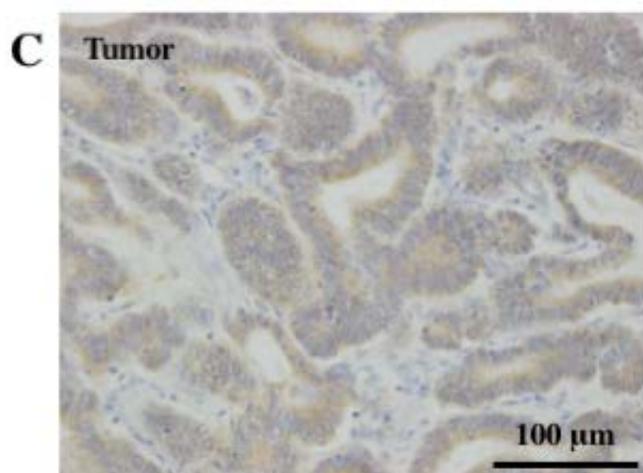
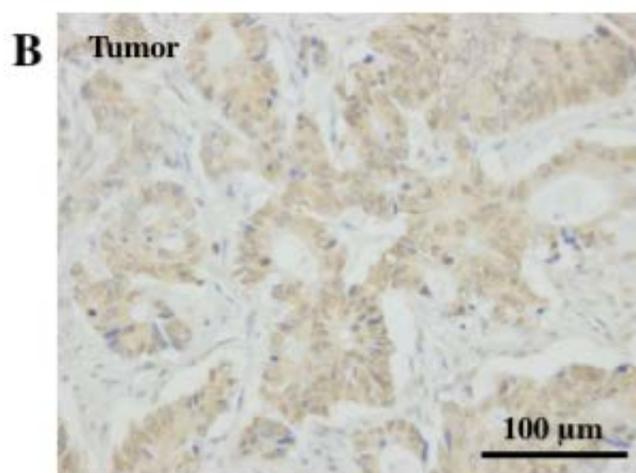
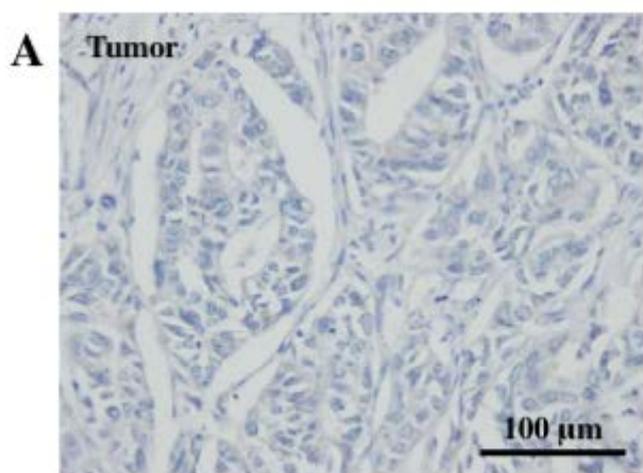
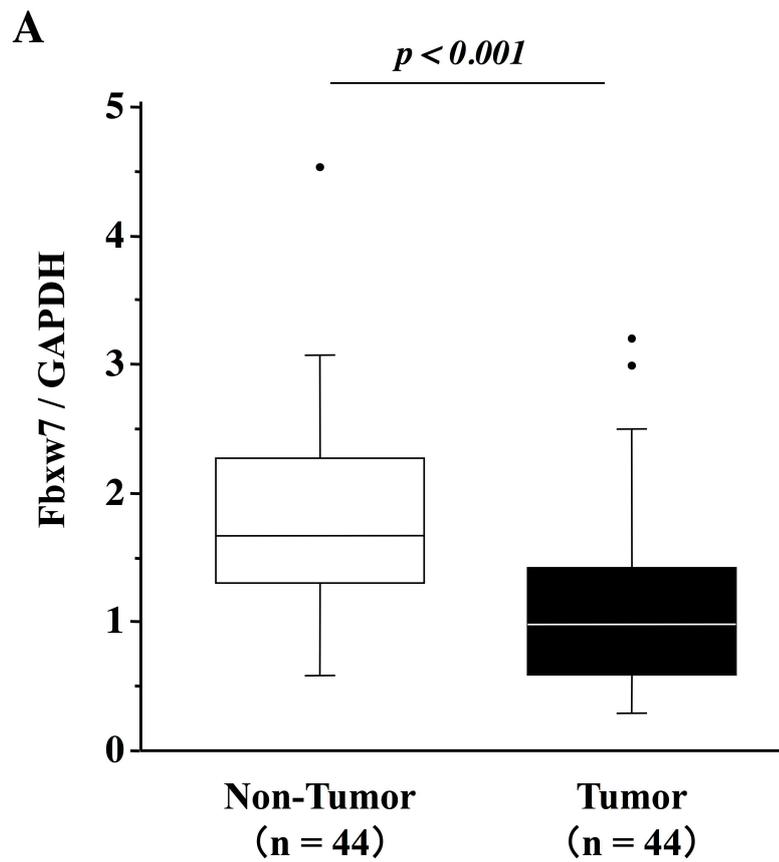
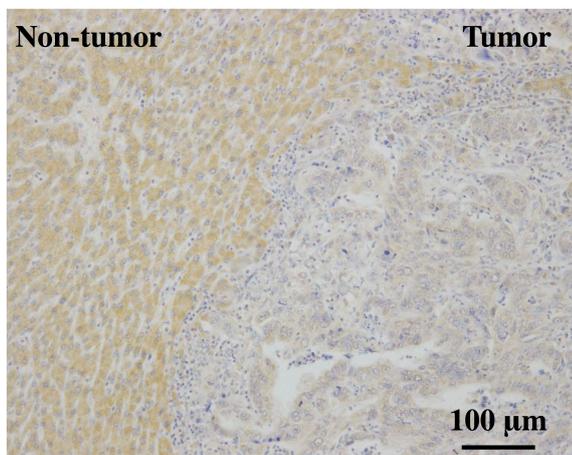


Figure 2



B



C

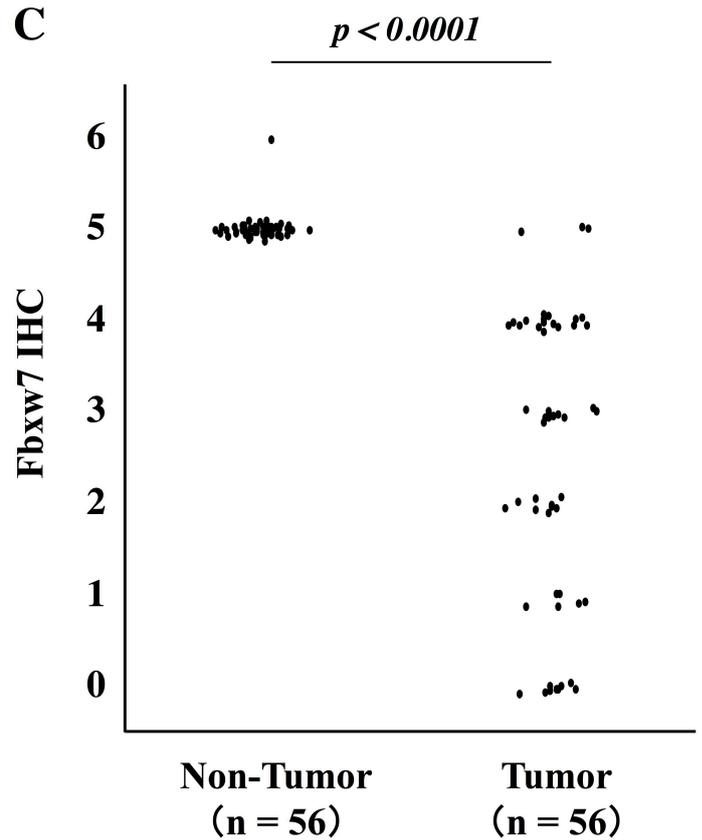


Figure 3

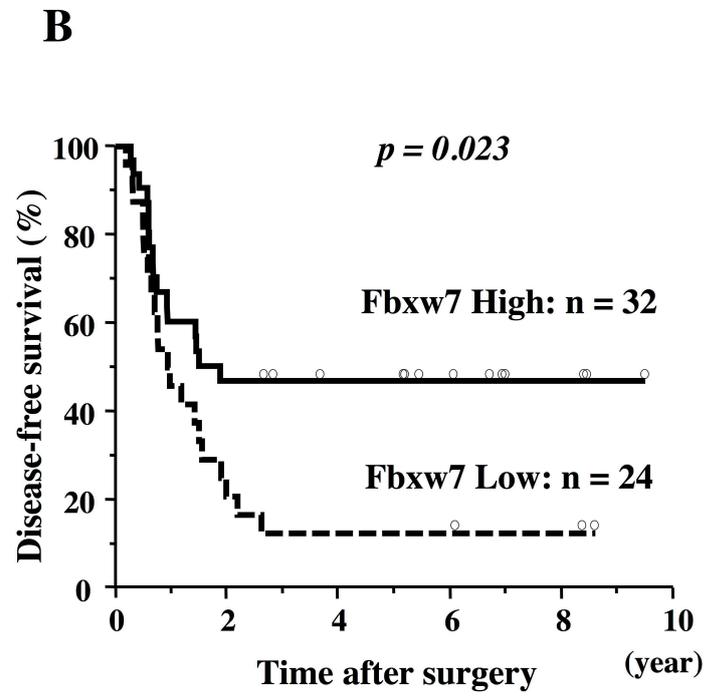
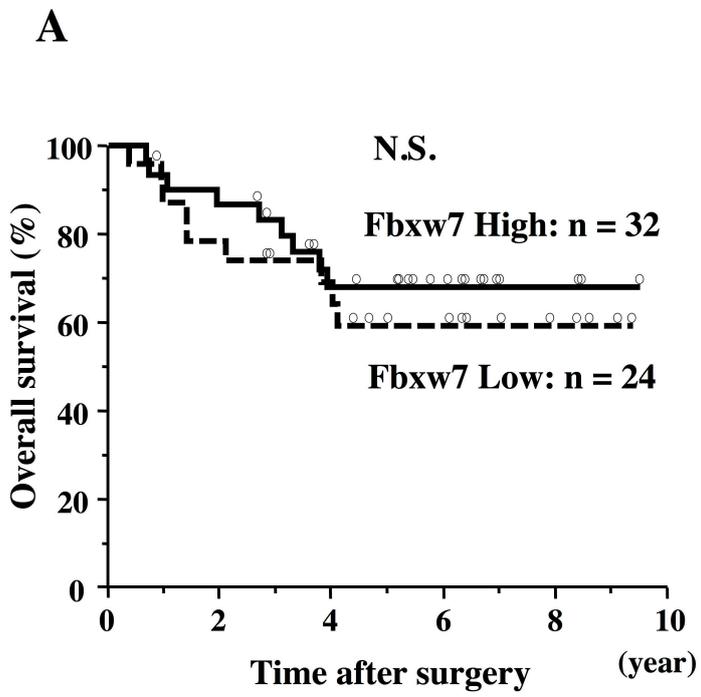


Figure 4

