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Low Expression of FBXO45 Is Associated with Gastric Cancer Progression and Poor Prognosis

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Low Expression of FBXO45 Is Associated with Gastric Cancer Progression and Poor Prognosis

NORIMICHI KOGURE¹, TAKEHIKO YOKOBORI¹, KYOICHI OGATA¹, BOLAG ALTAN¹, ERITO MOCHIKI², TETSURO OHNO¹, YOSHITAKA TOYOMASU¹, MITSUHIRO YANAI¹, AKIHARU KIMURA¹, TORU YANOMA¹, MASAKI SUZUKI¹, TUYA BAI¹, TAKAYUKI ASAO³ and HIROYUKI KUWANO¹

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Abstract. *Background:* F-Box protein 45 (FBXO45) is reported to be associated with cancer aggressiveness. We investigated the relationship between FBXO45 and clinicopathological factors in gastric cancer (GC). *Materials and Methods:* We used immunohistochemistry to investigate FBXO45 expression in 104 GC samples; the prognostic value of FBXO45 was also calculated. *Results:* FBXO45 levels in GC were higher than in normal tissues. Patients with relatively low FBXO45 expression (n=58) had increased cancer progression and poorer prognosis than those with high expression (n=46). Low FBXO45 expression was an independent negative prognostic factor in patients with GC. Using the public Kaplan–Meier plotter database, we showed that survival in patients with low expression of FBXO45 in GC was shorter than that in those with high FBXO45 expression, regardless of lymph node metastasis. *Conclusion:* A low FBXO45 expression level in GC tissues may be a powerful predictor of poor prognosis. FBXO45 might, therefore, be a promising candidate for a molecular targeted therapy in GC.

Gastric cancer (GC) rates have been decreasing recently due to better management and treatment of *Helicobacter pylori* (1), and many patients with GC have achieved disease-free status following curative resection and postoperative adjuvant

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chemotherapy (2). However, the prognosis for patients with advanced disease remains poor and unsatisfactory. To improve the outcomes of individual patients, identification of reliable predictors of poor prognosis or recurrence is extremely important in patients with advanced GC. Moreover, such markers may be promising therapeutic targets for refractory GC. We previously re-analyzed the GSE13911 expression microarray data of GC and normal gastric mucosa registered with the Gene Expression Omnibus (GEO) (3) and reported specific genes that we found to be highly expressed in GC compared to normal mucosa (4). Following that microarray analysis, we focused on F-box protein 45 (FBXO45), a gene of particular interest, in this study. F-Box proteins including FBXO45 are currently drawing attention in the field of cancer research because their expression levels are associated with several cellular processes, including those that are associated with tumorigenic pathways. These include cell-cycle progression, apoptosis, transcription, migration, invasion, and metastasis (5). Some F-box family members have already been shown to be related to cancer progression and poor prognosis. GC-specific FBXO45 is part of a ubiquitin ligase complex and plays a role in neuronal development (6). Moreover, FBXO45 was reported to play a role in the degradation of tumor suppressors or cancer-related proteins *via* ubiquitin-mediated proteolysis (5, 7). However, the relationships between FBXO45 expression, clinicopathological factors, and prognosis have not yet been investigated in clinical cancer samples, including those of patients with GC.

In this study, we investigated the cancer-specific expression of FBXO45 by re-analyzing a GEO database. We also examined the protein expression level of FBXO45 in GC samples to determine whether it is a suitable prognostic marker, and validated our findings using a public database.

Materials and Methods

Clinical samples. We used restricted primary GC tissues from 104 patients (67 men and 37 women) who underwent potentially curative surgery at the Department of General Surgical Science, Gunma University Hospital, between 1995 and 2000. All clinical GC samples in this study were used in accordance with institutional guidelines and the Helsinki Declaration after obtaining written informed consent from all participants. The clinicopathological findings were based on surgical records and pathology reports, and were determined according to the Japanese Classification of Gastric Carcinoma outlined by the Japanese Gastric Cancer Association (8).

Immunohistochemistry. Resected surgical specimens were fixed in 10% formaldehyde, embedded in paraffin blocks, cut into 4-µm-thick sections, and mounted on glass slides. The staining procedure was performed using standard methods as described previously (9). The sections were first incubated overnight at 4°C and then at room temperature for 30 min with goat polyclonal anti-FBXO45 antibody (sc-99418; Santa Cruz Biotechnology Inc. Heidelberg, Germany) at a dilution of 1:300 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. The sections were washed in PBS and then incubated with biotinylated anti-goat secondary antibody solution (Nichirei Co., Tokyo, Japan) for 30 min at room temperature. The chromogen (3,3'-diaminobenzidine tetrahydrochloride) was applied as a 0.02% solution containing 0.005% hydrogen peroxide in a 50 mM ammonium acetate-citrate acid buffer (pH 6.0). The sections were lightly counterstained with Mayer's hematoxylin and mounted on glass slides.

FBXO45 immunoreactivity was scored as follows: 0=no staining; score 1+=weak cytoplasmic staining; and score 2+=strong cytoplasmic staining. Immunohistochemically stained slides were examined and evaluated independently by two experienced researchers. The samples were classified into high (2+) or low (1+ and 0) expression groups.

Data mining. We used an online Kaplan–Meier plotter database to validate the association between *FBXO45* mRNA expression and overall survival in patients with GC. Kaplan–Meier plotter is an entirely independent patient database comprising large-scale survival data that can be stratified by selected genes and characteristics, such as intestinal or diffuse subtype of GC.

Statistical analysis. For continuous variables, the data are expressed as the means±standard deviation. The relationship between FBXO45 expression and clinicopathological factors was analyzed using the Wilcoxon test, chi-square test, and ANOVA. Postoperative survival curves were plotted according to the Kaplan–Meier method, and data were compared using the log-rank test. All differences were statistically significant at the level of *p*<0.05. The significance of potential prognostic factors was examined by using multivariate analysis. Cox proportional hazards regression analysis was used to test the independent prognostic contribution of FBXO45 expression. Statistical analysis was performed with the JMP software package (SAS Institute Inc., Cary, NC, USA).

Results

Expression of FBXO45 in clinical GC tissues. We used immunohistochemistry to investigate the cytoplasmic expression of FBXO45 in 104 GC specimens. Representative

Table I. *F-Box protein 45 (FBXO45) expression and clinicopathological factors of 104 patients with gastric cancer (GC).*

Factor	FBXO45 expression in GC (n=104)		
	Low (n=58)	High (n=46)	<i>p</i> -Value
Age (mean±SD)	62.6±11.0	62.3±11.6	0.81
Gender, n	35	32	0.3293
Male	23	14	
Female			
Histology, n	18	26	0.009
Well, moderate	40	20	
Poor, signet			
Lauren classification, n	17	28	0.0013
Intestinal type	41	18	
Diffuse type	62.6±28.0	51.2±30.6	0.008
Tumor size (mm)			
Depth	7	18	0.0013
sm, mp	51	28	
ss, se, si			
Lymph node metastasis	12	21	0.0066
Absent	46	25	
Present			
Lymphatic invasion	1	0	0.37
Absent	57	46	
Present			
Venous invasion	28	26	0.4
Absent	30	20	
Present			
Stage	20	36	<0.0001
I-II	38	10	
III-IV			

Well: Well-differentiated, moderate: moderately differentiated, poor: poorly differentiated, signet: signet ring cell, sm: submucosa, mp: *muscularis propria*, ss: subserosa, se: serosa-exposed, si: serosa-infiltrating, SD: standard deviation. Significant differences are shown in bold.

immunohistochemical stains are shown in Figure 1. FBXO45 was more highly expressed in cancerous tissues than in corresponding non-cancerous tissues, and was predominantly expressed in the cytoplasm (Figure 1A and B). Forty-six GC specimens were classified into the high FBXO45 expression group, while 58 were classified into the low FBXO45 expression group (Figure 1B-D).

Association between FBXO45 expression and clinicopathological factors in clinical GC samples. The correlations between FBXO45 expression in the GC specimens and clinicopathological characteristics of the corresponding patients (age, sex, histological type, Lauren classification, tumor size, tumor depth, lymph node metastasis, lymphatic invasion, venous invasion, and stage) are shown in Table I. Low FBXO45 expression levels in 104 GC samples were significantly associated with poor tumor differentiation and

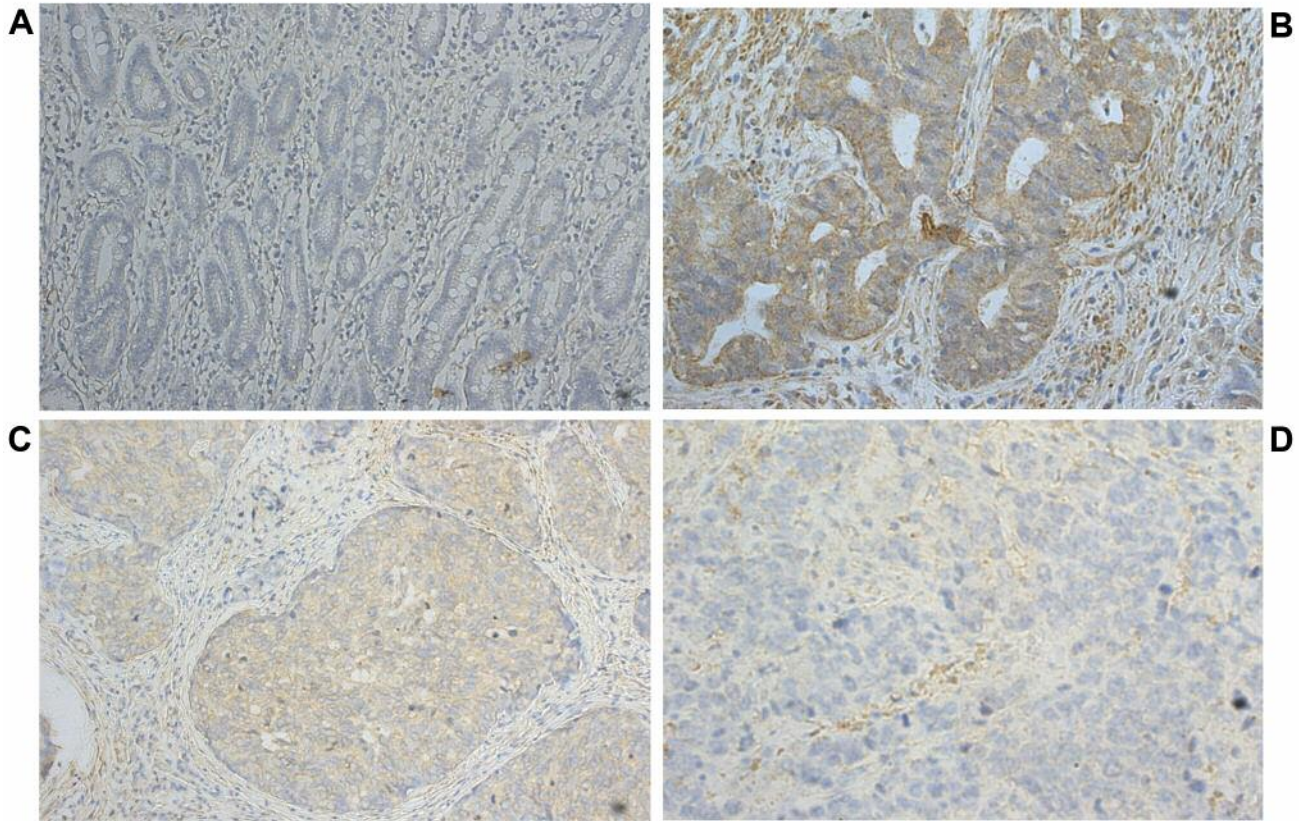


Figure 1. Immunohistochemical staining of F-box protein 45 (FBXO45) in representative primary gastric cancer samples: Normal gastric mucosa (A); high cytoplasmic expression of FBXO45 (2+; high expression group) (B); weak expression of FBXO45 (1+; low expression group) (C); no staining of FBXO45 (0; low expression group) (D); (all $\times 100$ magnification).

Table II. Results of univariate and multivariate analyses of clinicopathological factors affecting the overall survival rate following surgery.

Clinicopathological factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	<i>p</i> -Value	HR	95% CI	<i>p</i> -Value
Age: <62 vs. ≥ 62 years	2.52	1.37-4.94	0.0024	2.64	1.42-5.23	0.0018
Sex: Female/male	1.01	0.57-1.84	0.97	-	-	-
Depth: m, sm, mp vs. ss, se, si	3.81	1.66-11.0	0.0008	2.3	0.97-6.8	0.06
Lymph node metastasis: Negative vs. positive	3.58	1.77-8.25	0.0002	3.21	1.52-7.66	0.0016
Venous invasion: Negative vs. positive	1.96	1.12-3.52	0.019	1.36	0.77-2.47	0.3
FBXO45 expression: High vs. low	3.1	1.70-5.96	0.0001	1.94	1.03-3.86	0.039

HR: Hazard ratio, CI: confidence interval, m: mucosal, sm: submucosa, mp: *muscularis propria*, ss: subserosa, se: serosa-exposed, si: serosa-infiltrating. Significant values are shown in bold.

signet ring cell histotype ($p=0.009$), diffuse type of Lauren classification ($p=0.0013$), progression of tumor size ($p=0.008$), tumor depth ($p=0.0013$), lymph node metastasis ($p=0.0066$), and clinical stage ($p<0.0001$). No significant differences were observed with respect to age, sex, lymphatic invasion, and venous invasion.

Prognostic significance of FBXO45 expression in patients with GC. The overall and cancer-specific survival rates of patients with GC with low FBXO45-expressing tumors were significantly lower than those with high FBXO45-expressing tumors ($p=0.0002$ and $p=0.0004$, respectively; Figure 2A and B). Among patients with GC without pathological lymph

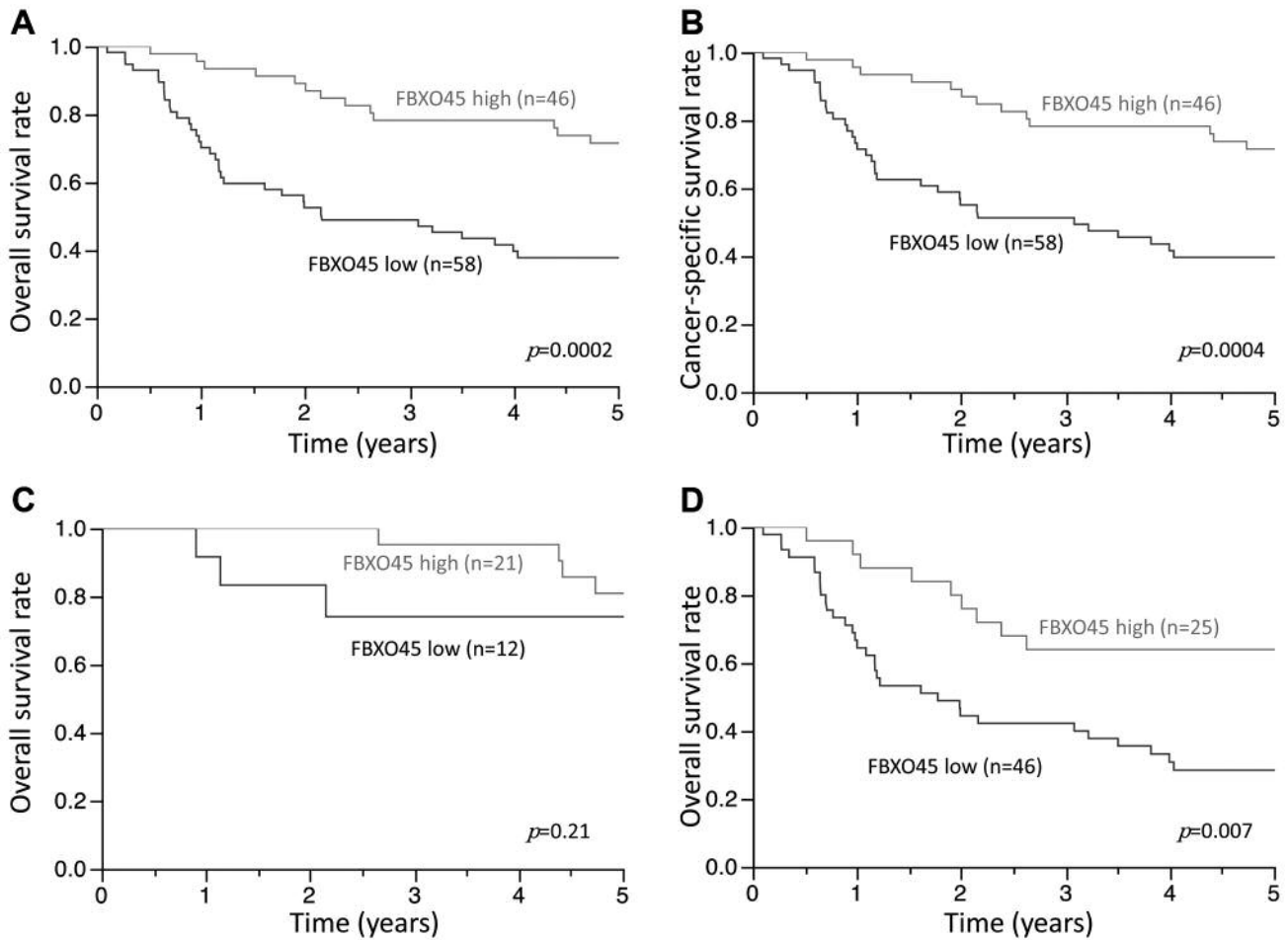


Figure 2. Kaplan–Meier curves showing survival in patients with gastric cancer (GC) according to the level of F-box protein 45 (FBXO45) expression. Overall (A) and cancer-specific (B) survival curves for patients with GC (n=104) based on the level of FBXO45 expression. C: Overall survival curves for patients with GC without lymph node metastasis (n=33) based on the level of FBXO45 expression. D: Overall survival curves for patients with GC with lymph node metastasis (n=71) based on the level of FBXO45 expression.

node metastasis (n=33), the prognosis was not significantly different in the low FBXO45 expression group than in the high expression group ($p=0.21$; Figure 2C). However, among patients with lymph node metastasis (n=71), those with low FBXO45 expression had poorer prognoses than those with high expression ($p=0.007$; Figure 2D). On multivariate analyses, low expression of FBXO45 in GC tissues was an independent prognostic factor for poor survival (relative Risk=1.94, 95% Confidence Interval=1.03-3.86, $p=0.039$), as were age and lymph node metastasis (Table II).

To validate our findings regarding the prognostic significance of FBXO45 in a large cohort of patients with GC, we examined the correlation of *FBXO45* mRNA expression and prognosis using the public Kaplan–Meier plotter database. Low *FBXO45* mRNA expression was correlated with poor overall and progression-free survival in 631 and 522 patients with GC, respectively ($p<0.0001$ and

$p=0.00012$, respectively; Figure 3A and B). In this large cohort, patients with GC with or without lymph node metastasis but low *FBXO45* expression exhibited poorer prognoses than those with high *FBXO45* expression ($p=0.032$ and $p<0.0001$, respectively; Figure 3C and D).

Discussion

In this study, we found the expression level of FBXO45 in primary GC to be higher than in corresponding normal gastric tissues, which was consistent with data from a previous expression microarray study. We showed that low relative expression levels of FBXO45 are associated with cancer progression and are an independent prognostic factor in patients with GC. We also validated the prognostic significance of low *FBXO45* expression in GC, which was true regardless of lymph node metastasis status, using a public database.

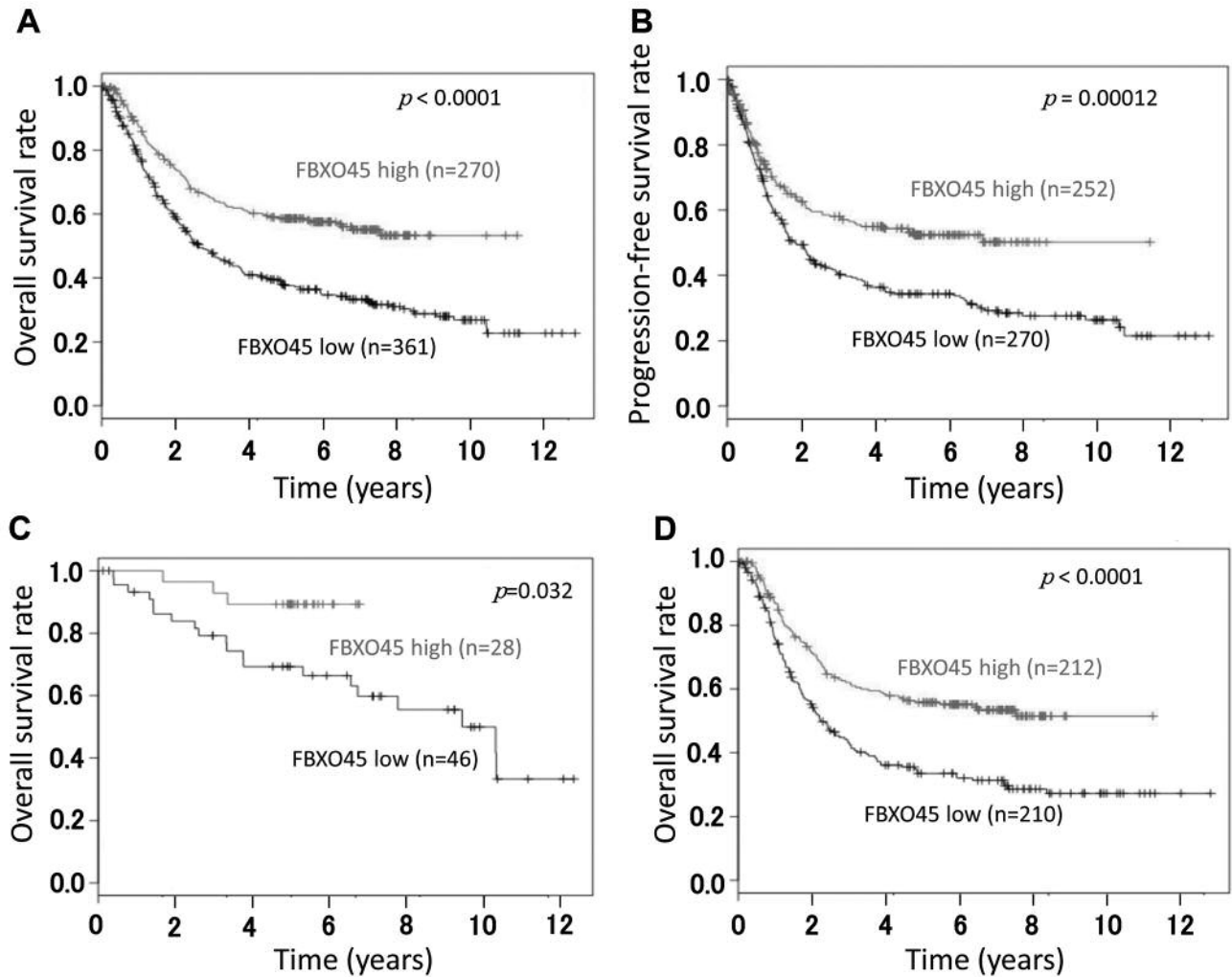


Figure 3. Prognostic significance of F-box protein 45 (FBXO45) expression in patients with gastric cancer (GC) in a public database. Kaplan–Meier survival curves for patients expressing a high or low level of FBXO45; adapted from KM plotter: <http://kmplot.com>. Overall (A) and progression-free (B) survival curves. C: Overall survival curves for patients with gastric cancer expressing high or low FBXO45; adapted from KM plotter: <http://kmplot.com>. Data for patients with GC without (C) and with (D) lymph node metastasis.

Patients with GC with high FBXO45 expression had better prognoses than those with relatively low expression (although levels considered low expression FBXO45 in GC remained higher than those in normal tissue). FBXO45 functions to degrade target proteins *via* ubiquitination (10); therefore, it may also target cancer-related proteins. Many epithelial–mesenchymal transition (EMT)-associated proteins are substrates of FBXO45, including snail family transcriptional repressor 1 (SNAIL1), SNAIL2, twist family BHLH transcription factor 1, and zinc finger E-box binding homeobox 1; these proteins induce EMT by repressing the epithelial marker E-cadherin. In several types of cancers, EMT-induced cancer cells develop invasive phenotypes and penetrate the surrounding stromal tissues, resulting in the

creation of a permissive microenvironment for cancer invasion and metastasis formation (11). Furthermore, EMT of cancer cells has been associated with resistance to chemotherapy and radiation therapy, which could give rise to recurrence and distant metastasis following standard adjuvant treatment (12). Therefore, it is plausible that the EMT that ensues owing to down-regulation of FBXO45 may be responsible for cancer aggressiveness and poor prognosis of patients with GC with low FBXO45 expression.

According to our immunohistochemical analysis and microarray data, FBXO45 expression was higher in GC than in corresponding normal tissues. FBXO45 was originally identified as an estrogen-induced F-box protein in the MCF-7 breast cancer cell line. However, there are few studies that

have examined the mechanisms of regulating FBXO45 expression. On the other hand, comparative genomic hybridization studies showed that chromosome 3q, which harbors *FBXO45*, was amplified in clinical GC samples (13, 14). In this study, we showed that the overexpression of FBXO45 in GC occurred in parallel at both the mRNA and protein levels. Therefore, we posit that *FBXO45* expression in GC is regulated at least partly by genomic alterations.

Tools to predict the high risk of recurrence and poor prognosis are urgently needed for clinical patients with GC who undergo curative resections. It was reported that adjuvant chemotherapy for patients with lymph node metastasis of GC is effective for preventing recurrence after curative surgery (15). While patients with GC normally treated with adjuvant chemotherapy can be cured with surgery alone, adjuvant therapy is still recommended for those with lymph node metastasis. Adjuvant chemotherapy has contributed to the decline of GC recurrence rates, but not sufficiently. In this study, we found that low cytoplasmic FBXO45 expression in GC tissues correlated with poor prognosis on subgroup analysis of patients with lymph node metastasis. Hence, low FBXO45 may be predictive of a higher risk of GC recurrence in patients with lymph node metastasis who are recommended for adjuvant therapy. Such a marker would therefore be critical for developing personalized treatments for patients according to their risk assessment.

In conclusion, we showed that relatively low FBXO45 expression in GC tissues is associated with cancer progression and constitutes an independent prognostic factor. The evaluation of FBXO45 in GC tissues might, therefore, be crucial for predicting aggressive phenotypes and poor prognoses in patients with GC. Further studies are warranted to evaluate the role of FBXO45 and its clinical application in GC treatment.

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