**ORIGINAL ARTICLE**

# FBXO50 Enhances the Malignant Behavior of Gastric Cancer Cells

**Short title:** *FBXO50* in gastric cancer

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**Key words:** gastric cancer, F-box-only 50, progression, biomarker

## Synopsis

The expression and functions of F-box-only 50 (*FBXO50*) were evaluated in gastric cancer. *FBXO50* enhances the malignant phenotype of tumor cells and may represent a marker for monitoring disease and a potential target of molecular therapy in gastric cancer.

## ABSTRACT

**Background.** Challenges to our understanding the molecular mechanisms of the progression of gastric cancer (GC) must be overcome to facilitate the identification of novel biomarkers and therapeutic targets. Here we analyzed the expression of the gene encoding F-box-only 50 (FBXO50) and determined whether it contributes to the malignant phenotype of GC.

**Methods.** *FBXO50* mRNA levels and copy numbers of the *FBXO50* locus were determined in 10 GC cell lines and a nontumorigenic epithelial cell line. PCR array analysis was performed to identify genes coordinately expressed with *FBXO50*. The effects of inhibiting *FBXO50* on GC cell proliferation, adhesion, invasiveness, and migration were evaluated using a small interfering RNA targeted to *FBXO50* mRNA. To evaluate the clinical significance of *FBXO50* expression, we determined the levels of *FBXO50* mRNA in tissues acquired from 200 patients with GC.

**Results.** The levels of *FBXO50* mRNA were increased in five GC cell lines and positively correlated with those of *ITGA5*, *ITGB1*, *MMP2*, *MSN*, *COL5A2*, *GNG11*, and *WNT5A*. Copy number gain of the *FBXO50* locus was detected in four GC cell lines. Inhibition of *FBXO50* expression significantly decreased the proliferation, adhesion, migration, and invasiveness of GC cell lines. In clinical samples, high *FBXO50* expression correlated with increased+ pT4, invasive growth, lymph node metastasis, and positive peritoneal lavage cytology. Patients with high *FBXO50* expression had a significantly higher prevalence of recurrence after curative gastrectomy and were more likely to experience shorter overall survival.

**Conclusions.** *FBXO50* may represent a biomarker for GC phenotypes and as a target for therapy.

Despite its declining incidence, gastric cancer (GC) is the fifth most frequent cancer and the third leading cause of cancer-related death worldwide.[1] Although therapeutic strategies have improved, the 5-year survival rates of patients with GC are <30%.[2,3] Metastasis and disease recurrence are two major obstacles to increasing long-term survival, and early intervention is required.[4,5] A better understanding of the molecular mechanisms of GC progression is therefore required to identify biomarkers and therapeutic targets to improve diagnosis and treatment strategies.

F-box proteins contain at least one F-box domain that mediates protein-protein interactions and is associated with signal transduction and the regulation of the cell cycle.[6-8] Little evidence is available that illuminates the role of genes that encode F-box only proteins (FBXO). For example, FBXO28 controls MYC-dependent transcription, is regulated during the cell cycle by cyclin-dependent kinase 1/2-mediated phosphorylation, and overexpression of *FBXO28* is strongly associated with poor prognosis of patients with breast cancer.[9] We identified *FBXO50* (alternatively called non-specific cytotoxic cell receptor protein 1, *NCCRP1*) as a gene that is aberrantly expressed in metastatic GC by our previous transcriptome analysis.[10,11] However, to our knowledge, no study reports the analysis of the expression and function of *FBXO50* in GC.[12]

These findings led us to hypothesize that *FBXO50* is associated with the malignant phenotype of GC. Therefore, the present study was conducted to analyze the expression of *FBXO50* in GC tissues and cell lines and to determine whether *FBXO50* enhances the malignant phenotype of GC cells.

## MATERIALS AND METHODS

### *Sample Collection*

The GC cell lines MKN1, MKN45, MKN74, NUGC2, NUGC3, NUGC4, and SC-6-JCK were obtained from the Japanese Collection of Research Bio Resources Cell Bank (JCRB) (Osaka, Japan), and the AGS, KATOIII, and N87 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas,VA). Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 5% $CO_2$. As a control, the nontumorigenic epithelial cell line (FHs74) was purchased from the ATCC.[13] The cell lines were analyzed using the short tandem repeat-polymerase chain reaction (PCR) method and authenticated by the JCRB Cell Bank during June 2015.

Primary GC tissues and the corresponding noncancerous adjacent tissues were collected from 200 patients who underwent gastric resection for GC at the Department of Gastroenterological Surgery, Nagoya University Hospital between 2001 and 2014. The tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C. Since 2010, specimens were classified histologically according to the 7th edition of the Union for International Cancer Control (UICC) classification system. The patients recruited before 2010 were reclassified according to the 7th edition of the UICC classification system. Since 2006, adjuvant chemotherapy using S-1 (an oral fluorinated pyrimidine) is administered to all patients with UICC stage II/III GC, unless contraindicated by the patient's condition.[14,15] Written informed consent for the use of clinical samples and data, as required by the Institutional Review Board at Nagoya University, Japan, was obtained from all patients.

*Analysis of FBXO50 Transcription*

*FBXO50* mRNA levels in cell lines and clinical samples were determined using a quantitative real-time reverse-transcription PCR (qRT-PCR) assay. Total RNAs (10 μg per sample) were used to generate cDNAs that were amplified with specific primers for *FBXO50* (Supplemental Table S1) as follows: initial denaturation at 95 °C for 10 min, 40 cycles at 95 °C for 10 s and 60 °C for 30 s. Samples were tested in triplicate, and samples without template were included in each PCR plate as negative controls. The ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used for real-time detection of the emission intensity of SYBR-Green fluorescence. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA served as an internal standard, and the expression level of each sample was calculated as the value of *FBXO50* mRNA divided by that of *GAPDH* mRNA.[16] High expression of *FBXO50* mRNA was defined as follows: expression level in a GC tissue was higher compared with that of the corresponding normal adjacent tissue.

*Copy Number Analysis of the FBXO50 Locus*

Genomic DNA was purified from GC cell lines and analyzed by the TaqMan Copy Number Assay (Applied Biosystems) to detect copy number alterations of the *FBXO50* locus according to the manufacturer's instructions. The assay sequence (assay ID: Hs02638838_cn, within exon 6) was employed, and data analysis was conducted using CopyCaller Software (Life Technologies).[17]

*PCR Array Analysis*

To identify genes coordinately expressed with *FBXO50* in GC cell lines, we used the Human Epithelial to Mesenchymal Transition (EMT) $RT^2$ Profiler PCR Array (Qiagen, Hilden, Germany). This array includes 84 “key” genes that encode proteins with the functions as follows: transcription factors, extracellular matrix proteins as well as proteins involved in the epithelial mesenchymal transition (EMT), cell differentiation, morphogenesis, growth, proliferation, migration, cytoskeleton, and signaling pathways.[16]

*Small Interfering RNA (siRNA)-mediated Knockdown of FBXO50*

MKN1 cells were cultured in a 24-well plate ($5 \times 10^4$ cells /ml). Cells were transiently transfected the next day with 30 nM siRNA specific for *FBXO50* (Supplemental Table S1) or a control siRNA (siControl) combined with LipoTrust EX Oligo (Hokkaido System Science, Sapporo, Japan). After transfection, cells were cultured in serum-free DMEM for 72 h and used in functional assays.[18]

*Cell Proliferation, Adhesion, Migration, and Invasion Assays*

Briefly, cell proliferation was evaluated using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells ($5 \times 10^3$ cells per well) were incubated, and the optical density of the solution in each well was measured on days 1, 3, and 5 after the addition of 10 mL of

Cell Counting Kit-8 solution.[19] The CytoSelect 48-Well Cell Adhesion Assay (Cell Biolabs, Inc., San Diego, CA) was used to determine adherence of the cells to the extracellular matrix components fibronectin, collagen I, collagen IV, laminin I, and fibrinogen. Cells ($7.5 \times 10^4$ cells per well) were added to each well, and optical density at 560 nm of each well was measured after 1 h. The ability of GC cells to invade Matrigel was determined using BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA) according to the manufacturer's protocol. Cells ($2.5 \times 10^4$) in serum-free DMEM were added to each upper well of the chamber. After 48 h, cells on the lower surface of the membrane were fixed, stained, and a microscope (200× magnification) was used to count the cells in eight randomly selected fields.[5] The migration of cells was evaluated using wound-healing assays as previously described. The width of the wound was measured at 100-mm intervals (20 measurements per well, 40× magnification).[11]

*Evaluation of the Clinical Significance of FBXO50 Expression*

*FBXO50* mRNA levels were determined in 200 matched pairs of resected gastric tissues. Patients were stratified into two groups; high (increased in GC tissues) and low (unchanged or decreased in GC tissues) *FBXO50* expression groups.

*Statistical Analysis*

Differences in the data for qualitative variables were compared between the two groups using the

$\chi^2$ test, and quantitative variables were compared using the Mann–Whitney test. The significance of the difference between two variables was assessed using Spearman's rank correlation coefficient. Overall and disease-free survival rates were calculated using the Kaplan–Meier method, and the difference between survival curves was analyzed using the log-rank test. Statistical analyses were performed using JMP 10 software (SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered statistically significant.

## RESULTS

### *Analyses of FBXO50 Expression and Copy Number in GC Cell Lines*

The levels of *FBXO50* mRNA were >2-fold higher in AGS, MKN1, N87, NUGC3, and SC-6-JCK cells compared with those of FHs74 cells (Fig. 1a). *FBXO50* mRNA levels did not differ according to the extent of differentiation of the GC cells. Copy number gain of the *FBXO50* locus was detected in four GC cell lines, and three (75%) cell lines expressed increased levels of *FBXO50* mRNA compared with that of FHs74 cells (Fig. 1a). PC array analysis revealed that EMT-related genes encoding integrin subunit alpha 5 (*ITGA5*), integrin subunit beta 1 (*ITGB1*), matrix metallopeptidase 2 (*MMP2*), moesin (*MSN*), collagen type V alpha 2 chain (*COL5A2*), G protein subunit gamma 11 (*GNG11*), and Wnt family member 5A (*WNT5A*) were expressed at levels with correlation coefficients ≥0.6 compared with that of *FBXO50* (Fig. 1b).

*Effect of FBXO50 Knockdown on the Malignant Phenotype of GC Cells*

To evaluate the function of *FBXO50* in GC cells, we used an *FBXO50* siRNA. We selected MKN1 cells for the analyses, because qRT-PCR assays revealed that it exhibited the highest levels of *FBXO50* mRNA expression among GC cells examined. After determining knockdown efficacy (Fig. 2a), we evaluated cell proliferation, adhesion, migration, and invasiveness. Inhibition of *FBXO50* expression significantly decreased the proliferation of MKN1 cells (30% and 33% decrease on days 3 and 5, respectively) (Fig. 2b). Further, knockdown of *FBXO50* expression decreased the adhesive capacity to four components of the extracellular matrix (fibronectin, collagen I, collagen IV and fibrinogen) in MKN1 cells (Fig. 2c). Invasiveness (Fig. 3a) and migration (Fig. 3b) of MKN1 cells were reduced by inhibiting *FBXO50* expression compared with the untransfected and siControl-transfected cells.

*Patient Characteristics*

The patient population included 150 males and 50 females aged from 20 to 86 years (65.6 ± 11.6 years, mean ± standard deviation). Pathologically, 124 and 76 patients were diagnosed with undifferentiated and differentiated GC, respectively. According to the 7th edition of the UICC classification, 38, 37, 58, and 67 patients were in stages I, II, III, and IV, respectively, of which 133 patients in stages I–III underwent R0 resection.

*Clinical Significance of FBXO50 Expression*

Expression levels of FBXO50 mRNA in GC tissues compared with the corresponding adjacent noncancerous tissues from 200 patients were shown in Supplementary FIG. 1. High *FBXO50* expression was significantly associated with tumor depth, invasive growth, lymph node metastasis, and positive peritoneal lavage cytology (Table 1). Patients with high *FBXO50* mRNA expression ($n$ = 75) were more likely to experience shorter overall survival compared to those with low *FBXO50* mRNA expression ($n$ = 125), and their 5-year survival rates were 45% and 68%, respectively ($P$ = 0.002) (Fig. 4a). Further, disease-free survival after curative gastrectomy was administered to 133 patients with stage I-III GC was significantly shorter in the high *FBXO50* expression group compared with the low *FBXO50* expression group (3-year survival rates, 63% and 76%, respectively, $P$ = 0.033) (Fig. 4b). The frequency of overall recurrence after curative gastrectomy in the high *FBXO50* expression group was higher compared with that of the low *FBXO50* expression group (43% and 26%, respectively, $P$ = 0.045) (Fig. 4c). The high *FBXO50* expression group had a higher frequency of hematogenous and peritoneal metastases as initial recurrence (Fig. 4c).

## DISCUSSION

The human *FBXO50* gene, located on chromosome 19q13.2, encodes a 31-kDa protein composed

275 amino acid residues.[12] *FBXO50* is ubiquitously expressed and is relatively abundant in the squamous epithelium, including the esophagus and skin.[12] FBXO family members are involved in the regulation of the cell cycle;[20-22] however, information on the physiological functions of *FBXO50* is limited. We reasoned therefore that analysis of *FBXO50* expression and function might identify its role in GC.

We show here that five out of 10 GC cell lines expressed increased levels of *FBXO50* mRNA and that such cells frequently (75%) harbored additional copies of *FBXO50.* Copy number gain up-regulates the expression of certain proto-oncogenes and oncogenes. Several studies report frequent copy number gains in colorectal and pancreatic cancers at chromosome 19q13,[23-25] and the present findings provide new information on copy number alteration of this locus in GC. The role of *FBXO50* in cancer is unknown, and it is therefore important to acquire knowledge about the involvement of *FBXO50.* In contrast, some of GC cell line had even lower levels of *FBXO50* mRNA than FHs74. Although *FBXO50* mRNA levels did not differ according to the extent of differentiation of the GC cells, all GC cell lines with the top 3 highest expression levels of *FBXO50* (MKN1, SC-6-JCK and N87) were derived from metastatic GC, suggesting *FBXO50* tended to be overexpressed in aggressive GC cells. Besides, analysis using clinical samples also revealed that *FBXO50* expression in gastric tissues is quite diversified, and it was aberrantly overexpressed in a certain population of GC tissues. From these findings, it was suggested that overexpression of *FBXO50* is not a ubiquitous event in GC.

The EMT plays an important role in cancer progression, and this switch in cell differentiation and behavior is mediated by numerous transcription factors.[26,27] Therefore, we conducted PCR array analysis that focused on the expression of EMT-related genes in GC cell lines. This analysis revealed that the expression levels of *FBXO50* mRNA positively correlated with genes upregulated during the EMT as follows: *ITGA5*, *ITGB1*, *MMP2*, *MSN*, *COL5A2*, *GNG11*, and *WNT5A*.[28,29] Although further investigations are required, including pathway analysis, these results support the possibility of interactions between FBXO50 and proteins that mediate the EMT. Moreover, there were some more EMT-related genes, such as *BMP1*, *SPARC*, *CALD1* and *TGFB2*, positively correlated to *FBXO50*, but their correlation coefficients to *FBXO50* were ranged 0.5 to 0.6 (data not shown). Data from larger number of GC cell lines examined would contribute to further understanding of the involvement of *FBXO50* in the EMT cascade.

These findings led us to determine whether *FBXO50* contributes to the pathogenesis of GC. We chose the MKN1 cell having the highest *FBXO50* mRNA levels to obtain reliable data on the phenotypic effects of siRNA-mediated knockdown of *FBXO50*, and found that inhibition of *FBXO50* expression significantly decreased in the proliferation, adhesion, migration, and invasiveness of GC cells. Such properties are required for cancer cells to become metastatic. These findings support the conclusion that *FBXO50* facilitates malignant behavior of GC. This conclusion is also supported by our present findings showing that increased expression of *FBXO50* was significantly associated with the more aggressive phenotypes of GC such as increased tumor depth, lymph node metastasis, and

positive peritoneal lavage cytology. Moreover, patients with high *FBXO50* mRNA expression experienced a higher incidence of postoperative recurrence and poorer prognosis, highlighting the potential utility of *FBXO50* expression as a novel biomarker for GC progression. There have been numerous molecular biomarkers for cancer progression. Both expression levels in cancerous tissues and differences between non-cancerous and cancerous tissues would be important abnormalities, depending on each biomarker. We found that increased *FBXO50* expression in GC tissues compared to the corresponding normal adjacent gastric tissues can be used for patient stratification to predict prognosis. To translate our results to the clinic, evaluation of *FBXO50* expression must show that it is effective for monitoring postoperative disease and for selecting an optimal multimodal management strategy.

There are some limitations of this study. Extensive expression analyses identified several proteins that contribute to the EMT. These proteins may interact with FBXO50, and pathway analyses should therefore be conducted to further understand the biological functions of *FBXO50* in GC. Second, the expression levels and prognostic value of *FBXO50* in GC were evaluated in retrospectively collected tissues from a relatively small number of patients. External validation using other large cohorts is required to validate our present findings.

Taken together, our findings indicate that *FBXO50* enhances the malignant phenotype and that the transcriptional or translational product of *FBXO50*, or both, will serve as potential biomarkers and targets of therapy, which may facilitate monitoring of GC and developing effective therapeutic

strategies.

## REFERENCES


**1.** Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin.* 2015;65:5-29.

**2.** Kanda M, Murotani K, Kobayashi D, et al. Postoperative adjuvant chemotherapy with S-1 alters recurrence patterns and prognostic factors among patients with stage II/III gastric cancer: A propensity score matching analysis. *Surgery.* 2015;158:1573-1580.

**3.** Van Cutsem E, Sagaert X, Topal B, Haustermans K, Prenen H. Gastric cancer. *Lancet.* 2016.

**4.** Songun I, Putter H, Kranenbarg EM, Sasako M, van de Velde CJ. Surgical treatment of gastric cancer: 15-year follow-up results of the randomised nationwide Dutch D1D2 trial. *Lancet Oncol.* 2010;11:439-449.

**5.** Kanda M, Shimizu D, Tanaka H, et al. Significance of SYT8 For the Detection, Prediction, and Treatment of Peritoneal Metastasis From Gastric Cancer. *Ann Surg.* 2016.

**6.** Radke S, Pirkmaier A, Germain D. Differential expression of the F-box proteins Skp2 and Skp2B in breast cancer. *Oncogene.* 2005;24:3448-3458.

**7.** Yu Y, Sun L, Ren N, Li Y, Rong L, Zhao G. Down-expression of F box only protein 8 correlates with tumor grade and poor prognosis in human glioma. *Int J Clin Exp Pathol.* 2014;7:8071-8076.

**8.** Zhang C, Li X, Adelmant G, et al. Peptidic degron in EID1 is recognized by an SCF E3 ligase complex containing the orphan F-box protein FBXO21. *Proc Natl Acad Sci U S A.* 2015;112:15372-15377.

**9.** Cepeda D, Ng HF, Sharifi HR, et al. CDK-mediated activation of the SCF(FBXO) (28) ubiquitin ligase promotes MYC-driven transcription and tumourigenesis and predicts poor survival in breast cancer. *EMBO Mol Med.* 2013;5:1067-1086.

**10.** Kanda M, Shimizu D, Tanaka H, et al. Metastatic pathway-specific transcriptome analysis identifies MFSD4 as a putative tumor suppressor and biomarker for hepatic metastasis in patients with gastric cancer. *Oncotarget.* 2016;7:13667-13679.

**11.** Kanda M, Tanaka C, Kobayashi D, et al. Epigenetic suppression of the immunoregulator MZB1 is associated with the malignant phenotype of gastric cancer. *Int J Cancer.* 2016;139:2290-2298.

**12.** Kallio H, Tolvanen M, Janis J, et al. Characterization of non-specific cytotoxic cell receptor protein 1: a new member of the lectin-type subfamily of F-box proteins. *PLoS One.* 2011;6:e27152.

**13.** Kanda M, Shimizu D, Fujii T, et al. Neurotrophin Receptor-Interacting Melanoma Antigen-Encoding Gene Homolog is Associated with Malignant Phenotype of Gastric Cancer. *Ann Surg Oncol.* 2016.

**14.** Sasako M, Sakuramoto S, Katai H, et al. Five-year outcomes of a randomized phase III trial comparing adjuvant chemotherapy with S-1 versus surgery alone in stage II or III gastric cancer. *J Clin Oncol.* 2011;29:4387-4393.

**15.** Kanda M, Kobayashi D, Tanaka C, et al. Adverse prognostic impact of perioperative allogeneic transfusion on patients with stage II/III gastric cancer. *Gastric Cancer.* 2016;19:255-263.

**16.** Kanda M, Shimizu D, Fujii T, et al. Function and diagnostic value of Anosmin-1 in gastric cancer progression. *Int J Cancer.* 2016;138:721-730.

**17.** Tanaka H, Kanda M, Shimizu D, et al. FAM46C Serves as a Predictor of Hepatic Recurrence in Patients with Resectable Gastric Cancer. *Ann Surg Oncol.* 2016.

**18.** Kanda M, Shimizu D, Fujii T, et al. Protein arginine methyltransferase 5 is associated with malignant phenotype and peritoneal metastasis in gastric cancer. *Int J Oncol.* 2016;49:1195-1202.

**19.** Oya H, Kanda M, Sugimoto H, et al. Dihydropyrimidinase-like 3 is a putative hepatocellular carcinoma tumor suppressor. *J Gastroenterol.* 2015;50:590-600.

**20.** Chiorazzi M, Rui L, Yang Y, et al. Related F-box proteins control cell death in Caenorhabditis elegans and human lymphoma. *Proc Natl Acad Sci U S A.* 2013;110:3943-3948.

**21.** Tian X, Dai S, Sun J, et al. F-box protein FBXO22 mediates polyubiquitination and degradation of KLF4 to promote hepatocellular carcinoma progression. *Oncotarget.* 2015;6:22767-22775.

**22.** Yumimoto K, Akiyoshi S, Ueo H, et al. F-box protein FBXW7 inhibits cancer metastasis in a non-cell-autonomous manner. *J Clin Invest.* 2015;125:621-635.

**23.** Kuuselo R, Simon R, Karhu R, et al. 19q13 amplification is associated with high grade and stage in pancreatic cancer. *Genes Chromosomes Cancer.* 2010;49:569-575.

**24.** Smith DH, Christensen IJ, Jensen NF, et al. An explorative analysis of ERCC1-19q13 copy number aberrations in a chemonaive stage III colorectal cancer cohort. *BMC Cancer.* 2013;13:489.

**25.** Orsetti B, Selves J, Bascoul-Mollevi C, et al. Impact of chromosomal instability on colorectal cancer progression and outcome. *BMC Cancer.* 2014;14:121.

**26.** Wu Q, Hou X, Xia J, et al. Emerging roles of PDGF-D in EMT progression during tumorigenesis. *Cancer Treat Rev.* 2013;39:640-646.

**27.** Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol.* 2014;15:178-196.

**28.** Bae GY, Choi SJ, Lee JS, et al. Loss of E-cadherin activates EGFR-MEK/ERK signaling, which promotes invasion via the ZEB1/MMP2 axis in non-small cell lung cancer. *Oncotarget.* 2013;4:2512-2522.

**29.** Prasad CP, Chaurasiya SK, Guilmain W, Andersson T. WNT5A signaling impairs breast cancer cell migration and invasion via mechanisms independent of the epithelial-mesenchymal transition. *J Exp Clin Cancer Res.* 2016;35:144.

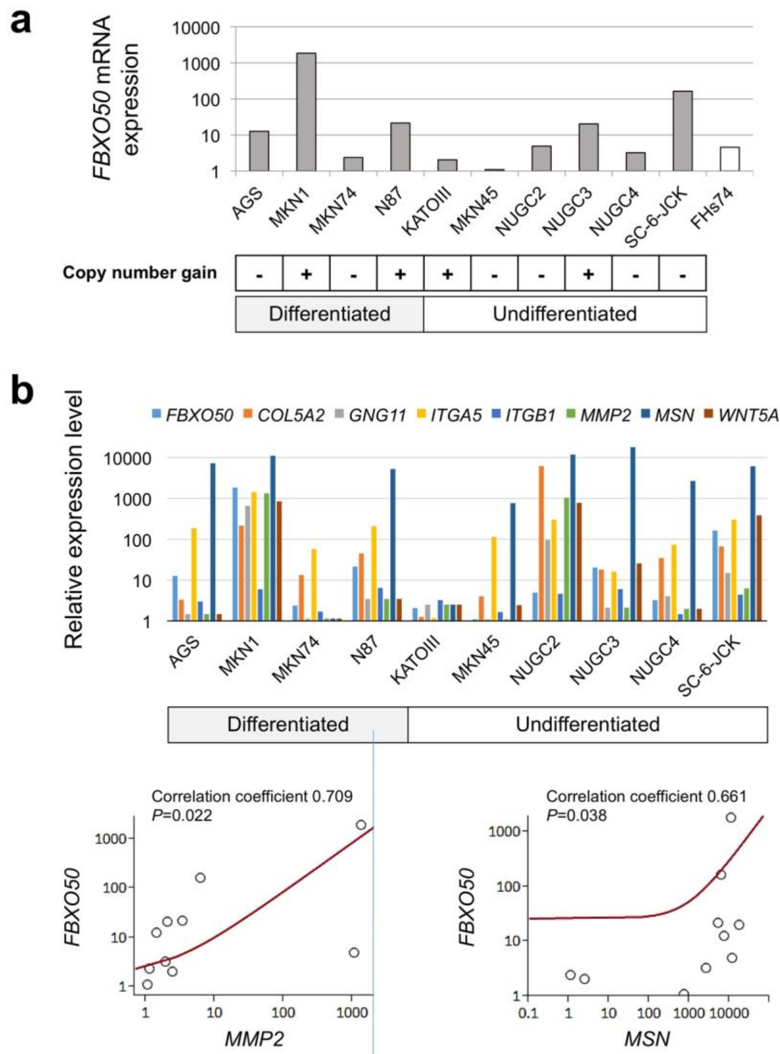



**FIG. 1**

Expression analysis of cell lines. (**a**) Different expression levels of *FBXO50* mRNA were observed among GC cell lines and between control cell lines. Copy number gain was detected in MKN1, N87, KATOIII, and NUGC3 cells. (**b**) Expression of *FBXO50* and genes expressed at similar differential levels were identified using PCR array analysis. Representative data for *MMP2* and *MSN* are shown.

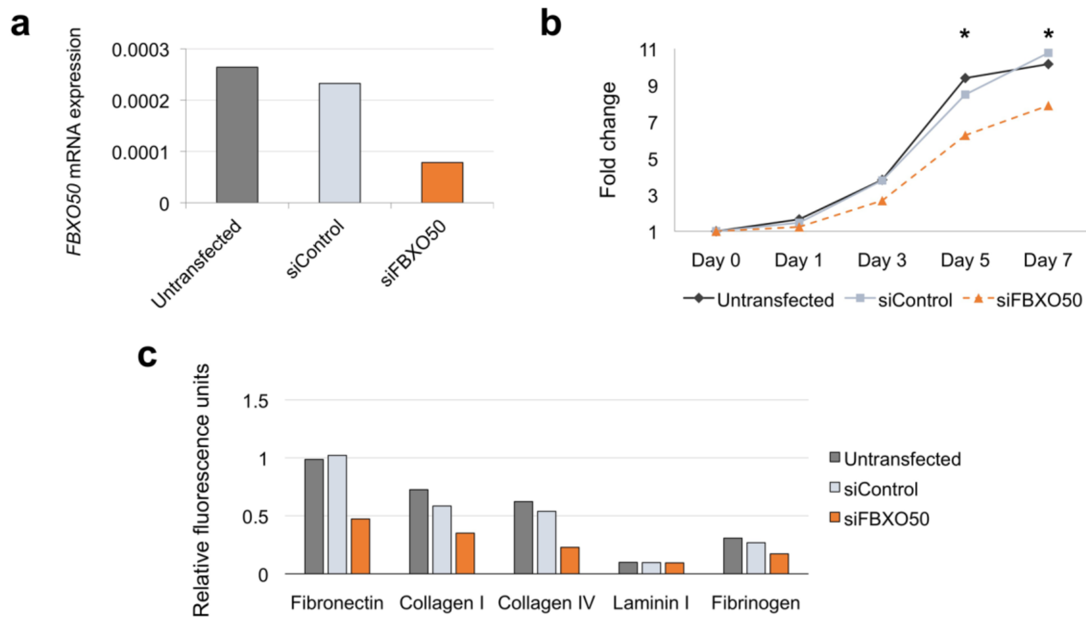



**FIG. 2**

Effects of siRNA-mediated knockdown of *FBXO50*. (**a**) Confirmation of siRNA-mediated *FBXO50* knockdown was determined using a qRT-PCR assay. (**b**) Cell proliferation assay. *FBXO50* siRNA significantly inhibited the proliferation of MKN1 cells. $*P < 0.05$. (**c**) Cell adhesion assay. Adhesion to four components of the extracellular matrix (fibronectin, collagen I, collagen IV and fibrinogen) was decreased by knockdown of *FBXO50* expression.

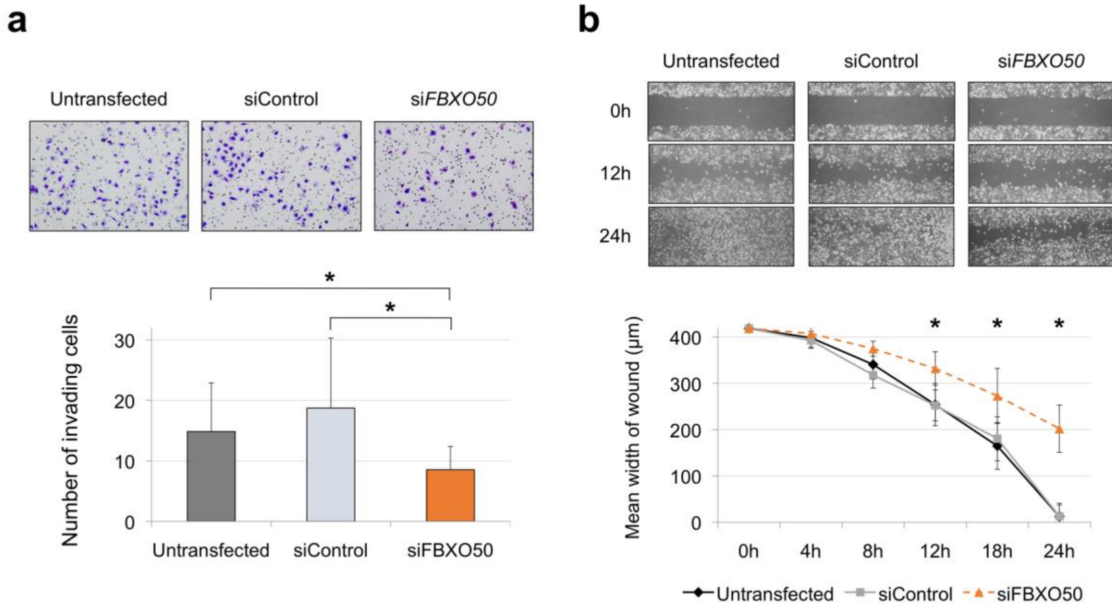



**FIG. 3**

Effects of siRNA-mediated knockdown of *FBXO50* expression. (**a**) Cell invasion assay. Invasion of Matrigel was decreased by inhibition of *FBXO50* expression. Number of invading cells indicated a mean number of invading cells in the eight randomly selected fields (200× magnification). (**b**) Wound healing assays of MKN1 cells. Knockdown of *FBXO50* mRNA significantly reduced migration. *$P < 0.05$. Error bars indicate standard deviation.

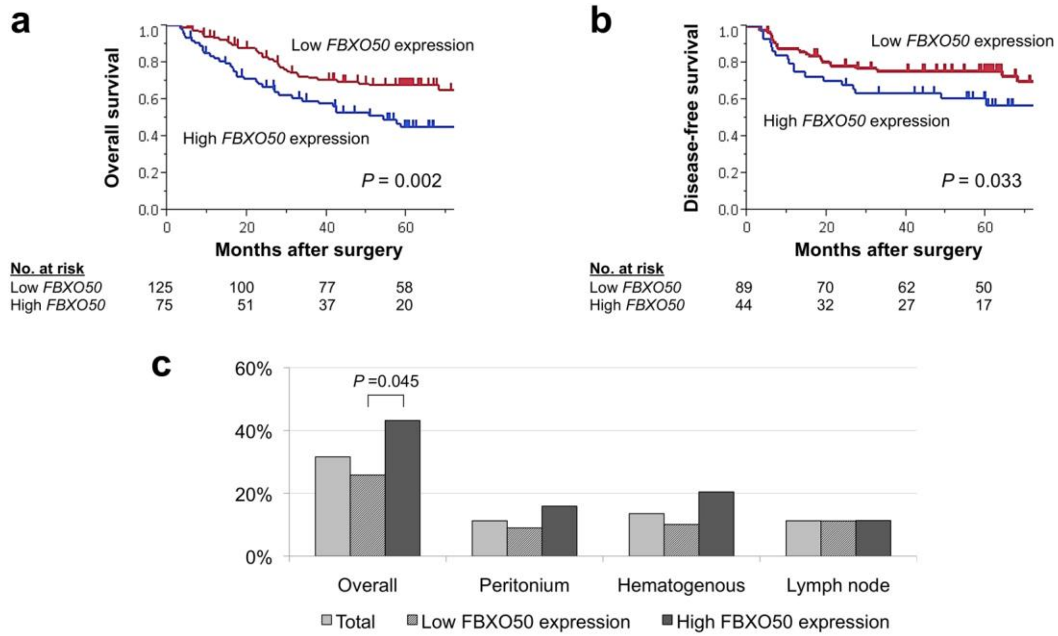
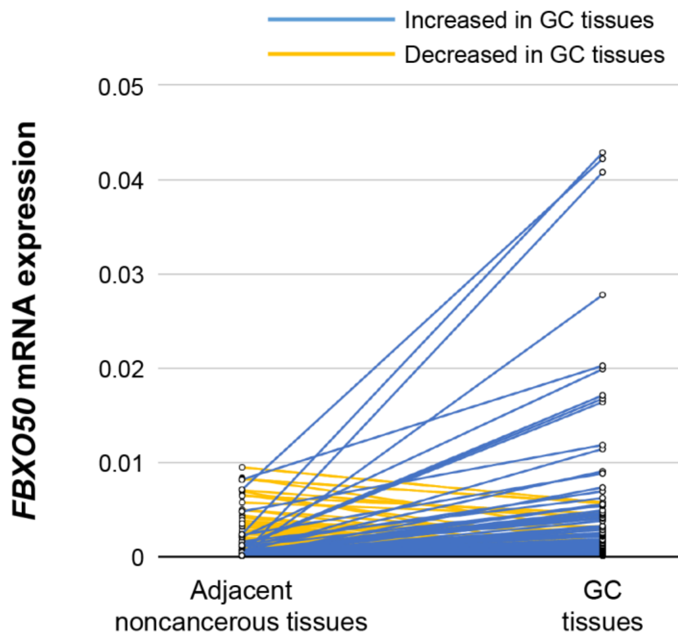



**FIG. 4**

*FBXO50* expression in clinical samples. Patients in the high *FBXO50* group were more likely to have poorer (**a**) overall and (**b**) disease-free survival compared with the others. (**c**) Frequencies of the sites of initial recurrence.




**Supplementary FIG. 1**

FBXO50 mRNA expression levels in clinical tissues.

**Table 1** Association between *FBXO50* mRNA expression levels and clinicopathological parameters of 200 patients with GC.

| Variables | High *FBXO50* expression (n) | Low *FBXO50* expression (n) | *P* value |
|---|---|---|---|
| Age | | | |
| < 65 year | 34 | 49 | 0.395 |
| ≥ 65 year | 41 | 76 | |
| Sex | | | |
| Male | 56 | 94 | 0.933 |
| Female | 19 | 31 | |
| Carcinoembryonic antigen (ng/ml) | | | |
| ≤ 5 | 55 | 101 | 0.221 |
| > 5 | 20 | 24 | |
| Carbohydrate antigen 19-9 (IU/ml) | | | |
| ≤ 37 | 59 | 102 | 0.614 |
| > 37 | 16 | 23 | |
| Tumor location | | | |
| Entire | 6 | 13 | |
| Upper third | 21 | 23 | 0.443 |
| Middle third | 19 | 38 | |
| Lower third | 29 | 51 | |
| Tumor size (mm) | | | |
| < 50 | 26 | 56 | 0.157 |
| ≥ 50 | 49 | 69 | |
| Tumor depth (UICC 7th) | | | |
| pT1 | 6 | 23 | |
| pT2 | 5 | 19 | 0.008 |
| pT3 | 15 | 30 | |
| pT4 | 49 | 53 | |
| Differentiation | | | |
| Differentiated | 24 | 52 | 0.173 |
| Undifferentiated | 51 | 73 | |
| Lymphatic involvement | | | |
| Absent | 7 | 21 | 0.131 |
| Present | 68 | 104 | |
| Vessel invasion | | | |
| Absent | 25 | 58 | 0.068 |
| Present | 50 | 67 | |

| | | | |
|---|---|---|---|
| Infiltrative growth type | | | |
| Invasive growth | 38 | 34 | 0.001 |
| Expansive growth | 37 | 91 | |
| Lymph node metastasis | | | |
| Absent | 15 | 52 | 0.001 |
| Present | 60 | 73 | |
| Peritoneal lavage cytology | | | |
| Negative | 50 | 103 | 0.012 |
| Positive | 25 | 22 | |

*UICC*, Union for International Cancer Control.

**Supplementary Table S1** Sequences of primers and siRNAs

| | Experiment | Type | Sequence (5´ - 3´) | Product size | Annealing temperature |
|---|---|---|---|---|---|
| ***FBXO50*** | qRT-PCR | forward | AAAGCTCCAGCAGAACCAAA | 104 bp | 60 °C |
| | | reverse | TAATGGCTGGTTGTTCGTCA | | |
| | siRNA | si*FBXO50*-1 | CCGAAGGCAUCAACAUUUA | | |
| | | si*FBXO50*-2 | GCUGCUGGAUGACGAACAA | | |
| | | si*FBXO50*-3 | UUUUCAGUUCUAAUGUACCAG | | |
| | | si*FBXO50*-4 | UUUCAACACACAUUUGCUGCC | | |
| ***GAPDH*** | qRT-PCR | forward | GAAGGTGAAGGTCGGAGTC | 226 bp | 60 °C |
| | | probe | CAAGCTTCCCGTTCTCAGCC | | |
| | | reverse | GAAGATGGTGATGGGATTTC | | |

*FBXO50*, F-box only 50; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *qRT-PCR*, quantitative real-time reverse-transcription polymerase chain reaction; *siRNA*, small interfering RNA.