Decreased expression of *FBXW7* is correlated with poor prognosis in patients with esophageal squamous cell carcinoma

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Abstract. *FBXW7* is a tumor suppressor gene that induces the degradation of positive cell-cycle regulators such as c-Myc, cyclin E, c-Jun and Notch. The loss of *FBXW7* promotes cell-cycle progression and cell proliferation. In the present study, we investigated the relationship between *FBXW7* expression and the clinicopathological characteristics of patients with esophageal squamous cell carcinoma (ESCC). The expression of *FBXW7* was quantified by real-time reverse transcription polymerase chain reaction in 43 primary ESCCs and their paired normal esophageal mucosa in patients who had not received preoperative therapy. *FBXW7* expression levels were significantly correlated with the progression of the cancer and with local invasiveness. In muscle-invasive tumor cases (T2-4), lymphatic invasive tumor cases and stage II-IV cases, *FBXW7* expression levels were significantly decreased (P=0.0315, P=0.0336 and P=0.0289, respectively). Decreased expression of *FBXW7* was correlated with poor prognosis (P=0.0255). In conclusion, this study examined the relationship between *FBXW7* expression and tumor progression in ESCC. We suggest that *FBXW7* is a molecular prognostic marker and can be used to elucidate the mechanism of carcinogenesis.

Introduction

Esophageal cancer is the sixth most common cancer worldwide. The prognosis of patients with esophageal cancer remains poor, prompting the search for new treatment strategies. The overall 5-year survival rate is less than 50%, despite the use of multi-modality therapy. Many patients in the early stage of the disease develop local tumor recurrence or distant metastasis within a short period after surgery. Therefore, more effective therapies for esophageal cancer must be developed.

The control of cellular proliferation is extremely important to an individual, therefore the cell cycle is strictly controlled during individual development and cell differentiation. Malignant tumors result from the loss of normal cell-cycle control, and tumor cells multiply in a disordered manner. Several genes and molecules are involved in the origin and/or progression of esophageal cancer, including TP53 (1,2), deleted in esophageal cancer 1 (DEC1) (3-5), deleted in colorectal cancer (DCC) (6-8), deleted in lung cancer 1 (DLC1) (9), cyclin D1 (10-12), transforming growth factor-β receptor type II (TGFBRII) (13,14), adenomatous polyposis coli (APC) (15), survivin (16,17) and murine double minute 2 (MDM2) (18). However, the precise mechanisms that underlie the development and progression of esophageal squamous cell carcinoma (ESCC) remain unclear.

The ubiquitin-proteasome system regulates important cellular processes including development and differentiation, apoptosis, protein transportation, immunologic and inflammatory responses, cell-cycle progression and cellular division (19). The cell cycle and cellular division are primarily coordinated by two ubiquitin ligases, SCF ubiquitin ligase and anaphase-promoting complex (19,20). The SCF ubiquitin ligases are comprised of the F-box protein family, Cul1, Rbx1 and Skp1. Approximately 70 F-box proteins are present in humans and provide substrate specificity (19,20).

FBXW7, one of the F-box proteins, induces the degradation of positive cell-cycle regulators such as c-Myc, cyclin E, c-Jun and Notch (19,20). These regulators are known as oncoproteins, and the genes encoding these proteins are oncogenes whose mutation and overexpression have been reported in humans. *FBXW7* is therefore focused on as a tumor suppressor gene (21-23), and has been reported to be a clinicopathologic factor in glioma (24), prostate cancer (25), colorectal cancer (26), gastric cancer (27) and T-cell acute lymphocytic leukemia (28,29). However, the role of *FBXW7* in esophageal cancers is uncertain. In the present study, we examined the relationship between the expression of *FBXW7* and the clinicopathological factors and prognosis of patients with ESCC.

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Materials and methods

Cell lines and cell culture. Esophageal cancer cell lines (TE1-15 and KYSE30-520) were purchased from the Japanese Collection of Research Bioresources (JCRB). The normal human esophageal mucosa Het-1A cell line was purchased from the American Type Culture Collection (ATCC). Esophageal cancer cell lines were grown in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Gibco) in tissue culture dishes at 37˚C in a humidified 5% CO_2 incubator. Het-1A cells were grown in LHC-9 serum-free medium (Biofluids, Rockville, MD, USA) at 37˚C in a humidified 5% CO_2 incubator.

Tissue samples. Esophageal cancer (primary ESCC) samples and paired non-cancerous samples were obtained from 43 patients who had undergone a radical esophagectomy at Nagoya City University Hospital between 1996 and 2005 without preoperative chemotherapy or radiation. The study design was approved by the Institutional Review Board of Nagoya City University Hospital, and written consent was obtained from all patients. Samples were snap frozen in liquid nitrogen and stored at -80˚C until RNA and DNA extraction. Patient characteristics are presented in Table I.

RNA extraction and real-time reverse transcription polymerase chain reaction analysis. Total RNA was extracted from the esophageal cancer tissue and the corresponding normal esophageal mucosa using the real-time reverse transcription polymerase chain reaction (RT-PCR) Absolutely RNA™ Miniprep kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The concentration of total RNA was adjusted to 200 ng/ml using a spectrophotometer. Reverse transcription reactions were performed at 42˚C for 90 min and at 95˚C for 5 min followed by incubation at 72˚C for 15 min using 1 μg of total RNA, 0.5 μg oligo (dT) primer and Superscript II enzyme (Gibco BRL, Gaithersburg, MD, USA).

Real-time quantitative reverse transcription polymerase chain reaction with TaqMan probes. Real-time quantitative RT-PCR amplification of the cDNA template corresponding to 20 ng of total RNA was performed using TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). PCR was conducted at 95˚C for 10 min, followed by 40 cycles at 95˚C for 3 sec and 60˚C for 30 sec. *FBXW7*-specific TaqMan probes were designed to amplify a 70-bp PCR product encoding the common region among three *FBXW7* isoforms (*FBXW7α*, *FBXW7β*, *FBXW7γ*) (TaqMan® Gene Expression Assays, assay ID: Hs01015623_m1, Applied Biosystems). The relative expression levels of *FBXW7* mRNA were obtained by normalizing the amount of *FBXW7* mRNA to that of GAPDH mRNA (TaqMan® Gene Expression Assays, assay ID: Hs99999905_m1, Applied Biosystems) as an endogenous control in each sample.

Immunohistochemistry. Immunohistochemical staining was performed on formalin-fixed paraffin-embedded primary human ESCC tissues using a 1:150 dilution of monoclonal anti-Ki-67 antibodies (Dako, Denmark A/S). Paraffin-embedded sections of tumor were deparaffinized and rehydrated. The sections were then heat-treated by microwaving in 10 mM citrate buffer for 10 min to facilitate antigen retrieval, then cooled to room temperature. The sections were treated with 0.3% H_2O_2 in methanol for 30 min to neutralize endogenous peroxidases, blocked with non-specific goat serum for 10 min and incubated with anti-Ki-67 antibody overnight at room temperature in a humidified chamber. Immunoreactive protein was detected with a Dako Envision™+ System, HRP (DAB). The sections were then counterstained with hematoxylin. The expression of Ki-67 was scored using light microscopy according to the proportion of positive staining throughout the entire slide: (-), negative or <5%; (+), <33%; and (++), >33%. Ki-67 immunohistochemical staining was classified as negative for scores of (-) and positive for scores of (+) or (++).

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the Stat-View software package (Abacus Concepts, Berkeley, CA, USA). The Mann-Whitney U test was used to evaluate the significance of differences in the expression levels of *FBXW7*/*GAPDH* mRNA. The survival of patients with ESCC was examined by the Kaplan-Meier method, and the survival time was compared using the log-rank test. Survival was measured from the day of surgery. Multivariate analysis was performed using Cox's regression model and the logistic multivariate regression model. Differences were considered statistically significant at $P<0.05$, and a tendency was determined at $P<0.1$.

Results

Quantitative RT-PCR was used to examine the relative expression level of *FBXW7* mRNA by normalization to *GAPDH* in two esophageal cancer cell lines (TE1-15, KYSE30-520) and one normal human esophageal mucosa cell line (Het-1A). *FBXW7* mRNA was detectable in all cell lines except TE14. KYSE50 had the highest expression level of *FBXW7* mRNA. Only this cell line exhibited increased expression of *FBXW7* mRNA compared to Het1A; the other cell lines had lower expression (Fig. 1). The expression of *FBXW7* mRNA was examined in the 43 ESCC tissue specimens and the paired normal esophageal mucosal tissue of patients who had not received preoperative therapy. The mean expression level of *FBXW7* mRNA in the ESCC tissue was lower than that of the corresponding normal tissue, although the difference was not statistically significant.

Next, the relationship between the ratio of *FBXW7* mRNA expression in the tumor to that in the normal esophageal mucosa (T/N ratio) and the clinicopathological factors of the 43 patients were examined. There were no significant differences in *FBXW7* mRNA expression levels with respect to age, gender, lymph node status or blood vessel invasion. However, the expression levels were significantly correlated with the progression of the cancer and its local invasiveness (stage, t-factor and lymphatic invasion) (Table 1, Fig. 2). The *FBXW7* mRNA expression levels in patients with muscle-invasive tumors (T2-4) were significantly lower than those in patients with less invasive T1 tumors (1.218±1.339 vs. 1.848±1.124;

Table I. Correlation of *FBXW7* mRNA expression in esophageal cancer with clinicopathological factors.

Characteristics	No. of patients (n=43)	*FBXW7* expression	P-value
Tissue (samples)			
Normal	43	16.293±64.896[a]	
Tumor	43	2.793±2.648[a]	0.9003
Age at surgery			
≤65	22	1.405±1.317[b]	
<65	21	1.442±1.303[b]	0.8841
Gender			
Male	37	1.345±1.148[b]	
Female	6	1.903±2.067[b]	0.8334
pStage			
0-I	8	2.154±1.204[b]	
II-IV	35	1.256±1.272[b]	0.0289
Primary tumor			
T1	14	1.848±1.124[b]	
T2-T4	29	1.218±1.339[b]	0.0315
Lymph node metastasis			
Negative	13	1.421±0.977[b]	
Positive	30	1.424±1.425[b]	0.6154
Lymphatic invasion			
-	10	1.957±1.163[b]	
+	31	1.252±1.308[b]	0.0336
Unknown	2		
Vessel invasion			
-	16	1.633±1.179[b]	
+	25	1.276±1.379[b]	0.1608
Unknown	2		
Ki-67			
-	16	1.659±1.124[b]	
+	25	1.171±1.146[b]	0.0733
Unknown	2		

[a]Expression relative to GAPDH; [b]ratio of tumor/normal tissue. Expression is presented as the mean ± standard deviation (SD). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

P=0.0315, Mann-Whitney U test) (Table I, Fig. 2A). The *FBXW7* mRNA expression levels were significantly lower in stage 0-I ESCC than in stage II-IV ESCC (1.256±1.272 vs. 2.154±1.204; P=0.0289, Mann-Whitney U test) (Table I, Fig. 2B). Moreover, *FBXW7* mRNA expression levels were significantly lower in patients with lymphatic invasion than in those without (1.252±1.308 vs. 1.957±1.163; P=0.0336) (Table I). Ki-67 as a proliferation marker was also detected by immunostaining, and its correlation with the expression levels of *FBXW7* was evaluated in the 41 cases investigated in this study. The Ki-67-positive cases [scored as (+) or (++)] tended to express decreased levels of *FBXW7* mRNA as compared to the negative cases (1.171±1.146 vs. 1.659±1.124; P=0.0733) (Table I, Fig. 3).

The correlation between the expression levels of *FBXW7* and the postoperative survival period of patients with ESCC was investigated (median follow-up, 30.0 months). The 43 cases were divided into the high *FBXW7* mRNA expression group [the ratio of *FBXW7* mRNA expression in the tumor to that in normal esophageal mucosa (T:N ratio) >1.0] and the low *FBXW7* mRNA expression group (T:N ratio <1.0). The patients with low levels of *FBXW7* mRNA expression had a significantly shorter postoperative survival time than the patients with high levels of *FBXW7* mRNA expression [24.9±19.5 (n=21) vs. 34.3±19.5 months (n=22); P=0.0255, log-rank test) (Fig. 4)].

Univariate analysis (Table II) revealed the following prognostic factors to be statistically significant: the extent of

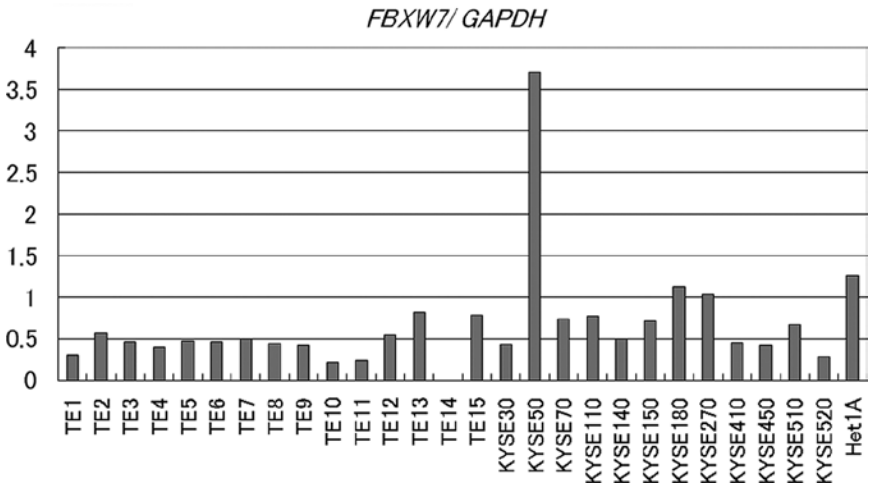

Figure 1. *FBXW7* mRNA was detectable in all cell lines except TE14. KYSE50 had the highest expression level of *FBXW7* mRNA, followed by Het-1A.

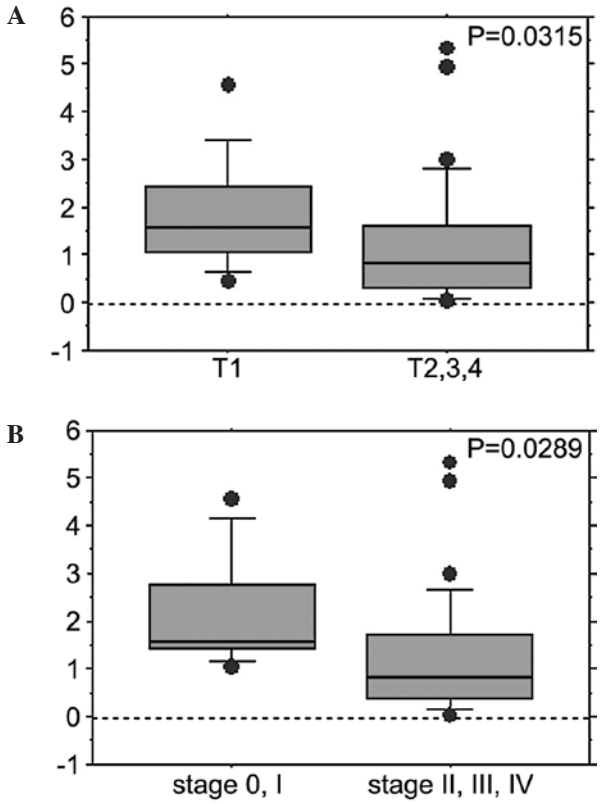

Figure 2. (A) *FBXW7/GAPDH* mRNA expression levels (T/N) were significantly lower in patients with T2-4 tumors than in patients with T1 tumors (P=0.0315, Mann-Whitney U test). (B) *FBXW7/GAPDH* mRNA expression levels (T/N) were significantly lower in patients with histological stage II-IV tumors than in patients with histological tumor stages 0-I (P=0.0289, Mann-Whitney U test).

the primary tumor (risk ratio 9.524, P<0.0001), lymph node metastasis (risk ratio 7.752, P<0.0001), lymphatic invasion (risk ratio 6.061, P=0.0006), vessel invasion (risk ratio 3.175, P=0.0006) and *FBXW7* mRNA expression (risk ratio 2.688, P=0.0332). However, accoding to the multivariate analysis, *FBXW7* mRNA expression was not an independent prognostic factor (data not shown).

Discussion

FBXW7 is an F-box protein that contains components of the SCF ubiquitin ligase complexes, which are involved in the ubiquitin-proteasome pathway. *FBXW7* is localized to chromosome region 4q32 and has three isoforms (*FBXW7α*, *FBXW7β*, *FBXW7γ*). *FBXW7* is important for the degradation of positive cell-cycle regulators such as c-Myc, cyclin E, c-Jun and Notch (20,21,30-32). c-Myc is an oncoprotein that is an important substrate of *FBXW7*. c-Myc helps to control the G1-to-S phase transition. It promotes the entry of cells into the G1 phase, and its expression is maintained throughout the cell cycle. Degradation of c-Myc by *FBXW7* leads to cell-cycle exit (G0 phase) and maintains cell-cycle arrest. Conversely, the loss of *FBXW7* promotes cell-cycle progression and cell proliferation, and is therefore considered one of the major causes of carcinogenesis or carcinoma development (33-35). Onoyama *et al* reported that mice carrying an *FBXW7* T-cell conditional knockout developed thymic hyperplasia and thymic lymphomas (35). Furthermore, decreased expression of *FBXW7* is correlated with poor prognosis in gastric cancer (27) and colorectal cancer (26).

In the present study, we investigated the relationship between the expression of *FBXW7* and the clinicopathological factors and prognosis of patients with ESCC who did not receive preoperative therapy. *FBXW7* expression levels were significantly correlated with the progression of the cancer and local invasiveness. In cases with muscle-invasive (T2-4), lymphatic invasive and stage II-IV tumors, *FBXW7* expression was significantly lower than in other cases (1.218±1.339 vs. 1.848±1.124, P=0.0315; 1.252±1.308 vs. 1.957±1.163, P=0.0336; 1.256±1.272 vs. 2.154±1.204, respectively; P=0.0289, Mann-Whitney U test) (Table I).

These results indicate that decreased expression of *FBXW7* may contribute to tumor growth and invasion in ESCC. Tumors with decreased *FBXW7* expression may undergo active cellular division due to the unregulated cell cycle and may become more invasive. This concept is supported by our finding that *FBXW7* expression levels tended to be lower in Ki-67-positive cases. We also found that the low *FBXW7*

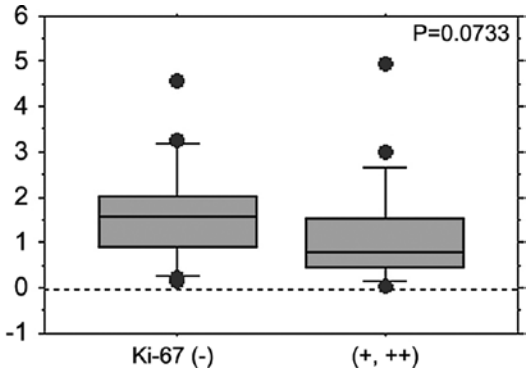

Figure 3. There was a trend towards lower *FBXW7/GAPDH* mRNA expression levels (T/N) in patients with Ki-67-positive compared to Ki-67-negative tumors (P=0.0733, Mann-Whitney U test).

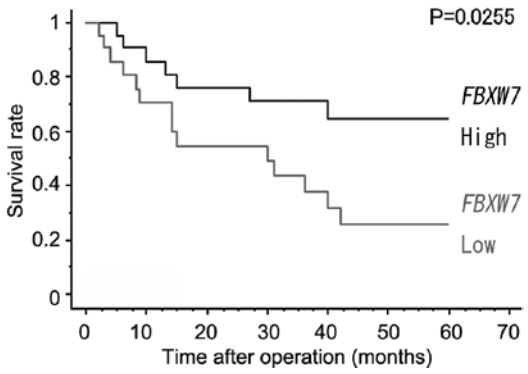

Figure 4. Patients with low levels of *FBXW7* mRNA expression had a significantly shorter postsurgical survival than those with high levels of *FBXW7* mRNA expression (24.9±19.5 vs. 34.3±19.5 months; P=0.0255, log-rank test).

Table II. Univariate analysis.

Parameter	Risk ratio	95% CI	P-value
Age at surgery			
≤65	1		
<65	0.933	0.547-1.591	0.7992
Gender			
Male	1		
Female	1.331	0.713-2.485	0.3688
Primary tumor			
T1	1		
T2-T4	9.524	3.401-27.027	<0.0001
Lymph node metastasis			
Negative	1		
Positive	7.752	3.067-19.608	<0.0001
Lymphatic invasion			
-	1		
+	6.061	2.169-16.949	0.0006
Vessel invasion			
-	1		
+	3.175	1.645-6.135	0.0006
FBXW7 expression (T/N)			
High	1		
Low	2.688	1.082-6.667	0.0332
Ki-67			
-	1		
+	1.447	0.762-2.747	0.2587

CI, confidence interval.

expression group had a significantly poorer prognosis than the high *FBXW7* expression group [24.9±19.5 (n=21) vs. 34.3±19.5 (n=22) months, P=0.0255, log-rank test; Fig. 4]. This was due to the increased proportion of advanced cases in the low *FBXW7* expression group as compared to the high *FBXW7* expression group.

Esophageal cancer has a very poor prognosis, and the molecular mechanism of carcinogenesis in this cancer is unclear. In the present study, we identified a relationship between *FBXW7* expression and tumor progression. It is therefore possible that *FBXW7* is a molecular prognostic marker that can be used to elucidate the mechanism of carcinogenesis.

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