Copy number loss of *FBXW7* is related to gene expression and poor prognosis in esophageal squamous cell carcinoma

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Abstract. *FBXW7* is a tumor suppressor gene that plays a role in cell cycle regulation via Myc degradation. However, the clinical significance of *FBXW7* in esophageal squamous cell carcinoma (ESCC) has not been evaluated. The purpose of this study was to assess the clinical significance of *FBXW7* for prognosis in human ESCC. Real-time RT-PCR was used to examine the expression of *FBXW7* to determine its clinicopathological significance in 75 cases of ESCC. Overall survival rate was calculated using the Kaplan-Meier method, while multivariate survival was analyzed with the Cox hazard model. *FBXW7* suppression analysis was performed to examine proliferation potency and Myc expression in the *FBXW7* siRNA groups. The relationship between *FBXW7* expression and the copy number loss of *FBXW7* was examined in clinical samples of ESCC. Finally, *FBXW7* copy number loss was linked to prognosis in 42 ESCC patients. *FBXW7* expression in cancer was lower compared to non-cancer tissues (P=0.003) and is an independent prognostic factor. The proliferation rates and Myc protein expression were significantly enhanced in *FBXW7* siRNA cells compared to the controls. Cases with a loss of *FBXW7* copy number had low *FBXW7* expression and a poorer prognosis than cases with no loss of copy number. Genetic alterations in esophageal cancer lead to the loss of *FBXW7* expression and increased cell proliferation. These genetic alterations of *FBXW7* status may provide a prognostic factor for ESCC patients.

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Introduction

Esophageal squamous cell carcinoma (ESCC) accounts for most esophageal cancers in East Asia. Although the sensitivity of ESCC to chemoradiotherapy is relatively high, patients with progressive ESCC have poorer prognoses (1, 2). Tumor suppressor inactivation (e.g. p53, p16, RB) and oncogene activation (e.g. Myc, and cyclin D) are involved in ESCC progression (3). In particular, the Myc transcription factor and its downstream targets (e.g., Cyclin D and CDKs) are associated with carcinogenesis and progression of many cancers, including ESCC. The finding that suppression of Myc greatly inhibits tumor formation, has made Myc a promising therapeutic target (4).

FBXW7 is an F-box protein that forms one of the four subunits of SCF ubiquitin ligase complexes, which induce the degradation of positive cell cycle regulators (oncoproteins) such as Myc (5-7). The degradation of Myc by *FBXW7* leads to cell cycle exit (G0 phase); therefore, altered *FBXW7* expression is considered a major cause of carcinogenesis or carcinoma development (8-10). Clinically, low expression of *FBXW7* in human solid tumors such as glioma, colorectal cancer and gastric cancer produces a poor prognosis (11-14). *FBXW7* mutation, transcriptional control by *p53* and copy number loss are the primary mechanisms of *FBXW7* inactivation (10,13-15), which is consistent with our finding that the low expression of *FBXW7* in colorectal cancer is due to copy number loss. A deletion of chromosome 4q, which includes *FBXW7*, has been reported in 30-40% of ESCC cases and is associated with a poor prognosis (16). These findings suggest that *FBXW7* contributes to carcinogenesis and tumor development in ESCC. However, few studies are available on the clinical significance and regulatory mechanism of *FBXW7* in ESCC.

The purpose of this study was to clarify the functional and clinical significance of the tumor suppressor *FBXW7* in ESCC: i) the effect of *FBXW7* function on proliferation potency in an ESCC cell line was evaluated using *FBXW7* siRNA, ii) the

clinical significance of *FBXW7* expression in ESCC was evaluated using real-time RT-PCR, and iii) the correlation between *FBXW7* expression and copy number loss was assessed in clinical samples.

Materials and methods

FBXW7 RNA interference. The following *FBXW7*-specific siRNA and negative control siRNA were purchased from Ambion/Applied Biosciences: Silencer Pre-designed siRNA1, sense: GCACAGAAUUGAUACUAACTT, antisense: GUUAGUAUCAAUUCUGUGCTG; Silencer Pre-designed siRNA2, sense: CCUUAUAUGGGCAUACUUCTT, antisense: GAAGUAUGCCCAUAUAAGGTG); and Silencer Negative Control no. 1 siRNA). Lipofectamine RNAi MAX (Invitrogen) and *FBXW7*-specific siRNA were incubated in 6-well flat bottom microtiter plates. After incubation, the KYSE70 cell line was seeded in these plates at $1.5x10^5$ cells/well in a volume of 2 ml and incubated in a humidified atmosphere (37˚C and 5% CO_2). The RNA interference assay was performed following a 24-h incubation.

Immunoblot analysis. Total protein was extracted from KYSE70 cells after *FBXW7* RNA interference reaction. Aliquots of total protein (35 μg) were electrophoresed in 7.5% concentrated Ready Gels J (Bio-Rad Laboratories, Japan). Western blot analysis was performed as previously described (17). Myc proteins were detected using anti-Myc (N-262, Santa Cruz Biotechnology), diluted 1:100. β-actin protein (cytoskeleton) diluted 1:1000 was used for normalization. ECL Detection Reagents (Amersham Biosciences) were used to detect antigen-antibody reactions.

In vitro proliferation assay. Proliferation was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche Diagnostics Corp., GmbH). After a 24-h incubation following siRNA addition, the cells were cultured further for 0-72 h and the absorbance of the samples was measured as previously described (18).

Clinical samples and ESCC cell lines. Seventy-five ESCC patients underwent surgical treatment at Kyushu University at Beppu and affiliated hospitals between 1992 and 2005. Resected tumor and paired non-tumor tissue specimens were immediately placed in RNAlater (Takara) and embedded in paraffin until RNA extraction. Written informed consent was obtained from all patients. The median follow-up period was 3.85 years.

The KYSE70 was a generous gift from Dr Y. Shimada (Kyoto University, Kyoto, Japan) (19). This cell line was maintained in RPMI-1640 containing 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 U/ml streptomycin sulfates, and cultured in a humidified 5% CO_2 incubator at 37˚C.

Real-time quantitative RT-PCR. The following primers were used for real-time qRT-PCR of *FBXW7*: 5'-AAAGAGTTGTT AGCGGTTCTCG-3' (sense) and 5'-CCACATGGATACCATC AAACTG-3' (antisense). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), the internal control, was amplified with the following primers: 5'-TTGGTATCGTGGAAGGACTCTA-3' (sense) and 5'-TGTCATATTTGGCAGGTT-3' (antisense). Real-time monitoring of PCR reactions was performed using the LightCycler System (Roche Applied Science) and SYBER-Green I (Roche Applied Science) according to the manufacturer's instructions (20). The amplification cycles consisted of an initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 62˚C (60˚C for *GAPDH*) for 10 sec, and elongation at 67˚C (65˚C for *GAPDH*) for 10 sec. Melting curve analysis was performed to distinguish specific products from non-specific products and primer dimers as previously described (21). The relative expression level of *FBXW7* was obtained by dividing the amount of *FBXW7* mRNA by the amount of *GAPDH* mRNA in each sample.

Immunohistochemical staining. Immunohistochemical analysis of FBXW7 and Myc was performed in the sequantial adjacent tissues from 18 ESCC samples. The avidin-biotin-peroxidase method (LSAB kit; Dako) was applied to detect the signal of the antigen-antibody reaction using antibodies against FBXW7 (1:100) (Abnova) and Myc (1:50) (Santa Cruz Biotechnology, Inc.) as previously described (14). All sections were counterstained with hematoxylin.

Laser microdissection. The tissues from another series of 38 patients with ESCC were collected for laser microdissection (LMD) using the LMD System (Leica Laser Microdissection System, Leica Microsystems) as previously described (22). Frozen sections were fixed in 70% ethanol for 30 sec, stained with hematoxylin and eosin and dehydrated as follows: 5 sec each in 70, 95 and 100% ethanol and a final 5 min in xylene. After air drying, target cells were excised (≥100 cells per section) and bound to the transfer film before total DNA and RNA were extracted.

Array CGH. The Agilent Human Genome CGH Microarray 244K platform is a high resolution 60-mer oligonucleotide-based microarray containing ~244,400 probes spanning coding and non-coding genomic sequences with median spacing of 7.4 and 16.5 kb, respectively. Labeling and array hybridization were performed according to the manufacturer's protocol. The arrays were analyzed using the Agilent DNA Microarray Scanner, and the raw signal intensities of tumor DNAs were transformed into log ratios against the reference DNA with Feature Extraction Software (v9.1) (Agilent Technologies). The log ratio was thereafter used as the signal intensity of each probe. One hundred and thirty samples from a different set of patients were subjected to circular binary segmentation (CBS) after median normalization (23). We wrote an R script for the median normalization, whereas an R program implemented in the 'DNA copy' package of the Bioconductor Project (http://www.bioconductor. org) was used for the CBS analysis. Instead of all of the CGH probes, 13,403 probes from chromosome 4 (NCBI Build 35) were analyzed in this study. An absolute log2 ratio >0.263 was used as the threshold for a gain or loss in DNA copy number for each probe.

cDNA microarray. Cyanine (Cy)-labeled cRNA was prepared using T7 linear amplification as described in the Agilent Low RNA Input Fluorescent Linear Amplification Kit Manual (Agilent Technologies). Labeled cRNA was fragmented and hybridized to the Whole Human Genome Oligo DNA Microarray Kit, 4x44K (Agilent Technologies). Fluorescence intensities

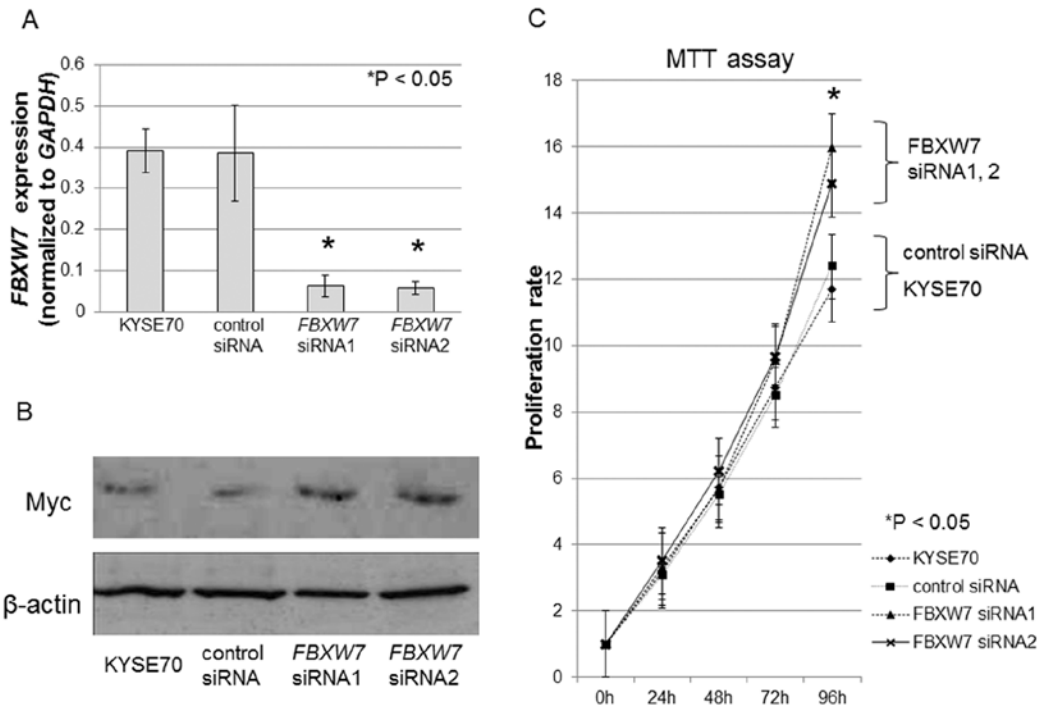

Figure 1. Proliferation assay with *FBXW7* siRNA in the KYSE70 ESCC cell line. (A) Quantitative RT-PCR was used to compare *FBXW7* expression in *FBXW7* siRNA cells and control siRNA. *FBXW7*, *GAPDH* expression was used for normalization. The data represent the mean ± SD. (B) Western blot analysis of Myc in *FBXW7* siRNA cells and control siRNA cells. Proteins were normalized to the level of β-actin. (C) MTT assay. The proliferation rate of *FBXW7* siRNA (1 and 2) cells was compared to that of control siRNA and parent KYSE70 cells. The data represent the mean ± SD.

were determined with an Agilent DNA Microarray Scanner and were analyzed using G2567AA Feature Extraction Software version A.7.5.1 (Agilent Technologies), which uses LOWESS (locally weighted linear regression curve fit) normalization (24). This microarray study followed the MIAME guidelines issued by the Microarray Gene Expression Data group (25). Further analyses were performed using GeneSpring version 7.3 (Silicon Genetics).

Analysis of copy number alteration. Among 75 ESCC samples (T) in which *FBXW7* mRNA levels were measured, copy number evaluation by PCR was performed with 42 genomic DNA samples (T and N) using the following primers: 5'-GGGATTCCC AGAGTGTGTATGT-3' (sense) and 5'-CTGTGCCTGAGAAGA TCAGATG-3' (antisense). β-globin was amplified and used as an internal control using 5'-TCTTATCTTCCTCCCACAGC TC-3' (sense) and 5'-GGACTTAGGGAACAAAGGAACC-3' (antisense). The amplification cycles consisted of an initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec, and elongation at 65˚C for 10 sec. Melting curve analysis was performed to distinguish specific products from non-specific products and primer dimers. The signal generated by *FBXW7* was divided by that of *β-globin* in each sample to obtain the copy number of *FBXW7*. Copy number ratio (CNR) = (T) copy number/(N) copy number. A CNR <0.8 defined the copy number loss (+) group, while a CNR ≥0.8 denoted the copy number loss (-) group.

Statistical analysis. Differences between two groups were estimated with Student's t-test, χ^2 analysis and ANOVA. Survival was measured using Kaplan-Meier analysis, with the log-rank test applied for comparison. Survival was measured from the day of the surgery. All differences were statistically significant at the level of P<0.05. Statistical analyses were performed using the JMP 5 for Windows software package (SAS Institute).

Results

FBXW7 RNAi in ESCC cells promotes proliferation in vitro. The ESCC cell line KYSE70 expresses a high level of *FBXW7* mRNA. Because Myc is a target of FBXW7, the effect of *FBXW7* suppression on the expression of Myc was evaluated. Western blot analysis found that the level of Myc protein was enhanced by the suppression of *FBXW7* using *FBXW7*-specific siRNA (Fig. 1A and B). An evaluation of the proliferative activity of KYSE70 cells treated with *FBXW7*-specific siRNA found a significant increase in proliferation rate compared to untreated cells (Fig. 1C).

Clinical significance of FBXW7 mRNA expression in ESCC cases. The expression levels of *FBXW7* mRNA in cancer (T) (n=75) and paired non-cancer (N) (n=75) tissues from ESCC patients were examined by real-time RT-PCR. The expression of *FBXW7* mRNA was significantly lower in cancer tissues than in non-cancer tissues (P=0.003) (Fig. 2A). The survival curve showed that patients in the low *FBXW7* expression group (n=38) had a significantly poorer prognosis than those in the high *FBXW7* expression group (n=37) (P=0.033) (Fig. 2B). Clinicopathological factors were significantly different in the low *FBXW7* expression group. Tumor size was more progressive compared to the high *FBXW7* expression group (P=0.025). However, no significant differences were observed regarding age, gender, histology,

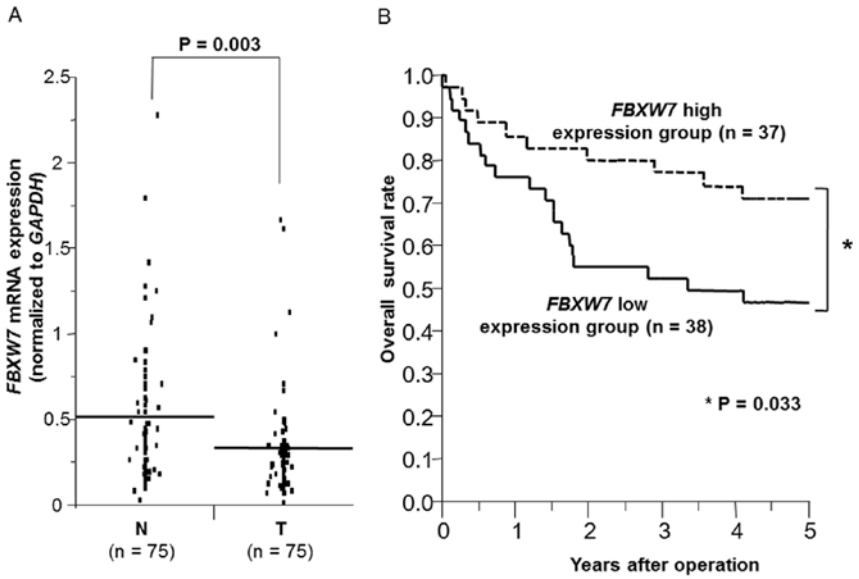

Figure 2. Clinical significance of *FBWX7* mRNA expression in clinical samples. (A) *FBXW7* mRNA expression in 75 cancer (T) and 75 non-cancer (N) tissues from ESCC patients by real-time RT-PCR. *FBXW7* (T), *FBXW7* mRNA (T)/*GAPDH* mRNA (T); *FBXW7* (N), *FBXW7* mRNA (N)/*GAPDH* mRNA (N). Horizontal lines indicate means. (B) Kaplan-Meier survival curves of ESCC patients according to the level of *FBXW7* mRNA expression, high *FBXW7* expression group (n=37); low *FBXW7* expression group (n=38).

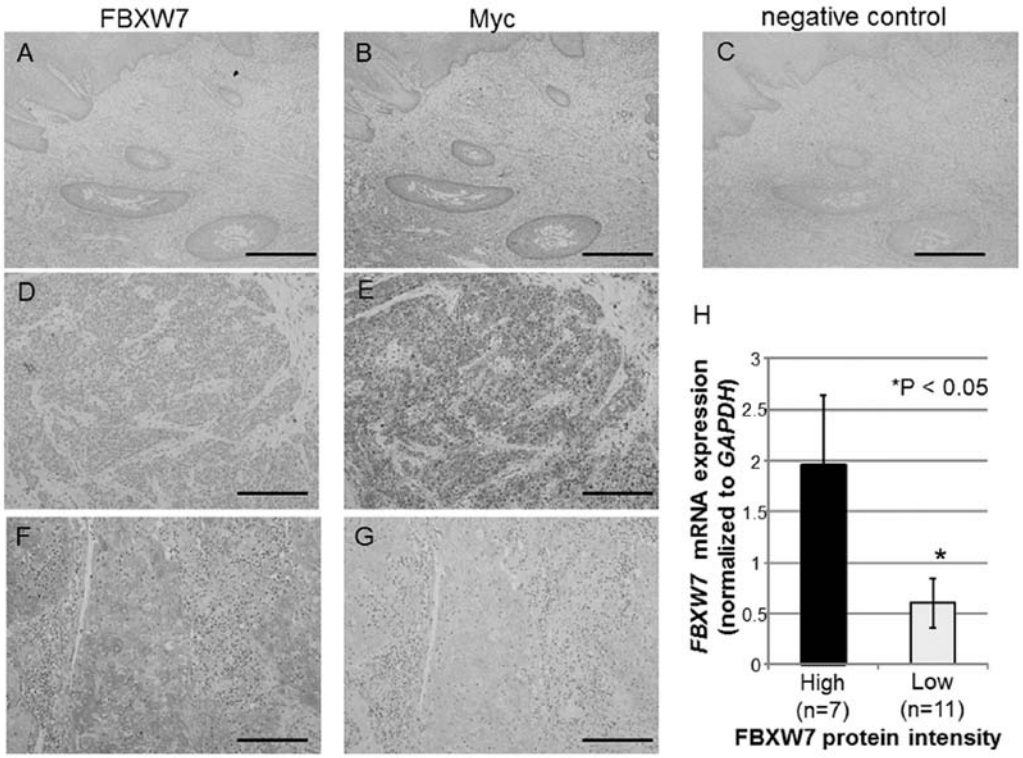

Figure 3. Immunohistochemical analysis of FBXW7 and Myc in sequantial adjacent tissues from 18 ESCC samples. (A, B, D and E) Myc expression levels were enhanced in a representative tissue from the group with a low level of FBXW7 expression. (C) Negative control [(same sample as (A)] was prepared using non-immune rabbit IgG as for the primary antibody. (F and G) Myc expression levels were suppressed in a representative tissue from the group with a high expression of *FBXW7* protein. Upper panel (magnification x40; scale bar, 500 μm), middle panel (magnification x100; scale bar, 200 μm), and lower panel (magnification x100; scale bar, 200 μm). (H) Relationship of *FBXW7* mRNA and FBXW7 protein expression. FBXW7 mRNA expression was normalized to *GAPDH* expression. The data represent the mean ± SD.

lymph node metastasis, lymphatic invasion or venous invasion (Table I). Multivariate analysis revealed that the level of *FBXW7* mRNA expression in cancer is an independent prognostic factor (Table II) (RR = 1.67, 95% CI 1.03-2.81, P=0.036).

We examined the relationship between FBXW7 and target protein Myc in clinical ESCC samples. As a result, the enhanced expression of Myc was validated in tissues with low levels of *FBXW7* in clinical ESCC samples (Fig. 3A, B, D and E).

Table I. *FBXW7* gene expression and clinicopathological factors for 75 ESCC patients.

Factors	*FBXW7/GAPDH* High expression n=37	*FBXW7/GAPDH* Low expression n=38	P-value
Age			
<70	18	25	0.33
≥70	12	10	
Gender			
Male	27	28	0.23
Female	3	7	
Histology			
Well, moderate	22	20	0.7
Poor	7	8	
Depth			
T1, T2	20	12	0.025[a]
T3, T4	14	25	
Lymph node metastasis			
Absent	9	11	0.7
Present	26	26	
Lymphatic invasion			
Absent	8	5	0.28
Present	26	32	
Venous invasion			
Absent	4	5	0.86
Present	29	32	
Neo adjuvant therapy			
Absent	30	29	0.95
Present	7	7	

Well, well differentiated; moderate, moderately differentiated; poor, poorly differentiated. Neo adjuvant therapy, radiation and/or chemotherapy. [a]P<0.05.

Conversely, Myc expression levels were suppressed in tissues in which FBXW7 was overexpressed (Fig. 3F and G). To confirm the correlation between *FBXW7* mRNA and FBXW7 protein, 18 ESCC samples were divided into two groups according to FBXW7 protein level (i.e., high or low). The high FBXW7 protein group (n=7) showed high *FBXW7* mRNA expression levels, while low mRNA levels corresponded to low protein levels (n=11) (P<0.05) (Fig. 3H).

Aberrations in FBXW7 copy number in ESCC specimens. To clarify the cause of suppression of *FBXW7* mRNA in ESCC, we investigated *FBXW7* copy number aberrations in laser-microdissected 38 ESCC specimens using CGH array analysis. These evaluations found *FBXW7* copy number loss rates of 44.7% (17/38) in the clinical samples (Fig. 4A). As shown in Fig. 4B, there was a significant correlation between *FBXW7* expression and copy number of the *FBXW7* region (Fig. 4B) (P<0.05). Therefore, the loss of *FBXW7* expression was caused by genetic alteration in the flanking region of *FBXW7*. In addition, copy number determination for 42 DNA samples that were used for the *FBXW7* mRNA expression assay revealed those with a copy number loss had a poorer prognosis (Fig. 5) (P=0.024).

Discussion

The finding that lower expression of *FBXW7* in the cancer esophageal tissue of ESCC patients is an independent prognostic factor was consistent with the results of previous studies. Moreover, we also showed that an *FBXW7* copy number loss regulates *FBXW7* mRNA expression and prognosis in clinical ESCC samples.

The regulatory mechanisms of *FBXW7* function and expression are primarily *FBXW7* mutation, transcriptional control by *p53* and genomic alteration (6,13-15). *FBXW7* mutation hot spots in the binding site of target proteins result in *FBXW7* hypofunction in T-cell lymphoma and the accumulation of several oncoproteins, including Myc, in these cells. In contrast, baseline expression of *FBXW7* mRNA is suppressed in *p53*$^{-/-}$ mice, and *FBXW7* mRNA expression was activated when *p53* expression was induced by radiation in WT mice (9). In gastric cancer and breast cancer, *FBXW7* expression is suppressed by *p53* mutation (14,15).

Table II. Univariate and multivariate analysis for overall survival (Cox proportional regression model).

Clinicopathologic variable	Univariate analysis RR	95% CI	P-value	Multivariate analysis RR	95% CI	P-value
Gender	1.13	0.58-2.87	0.74	-	-	-
Histology grade (well, moderate/poor)	1.39	0.87-2.16	0.16	-	-	-
Depth (T1,T2,T3/T4)	2.5	1.28-3.08	0.004[a]	1.64	0.6-3.59	0.29
Lymph node metastasis	2.32	1.23-5.85	0.006[a]	1.90	0.96-4.92	0.06[b]
Lymphatic invasion	2.13	1.43-3.25	0.0001[a]	-	-	-
Venous invasion	1.52	1.03-2.27	0.03[a]	1.94	1.05-3.88	0.032[a]
***FBXW7* expression (high/low)**	**1.46**	**1.03-2.15**	**0.035[a]**	**1.67**	**1.03-2.81**	**0.036[a]**

RR, relative risk; CI, confidence interval; [a]P<0.05; [b]P<0.1.

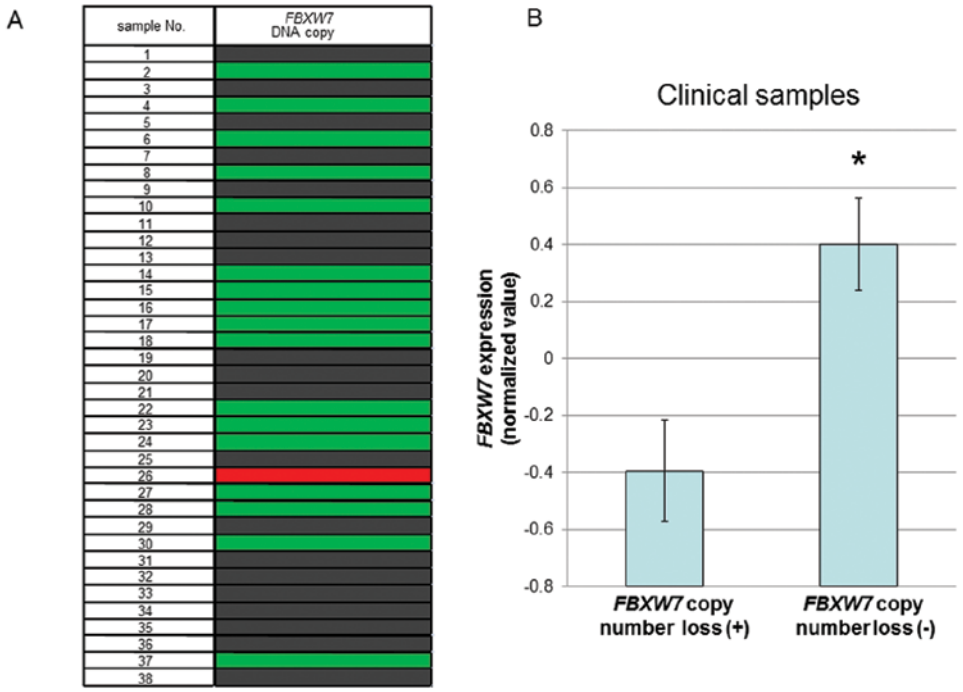

Figure4. Concordant loss of *FBXW7* expression and copy number alteration in the flanking region of *FBXW7*. (A) Seventeen ESCC cases (17/38, 44.7%) were examined for deletion of the *FBXW7* region. The green bar indicates gain, the red bar indicates loss, and the black bar indicates no change. (B) The expression of *FBXW7* in copy number loss (+) and (-) samples.

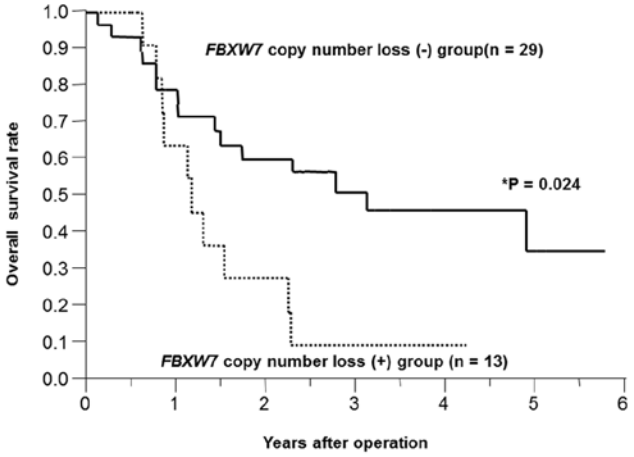

Figure 5. Kaplan-Meier survival of ESCC patients according to the *FBXW7* copy number. *FBXW7* copy number was normalized using the β-globin copy number as an endogenous control in each sample. Using matched normal (N) and cancer (T) tissue samples, the copy number ratio = (T) copy number/(N) copy number: if <0.8, the sample was placed in the copy number loss (+) group (n=13); if ≥0.8, the sample was put in the copy number loss (-) group (n=29).

In this study, genomic alteration as *FBXW7* regulatory mechanism in ESCC was investigated because a copy number loss had been detected at chromosome 4q in approximately 30-40% of ESCC samples in previous studies (26-28). *FBXW7* mRNA expression in ESCC was significantly associated with *FBXW7* copy number. From these observations, we conclude that the *FBXW7* mRNA expression level in ESCC is regulated by genomic alteration of *FBXW7*, as it is in colorectal cancers.

Although *FBXW7* was associated with ESCC progression through the degradation of Myc, *FBXW7* has other important degradation targets, including cyclin E and Jun (6). From these reports, the examination of *FBXW7* expression in cancer tissues might be able to indirectly evaluate the expression of several oncoproteins. In addition, *FBXW7* may be a therapeutic tool for cell cycle regulation and induction of dormancy in ESCC cells due to its ability to reduce the levels of several oncoproteins (e.g., Myc and cyclin E).

The *FBXW7* mRNA showed a positive correlation with the *FBXW7* protein. Therefore, when considering the clinical application of copy number evaluation, the use of stable genomic DNA may be more promising as a prognostic marker in clinical ESCC than the examination of *FBXW7* mRNA expression with unstable RNA.

In conclusion, we clarified that *FBXW7* mRNA expression is regulated by copy number loss in clinical ESCC cases. Evaluations of *FBXW7* expression and *FBXW7* copy number loss are potentially of use for the clinical prediction of cancer progression and prognosis in ESCC. Moreover, because *FBXW7* regulates proliferation ability through the degradation of several oncoproteins, including Myc, it could be a promising treatment tool of intractable ESCC.

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