# *FBXO31* determines poor prognosis in esophageal squamous cell carcinoma

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**Abstract.** *Cyclin D1* plays important roles in esophageal squamous cell carcinoma (ESCC) cases by amplification of the 11q13.3 locus. FBXO31 is a subunit of the SCF ubiquitin ligase, which targets cyclin D1 for degradation. In this study, we clarified the clinical significance of *FBXO31* and characterized the association between cyclin D1 and FBXO31 in ESCC cases. Total RNA was extracted from tumor tissues obtained from 68 ESCC patients who underwent surgical resection. *FBXO31* expression levels were determined by quantitative RT-PCR, and both FBXO31 and cyclin D1 protein expression and localization were evaluated by immunohistochemistry (IHC). Furthermore, using CGH and gene expression array data of another subset, we validated the association between *cyclin D1* genomic amplification and *FBXO31* expression levels. Higher *FBXO31* expression levels significantly correlated with depth of tumor invasion and clinical stage (P<0.05). In addition, the *FBXO31* high expression group showed a significantly poorer prognosis than the low expression group (P<0.001). Multivariate analysis indicated that *FBXO31* expression was an independent prognostic factor [relative risk (RR): 1.79, confidence interval (CI): 1.14-3.01, P=0.01]. Using IHC, concordant expression was observed between cyclin D1 and FBXO31 in the nucleus and cytoplasm, respectively. CGH array indicated that cases having *cyclin D1* with increased copy number were significantly associated with elevated *FBXO31* expression levels (P<0.05). *FBXO31* could be a novel and robust prognostic marker for ESCC.

## Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignant tumors because ESCC frequently involves lymph node metastasis and tumor invasion to adjacent organs at early stages (1). Recently, therapeutic modalities for ESCC have been developed that improved clinical outcomes to some extent, although the 5-year probability of survival still remains at only was still 30-40% (2-5). Therefore, identification of novel molecular markers to predict prognosis and susceptibility to adjuvant therapy is still required.

In the current study, we focused on a cell cycle regulator gene that should play a role in determining the fate of cancer cells. Dysregulation of cell cycle related molecules promotes unlimited cancer cell proliferation and enhances the capability of tumor invasion. Among diverse cell cycle regulating molecules in malignant cells, elevated cyclin D1 expression accelerates the cell cycle and promotes proliferation (6,7). In general, it is well-recognized that genomic amplification of *cyclin D1* (11q13) in ESCC patients contributes to oncogenesis and disease progression, leading to poor prognosis (8-10). As such, determination of how cyclin D1 activity is regulated in ESCC is of great importance.

Recently, Santra *et al* reported that FBXO31 is one of the F-box proteins comprising the SCF (Skp2/Cull/F-box protein) ubiquitin ligase complex, which mediates ubiquitination and degradation of cyclin D1 (11-14). Therefore, we focused on the response of *FBXO31* against *cyclin D1* overexpression following genomic amplification in ESCC cases. The purpose of the current study is to clarify the association between *FBXO31* level and clinicopathologic factors in ESCC cases and to characterize the association between cyclin D1 and FBXO31 levels in ESCC cases.

## Materials and methods

*Clinical samples and cell lines.* Sixty-eight ESCC samples were obtained during surgery after obtaining written informed consent. All patients underwent primary tumor resection between 1992 and 2000 at Kyushu University Hospital at Beppu and affiliated hospitals, Kurume University Hospital and Kagoshima University Hospital. There were no neoadjuvant chemoradiotherapy cases. Resected cancer tissues were immediately cut and embedded in Tissue-Tek OCT medium (Sakura), frozen in liquid nitrogen, and kept at -80˚C until RNA extraction. Following isolation of RNA and DNA, cDNA was synthesized from 8.0 $\mu$g total RNA as described previously (15).

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*Real-time quantitative RT-PCR*. *FBXO31*-specific oligonucleotide primers were designed to amplify a 291-bp PCR product. The following primers were used: *FBXO31* sense primer 5'-AATCCGGCCTTTTGACCAGA-3' and antisense primer 5'-TCCGCTCACAGGAAGAGCAC-3' and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 270 bp) sense primer 5'-GTCAACGGATTTGGTCTGTATT-3' and antisense primer 5'-AGTCTTCTGGGTGGCAGTGAT-3'. PCR amplification for quantification of *FBXO31* and *GAPDH* mRNA in clinical samples was done using the LightCycler system (Roche Applied Science) and the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Applied Science) as described previously (16). The amplification conditions consisted of initial denaturation at 95˚C for 10 min followed by 35 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec, and elongation at 65˚C for 10 sec. The relative expression levels of *FBXO31* were obtained by normalizing the amount of *FBXO31* mRNA with respect to *GAPDH* mRNA, which served as an endogenous control for each sample.

*Statistical analysis*. Differences between the two groups were estimated with Student's t-test, $\chi^2$ test. Overall survival curves were plotted according to the Kaplan-Meier method, with the log-rank test applied for comparison. Survival times were measured from the day of the surgery. Data for *FBXO31* mRNA expression levels in the two groups were analyzed with ANOVA. Variables with a value of $P<0.05$ by univariate analysis were used in subsequent multivariate analyses based on Cox's proportional hazards model. All differences were statistically significant at the level of $P<0.05$ and a tendency was indicated at the level of $P<0.1$. Statistical analyses were done using the JMP 5 for Windows software package (SAS Institute).

*Immunohistochemistry*. Immunohistochemical analysis of FBXO31 and cyclin D1 was performed on formalin-fixed, paraffin-embedded surgical sections obtained from ESCC cases. Tissue sections were deparaffinized, soaked in 0.01 M sodium citrate buffer and boiled in a microwave for 5 min at 500 W to retrieve cell antigens. Next, tissue sections were blocked, and then incubated with specific antibodies overnight at 4˚C. Primary mouse monoclonal antibody against FBXO31 (Abnova, B01P) mouse polyclonal antibody against cyclin D1 (Santa Cruz, H-295) were both used at a dilution of 1:100. Tissue sections were immunohistochemically stained using Envision reagents (Envision+Dual link/HRP; Dako, Denmark). All sections were counterstained with hematoxylin.

*Laser microdissection*. The tissues from another subset of 77 ESCC cases were collected for laser microdissection (LMD). ESCC tissues were microdissected using the LMD system (Leica Laser Microdissection System, Leica Microsystems, Wetzlar, Germany) as previously described (17). For LMD, 5 $\mu$m 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 final 5 min in xylene. Sections were air-dried, then microdissected with the LMD system. Target cells were excised, with each section having at least 100 cells, bound to transfer film, and total DNA and RNA extracted.

*cDNA microarray*. We used the commercially available Human Whole Genome Oligo DNA Microarray Kit (Agilent Technologies, Santa Clara, CA). Cyanine (Cy)-labeled cRNA was prepared using T7 linear amplification as described in the Agilent Low RNA Input Fluorescent Linear Amplification Kit Manual (Agilent Technology). Labeled cRNA was then fragmented and hybridized to an oligonucleotide microarray (Whole Human Genome 4x44 Agilent G4112F). Fluorescence intensities were determined with Agilent DNA microarray scanner and analyzed using G2567AA Feature Extraction Software version A7.5.1 (Agilent Technologies), which used the LOWESS (locally weighted linear regression curve fit) normalization method (18). This microarray study followed MIAME guidelines issued by the Microarray Gene Expression Data group (19). Further analyses were performed using GeneSpring version 7.3 (Silicon Genetics, San Carlos, CA).

*Array-CGH*. Array-CGH was performed using the Agilent Human Genome Microarray Kit 244K (Agilent Technologies). The array-CGH platform is a high resolution 60-mer oligonucleotide based microarray containing about 244,400 probes spanning coding and non-coding genomic sequences with median spacing of 7.4 and 16.5 kb, respectively. Labeling and hybridization were performed according to the protocol provided by Agilent (Protocol version 4.0 June 2006). Arrays were analyzed using the Agilent DNA microarray scanner.

*Array CGH data analysis*. The raw signal intensities of tumor DNA were measured with Human Genome CGH Microarray 244K (Agilent Technologies), which were then transformed into log ratio to reference DNA with 'Feature Extraction' software (version 9.1) from Agilent Technologies. The log ratio was thereafter used as the signal intensity of each probe. Forty ESCC samples from different patients were subjected to ADM-1 after median normalization (20). An R script written by us was used for the median normalization, whereas an R implemented in the 'DNA copy' package of the Bioconductor project was used for ADM-1 analysis. An absolute log2 ratio >0 was used as the threshold for the amplification in DNA copy number for each probe.

## Results

*FBXO31 levels and clinicopathologic characteristics*. We classified the 68 ESCC cases into two groups using the median of *FBXO31* mRNA level in cancer tissue as determined by qRT-PCR. The clinicopathologic significance of *FBXO31* levels is summarized in Table I. In the *FBXO31* high expression group (n=34), depth of tumor invasion and clinical stage were elevated compared to the *FBXO31* low expression group (n=34) ($P<0.05$). There were no significant differences between age, gender, histology, lymph node metastasis, lymphatic and venous invasion (a part of data not shown).

The 5-year probability of survival for patients in the *FBXO31* high and low expression group was 33.4 and 77.6%, respectively. The *FBXO31* high expression group showed a significantly poorer prognosis than in the low expression group ($P<0.001$) (Fig. 1).

Univariate analysis identified *FBXO31* level, depth of tumor invasion and lymph node metastasis as prognostic factors

Table I. *FBXO31* expression and clinicopathologic factors in 68 ESCC cases.

| Factors | *FBXO31* low expres. (n=34) No. | % | *FBXO31* high expres. (n=34) No. | % | P-value |
|---|---|---|---|---|---|
| Depth of tumor invasion | | | | | |
| T1, T2 | 22 | 64.7 | 10 | 29.4 | 0.003[a] |
| T3, T4 | 12 | 35.3 | 24 | 70.6 | |
| Lymph node metastasis | | | | | |
| Absent | 12 | 35.3 | 7 | 20.6 | 0.17 |
| Present | 22 | 64.7 | 27 | 79.4 | |
| Lymphatic invasion | | | | | |
| ly0, ly1 | 17 | 50.0 | 17 | 50.0 | 1.00 |
| ly2, ly3 | 17 | 50.0 | 17 | 50.0 | |
| Venous invasion | | | | | |
| v0, v1 | 14 | 41.2 | 18 | 52.9 | 0.33 |
| v2, v3 | 20 | 58.8 | 16 | 47.1 | |
| Stage | | | | | |
| I, II | 22 | 64.7 | 12 | 35.3 | 0.015[a] |
| III, IV | 12 | 35.3 | 22 | 64.7 | |

[a]P<0.05; low expres., low expression; high expres., high expression.

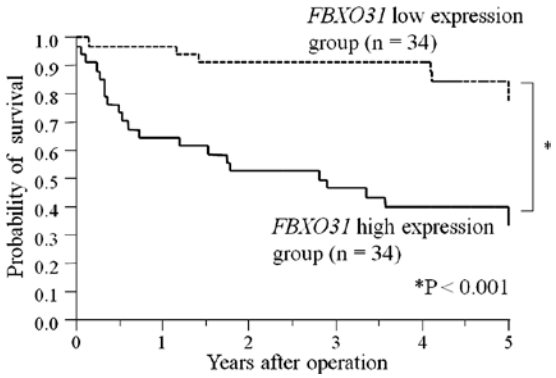


Figure 1. Kaplan-Meier overall survival curves according to *FBXO31* level. The overall survival rate for the *FBXO31* high expression group (n=34) was significantly poorer than that of the low expression group (n=34).

for 5-year overall survival after surgery. Variables having a P-value <0.05 by univariate analysis were selected for multivariate analysis using Cox's proportional hazards model. By multivariate analysis, we found that *FBXO31* level was an independent prognostic factor for 5-year overall survival after surgery [relative risk (RR): 1.79, confidence interval (CI): 1.14-3.01, p=0.01] (Table II).

*The association between FBXO31 and cyclin D1 expression by IHC analysis*. IHC analysis of cancer tissues from adjacent sections showed that cyclin D1 expression had a nuclear localization, while FBXO31 resided in the cytoplasm (Fig. 2). Moreover, concordant expression was observed between cyclin D1 and FBXO31 in the nucleus and cytoplasm, respectively. We also validated ten resected specimens, which showed a staining pattern similar to the representative cases. As for these intriguing findings, there is no direct evidence to explain it. However, this result supports previous study demonstrating that other F-box protein targeting cyclin D1, FBXW8, also had a cytoplasmic localization, and degraded cyclin D1 as it moved from the nucleus to the cytoplasm during the subsequent S-phase (21,22).

*Relationship between cyclin D1 genomic amplification and FBXO31 level*. To clarify the mechanism of the correlation between FBXO31 and cyclin D1 protein levels in ESCC cases, we investigated whether *cyclin D1* genomic amplification related to *FBXO31* level in ESCC cases. Seventy-seven ESCC cases from another subset were analyzed. These samples were obtained by laser-microdissection (LMD). 51.9% (40/77) showed significantly higher copy number of *cyclin D1* localized genomic loci (11q13). CGH and gene expression array indicated that *cyclin D1* genomic amplification level was correlated with *cyclin D1* expression in 40 *cyclin D1* amplification cases (Fig. 3A). Furthermore, we classified the 40 *cyclin D1* amplification cases into two groups according to the median *cyclin D1* amplification level in cancer tissue as determined by CGH array data. The *cyclin D1* high amplifica-

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

| Factors | Univariate analysis RR | 95% CI | P-value | Multivariate analysis RR | 95% CI | P-value |
|---|---|---|---|---|---|---|
| Depth of tumor invasion (T1, T2/T3, T4) | 6.06 | 2.79-25.6 | <0.001[a] | 5.21 | 1.00-6.20 | <0.001[a] |
| Lymphatic metastasis (negative/positive) | 3.87 | 1.78-16.4 | <0.001[a] | 3.6 | 0.61-6.91 | 0.002[a] |
| Lymphatic invasion (ly0, ly1/ly2, ly3) | 1.98 | 1.32-3.17 | <0.001[a] | 0.97 | 0.79-4.14 | 0.93 |
| Venous invasion (v0, v1/v2, v3) | 1.58 | 1.06-2.47 | 0.02[a] | 1.35 | 1.54-16.9 | 0.25 |
| *FBXO31* expression (low/high) | 2.31 | 1.50-3.82 | <0.001[a] | 1.79 | 1.22-3.84 | 0.01[a] |

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

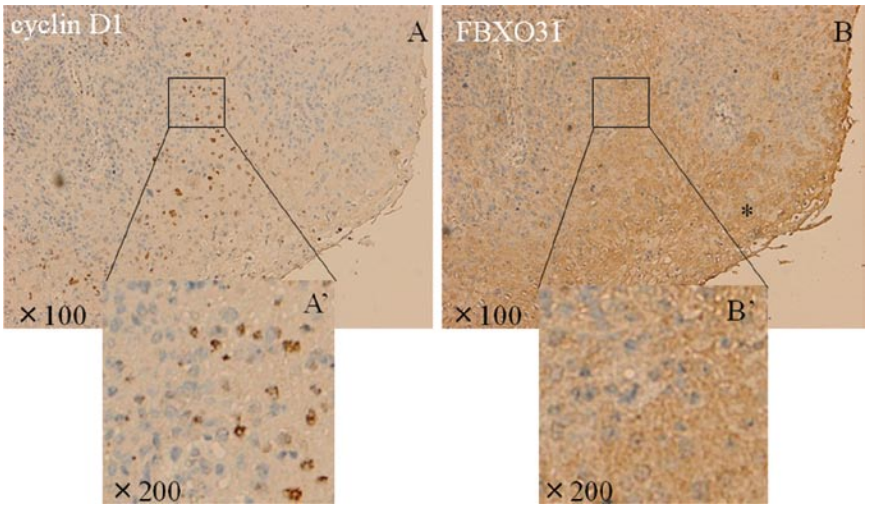


Figure 2. Immunohistochemical analysis of cyclin D1 (A) and FBXO31 (B). Cyclin D1 expression was localized to the nucleus and FBXO31 expression was localized to the cytoplasm. The concordant expression between cyclin D1 and FBXO31 was observed in cancer tissue of the adjacent section. Original magnification: A, B: x100; A', B': x200.

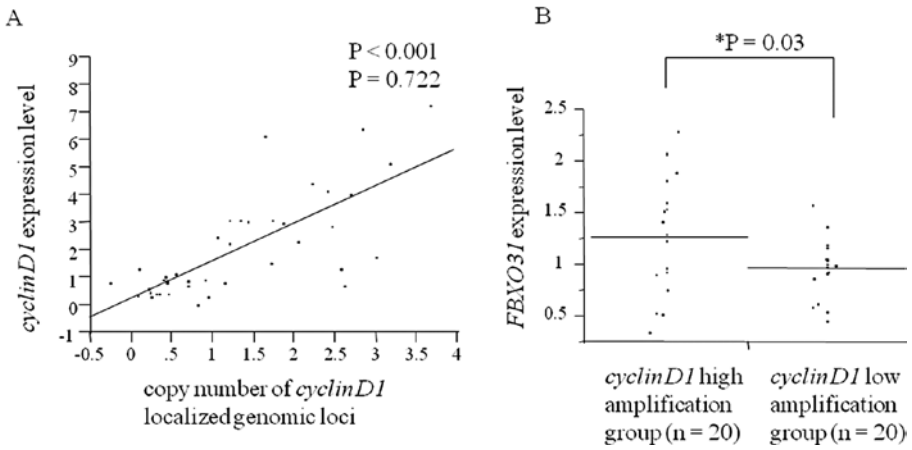


Figure 3. Relationship between *cyclin D1* genomic amplification and *FBXO31* level. A, Correlation between copy numbers of *cyclin D1* localized genomic loci (11q.13) and *cyclin D1* mRNA level (P<0.001, ϱ=0.722). B, In *cyclin D1* high amplification group (n=20), *FBXO31* level was significantly higher compared to the low amplification group (n=20).

tion group (n=20) had significantly elevated *FBXO31* level (P<0.05) (Fig. 3B).

## Discussion

Kumar *et al* described *FBXO31* as a senescence gene, and a candidate breast tumor suppressor (12). In contract to that study, our results indicate that *FBXO31* overexpression correlated with poor prognosis in ESCC patients. There is no clear explanation for the difference between these two solid malignancies, while survival studies might provide some clarification. F-box proteins, such as FBXO7, FBX4, and FBXW8 participate in ubiquitination and subsequent degradation of cyclin D1 (22-25). Therefore, overexpression of these F-box proteins could induce cell cycle arrest and apoptosis, especially in cancer cells. On the other hand, Okabe *et al* reported that one F-box protein targeting cyclin D1, FBXW8, positively regulates the cell cycle in HCT116 colorectal cancer cells and plays an essential role in cancer cell proliferation by promoting proteolysis and rapid turnover of cyclin D1 (22). This rapid turnover of cyclin D1 in the nucleus might require the degradation of cytoplasmic cyclin D1 during the S-phase. They also demonstrated that FBXW8 knockdown inhibits cancer cell proliferation (22). Furthermore, the high amplification of the *cyclin D1* locus group was significantly associated with elevated *FBXO31* level. Therefore, we suggest that *FBXO31* overexpression accelerates the degradation of cyclin D1 in cytoplasm, which might contribute to the elevation of malignant potential upon *cyclin D1* genomic amplification in ESCC cases. This mechanism was supported by our IHC results. Previous studies have demonstrated that F-box proteins targeting cyclin D1, such as FBXO7, FBX4, and FBXW8, localize to the cytoplasm while activated cyclin D1 localizes to the nucleus during the G1 phase, but degrades cytoplasmic cyclin D1 during the S-phase (22-26). Our current study then revealed concordant expression between cyclin D1 in the nucleus and FBXO31 in the cytoplasm of ESCC cells.

These results suggest *cyclin D1* overexpression may induce *FBXO31* expression in ESCC cases. In a clinicopathological context, the correlation between depth of tumor invasion and *FBXO31* level may be due rapid turnover of cyclin D1.

We confirmed that *cyclin D1* amplification increased *FBXO31* transcriptional activity in ESCC patients. Since the mechanisms by which *FBXO31* transcription is regulated remain unclear, further experimental study will be required to elucidate the direct findings of FBXO31 and amplificated *cyclin D1* in ESCC.

In this study, *FBXO31* level was significantly associated with genomic amplification of *cyclin D1*. The IHC study results suggest, that FBXO31 ubiquitinates cyclin D1 in the cytoplasm of ESCC cells. *FBXO31* expression was also found to be independent prognostic factor in ESCC. Therefore, *FBXO31* mRNA expression in the cancer tissue may be promising novel prognostic marker, as well as a guide for therapeutic strategies.

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