FBXW7 expression affects the response to chemoradiotherapy and overall survival among patients with oral squamous cell carcinoma: A single-center retrospective study

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Abstract
FBXW7 (F-box and WD repeat domain containing-7) is a tumor suppressor protein that regulates the degradation of various oncoproteins in several malignancies. However, limited information is available regarding FBXW7 expression in oral squamous cell carcinoma. Therefore, this study aimed to determine the clinical significance of FBXW7 expression in oral squamous cell carcinoma. The FBXW7 expression patterns in oral squamous cell carcinoma and adjacent normal tissues from 15 patients who underwent radical resection were evaluated using quantitative real-time polymerase chain reaction and immunohistochemical staining. In addition, immunohistochemistry was performed using paraffin-embedded sections from biopsy specimens obtained from 110 patients with oral squamous cell carcinoma who underwent surgery after 5-fluorouracil-based chemoradiotherapy. The associations of FBXW7 expression with various clinicopathological features and prognosis were evaluated in these patients. As a result, in the 15 matched samples, the FBXW7 expression was significantly decreased in the oral squamous cell carcinoma tissues compared to that in the adjacent normal tissues. In the clinicopathological analysis, compared to high protein expression, low FBXW7 expression was found to significantly associate with a poor histological response to preoperative chemoradiotherapy. Kaplan–Meier curve analysis revealed that low FBXW7 expression was significantly associated with a poor prognosis, and FBXW7 expression was found to be an independent predictor of overall survival in the multivariate analysis. Our results suggest that FBXW7 may function as a tumor suppressor protein in oral squamous cell carcinoma. In addition, FBXW7 could be a potential biomarker for predicting not only the clinical response to chemoradiotherapy but also overall survival in patients with oral squamous cell carcinoma.

Keywords
Oral squamous cell carcinoma, F-box and WD repeat domain-containing 7, chemoradiotherapy, resistance, prognosis

Date received: 3 July 2017; accepted: 24 August 2017
**Background**

Oral squamous cell carcinoma (OSCC) is a common oral cavity disease that negatively affects the quality of life and survival of the patients. Despite recent improvements and innovations in diagnostic techniques and multimodality treatments, the prognosis of OSCC remains poor, with a 5-year survival rate of approximately 60%. Therefore, to further improve the patient outcomes, it is imperative to identify novel biomarkers that can predict the efficacy and outcomes of chemotherapy and/or radiotherapy among patients with OSCC.

F-box and WD repeat domain-containing 7 (FBXW7), also known as AGO, CDC4, or SEL10, is a component of the Skp, Cullin, F-box containing complex (SCF)-type ubiquitin ligase (a complex of the SKP1, CUL1, and F-box proteins) that targets various oncoproteins for ubiquitination and is generally accepted as a tumor suppressor protein. Approximately 6% of all primary human tumors harbor mutations in the FBXW7 gene. Furthermore, inactivation of FBXW7 through mutation, deletion, or promoter hypermethylation is common in various malignancies such as leukemia, breast cancer, and colon cancer. Moreover, mutations and deletions in FBXW7 have been shown to lead to the accumulation of proliferation-related proteins such as c-Myc, cyclin E, and c-Jun. Therefore, FBXW7 can modulate the cell cycle, proliferation, and differentiation by degrading proteins that are related to cell growth and differentiation.

Many researchers have reported that low expression of FBXW7 is associated with poor clinicopathological characteristics and prognosis of solid tumors such as gastric cancer, colorectal cancer, breast cancer, and glioma. In one previous study, loss of FBXW7 expression was demonstrated to be associated with aggressive malignant phenotypes in a tongue cancer cell line. Others have shown that FBXW7 plays an important role in the development of treatment resistance of cancer cells in a variety of malignancies, and the mutation profile of the FBXW7 gene has been reported to have adverse effects on the clinical outcomes of induction chemotherapy for head and neck cancer.

Despite the existing research, few studies have evaluated the expression of FBXW7 in OSCC and adjacent normal tissues. Moreover, little is currently known regarding the clinical significance of FBXW7 expression in OSCC and whether it leads to treatment resistance phenotypes. Therefore, this study aimed to evaluate the expression patterns of FBXW7 in normal and cancerous oral tissues and the clinical significance of FBXW7 expression among patients with advanced OSCC.

**Methods**

**Patients and tissue specimens**

First, to evaluate the differences in expression patterns of FBXW7 between cancerous and adjacent normal tissues in the oral cavity, tissue samples obtained from 15 patients who underwent radical resection without preoperative chemoradiotherapy (CRT) were used for analyses of the messenger RNA (mRNA) and protein levels of FBXW7. Next, for the clinicopathological analysis, biopsy and postoperative tissue samples were obtained from 110 patients with locally advanced OSCC who were treated at Kumamoto University Hospital between October 2003 and January 2014 (Supplementary Table S1). All 110 patients had received preoperative CRT and subsequently underwent curative surgery. Radiotherapy was administered 5 days per week with a daily dose of 2 Gy, for a total of 3 weeks (total dose: 30 Gy). After starting the radiotherapy, each patient concurrently received S1 (an oral 5-fluorouracil (FU)-based anti-cancer agent) at 80, 100, or 120 mg/day, according to their body surface area, for 14 days. All tumors were staged according to the 2002 tumour–node–metastasis (TNM) classification of the Union of International Cancer Control, and the degree of differentiation was determined according to the World Health Organization’s grade classification. The untreated biopsy specimens obtained before the administration of preoperative CRT were used for the immunohistochemical analyses. The surgical specimens were used to evaluate the histological responses to CRT. All samples were fixed in 10% formalin and embedded in paraffin.

This study was performed with the approval of the Ethics Committee of Kumamoto University (approval number 174) and in accordance with the Good Clinical Practice and the Declaration of Helsinki guidelines.

**RNA isolation, reverse transcription, and quantitative real-time polymerase chain reaction**

Total RNA was isolated from 15 primary OSCC specimens and adjacent normal tissues using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). The RNA quantity, purity, and integrity were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Complementary DNA was synthesized from the total RNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Life Science, Osaka, Japan). For the quantitative real-time reverse transcription polymerase chain reaction (PCR), each reaction mixture was diluted five-fold with DNase-/Rnase-free water (Invitrogen, Carlsbad, CA, USA), and 4 μL of each mixture was subjected to PCR. The reactions were run using the THUNDERBIRD SYBR qPCR Mix (Toyobo Life Science) and Light Cycler 1.5 instrument (Roche Diagnostics, Basel, Switzerland). The comparative Ct (ΔΔCt) method was used to determine the fold changes in expression, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization, and each sample was run in triplicate. The following primers were used to...
amplify *Fbxw7*: forward: 5′-ACTGGGCTTGTACCATGTTCA-3′ and reverse: 5′-TGAGGTCCCCCAAGTTGGTG-3′ and *GAPDH*: forward: 5′-TGTTGCACCATACTGACCCCTT-3′ and reverse: 5′-CTCCACGACGTACCTG-3′. The cycling conditions included initial denaturation at 98°C for 5 min, followed by 45 cycles of 98°C for 15 s, 60°C for 30 s, and 72°C for 60 s.

### Immunohistochemical staining and histopathological evaluation

Formalin-fixed paraffin-embedded specimens were cut into 4-μm sections and mounted on MAS-GP-coated slides (Matsunami Glass Industry Ltd., Osaka, Japan). To retrieve the FBXW7 antigen, the sections were subjected to deparaffinization and rehydration and then heated in an autoclave in 0.01 mol/L citrate buffer (pH 7.0) for 15 min at 121°C. Subsequently, the sections were incubated with 3% H2O2 in absolute methanol for 15 min to block endogenous peroxidase activity. The sections were also incubated with Protein Block Serum Free Reagent (Dako, Glostrup, Denmark) for 10 min to block non-specific staining. After the blocking step, the sections were incubated at 4°C overnight with an anti-FBXW7 antibody (clone 3D1; Abnova Corporation, Taipei, Taiwan), followed by sequential 60-min incubations with the secondary antibody (EnVision + System-HRP Labelled Polymer; Dako) and the Liquid DAB + Substrate Chromogen System (Dako). All slides were counterstained using hematoxylin for 60 s before the dehydration and mounting steps. The surgical specimens were used for evaluation of the histological responses to CRT and were graded according to the criteria proposed by Shimosato et al.19 as follows: grade I, no destruction of the tumor structure; grade IIa, mild destruction of the tumor structure (i.e. “viable tumor cells” are frequently observed); grade IIb, severe destruction of the tumor structure (i.e. few “viable tumor cells” are observed); grade III, nonviable tumor cells are present; and grade IV, no tumor cells remain.

### Assessment of immunohistochemical staining

Three independent observers, blinded to the patients’ clinical status, interpreted the immunohistochemical data. We assessed the expression levels of FBXW7 in cancer cells and the adjacent normal epithelial cells. Each specimen was scored according to the percentage of FBXW7-positive epithelial cells: <5%, 6–35%, 36–70%, and ≥71% were scored as 1, 2, 3, and 4 points, respectively. A second score was assigned according to the FBXW7 staining intensity: 1, 2, 3, and 4 points for negative, weak, moderate, and strong staining, respectively. The overall score for FBXW7 expression was calculated by multiplying both scores, and a result of ≥4 was considered indicative of high FBXW7 expression. Disagreements regarding the immunohistochemical staining scores were resolved using discussion and consensus.

### Statistical analysis

The chi-square test was used to evaluate the association between the FBXW7 expression status and the patients’ clinical parameters. Survival analysis was performed using the Kaplan–Meier method and the log-rank test. Multivariate survival analyses were performed using Cox regression models to evaluate the associations of FBXW7 expression with overall survival (OS) and disease-free survival (DFS). All p values were based on two-tailed tests, and p values of <0.05 were considered statistically significant. All statistical analyses were performed using JMP software (version 9; SAS Institute Inc., Cary, NC, USA).

### Results

#### FBXW7 expression is decreased in OSCC specimens

To clarify the expression of FBXW7 in the OSCC and adjacent normal tissues, the mRNA and protein levels of FBXW7 were examined using quantitative real-time reverse transcription PCR and immunohistochemical staining in 15 specimens of OSCC and paired adjacent normal tissues. As seen in Figure 1, the expression of FBXW7 mRNA was significantly lower in the OSCC tissues compared to that in the adjacent normal tissues (p = 0.004; Figure 1(a)). As shown in the representative pictures of immunohistochemical staining of the OSCC (Figure 2(A) and (B)) and adjacent normal epithelial cells (Figure 2(C) and (D)), the FBXW7 immunostaining scores were significantly lower in the OSCC cells compared to those in the adjacent normal epithelial cells (p = 0.017; Figure 1(b)).

#### Expression of FBXW7 in OSCC cases

The expression of FBXW7 from the 110 patients with OSCC was evaluated using immunohistochemical staining of their biopsy specimens. FBXW7 immunoreactivity was mainly observed in the nucleus of the cancer cells (Figure 3(a) and (b)), and the expression of FBXW7 was diverse (Figure 3(c) and (d)). Therefore, we classified the patients as having either low (score <4) or high FBXW7 expression (score ≥4). Among the 110 OSCC cases, 74 cases (67.3%) had low FBXW7 expression and 36 cases (32.7%) had high FBXW7 expression.

#### Low FBXW7 expression is associated with a poor response to preoperative CRT

We also examined the associations of FBXW7 expression with the patients’ clinicopathological variables in 110
patients who underwent preoperative CRT (Table 1). Tumors with low FBXW7 expression were significantly more common among patients with a poor histological response to preoperative CRT \( (p=0.021) \). However, no significant association was observed between FBXW7 expression and age, sex, primary tumor site, pT-stage, pN-stage, clinical stage, differentiation, recurrence, or metastasis.

**Low FBXW7 expression is associated with a poor prognosis among OSCC cases**

The Kaplan–Meier method was used to evaluate the OS and DFS rates of the 110 patients with OSCC. The 5-year OS rates were significantly lower in patients with low FBXW7 expression compared to patients with high FBXW7 expression \( (p=0.038; \text{Figure 4(a)}) \). Although the 5-year DFS rate tended to be lower in patients with low FBXW7 expression, the difference was not statistically significant \( (p=0.526; \text{Figure 4(b)}) \).

**Low FBXW7 expression independently predicts OS in OSCC cases**

Multivariate analysis using a Cox proportional hazards regression model in the 110 patients who underwent preoperative CRT and subsequently underwent curative surgery revealed that the FBXW7 expression status (hazard ratio (HR): 4.26, 95% confidence interval (CI): 1.52–13.8; \( p=0.0049 \)), the pathological response to preoperative CRT (HR: 0.331, 95% CI: 0.102–0.905; \( p=0.0304 \)), and recurrence and/or metastasis after the first operation (HR: 25.3, 95% CI: 8.26–112; \( p<0.0001 \)) were significant prognostic factors for OS (Table 2). However, for DFS, the same multivariate analysis revealed that the FBXW7 expression status (HR: 0.765, 95% CI: 0.347–1.74; \( p=0.515 \)) was not a significant prognostic factor (Table 2). On the other hand, the pathological response to preoperative CRT (HR: 0.254, 95% CI: 0.101–0.579; \( p=0.0008 \)) and pStage (HR: 11.2, 95% CI: 1.91–66.8; \( p<0.0079 \)) were significant prognostic factors for DFS (Table 2).

**Discussion**

In accordance with previous reports, \(^3\) \(^4\) \(^20\) \(^22\) our results indicated that the expressions of FBXW7 mRNA and protein were significantly decreased in human OSCC tissues compared to those in the adjacent normal tissues. In this context, the tumor suppressor function of FBXW7 is exerted by increasing genetic stability and degrading oncoproteins, such as c-Myc, cyclin E, c-Jun, and Notch1. \(^3\) \(^20\) \(^23\) \(^24\) Interestingly, Agrawal et al. \(^21\) reported that FBXW7 mutations were observed in a hotspot that is known to disrupt the degradation of Notch1. \(^25\) In addition, recent studies have revealed that Notch1 may play an important role in a variety of malignant phenotypes, including in mediating proliferation and invasion in a subpopulation of OSCC cases. \(^26\) \(^28\) Thus, aberrant FBXW7 may modulate oncoproteins, including Notch1, and contribute to the development and progression of OSCC, although further studies are needed to clarify the detailed mechanism of FBXW7-mediated tumor suppression and its function in OSCC.
In this study, the low FBXW7 expression group showed a poorer response to preoperative 5-FU-based CRT compared to the high expression group. Similarly, an intimate relationship between reduced FBXW7 expression and resistance to chemotherapy has been observed in several other malignancies.\(^\text{29}\) Inuzuka et al.\(^\text{8,15}\) reported that the loss of FBXW7 decreased the sensitivity of T-cell acute lymphoblastic leukemia to sorafenib and a pan-BCL-2 inhibitor through increased Mcl-1 expression (a pro-survival protein). Wertz et al.\(^\text{16}\) reported that FBXW7 suppression also reduced the sensitivity of colon and ovarian cancer cells to anti-tubulin chemotherapeutic agents. In addition, FBXW7-dependent chemotherapeutic resistance has been reported in breast cancer\(^\text{10}\) and colorectal cancer cells, with loss of FBXW7 expression significantly associating with tolerance to 5-FU.\(^\text{31}\) Moreover, Ock et al.
reported that FBXW7 mutations influence the effects of induction chemotherapy (e.g. docetaxel, cisplatin, or 5-FU). Therefore, although the precise mechanisms underlying 5-FU resistance remain unclear, our data, along with previous findings, suggest that FBXW7 may, at least partly, mediate 5-FU resistance in OSCC.17 Our findings also suggest that FBXW7 may regulate the sensitivity of OSCC to radiation, although few studies have evaluated the direct relationship between FBXW7 and radioresistance. In contrast to our current findings, Zhang et al.32 reported that FBXW7 facilitates non-homologous end-joining (NHEJ) and that FBXW7 inactivation abrogates NHEJ repair and sensitizes colon cancer cells to radiation. Nevertheless, other reports have indicated that accumulation of FBXW7 substrates (e.g. c-Myc, Mcl-1, and Notch1) confers radioresistance in various types of cancer cells.33–35 Thus, based on these inconsistent findings, we speculate that FBXW7 suppression more strongly activates oncogenic signaling and induces radioresistance, as compared to NHEJ suppression, in cases of OSCC.36 The prognostic significance of low FBXW7 expression has been reported in many types of solid tumors, such as breast, colorectal, cervical, and renal cancers.12,13,38,39 We also observed that low FBXW7 expression was associated with shorter OS among patients with advanced OSCC, and for the first time, we demonstrated this relationship among patients with OSCC who underwent CRT. As the FBXW7 expression levels were significantly associated with the pathological response to preoperative CRT (Table 1), the difference in OS between the groups in the multivariate analysis may be partially due to the different responses to preoperative CRT in the groups with low and high FBXW7 expression.

In this study, we showed the clinical significance of FBXW7 in OSCC. However, there are some limitations to this study. Most importantly, our data are based only on analyses according to differences in the level of FBXW7 using clinical tissue samples. Therefore, additional in vitro analyses are warranted to examine the molecular basis and exact function of FBXW7 in OSCC.
Conclusion

This study is the first report to indicate that low FBXW7 expression associates with CRT resistance and a poor prognosis among patients with locally advanced OSCC. Therefore, our data indicate that FBXW7 could be useful as a therapeutic and prognostic biomarker of OSCC. However, further studies will be needed to clarify the functional roles of FBXW7 in chemoresistance and radioresistance, as well as the most effective method for therapeutically targeting FBXW7.

Acknowledgements

We thank Editage (http://www.editage.jp) for English language editing. R.Y., M.N., and A.H. conceived the study and devised the experimental design. H. Nakay participated in the study’s design, study coordination, and drafting of the manuscript. All authors have read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

The study followed the guidelines of the Ethics Committee of Kumamoto University. Informed consent (general consent to retrospective data analysis) was obtained from all patients prior to the biopsy and operation based on the guidelines of the

Table 1. Correlation between the FBXW7 expression status and clinicopathological factors in 110 OSCC patients.

| Characteristics          | Total FBXW7 status | p value |
|-------------------------|--------------------|---------|
|                         | Low expression     | High expression |
|                         | n (%)              | n (%)   |
| Age (years)             |                    |         |
| Median                  | 66.4               |         |
| Range                   | 30–87              | 33–85   |
| ≤65                     | 45 (75.6)          | 11 (24.4) | 0.123 |
| >65                     | 40 (61.5)          | 25 (38.5)   |
| Sex                     |                    |         |
| Male                    | 46 (70.8)          | 19 (29.2)  | 0.348 |
| Female                  | 28 (62.2)          | 17 (37.8)  |
| Primary site            |                    |         |
| Tongue                  | 25 (73.5)          | 9 (26.5)  | 0.302 |
| Mandible                | 16 (66.7)          | 8 (33.3)  |
| Maxilla                 | 13 (61.9)          | 8 (38.1)  |
| Oral floor              | 9 (75.0)           | 3 (25.0)  |
| Buccal mucosa           | 7 (46.7)           | 8 (53.3)  |
| Hard palate             | 4 (100)            | 0 (0)    |
| pT-stage                |                    |         |
| T2                      | 28 (66.7)          | 14 (33.3) | 0.696 |
| T3                      | 18 (62.1)          | 11 (37.9)  |
| T4                      | 28 (71.8)          | 11 (28.2)  |
| pN-stage                |                    |         |
| N=0                     | 42 (66.7)          | 21 (33.3) | 0.875 |
| N≥1                     | 32 (68.1)          | 15 (31.9)  |
| pStage                  |                    |         |
| I, II                   | 17 (65.4)          | 9 (34.6)  | 0.855 |
| III                     | 22 (64.7)          | 12 (35.3)  |
| IV                      | 30 (70.0)          | 15 (30.0)  |
| Differentiation         |                    |         |
| Well                    | 55 (67.1)          | 27 (32.9) | 0.932 |
| Moderate                | 19 (67.9)          | 9 (32.1)   |
| Pathological response   |                    |         |
| Grade=0, I, II          | 44 (77.2)          | 13 (22.8) | 0.021* |
| Grade ≥ III             | 30 (56.6)          | 23 (43.4)  |
| Recurrence/metastasis   |                    |         |
| No                      | 42 (65.3)          | 25 (34.7) | 0.957 |
| Yes                     | 25 (65.8)          | 13 (34.2)  |

The chi-square test was used to examine the correlation between FBXW7 expression and clinicopathological factors (*p < 0.05).

Figure 4. Relationship between F-box and WD repeat domain-containing 7 (FBXW7) expression and survival among patients with oral squamous cell carcinoma. Kaplan–Meier survival analysis was used to compare 110 patients with OSCC according to low or high FBXW7 expression. (a) Overall survival (OS; *p < 0.05). (b) Disease-free survival (DFS). However, further studies will be needed to clarify the functional roles of FBXW7 in chemoresistance and radioresistance, as well as the most effective method for therapeutically targeting FBXW7.
All tissue specimens were handled and made anonymous according to the ethical and legal standards. The data supporting the conclusions of this article are included within the article and its additional file (Figure S1). The datasets used and/or analyzed during this study are available from the corresponding author upon reasonable request.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by a Grant-in-Aid for Young Scientists B (16K20591) from the Japanese Ministry of Education, Culture, Sport, Science and Technology.

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**Table 2.** The results of the multivariate analysis of prognostic factors by the Cox proportional hazards regression model.

| Variables               | OS Hazard ratio (95% CI) | p value | DFS Hazard ratio (95% CI) | p value |
|-------------------------|--------------------------|---------|---------------------------|---------|
| **FBXW7**               |                          |         |                           |         |
| High                    | 4.26 (1.52–13.8)         | 0.0049**| 0.765 (0.347–1.74)        | 0.515   |
| Low                     | 1.30 (0.431–3.90)        | 0.632   | 1.00 (0.448–2.22)         | 0.991   |
| **Sex**                 |                          |         |                           |         |
| Male                    | 1.07 (0.363–3.32)        | 0.897   | 1.40 (0.627–3.19)         | 0.41    |
| Female                  | 1.52 (0.506–5.03)        | 0.458   | 0.729 (0.408–1.37)        | 0.312   |
| **Age**                 |                          |         |                           |         |
| ≤ 65                    | 2.23 (0.763–6.88)        | 0.143   | 1.23 (0.498–3.05)         | 0.645   |
| > 65                    | 0.967 (0.132–6.90)       | 0.973   | 11.2 (1.91–66.8)          | 0.0079***|
| **Stage**               |                          |         |                           |         |
| Well                    | 1.26 (0.490–3.63)        | 0.634   | 1.50 (0.684–3.15)         | 0.298   |
| Moderate                | 0.331 (0.102–0.905)      | 0.0304* | 0.254 (0.101–0.579)       | 0.0008***|
| Pathological response   |                          |         |                           |         |
| Grade 0, I, II          | 0.331 (0.102–0.905)      | 0.0304* | 0.254 (0.101–0.579)       | 0.0008***|
| Grade III               | 25.3 (8.26–112)          | <0.0001**| –                         | –       |
| Recurrence/metastasis   |                          |         |                           |         |
| No                      |                          |         |                           |         |
| Yes                     |                          |         |                           |         |
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