Abstract. Low expression of p27Kip1 is associated with disease progression and an unfavorable outcome in several malignancies, including oral squamous cell carcinoma (OSCC). In addition, the p27Kip1 protein is thought to be degraded by the Jun activation domain-binding protein 1 (Jab1). The purpose of this study was to examine whether Jab1 expression can be a useful prognostic factor in OSCC patients treated by 1 M Tegafur and 4 M uracil (UFT) in combination with radiation. Jab1 expression was investigated by immunohistochemistry in biopsy samples from 102 OSCC patients who were treated by UFT in combination with radiation. The associations of each expression with the clinicopathological characteristics and patient survival were also analyzed. A significant association was found between Jab1 expression and cervical lymph node metastasis (p=0.0004), stage of disease (p=0.0011), therapeutic effect (p=0.0133) and patient outcome (p=0.0095). The 5-year survival rates of Jab1 high- and low-expression tumors were 53.0% and 80.6%, respectively and this difference was significant (p=0.0053) by the log-rank test. Multivariate analysis revealed that reduced term survival was related to high levels of Jab1 expression (p=0.0082). These results suggest that Jab1 may be a useful prognostic factor in OSCC patients treated by UFT in combination with radiation.

p27Kip1 is a cyclin-dependent kinase inhibitor, which regulates the progression of cells from the G1- into the S-phase of the cell cycle. A reduced expression level of p27Kip1 is associated with the high aggressiveness and poor prognosis of various malignant tumors, including breast, lung, gastric and oral cancer (1-4). Thus, p27Kip1 is thought to be one of the important prognostic factors in various carcinomas (5).

Mutation of the p27Kip1 gene seems to be uncommon in human malignancies (6). Briefly, the quantity of p27Kip1 protein is regulated by a post-transcriptional mechanism rather than p27Kip1 gene aberrations. Recently, the Jun activation domain-binding protein 1 (Jab1) has been shown to shuttle p27Kip1 from the nucleus to the cytoplasm and to decrease the amount of p27Kip1 in the cell by accelerating p27Kip1 degradation via the ubiquitin-proteasome system (7, 8).

Jab1 expression has been examined in various tumors, including ovarian, breast, pancreatic, thyroid, pituitary and oral squamous cell carcinoma (OSCC) (9-17). It is reported that the Jab1 protein was mainly expressed in invasive breast cancer (10, 11). Korbonits et al. (16) found that Jab1 was significantly associated with cervical lymph node metastasis and poor prognosis in OSCC. However, the prognostic significance of Jab1 expression in OSCC patients treated by 1 M Tegafur and 4 M uracil (UFT) in combination with radiation has not been directly investigated. OSCC is the sixth most common solid tumor, accounting for 5.5% of all malignancies worldwide (18). Squamous cell carcinoma accounts for 96% of all tumors of the oral cavity (19) and many patients with these tumors die from metastatic disease (20). In addition, the incidence of OSCC is increasing among the younger generation (21).

In our previous study, p27Kip1 expression was found to be one of the most important prognostic factors in OSCC patients low expression of p27Kip1 being associated with disease progression and an unfavorable outcome in these patients (4). Therefore, the Jab1 protein, as a negative regulator of the p27Kip1 protein, was hypothesized to play an important role in the disease progression of OSCC. To gain better insight into the
clinical significance of Jab1, Jab1 expression was investigated immunohistochemically in 102 OSCC patients, and whether this expression was associated with the clinicopathological parameters and prognosis of OSCC patients treated by UFT in combination with radiation was assessed.

Materials and Methods

Patients and specimens. A total of 102 patients with OSCC were examined from April 1993 to March 1999 at the Second Department of Oral and Maxillofacial Surgery of the Dental Hospital of Tokushima University, Tokushima, Japan. The International Union Against Cancer TNM (22) classification was used for tumor staging. The patients had stage II, III or IV lesions, without distant metastasis to the lung or intestinal system, at the first visit to our clinic. No patient had previously received any treatment. All of the 102 patients were histopathologically diagnosed with squamous cell carcinoma. The clinical data on the patient’s age, gender, T classification, N classification, stage of disease, therapeutic effect and outcome are shown in Table I.

All patients received chemoradiation as a primary treatment. Briefly, they were treated by chemotherapy (UFT; 300-400 mg/day for 4-6 weeks per os) in combination with radiotherapy (60Co γ-ray irradiation, a total dose of 50-60 Gy). Approximately 1 month after the completion of the chemotherapy in combination with radiotherapy, biopsy materials were taken again from the treated patients for the assessment of therapeutic effect. If tumor cells were not detected, the patients were followed as out-patients. In the case of detection, surgery was performed. Before the primary treatment, all specimens were obtained from 102 patients referred for biopsy. All the tissue samples were fixed in phosphate-buffered 10% formalin and paraffin-embedded.

Immunohistochemical staining and evaluation. Three serial sections of 4 μm were cut from formalin-fixed, paraffin-embedded tissues and mounted on poly-L-lysine-coated slides. The sections were dewaxed in xylene and rehydrated in graded ethanols, according to standard procedures. A serial section from each specimen was stained with hematoxylin and eosin for histological evaluation. Other sections were microwaved in a citrate buffer, pH 6.0, 2 times for 4-6 minutes, cooled to room temperature gradually, and then rinsed in distilled water. Endogenous peroxidase activity was blocked using 0.3% H2O2 in methanol for 30 min, followed by rinsing in distilled water and in phosphate-buffered saline (PBS) at room temperature. A 10% normal horse serum was applied to the primary treatment, all specimens were obtained from 102 patients referred for biopsy. All the tissue samples were fixed in phosphate-buffered 10% formalin and paraffin-embedded.

The immunostained sections were evaluated by 2 independent observers, using an Olympus light microscope (AH-2, Olympus, Tokyo, Japan) under low power (10X objective), without prior knowledge of the clinical or histological diagnosis. The mean percentage of positively-stained cells was estimated by counting 1000 cells per area from at least 3 varied areas. Jab1 protein-positive cells were stained dark brown on the nuclei. Tumor cells with less staining intensity than infiltrating lymphocytes were regarded as negative. The expression of Jab1 was graded as: high (more than 50% of cancer cells stained) or low (less than or equal to 50% stained) Jab1 expression.

Statistical analysis. The association between Jab1 and clinicopathological parameters was assessed using the Fisher’s exact test or the Chi-square test. The overall survival was calculated using the Kaplan-Meier method and comparison between groups was done by a log-rank test.
performed with the log-rank test. The Hazard Ratio and Confidence Interval (CI) relating to the expression of Jab1 were evaluated by the Cox proportional hazard regression model. All statistical significance was set at $p<0.05$. Statistical analyses were performed using the StatView software (version 5.0J, SAS Institute Inc. Cary, NC, USA).

Results

All OSCC samples showed Jab1 protein expression. The Jab1 protein was detected in the nuclei of both normal epithelium adjacent to cancer and cancer epithelium, myoepithelium, endothelium and lymphocytes (Figure 1). Moreover, 66 out of 102 (64.7%) patients showed high intensity Jab1 expression, while 36 out of 102 (35.3%) patients showed low-intensity Jab1 expression by immunohistochemical examination.

The relationship between Jab1 expression and clinicopathological features is shown in Table I. Significantly higher levels of Jab1 expression were seen in cervical lymph node metastasis ($p=0.0004$), stage ($p=0.0011$), therapeutic effect ($p=0.0133$) and patient outcome ($p=0.0095$).
was no significant difference in age, gender or tumor size between the low and high Jab1 expression groups. The 5-year post-therapeutic survival of patients according to the Jab1 expression are shown in Figure 2. Survival was analyzed by the Kaplan-Meier method. The 5-year-survival rates of Jab1 high and low expression tumors were 53.0% and 80.6%, respectively and this difference was significant \( (p=0.0053) \) by the log-rank test. Multivariate analysis revealed that reduced-term survival was related to large tumor size (T3 and T4) \( (p=0.0001) \), cervical lymph node metastasis(N+) \( (p=0.0001) \), advanced stage (stage III and IV) \( (p=0.0016) \), poor therapeutic effect (PR+NC) \( (p=0.0075) \) and high levels of Jab1 expression \( (p=0.0082) \), although no other variants were identified (Table II).

### Discussion

We have continued to treat OSCC patients at T2 (>3 cm), T3 and T4 cases by chemotherapy (UFT; 300-400 mg/day for 4-6 weeks per os) in combination with radiotherapy (\(^{60}\)Co \( \gamma \)-ray irradiation, a total dose of 50-60 Gy). \( \text{p}27\text{Kip1} \) is thought to be one of the important prognostic factors in various carcinomas because loss of \( \text{p}27\text{Kip1} \) has been associated with disease progression and an unfavorable outcome in several malignancies (23). We also reported that \( \text{p}27\text{Kip1} \) is one of the most important prognostic factor in OSCC (4). In addition, we have found that high expression of S-phase kinase-interacting protein 2 (Skp2) is a strong prognostic marker in OSCC patients treated by UFT in combination with radiation (24). Previous reports indicated that Skp2 is a specific ubiquitin ligase subunit that targets \( \text{p}27\text{Kip1} \) for degradation (7, 25) and Jab1 controls the activity of \( \text{p}27\text{Kip1} \) by facilitating its degradation (26). Briefly, it was thought that Jab1 expression as well as Skp2 expression must be associated with reduced expression of the \( \text{p}27\text{Kip1} \) protein. We, thus, attempted to tried to assess whether Jab1 expression was associated with the clinicopathological parameters and prognosis of OSCC patients treated by UFT in combination with radiation.

A high expression of Jab1 was well-correlated with poor prognosis in OSCC patients in the present study. The correlation between the expression of Jab1 and the clinicopathological parameters was also evaluated in the same patients, and high expression of Jab1 was found to be significantly associated with cervical lymph node metastasis, stage of disease, therapeutic effect, patient outcome and low survival rates (Table I). These findings suggested that the immunohistochemical evaluation of Jab1 might be a reliable indication of the prognosis of OSCC patients. Moreover, multivariate analysis also revealed that the intensity of Jab1 expression was useful as a prognostic marker.

Seventy-nine percent of cases without \( \text{p}27\text{Kip1} \) expression showed high Jab1 expression, while 18 cases had high expression of Jab1 protein despite showing high \( \text{p}27\text{Kip1} \) expression (data not shown). Reduction of \( \text{p}27\text{Kip1} \) protein levels should be related to Skp2 as well as to Jab1. Interestingly, these 18 cases did not always have low expression of Skp2. Recently, the Kip1-ubiquitylation promoting complex (KPC)1/2 was found as a new cytoplasmic ubiquitin ligase for \( \text{p}27\text{Kip1} \) protein degradation (27-29). Therefore, we suggest that the decreased levels of \( \text{p}27\text{Kip1} \) were caused by some factors including Jab1, Skp2, KPC1/2 and others. Whether KPC1/2 expression could be a useful prognostic factor for OSCC patients should be examined next.

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