ANTICANCER RESEARCH *25*: 2471-2476 (2005)

High Expression of S-phase Kinase-associated Protein 2 (Skp2) is a Strong Prognostic Marker in Oral Squamous Cell Carcinoma Patients Treated by UFT in Combination with Radiation

KOJI HARADA[1], SUPRIATNO[1], SHIN-ICHI KAWAGUCHI[2], YUICHIRO KAWASHIMA[1], YASUTAKA ITASHIKI[1], HIDEO YOSHIDA[1] and MITSUNOBU SATO[1]

[1]Second Department of Oral and Maxillofacial Surgery and [2]Department of Oral and Maxillofacial Radiology, University of Tokushima, School of Dentistry, 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan

Abstract. *Low expression of $p27^{Kip1}$ is associated with disease progression and an unfavorable outcome in several malignancies including oral squamous cell carcinoma (SCC). In addition, $p27^{Kip1}$ protein is thought to be degraded by Skp2 (S-phase kinase-associated protein 2). The purpose of this study was to examine whether Skp2 expression can be a useful prognostic factor in oral SCC patients treated by UFT in combination with radiation. The Skp2 expression was investigated by immunohistochemistry in biopsy samples from 102 oral SCC patients, who were treated by UFT in combination with radiation. Associations of each expression with the clinicopathological characteristics and patient survival were also analyzed. A significant association was found between Skp2 expression and tumor size ($p=0.0462$), cervical lymph node metastasis ($p=0.0209$), therapeutic effect ($p=0.0490$) and patient outcome ($p=0.0002$). The 5-year survival rates of Skp2 high and low expression tumors were 40.5 % and 78.5 %, respectively, and this difference was significant ($p=0.0001$) by log-rank test. Multivariate analysis revealed that reduced term of survival was related to high levels of Skp2 expression ($p=0.0001$). These results suggest that Skp2 may be a useful prognostic factor in oral SCC patients treated by UFT in combination with radiation.*

$p27^{Kip1}$ is a cyclin-dependent kinase inhibitor which regulates progression of cells from G1- into S-phase in a cell cycle. It has been reported that low expression of $p27^{Kip1}$ is associated with disease progression and an unfavorable outcome in several malignancies including breast, lung, gastric and oral cancer (1-4). Thus, $p27^{Kip1}$ is thought to be one of the important prognostic factors in various carcinomas (5).

$p27^{Kip1}$ gene mutation seems to be uncommon in human malignancies (6). In short, the quantity of $p27^{Kip1}$ protein is regulated by a post-transcriptional mechanism rather than $p27^{Kip1}$ gene aberrations. Recent studies showed that Skp2 (S-phase kinase-associated protein 2) can degrade the $p27^{Kip1}$ protein by a ubiquitin-proteasomal system (7). Recently, overexpression of Skp2 has been observed in transformed cells (8) and in various types of human tumors (9-14). The expression of Skp2 correlates with the grade of malignancy in lymphomas (9) and oral squamous cell carcinomas (12). An elevated expression of Skp2 indicates poor prognosis for patients with oral squamous cell carcinomas (13) and gastric cancers (14). However, the prognostic significance of Skp2 expression in oral SCC patients treated by UFT in combination with radiation has not been directly investigated.

Oral squamous cell carcinoma (SCC) is the sixth most common solid tumor, accounting for 5.5% of all malignancies worldwide (15). SCC accounts for 96% of all tumors of the oral cavity (16), and many patients with these tumors die from metastatic disease (17). In addition, the incidence of oral SCC is increasing among the younger generation (18). In this study, we examined whether Skp2 expression can be a useful prognostic factor in oral SCC patients treated by UFT in combination with radiation.

Patients and Methods

Patients and specimens. A total of 102 patients with oral SCC 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 (19) classification was used for tumor staging.

Correspondence to: Dr Koji Harada, Second Department of Oral and Maxillofacial Surgery, University of Tokushima, School of Dentistry, 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan. Tel: +81 88 633 7354, Fax: +81 88 633 7462, e-mail: harako@dent.tokushima-u.ac.jp

Key Words: Skp2, UFT, radiation, prognosis, immunohistochemistry.

Table I. *Skp2 expression in relation to clinical characteristics.*

Characteristics	Total (n=102)	High (n=37)	Low (n=65)	*p*-value
Age (mean)	23-87 (65.3)	48-85 (67.3)	23-87 (64.2)	NS
Sex (M:F)	61:41	22:15	40:25	NS
T classification				
T2	69	20	49	0.0462
T3	18	10	8	(T2 *vs*
T4	15	7	8	T3+T4)
N classification				
N(-)	47	11	36	0.0209
N(+)	55	26	29	
Stage				
II	36	9	27	0.1318
III	28	7	21	(stageII *vs*
VI	38	21	17	stageIII+IV)
Therapeutic effect				
CR	25	5	20	0.0490
PR	53	12	41	(CR *vs*
NC	24	20	4	PR+NC)
Outcome				
Alive	64	14	50	0.0002
Dead	38	23	15	
5-year survival rate (Kaplan-Meier, %)	64.7	40.5	78.5	0.0001 (log-rank)

NS: not significant; M, F: male, female.
Complete response (CR) means disappearance of all lesion and no occurrence of new lesion by therapy for 4 weeks or more.
Partial response (PR) means reduction of 50% or more lesion and no occurrence of new lesion by therapy for 4 weeks or more.
No change (NC) means reduction of 50% or less lesion without occurrence of new lesion by therapy for 4 weeks or more.
The immunohistochemical evaluation was performed according to our criteria of high (more than 20% of cancer cells stained), and low (less than or equal to 20% stained), Skp2 expression.
T, N and stage grouping were classified according to the 1997 International Union Against Cancer criteria. *p*-value was identified by a Fisher's exact test without age, sex and 5-year survival rate. Age and sex was evaluated by a Chi-square test. The 5-year survival rate was evaluated by the log-rank test.

They had stage II, III or IV lesions, without distant metastasis such as lung or intestinal system, at the first visit to our clinic. No patient had previously received any treatments. All of the 102 patients were histopathologically diagnosed as squamous cell carcinoma. Clinical data on patient's age, sex, 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 ($_{60}$Co γ-ray irradiation, a total dose of 50-60 Gy). About 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 we could not detect tumor cells, the patients were followed as outpatients. In the case of detection of tumor cells, surgical operation was done. 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. Two 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 haematoxylin and eosin for histological evaluation. Other sections were microwaved in a citrate buffer, pH 6.0, two times for 5 min, cooled to room temperature gradually, and then rinsed in distilled water. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 30 min, and sections were rinsed in distilled water and in phosphate-buffered saline (PBS) at room temperature. A 10 % normal horse serum was applied to the sections for 30 min as a blocking reagent to reduce nonspecific binding. A 1:100 dilution of the polyclonal antibody against Skp2 protein (H-435, Santa Cruz, CA, USA) was used as the primary antibody. The sections were incubated at 37˚C for 90 min, then rinsed in PBS and incubated with biotinylated secondary antibody for 30 min, followed by incubation with streptavidin peroxidase reagents (Vector, CA, USA) for 30 min. After being washed in PBS, they were incubated in diaminobenzidine solution with H_2O_2 for 3 min. Finally, the sections were counterstained with haematoxylin for 30 sec, washed in water, dehydrated and mounted, according to standard procedures. Negative controls for each material were processed in the same manner, using PBS instead of the primary antibody.

The immunostained sections were evaluated by two 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 500 cells per area from at least three varied areas. $p27^{Kip1}$ and Skp2 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 Skp2 was graded as: high (more than 20% of cancer cells stained) and low (less than or equal to 20% stained).

Statistical analysis. The associations between Skp2 and clinicopathological parameters were assessed using the Fisher's exact test or Chi-square test. Overall survival was calculated using the method of Kaplan-Meier, and comparison between groups was performed with the log-rank test. The Hazard Ratio and the Confidence Interval (CI) relating to the expression of Skp2 was 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 oral SCC samples showed Skp2 protein expression. Skp2 protein was detected in the nuclei of both normal epithelium

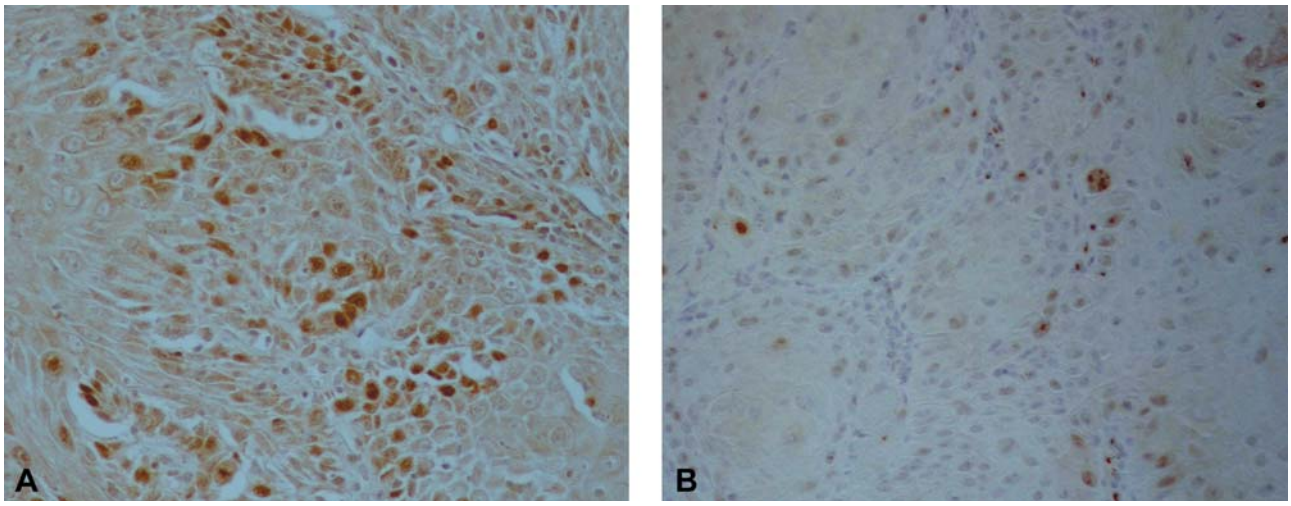

Figure 1. *Immunohistochemical staining of Skp2 in oral squamous cell carcinoma. (A) High Skp2 expression case, (B) low Skp2 expression case. Dark brown staining on the nuclei is positive (original magnification x300: A and B).*

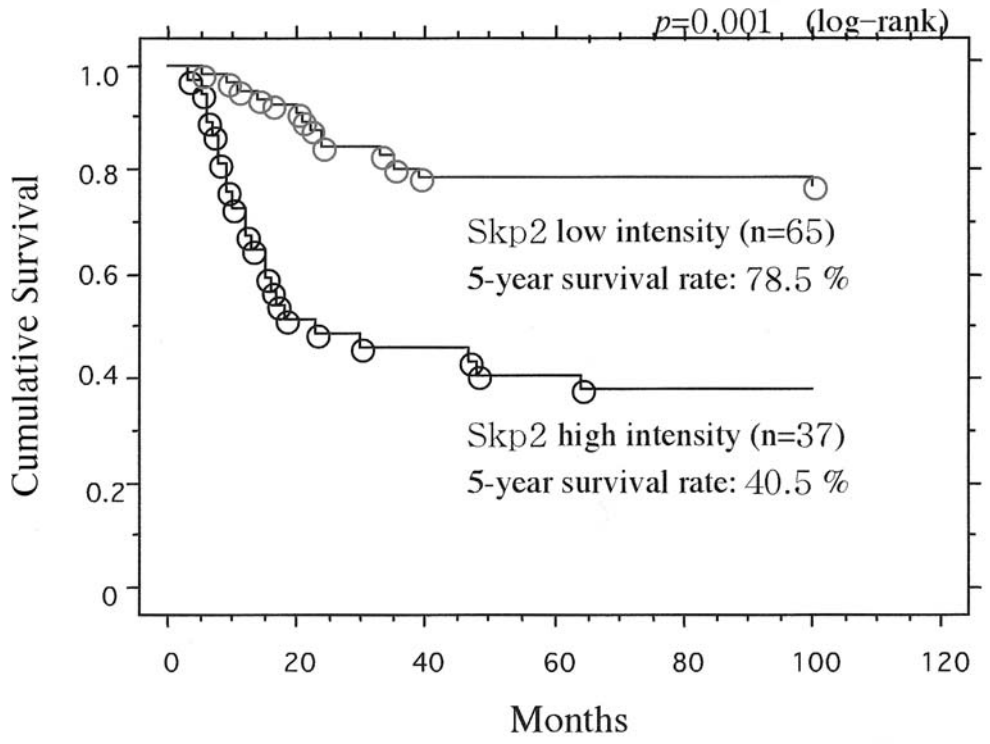

Figure 2. *Kaplan-Meier plot demonstrates the 5-year survival rates of patients with high Skp2 expression (more than 20% tumor cell nuclei positive) and with low Skp2 expression (less than 20%). The 5-year survival rates of the low Skp2 expression group (40.5 %) is significantly higher than that of the high Skp2 expression group (78.5 %; p=0.0001).*

adjacent to cancer and cancer epithelium, myoepithelium, endothelium and lymphocytes (Figure 1). Moreover, 37 out of 102 (36.3%) patients showed high intensity Skp2 expression, while 65 out of 102 (63.7%) patients showed low intensity Skp2 expression by immunohistochemical examination.

The relationship between Skp2 expression and histopathological features is shown in Table I. Significantly higher levels of Skp2 expression were seen in tumor size ($p=0.0462$), cervical lymph node metastasis ($p=0.0209$), therapeutic effect ($p=0.0490$) and patient outcome

Table II. *Risk factors affecting overall survival rate determined by Cox's proportional hazard model.*

Variable	Hazard ratio	95%CI	*p*-value
T classification			
T2 *vs* T3+T4	0.267	0.140-0.509	0.0001
N classification			
N2 *vs* N3+N4	0.196	0.086-0.447	0.0001
Stage			
StageII *vs* Stage III+IV	0.245	0.102-0.586	0.0016
Effect			
CR *vs* PR+NC	0.067	0.009-0.486	0.0075
Skp2 expression			
High *vs* Low	3.954	2.056-7.604	0.0001

CI: Confidence Interval

($p=0.0002$). There was no significant difference in age, sex and stage classification between the low and high Skp2 expression groups.

The five-year post-therapeutic survival of patients according to the Skp2 expression are shown in Figure 2. Survival was analyzed by the Kaplan-Meier method. The 5-year survival rates of Skp2 high and low expression tumors were 40.5 % and 78.5 %, respectively, and this difference was significant ($p=0.0001$) by log-rank test.

Multivariate analysis revealed that reduced term of survival was related to large tumor size (T3 and T4) ($p=0.0001$), cervical lymph node metastasis ($p=0.0001$), advanced stage (stage III and IV) ($p=0.016$) and high levels of Skp2 expression ($p=0.0001$), although no other variants were identified (Table II).

Discussion

We have continued to treat oral SCC 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 γ -ray irradiation, a total does of 50-60 Gy). $p27^{Kip1}$ is thought to be one of the important prognostic factors in various carcinomas, because loss of $p27^{Kip1}$ has been associated with disease progression and an unfavorable outcome in several malignancies (19). We have already reported that $p27^{Kip1}$ is a most important prognostic factor in oral SCC (20). Moreover, it has already been reported that Skp2 is a specific ubiquitin ligase subunit that targets $p27^{Kip1}$ for degradation (21, 22). So, we supposed that reduced expression of $p27^{Kip1}$, associated with high aggressiveness and poor prognosis in various carcinomas, is caused by a high expression of Skp2. In this study, we examined whether Skp2 expression can be a useful prognostic factor in oral SCC patients treated by UFT in combination with radiation.

An inverse correlation between the expression of Skp2 and $p27^{Kip1}$ in oral SCC has already been reported (12, 13, 23); however, in one report there was no statistical difference between the patients with and without Skp2 expression on overall survival rate by Kaplan-Meier survival curves (23), while in another report the overall survival rate was higher in patients without Skp2 expression (13). We demonstrated that high expression of Skp2 was well correlated with poor prognosis in oral SCC patients in the present study (Figure 1). We also evaluated the correlations between the expression of Skp2 and clinicopathological parameters in the same patients, and we found that high expression of Skp2 was significantly associated with cervical lymph node metastasis, therapeutic effect, patient outcome and low survival rates (Table I). These findings suggested that the immunohistochemical evaluation of Skp2 might be a reliable indication of the prognosis of oral SCC patients. Moreover, multivariate analysis also revealed that the intensity of Skp2 expression was useful as a prognostic marker.

Seventy-seven percent of cases without $p27^{Kip1}$ expression showed high Skp2 expression, but 8 cases had high expression of Skp2 protein despite showing high $p27^{Kip1}$ expression (data not shown). Reduction of $p27^{Kip1}$ protein should be related to Jab1 (Jun activation domain-binding protein 1) as well as Skp2. Interestingly, these 8 cases had low expression of Jab1. Therefore, we think that the decreased levels of $p27^{Kip1}$ are caused by factors other than Skp2 and Jab1.

In conclusion, we found that Skp2 as well as $p27^{Kip1}$ may be useful prognostic factors in oral SCC patients treated by UFT in combination with radiation.

Acknowledgements

This study was supported in part by Grants-in-Aid for Cancer Research and for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

1 Catzavelos C, Bhattacharya N, Ung YC, Wilson JA, Roncari L, Sandhu C *et al*: Decreased levels of the cell-cycle inhibitor $p27^{Kip1}$ protein: prognostic implications in primary breast cancer. Nature Med *3*: 227-230, 1997.

2 Esposito V, Baldi A, De Luca A, Groger AM, Loda M, Giordano GG *et al*: Prognostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. Cancer Res *57*: 3381-3385, 1997.

3 Mori M, Mimori K, Shiraishi T, Tanaka S, Ueo H, Sugimachi K *et al*: p27 expression and gastric carcinoma. Nature Med *3*: 593, 1997.

4 Harada K, Supriatno, Yoshida H and Sato M: Low $p27^{Kip1}$ expression is associated with poor prognosis in oral squamous cell carcinoma. Anticancer Res *22*: 2985-2989, 2002.
5 Lloyd RV, Erickson LA, Jin L, Kulig E, Qian X, Cheville JC and Scheithauer BW: $p27^{Kip1}$: A multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers. Am J Pathol *154*: 313-323, 1999.
6 Kawamata N, Morosetti R, Miller CW, Park D, Spirin KS, Nakamaki T *et al*: Molecular analysis of the cyclin-dependent kinase inhibitor gene p27/ in human malignancies. Cancer Res *55*: 2266-2269, 1995.
7 Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V *et al*: Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science *269*: 682-685, 1995.
8 Zhang H, Kobayashi R, Galaktionov K and Beach D: p19Skp1 and p45Skp2 are essential elements of the cyclin A-CDK2 S phase kinase. Cell *82*: 915-925, 1995.
9 Latres E, Chiarle R, Schulman BA, Pavletich NP, Pellicer A, Inghirami G and Pagano M: Role of the F-box protein Skp2 in lymphomagenesis. Proc Natl Acad Sci USA *98*: 2515-2520, 2001.
10 Hershko D, Bornstein G, Ben-Izhak O, Carrano A, Pagano M, Krausz MM and Hershko A: Inverse relation between levels of $p27^{Kip1}$ and of its ubiquitin ligase subunit Skp2 in colorectal carcinomas. Cancer *91*: 1745-1751, 2001.
11 Chiarle R, Fan Y, Piva R, Boggino H, Skolnik J, Novero D, Palestro G, De Wolf-Peeters C, Chilosi M, Pagano M and Inghirami G: S-phase kinase-associated protein 2 expression in non-Hodgkin's lymphoma inversely correlates with p27 expression and defines cells in S phase. Am J Pathol *160*: 1457-1466, 2002.
12 Gstaigert M, Jordan R, Lim M, Catzavelos C, Mestan J, Slingerland J and Krek W: Skp2 is oncogenic and overexpressed in human cancers. Proc Natl Acad Sci USA *98*: 5043-5048, 2001.
13 Kudo Y, Kitajima S, Sato S, Miyauchi M, Ogawa I and Takata T: High expression of S-phase kinase-interacting protein 2, human F-box protein, correlates with poor prognosis in oral squamous cell carcinomas. Cancer Res *61*: 7044-7047, 2001.
14 Masuda TA, Inoue H, Sonoda H, Mine S, Yoshikawa Y, Nakayama K, Nakayama K and Mori M: Clinical and biological significance of S-phase kinase-associated protein 2 (Skp2) gene expression in gastric carcinoma: modulation of malignant phenotype by Skp2 overexpression, possibly *via* p27 proteolysis. Cancer Res *62*: 3819-3825, 2002.
15 Johnson NW: Epidemiology of oral cancer in risk markers of oral disease. *In:* Johnson NW, ed. Oral Cancer, vol.2. Cambridge, UK: Cambridge University Press, pp. 3-26, 1991.
16 Thomas GJ, Jones J and Speight PM: Integrins and oral cancer. Oral Oncol *33*: 381-388, 1997.
17 Ramos DM, Chen BL, Boylen K, Stern M, Kvamer RH, Sheppard D, Nishimura SL, Greenspan D, Zardi L and Pytela R: Stromal fibroblast influence oral squamous-cell carcinoma cell interactions with tenascin-C. Int J Cancer *72*: 369-372, 1997.
18 Silverman S Jr: Oral Cancer, 4th.ed, BC Becker, Ontario, 1998.
19 Sobin LH and Wittekind Ch: TNM Classification of Malignant Tumours. International Union Against Cancer (UICC), Ed. 5. New York: Wiley-Liss, pp. 20-37, 1997.
20 Lloyd RV, Erickson LA, Jin L, Kulig E, Qian X, Cheville JC and Scheithauer BW: $p27^{Kip1}$: a multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers. Am J Pathol *154*: 313-323, 1999.
21 Carrano AC, Eytan E, Hershko A and Pagano M: Skp2 is required for ubiquitin-mediated degradation of the Cdk inhibitor p27. Nat Cell Biol *1*:193-197, 1999.
22 Sutterluty H, Chatelain E, Mart A, Wirbeluauer C, Senften M, Muller U and Krek W: $p45^{Skp2}$ promotes $p27^{Kip1}$ degradation and induces S phase in quiescent cells. Nat Cell Biol *1*:207-214, 1999.
23 Shintani S, Li C, Mihara M, Hino S, Nakashiro K and Hamakawa H: Skp2 and Jab1 expression are associated with inverse expression of $p27^{KIP1}$ and poor prognosis in oral squamous cell carcinomas. Oncology *65*: 355-362, 2003.

Received September 20, 2004
Revised February 7, 2005
Accepted February 21, 2005