High Expression of S-Phase Kinase-interacting Protein 2, Human F-box Protein, Correlates with Poor Prognosis in Oral Squamous Cell Carcinomas

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Abstract

Reduced expression of p27Kip1, a cyclin-dependent kinase (Cdk) inhibitor, is frequently found in various cancers, including oral squamous cell carcinoma (OSCC), and is attributable to an enhancement of its degradation. Skp2, an F-box protein necessary for DNA replication, is required for the ubiquitinylation and subsequent degradation of p27Kip1. In the present study, we examined the expression of Skp2 and its correlation with the expression of p27Kip1 protein or p27Kip1 degradation in OSCC. Using immunohistochemistry, we found that high expression of Skp2 was present in 49% of OSCCs and only 20% of epithelial dysplasias. Significantly, high expression of Skp2 was correlated with poor prognosis of OSCC patients. We also found an inverse correlation between the expression of Skp2 and p27 by immunohistochemical analysis. A similar correlation was observed in OSCC cell lines and OSCC tissues by Western blot analysis. Interestingly, OSCC tissues with Skp2 expression had high p27Kip1 degradation activity. These findings indicate that (a) Skp2 may play an important role for the development of OSCC, (b) Skp2 can be a novel target for OSCC treatment as well as a strong prognostic marker, and (c) the reduction in p27Kip1 protein may be brought about by enhancement of its degradation mediated by increased levels of Skp2 protein.

Introduction

The proliferation and progression of cancer cells have been known to closely relate to abnormalities of various positive and negative cell cycle regulators. p27Kip1, a Cdk inhibitor, mediates G1 arrest induced by transforming growth factor β, contact inhibition, or serum deprivation in epithelial cell lines (1, 2). The increase in the cellular abundance of p27Kip1 upon induction of cell quiescence is primarily attributable to a decrease in the rate of its degradation (3). p27Kip1 is polyubiquitinated both in vivo and in vitro, and less p27Kip1 ubiquitination activity is present in proliferating cells than in quiescent cells (3). Furthermore, p27Kip1 ubiquitination requires its phosphorylation on threonine-187 (4). Reduced expression of p27Kip1 has been found frequently in various cancers, and the lack of p27Kip1 is suggested to be the result of an enhancement of its degradation (5). Aggressive human cancers express low levels of p27Kip1 because of its decreased stability (5). We also found that reduced expression of p27Kip1 was shown in 87% of OSCC cases and was correlated with its malignancy (6). Importantly, reduced p27Kip1 levels represent a powerful prognostic marker for poor survival in cancer patients.

Skp2, an F-box protein necessary for DNA replication, was originally identified as a protein that interacts with cyclin A (7). Recently, it has been reported that p27Kip1 is specifically recognized and targeted for ubiquitination by Skp2 (8–10). Skp2 is required for the ubiquitination and subsequent degradation of p27Kip1 both in vivo and in vitro (8–10). Skp2 is a rate-limiting component of the machinery that ubiquititates and degrades phosphorylated p27Kip1 (8). Skp2 frequently is overexpressed in tumor cell lines, and forced expression of Skp2 in quiescent fibroblasts induces DNA synthesis (7, 9). These findings led us to hypothesize that the enhanced p27Kip1 degradation observed in many aggressive human tumors might be attributable to increased levels of Skp2. However, the abnormal expression of Skp2 and the correlation between Skp2 and p27Kip1 expression in cancer remain unclear. Therefore, in the present study, we examined the expression of Skp2 protein and its correlation with the expression of p27Kip1 protein or p27Kip1 degradation in OSCC.

Materials and Methods

Tissue Samples. Tissue samples from 15 patients with epithelial dysplasias (12 men and 3 women) and 37 patients with OSCC (21 men and 16 women) were retrieved from the Surgical Pathology Registry of Hiroshima University Dental Hospital from 1976 to 1997. At the time of diagnosis, the ages of the patients with epithelial dysplasia and OSCC were 37–81 years (mean, 60.7 years) and 31–87 years (mean, 61.4 years), respectively. For the present analysis, only biopsied specimens from the tongue before radiotherapy were selected to avoid possible influences of the lesion sites and treatment modalities on data. Among the 37 OSCCs, follow-up data were available in 27 patients (15 men and 12 women). The mean follow-up period for these OSCC patients was 70.37 months (range, 4–209 months). For immunohistochemical examination, tissues were fixed in 10% buffered formalin and embedded in paraffin. The numbers of cases for each degree of epithelial dysplasia and histological grade of OSCC are listed in Table 1. Histological grade and stage of tumor were classified according to the criteria of the Japan Society for Head and Neck Cancer (11). Fresh samples were taken from neoplastic tissues and from nonneoplastic tissues for Western blot analysis.

Immunohistochemistry. Immunohistochemical detection of Skp2 or p27Kip1 was performed using a streptavidin-biotin peroxidase technique as described previously (6, 12). An antihuman Skp2 rabbit polyclonal antibody (diluted 1:100; Transduction Laboratories, Lexington, KY) was used. Nuclear staining of Skp2 and p27Kip1 was scored on a semiquantitative scale (see below) by evaluating the percentage of stained nuclei within representative areas of each tumor. For superficial carcinomas, stained sections were observed throughout the lesion. For advanced large tumors, at least 10 fields, including superficial, central, and deep invasive areas, were observed, and the number of stained cells and staining intensity were evaluated. In each field, we counted ≥300 cells, using an eyepiece graticule to prevent recounting. Although qualitative differences in staining intensity were observed with considerable intratumoral heterogeneity, all positive cases showed obvious nuclear staining, at least focally. The expression of Skp2 was graded as ++ (+30% of tumor cells showed strong or diffuse immunopositivity), + (5–30% of tumor cells showed moderate or patchy immunopositivity), and − (<5% of the tumor cells showed weak or focal immunopositivity or no staining). p27Kip1 expression was graded as described previously (6, 12).

Statistical Analysis. Patients’ survival data were used to determine possible correlation between Skp2 or p27Kip1 expression levels and disease-free
High Expression of Skp2 in OSCC

Table 1 Expression of Skp2 in OSCC and its correlation with clinicopathological parameters

<table>
<thead>
<tr>
<th></th>
<th>Number of cases</th>
<th>Skp2 expression, n (%)</th>
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<tr>
<td></td>
<td></td>
<td>− and +</td>
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<tr>
<td>Epithelial dysplasia</td>
<td>15</td>
<td>12 (80%)</td>
</tr>
<tr>
<td>Historya</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild to moderate</td>
<td>11</td>
<td>10 (91%)</td>
</tr>
<tr>
<td>Severe</td>
<td>4</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>p27 expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ and +</td>
<td>15</td>
<td>12 (80%)</td>
</tr>
<tr>
<td>−</td>
<td>0</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Cancer</td>
<td>37</td>
<td>19 (51%)</td>
</tr>
<tr>
<td>Historyb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>14</td>
<td>8 (57%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>17</td>
<td>8 (47%)</td>
</tr>
<tr>
<td>Poor</td>
<td>6</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>Stagec</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>II + III</td>
<td>8</td>
<td>5 (62.5%)</td>
</tr>
<tr>
<td>IV</td>
<td>25</td>
<td>11 (44%)</td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>8 (73%)</td>
</tr>
<tr>
<td>Positive</td>
<td>26</td>
<td>11 (42%)</td>
</tr>
<tr>
<td>p27 expressiond</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ and +</td>
<td>18</td>
<td>11 (61%)</td>
</tr>
<tr>
<td>−</td>
<td>19</td>
<td>17 (42%)</td>
</tr>
</tbody>
</table>

* Grades of Skp2 or p27 expression were classified as + + (>30%); + (5–30%); and − (<5%).

a According to the criteria of the Japanese Classification of Head and Neck Cancer (11). Stage grouping as follows: stage 1, includes T<sub>n</sub>, N<sub>0</sub>, M<sub>0</sub>; stage 2 includes T<sub>n</sub>, N<sub>0</sub>, M<sub>0</sub>; stage 3 includes T<sub>n</sub>, N<sub>0</sub>, M<sub>0</sub>; stage 4 includes T<sub>n</sub>, N<sub>0</sub>–1, M<sub>0</sub> or any T, any N, M<sub>0</sub>.
b Only p < 0.05. Correlation was analyzed by Fisher’s exact test.

d For the degradation assay. Purified histidine-tagged p27<sub>Kip1</sub> was incubated at 37°C for different times in 30 µl of a degradation mixture containing 100 µg of cell extract, 50 m M Tris-HCl (pH 8.5), 5 mM MgCl<sub>2</sub>, 1 mM DTT, 2 mM ATP, 10 mM creatine phosphokinase (Sigma), 10 mM creatine phosphate (Sigma), and 5 µM ubiquitin (Sigma). p27<sub>Kip1</sub> degradation was analyzed by immunoblotting with an antihuman p27<sub>Kip1</sub> antibody.

Results

Immunohistochemical Expression of Skp2 in Epithelial Dysplasia and OSCC. Fifteen cases of epithelial dysplasia of the tongue were analyzed, including 11 cases of mild to moderate, and 4 cases of severe dysplasia. Thirty-seven OSCC cases were also analyzed. The incidence of Skp2 expression in epithelial dysplasia and OSCCs is summarized in Table 1. Of 15 epithelial dysplasia cases, 12 (80%) showed <30% positive cells (Fig. 1A). Only 1 (9%) of 11 mild-to-moderate dysplasia cases and 2 (50%) of 4 severe dysplasia cases showed high expression of Skp2 (>30% positive cells; +; Fig. 1B). We could not find loss of p27<sub>Kip1</sub> expression in the epithelial dysplasias.

In contrast to epithelial dysplasia, 49% (18 of 37) of OSCC cases showed high expression of Skp2 (Fig. 1C and Table 1). High expression of Skp2 was found in 58% of cases with metastasis, and in 17% of cases without metastasis. Expression of Skp2 seemed to be associated with metastasis, but there was no statistically significant correlation (P = 0.09). We found no correlation between Skp2 expression and histology or stage grouping. Although we also examined the influence of the age, sex, and smoking habits of OSCC patients on Skp2 expression, there were no correlations among them. Fifty-eight percent of cases without p27<sub>Kip1</sub> expression showed high expression of Skp2. There was inverse correlation between Skp2 and p27<sub>Kip1</sub> expression in epithelial dysplasias and OSCCs (Table 2).

Correlation between Skp2 Expression and Survival Rate. The relationships between Skp2 expression and survival rates for 27 OSCC patients with follow-up data were also examined. Fig. 1, D–F, shows the Kaplan-Meier survival curves of the patients grouped by the immunoreactivity of Skp2 and/or p27<sub>Kip1</sub> in their tumors. The cumulative survival rate of the patients with high expression of Skp2 (>30%) was remarkably lower than that for patients with <30% positive cells, and there was a statistical significance between them (P < 0.01; Fig. 1D). The cumulative survival rate of the patients with loss of p27<sub>Kip1</sub> expression (<5%) was also lower than that of patients with positive expression of p27<sub>Kip1</sub> (>5%; Fig. 1E). Moreover, patients with high expression of Skp2 and loss of p27 expression (Skp2 >30%, p27<sub>Kip1</sub> <5%) showed poor prognosis in comparison with the other groups (Skp2 >30%, p27<sub>Kip1</sub> >5%; Skp2 <30%, p27<sub>Kip1</sub> <5%; Skp2 <30%, p27<sub>Kip1</sub> >5%; Fig. 1F).

Expression of Skp2 in OSCC Cell Lines and Tissues. The expression of Skp2 and p27<sub>Kip1</sub> proteins in seven OSCC cell lines (HSC2, HSC3, HSC4, Ca9-22, Ho-1-N-1, Ho-1-U-1, and KB) was examined by Western blot analysis as shown in Fig. 2A. OSCC cells, except for HSC4 cells, showed expression of Skp2 protein. Cells lines with low-level p27<sub>Kip1</sub> expression (HSC2, HSC3, Ho-1-N-1, and KB) showed high expression of Skp2. Cells with high expression of p27<sub>Kip1</sub> (HSC4 and Ho-1-U-1) showed low expression of Skp2.

In Vitro p27<sub>Kip1</sub> Protein Degradation Assay. The p27<sub>Kip1</sub> degradation assay was performed as described by Pagano et al. (3). Each frozen tissue sample and cells were prepared by the addition of three to five volumes of lysis buffer containing 20 mM Tris-HCl (pH 8.5) and 1 mM DTT to a cell pellet. The following protease inhibitors were added: 0.1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml t-1-chloro-3-(4-tosylamido)-4-phenyl-2-butannone; 10 µg/ml t-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride, and 1 µg/ml aprotonin. Each sample was frozen and thawed three times. The lysate was centrifuged at 13,000 rpm in an Eppendorf centrifuge for 15 min at 4°C. The supernatant was retrieved and stored at −80°C for the degradation assay. Purified histidine-tagged p27<sub>Kip1</sub> was incubated at 37°C for different times in 30 µl of a degradation mixture containing 100 µg of cell extract, 50 mM Tris-HCl (pH 8.5), 5 mM MgCl<sub>2</sub>, 1 mM DTT, 2 mM ATP, 10 mM creatine phosphokinase (Sigma), 10 mM creatine phosphate (Sigma), and 5 µM ubiquitin (Sigma). p27<sub>Kip1</sub> degradation was analyzed by immunoblotting with an antihuman p27<sub>Kip1</sub> antibody.
found an inverse correlation between Skp2 and p27 Kip1 expression in these cells. However, Ca9-22 cells showed high expression of Skp2 despite showing high expression of p27Kip1.

We next examined the expression of Skp2 and p27Kip1 proteins in OSCC tissues by Western blot analysis (Fig. 2B). Because the reduction in p27 Kip1 protein is brought about by ubiquitin-proteasome-mediated degradation, we also examined the p27 Kip1 degradation activity in these tissues. All OSCC tissues showed reduced expression of p27Kip1 protein in comparison with corresponding nonneoplastic tissues. Skp2 expression was found in three of five OSCC tissues. Accordingly, these three tissues showed high p27Kip1 degradation activity (Fig. 2C).

Discussion

Skp2, a human F-box protein, was originally identified as a protein that interacts with cyclin A (7). F-box proteins form an expanding family of eukaryotic proteins characterized by an 40-amino acid F-box motif that is necessary to bind the other subunits of the ubiquitin ligase (14). F-box proteins are components of ubiquitin protein ligases (E3), called the SCF complex, which contains the following basic subunits: Skp1 (S-phase kinase-associated protein 1), a cullin subunit (called Cul1 in metazoans), Roc1 (also called Hrt1 or Rbx1), and one of many F-box proteins. Skp2 frequently is overexpressed in tumor cell lines, and forced expression of Skp2 in quiescent fibroblasts induces DNA synthesis (7, 9). In the present study, we demonstrated that high expression of Skp2 was found in 49% of OSCCs in comparison with 20% of epithelial dysplasias (Table 1 and Fig. 1). Moreover, high expression of Skp2 was well correlated with poor prognosis in OSCC patients (Fig. 1, D–F). These findings are consistent with the following results indicating that Skp2 is an oncogene: (a) Skp2 was frequently overexpressed in tumor cell lines (7); (b) forced expression of Skp2 in quiescent fibroblasts induced DNA synthesis (9); (c) Skp2 cooperated with activated N-Ras in tumorigenesis in an in vivo model (15); (d) Skp2 cooperated with H-RasG12V to malignantly transform primary rodent fibroblasts as scored by colony formation in soft agar and tumor formation in nude mice (16); and (e) cotransfection with

Table 2  Correlation between the expression of Skp2 and p27Kip1 in epithelial dysplasias and OSCCs

Grades of p27- and Skp2-positive cells were classified as ++ (>30%), + (5-30%), and − (<5%). Correlation by Fisher’s exact test: P < 0.05.

<table>
<thead>
<tr>
<th>p27 expression</th>
<th>− and +</th>
<th>+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>++ and +</td>
<td>23</td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td>−</td>
<td>8</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>21</td>
<td>52</td>
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</table>
Skp2 and cyclin E promoted abundant hepatocyte replication and hyperplasia of the liver in vivo (17). There have been reports on overexpression of Skp2 protein in human tumors (7, 16); however, our report is the first to show a correlation with prognosis. On the other hand, Skp2 may play an important role for regulating normal cell proliferation, because Skp2-deficient mice grow slower than littermate controls and have smaller organs, with all tissues containing decreased numbers of cell (18). However, the mechanism of Skp2 overexpression in cancer cells is uncertain. Recently, it has been reported that Skp2 is required for the ubiquitination and subsequent degradation of p27Kip1 both in vivo and in vitro (8–10). Skp2–/– cells show high levels of p27 and free cyclin E (non-Cdk2 bound), polyploidy, and centrosome overduplication (18). It is well known that reduced expression of p27Kip1 is frequently found in various cancers and correlated with poor survival of cancer patients (5). Moreover, aggressive human cancers express low levels of p27Kip1 because of its decreased stability (5), but the detailed mechanism of abnormal degradation of p27Kip1 protein in cancer cells is still unclear. We previously demonstrated that (a) reduced expression of p27Kip1 was found frequently in OSCCs and was well correlated with poor prognosis (6), (b) a reduction in p27Kip1 protein was correlated with early invasion and metastasis of OSCC (12), (c) in OSCC cell lines, reduced expression of p27Kip1 protein was brought about by proteasome-mediated degradation (6), and (d) p27Kip1 accumulation by inhibition of proteasome function induced apoptosis in OSCC cell lines (13). We hypothesize that reduced expression of p27Kip1 protein observed in OSCC might be attributable to enhanced degradation mediated by increased levels of Skp2 protein. In this study, we found an inverse correlation between the expression of Skp2 and p27 by immunohistochemistry (Table 2). Fifty-eight percent of cases without p27Kip1 expression showed high Skp2 expression. We also found an inverse correlation in OSCC cell lines by Western blot analysis, but one of them (Ca9-22 cells) had high expression of Skp2 protein despite showing high p27Kip1 expression (Fig. 2A). In OSCC tissues, three of five cases showed an inverse correlation between the expression of Skp2 and p27 (Fig. 2B). Interestingly, these three cases had high p27Kip1 degradation activity (Fig. 2, B and C). We think that the reduction in p27Kip1 protein may be brought about by high Skp2-mediated degradation in some OSCC cases. It has recently been reported that human Cks1, a member of the Suc1/Cks family of proteins, which are essential for cell cycle progression, binds to Skp2 and greatly increases binding of T187-phosphorylated p27 to Skp2 (19). We previously found overexpression of Cks1 mRNA in 15 (62.5%) of 24 gastric cancers (20). Therefore, we think that another molecule such as Cks1 may be involved in p27Kip1 degradation in OSCC cases who did not show high expression of Skp2 and showed reduced expression of p27Kip1. To clarify the correlation between Skp2 expression and p27Kip1 degradation, we plan to do further studies using more OSCC tissues.

In the present study, we found that high Skp2 expression was significantly correlated with poor prognosis in OSCC and an inverse correlation between the expression of Skp2 and p27Kip1 in oral tissues. These findings indicate that (a) Skp2 may play an important role for the development of OSCC, (b) Skp2 can be a novel target for OSCC treatment as well as a strong prognostic marker, and (c) the reduction in p27Kip1 protein may be brought about by enhancement of its degradation mediated by increased Skp2 protein.

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References


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