Inverse Correlation Between Expression Levels of p27 and the Ubiquitin Ligase Subunit Skp2 in Early Esophageal Squamous Cell Carcinoma

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**Abstract.** Background: In esophageal squamous cell carcinoma (SCC), the relationship between expression of the cyclin-dependent kinase inhibitor p27 and tumor aggression and prognosis is still controversial. Moreover, the expression of S-phase kinase-interacting protein 2 (Skp2), the ubiquitin ligase subunit required for the ubiquitin-dependent degradation of p27, remains unknown. Materials and Methods: We used immunohistochemistry to analyze Skp2 and p27 expression in surgical specimens obtained from 32 patients with early esophageal SCC. We also used Western blotting to characterize the expression of Skp2 and p27 in 7 cell lines derived from esophageal SCC. Results: Expression of Skp2 showed an inverse topographic distribution and correlation to that of p27 in many esophageal carcinomas. Of the 7 cell lines, 6 showed an inverse relationship between Skp2 and p27 expression. Patients without an inverse correlation between Skp2 and p27 expression had a significantly unfavorable prognosis in the early stage (p=0.0493). Conclusion: These findings suggest that the target substrate of Skp2 in early esophageal SCC is mainly p27, and that failure of Skp2-induced degradation of p27 may influence tumor progression and lead to a poor prognosis.

S-phase kinase-interacting protein 2 (Skp2), the component of the SCF ubiquitin ligase complex composed of Skp1, Cullins and F-box proteins, plays a crucial role in cell cycle progression (1-3). In the SCF complex, the Cullins protein interacts with Skp1 and Skp1 in turn binds to an F-box protein. Recruitment of different F-box proteins into the SCF complex may be important for the specific ubiquitination of certain target proteins (4). Skp2, a member of the F-box protein family, was discovered to be a protein that interacted with S-phase kinase Cdk2/cyclinA (5, 6). It is also involved in the ubiquitin-dependent degradation of the cyclin-dependent kinase (CDK) inhibitor, p27 (1-3). Levels of Skp2 expression are low in G0/G1- and increase in the S-phase (6), while they are rate-limiting for the degradation of p27 and lead to G1 arrest (1). Skp2 has been shown to be involved in the degradation of other cell cycle regulators, including cyclinE (7), E2F-1 (8) and cyclinD1 (9). In addition, it mediates the degradation of both positive and negative cell cycle regulators. There may be other target substrates of Skp2 throughout the cell cycle.

Cell cycle progression is controlled by the sequential activation and inactivation of a series of cyclin-CDK complexes. p27 regulates progression from G1- to the S-phase by binding to and inhibiting the activity of a broad range of cyclin-CDK complexes required for entry into the S-phase (10-12). The level of p27 expression is controlled by ubiquitin-dependent degradation; Skp2 can promote the degradation of p27 (13). It has recently been reported that Skp2 overexpression may lead to accelerated p27 degradation and contribute to tumor progression in human oral (14, 15), colon (16), gastric (17) and prostate cancer (18). Generally, loss of p27 expression is associated with poor prognosis in patients with many cancers. In esophageal squamous cell carcinoma (SCC), low levels of p27 expression affect tumor progression or indicate a poor prognosis (19, 20), but so do high levels of p27 expression (21-23). Consequently the relationship between p27 expression and tumor aggression and prognosis is still controversial in esophageal SCC.

In this study, we examined the correlation between Skp2 and p27 expression in early esophageal SCC. In doing so we sought to elucidate the role of Skp2 in tumor progression through the system of ubiquitin-dependent degradation, as well as observation of the expression of p27 or other cell cycle regulators.
Materials and Methods

Patients. Surgical specimens were obtained from 32 patients who had esophageal SCC and underwent potentially curative surgery without preoperative therapy at the Department of General Surgical Science, Gunma University Graduate School of Medicine, Japan, between 1983 and 2000. The age of the patients ranged from 40 to 73 years with a mean age of 62.9 years. Tumor stage was classified according to the fifth edition of the TNM classification of the International Union Against Cancer.

Immunohistochemistry for Skp2 and p27. Resected specimens were fixed with 10% formaldehyde and embedded in paraffin blocks. Immunohistochemical staining of the sections was performed by the standard avidin-biotin peroxidase complex method described previously (24). Briefly, the sections were incubated with anti-Skp2 polyclonal antibody (Zymed Laboratories, San Francisco, CA, USA) at a dilution of 1:100 and anti-p27 monoclonal antibody (clone 57, Transduction Laboratories, Lexington, KY, USA) at a dilution of 1:1000 and counterstained lightly with hematoxylin. A negative control was prepared by substituting normal rabbit and mouse serum for each primary antibody. No staining was detected in any control section.

Evaluation of Skp2 and p27 expression. Skp2 and p27 immunoreactivity was observed in all samples, confined to the

Figure 1. Photographs of tissue sections immunostained for Skp2 and p27 (x100).
(A) Skp2 was detected in the nuclei of the parabasal regions in normal esophageal epithelium. Primary esophageal squamous carcinoma cells also stained positively for Skp2 in the nuclei.
(B) p27 was detected in the nuclei of suprabasal, terminally-differentiated keratinocytes in normal esophageal epithelium and stained positively in the nuclei of the carcinoma cells.
(C) Skp2 was detected in the periphery of the tumor cell nests invading to the submucosa level of primary esophageal squamous carcinoma.
(D) p27 was detected in the central areas of the tumors, showing an inverse topographic distribution to Skp2.
nuclei of tumor cells and normal epithelial cells. Skp2 and p27 immunostaining was evaluated by two of us (MF and MN) in a coded manner (at least 10 high-power fields at random, minimum of 1000 cells) and scored for the degree of expression. Normal squamous mucosa was always used as the positive control to ensure the quality of immunostains. The sections were graded for percentage of positive nuclei. The mean value of the scores of tumor cells was chosen as a cut-off to separate low and high expression of both Skp2 and p27, as described in previous studies (14, 22). Thus, we determined that the positive staining of 20% of Skp2 cells and 50% of p27 cells were the optimal cut-off points for discrimination of Skp2 and p27 immunostaining.

Cell culture. Seven established cell lines derived from esophageal SCC were used: TE-series 1, 2, 8, 13 and 15 (gift from Dr. T. Nishihira, Tohoku University, Japan) (25), T.T and T.Tn (JCRB0262 and 0261, gift from Dr. K. Takahashi). The TE-series were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin); T.T and T.Tn were cultured in a 1:1 Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (Sigma) containing 10% fetal bovine serum and antibiotics as described above.

Cell extraction and Western blotting. Lysates from exponentially growing cell lines were prepared in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% aprotinin and 1 mM phenylmethylsulfonyl fluoride and subjected to Western blotting, as described previously (24). The protein concentration was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Thirty micrograms of protein from each cell line was subjected to electrophoresis on a 10% Ready-Gel (Bio-Rad, Tokyo, Japan) followed by electroblotting onto a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Bucks., UK). The proteins were immunoblotted using anti-Skp2 (Zymed Laboratories), anti-p27 (Transduction Laboratories), anti-cyclinE (Neomarkers, Fremont, CA, USA), anti-cyclinD1 (Neomarkers), anti-cyclinA (Transduction Laboratories), anti-E2F-1 (Transduction Laboratories) and anti-Skp1 antibodies (Transduction Laboratories). Anti-‘-actin (Sigma) antibody served as the control.

RNA extraction and Northern blotting. Total RNA was extracted from the cells with TRIZOL (Gibco BRL, Rockville, MD, USA). Twenty micrograms of RNA per lane was electrophoresed in 1.2% agarose gels containing 2.2 mol/l formaldehyde and blotted onto a Biodyne B membrane ( Pall, Tokyo, Japan). The cDNA probe was

Table I. The correlation between Skp2 expression and clinicopathological characteristics of patients with early esophageal SCC.

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<td>High(n=24)</td>
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SD: standard deviation (%)

Table II. The correlation of Skp2 expression with p27 expression in early esophageal SCC.

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<tr>
<td></td>
<td>Low</td>
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<td>3</td>
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labeled using a Random Primed DNA Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany) and \([\cdot- 32P]\)-dCTP (Amersham Pharmacia Biotech). The human Skp2 and p27 probe were digested from pcDNA3-Skp2 (26) and -p27 with EcoR\(\pi\) and Xho\(\pi\) to yield 1308 bp and 580 bp cDNA fragments, respectively. Membranes were prehybridized at 42\(\degree\)C for more than 2 h and hybridized overnight at 42\(\degree\)C after staining with methylene blue to verify quality and quantity of the RNA. The membranes were washed in 2 x SSC, 0.1% SDS for 15 min and 0.2 x SSC, 0.1% SDS for 15 min at 42\(\degree\)C. The washed membrane was exposed to X-ray film using an intensifying screen.

Statistical analysis. Statistical analysis was performed using the Chi-squared test, Fisher's exact test and the Mann-Whitney \(U\)-test. Patient survival curves were calculated using the Kaplan-Meier method and analysis of survival was performed using the log rank test.

Results

Immunohistochemical correlation between Skp2 and p27 expression. In normal squamous epithelium of the esophagus, immunostaining of Skp2 was detected in the nuclei of the parabasal regions (Figure 1A) and immunostaining of p27 was detected in the nuclei of the suprabasal, terminally differentiated keratinocytes (Figure 1B). In primary esophageal squamous carcinoma, both Skp2 and p27 positive staining were in the nucleus (Figure 1A, B). Within almost all early esophageal squamous carcinomas, there was an inverse topographic distribution of Skp2- and p27-positive tumor cells, with positive immunostaining for Skp2 in the periphery of the tumor cell nests (Figure 1C) and p27 restricted to the central areas (Figure 1D).

Skp2 expression was high in 24 (75.0%) out of 32 patients with early esophageal SCC and low in 8 (25.0%). The level of p27 expression was high in 8 (25.0%) out of 32 patients and low in 24 (75.0%). There was no significant association between Skp2 or p27 expression and the clinicopathological characteristics of patients with early esophageal SCC (Table I). Survival rates did not differ significantly according to Skp2 or p27 expression (data not shown).

We attempted to analyze the relationship between Skp2 and p27 expression using the cut-off described above and found that Skp2 expression was inversely related to p27 expression \((p=0.0184, \text{Table II})\).

Moreover, the survival rates of patients with and without an inverse correlation between Skp2 and p27 expression were further analyzed. Survival rates of patients without an inverse correlation between Skp2 and p27 expression were significantly lower than those of patients with an inverse correlation \((p=0.0493; \text{Figure 2})\). The 5-year survival rates of patients with and without the inverse correlation were 87% and 60%, respectively. However, according to multivariate analysis with a Cox proportional hazards model, Skp2 and p27 inverse immunostaining was not identified as an independent prognostic factor (data not shown).

Expression of Skp2, p27 and other cell cycle regulators at the protein level in the cell lines. We characterized the expression of Skp2, p27 and other cell cycle regulators at the protein level in seven cell lines derived from esophageal SCC. Although all of these cell lines were originally derived from esophageal SCC, Western blotting revealed that the levels of expression of Skp2, p27, cyclin E, cyclin D1 and E2F-1 differed, whereas the expression of cyclin A and Skp1 was relatively steady. Apart from the TE2 cell line, the other cell lines showed an inverse relationship between the levels of Skp2 and p27 expression. Unlike the case of p27, there seemed to be no inverse relationship between the expression level of Skp2 and those of cyclin E, cyclin D1 and E2F-1 (Figure 3).

Skp2 and p27 expression at the mRNA level in the cell lines. In view of the variations shown among the seven cell lines in the level of expression of Skp2 and p27 protein, we performed Northern blotting to observe the level of expression of Skp2 and p27 mRNA. The level of expression of Skp2 mRNA was various and not related to the level of expression of Skp2 protein in the cell lines. However, the level of expression of p27 mRNA was relatively steady unlike the case of p27 protein (Figure 4).

Discussion

Generally, in many cancers, reduced p27 expression is associated with a worse clinical outcome and greater tumor invasiveness. In previous studies, high levels of expression
of Skp2, a specific ubiquitin ligase subunit that can promote the degradation of p27, have been found to be associated with tumor progression in human oral (14, 15), colon (16), gastric (17) and prostate cancer (18). However, the relationship between p27 expression and tumor aggression is still controversial in esophageal SCC, and the relationship between Skp2 and p27 expression has not been reported on in this cancer. Initially, we found by immunohistochemistry that Skp2 expression showed an inverse location and correlation to p27 expression, especially in early esophageal squamous carcinomas. Moreover, the survival rates of patients without the inverse correlation between Skp2 and p27 expression were significantly lower than those of patients with the inverse correlation (Figure 2). Our data indicate that the target substrate of Skp2 is mainly p27 in
esophageal SCC, but that reduction in the rate of Skp2-induced degradation of p27 may influence tumor progression and lead to a poor prognosis. Further analysis of p27 and Skp2 expression together may help shed light on the controversial relationship between p27 and tumor progression and prognosis in esophageal SCC.

Skp2 is known to be involved in degradation of not only the negative cell-cycle regulator p27, but also the positive regulators cyclin E (7), E2F-1 (8) and cyclin D1 (9). In 6 of the 7 cell lines we examined, Western blotting revealed an inverse relationship between the levels of expression of Skp2 and p27 (Fig. 3). However, there was no inverse relationship between the expression level of Skp2 and those of cyclin E, cyclin D1 and E2F-1. Thus, the target substrate of Skp2 also seems to be p27 in the cell lines.

Levels of Skp2 expression fluctuate in the normal cell cycle, decreasing in the G0/G1-phase and increasing in the S-phase (6). In esophageal SCC, the mechanism that alters the expression of Skp2 remains to be determined. Our study revealed that the level of expression of Skp2 protein varied markedly in the 7 lines. In contrast, the level of expression of p27 mRNA was relatively steady (Figure 4). Therefore, p27 expression must be regulated at both the translational and post-translational levels (27). Moreover, p27 expression may be involved in Skp2 expression at the post-translational level. Skp2 expression may be regulated at the transcriptional, translational and post-translational levels.

We found no statistically significant tendency for an inverse relationship between the expression of Skp2 and that of p27 in 49 patients with advanced esophageal SCC (data not shown). As there was no such inverse relationship in the advanced tumors, levels of both Skp2 and p27 expression were thought to be increased in the G1/S-phase, and thus staining for both markers could not be obtained in all samples because the tumor cells might have been at other phases of the cell cycle. Therefore, because the staining scores for Skp2 often seemed to approximate those for p27 in tumors at stages other than early ones, we considered that the inverse correlation between the markers might not be significant at these times.

In conclusion, Skp2 is inversely correlated with p27, which is probably the main target substrate, in primary esophageal SCC. Failure of Skp2-induced degradation of p27 may influence tumor progression and lead to a poor prognosis, especially in the early stages. Further studies of the effects of Skp2 are needed to elucidate the participation of various cell cycle regulators or other components of the SCF complex in esophageal SCC.

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References


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