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

Taka-aki Masuda, Hiroshi Inoue, Hideto Sonoda, Shinji Mine, Yasuji Yoshikawa, Keiko Nakayama, Kei-Ichi Nakayama, and Masaki Mori

INTRODUCTION

Dysregulation of the cell cycle is required for the formation of most malignant tumors. The importance of G1-S progression in the formation of malignant tumors has been highlighted by the incidence of aberrations in genes involved in this progression in a wide variety of tumors. Recently, the mechanisms that drive the cell from the G0-G1 phase into S-phase have been discovered. p27 is an inhibitor of the protein kinases cyclin-dependent kinase 2/cyclin E and cyclin-dependent kinase 2/cyclin A, which drive cells from the G1 to the S-phase of the cell division cycle (1, 2).

Many clinical studies have indicated that low levels of p27 are associated with high aggressiveness and poor prognosis in a large variety of malignant tumors (1, 2), including breast (3–6) and colorectal (7) carcinomas. The importance of G1-S progression in the formation of malignant tumors, including gastric carcinoma, has been highlighted by the incidence of aberrations in genes involved in this progression in a wide variety of malignant tumors (1, 2), including breast (3–6) and colorectal (7) carcinomas. The major regulatory machinery of p27 protein levels is posttranslational ubiquitin-mediated proteolysis (9, 10). In malignant tumors, reduced p27 protein expression is usually not caused by changes of the gene encoding this protein (11). Instead, increased degradation of p27 may be an important cause of the frequently observed loss of p27 in malignant tumors.

Recent studies have shown that one mechanism involved in p27 degradation is an SCF-type ubiquitin ligase complex (12, 13). Skp2 is a member of the F-box family of the specific substrate-recognition subunit of SCF ubiquitin-protein ligase complexes (14). Expression of Skp2 was required for the ubiquitination and subsequent degradation of p27 in vitro (15–17), and Skp2 knock-out cells show high levels of p27 and free cyclin E, polyplody, and centrosome overduplication, as we reported previously (18). Therefore, a decreased level of p27 expression in human malignant tumors may be caused by increased expression of Skp2, which targets p27 for degradation.

Recently, a line of evidence has indicated a possible relationship between Skp2 expression and the malignancy of tumors. Skp2 expression was shown to be greatly increased in malignantly transformed cells lines (14) including oral squamous cell carcinoma (19) and correlated directly with the grade of malignancy of lymphoma (20) and oral squamous cell carcinoma (19). The level of p27 was reported to be inversely related to that of Skp2 in lymphoma (20), oral squamous cell carcinoma (19, 21), and colorectal carcinoma (22). Kudo et al. (21) reported that high Skp2 expression was correlated with poor prognosis in oral squamous cell carcinoma. Thus, Skp2 may have a great significance in human carcinogenesis. However, there have not been any studies regarding the clinical significance or the biological behavior of Skp2 expression in human gastric carcinomas.

We therefore investigated Skp2 expression in human gastric carcinomas, the significance of Skp2 expression, and the relationship between Skp2 and p27 in vivo. We then established Skp2 stably transfected human gastric carcinoma cell lines and examined the biological behavior of Skp2-transfected cells and the relationship between Skp2 and p27 expression in vitro.

MATERIALS AND METHODS

Clinical Samples. Fresh surgical specimens were obtained from 98 patients with primary gastric carcinoma and their paired adjacent normal gastric mucosa after informed consent was obtained. The patients had undergone surgery at the Department of Molecular and Surgical Oncology, Medical Institute of Bioregulation, Kyushu University (Beppu, Japan) from 1993 to 2000. None of these patients received preoperative treatment such as radiation or chemotherapy. Data concerning patient outcome, including overall survival and development of metastasis, were available for all 98 patients, and the observation period ranged from 3 months to 77 months (the median follow-up period was 36.6 months). Of the 98 patients, 45 died of gastric carcinoma.
Cell Culture. The human gastric cancer cell lines AZ-521, KATOIII, MKN7, MKN28, MKN45, NUGC3, and NUGC4 were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan), and maintained in RPMI 1640 supplemented with 10% FBS at 37°C in a 5% humidified CO2 atmosphere.

Antibodies. Mouse monoclonal antibodies to Skp2 and p27 were purchased from Zymed Laboratory (San Francisco, CA) and Transduction Laboratories (Lexington, KY), respectively. These antibodies were used for immunohistochemistry and Western blot analysis.

Total RNA Isolation. Frozen tissue specimens or cultured cell lines in a state of subconfluence were homogenized, and the total RNA was extracted using the modified acid-guanidine-phenol-chloroform method as described previously (23).

RT-PCR and Northern Blot Analysis. The reverse transcriptase reaction was performed as described previously (23). PCR amplification was performed by 24 cycles of incubation at 94°C for 1 min, at 54°C for 1 min, and at 72°C for 1 min. The following primers were used (all 5’ to 3’ direction): Skp2 sense primer, GCTGCCTAAGGTCTGTGGTG; and antisense primer AGGCTTGATACCTGCAACTGT; and GAPDH sense primer GTAACGCGATTGGTGTGCATTGTA and antisense primer AGTCTGTGGTGGCAGT.

Northern blot analysis was performed as described previously (24). In brief, total RNA was electrophoresed in 1.0% formaldehyde-agarose gels, transferred to Hybond N nitrocellulose filters (Amersham, Tokyo, Japan), and then hybridized with randomly primed 32P-labeled cDNA probes for Skp2. Filters were exposed to autoradiography for 2 h, and the mRNA levels were quantitated using a Bio-Image analyzer BAS 2500 (Fuji Film, Inc., Tokyo, Japan).

Immunohistochemistry. Immunohistochemical studies of Skp2 and p27 in 32 gastric carcinoma cases were performed using the avidin-biotin-peroxidase method (LSAB kit; DAKO, Kyoto, Japan) on formalin-fixed, paraffin-embedded tissues as described previously (5). All sections were counterstained with hematoxylin. The primary monoclonal antibodies against Skp2 and p27 were used at dilutions of 1:5000 and 1:1000, respectively. p27 scores were determined by observing 1000 cancer cells in at least five high-power fields and were classified as high (staining in >50% of cells) or low (staining in ≤50% of cells) as described previously (8). The scoring was independently determined by two observers (T-a. M. and Y. Y).

Western Blot Analysis. Total protein was extracted from samples with RIPA buffer. Aliquots of total protein were applied to 10% acrylamide gradient gels. After electrophoresis, samples were electrobotted onto a polyvinylidene membrane (Immobilon; Millipore, Inc., Bedford, MA) at 0.5 A for 40 min at 4°C. gels. After electrophoresis, samples were electroblotted onto a polyvinylidene membrane (Immobilon; Millipore, Inc., Bedford, MA) at 0.5 A for 40 min at 4°C. Western blot analysis was performed as described previously (25). Briefly, after Skp2-transfected cells and mock-transfected cells (2.0 × 105/plate) were incubated for 72 h in serum-free medium at 37°C and then were kept in medium with serum (10% FBS) for 18 h at 37°C. The cells were harvested and fixed in 70% ethanol at −20°C. Then, the cells were washed and resuspended in PI staining buffer (5 µg/ml PI and 0.25 mg/ml RNase) in PBS. DNA content was evaluated using an EPICS XL flow cytometer (Beckman Coulter Corp., Tokyo, Japan) as described previously (18) and then transfected into the cell lines by the lipofection method (Life Technologies, Inc., Tokyo, Japan) as described previously (25). Then, three stably transfected clones expressing abundant Skp2 protein were selected after G418 (800 µg/ml) treatment and used for the subsequent experiments. A mock vector-transfected clone of each cell line was used for the control.

In Vitro Proliferation Assay. Skp2-transfected cells and mock-transfected cells were plated at a density of 1.0 × 105 cells/well in three 6-cm plates and were harvested and counted on days 3, 7, and 10. The medium was changed every 72 h. This experiment was repeated three times.

Cell Cycle Analysis. Skp2-transfected cells and mock-transfected cells (2.0 × 105) were preincubated for 72 h in serum-free medium at 37°C and then were kept in medium with serum (10% FBS) for 18 h at 37°C. The cells were harvested and fixed in 70% ethanol at −20°C. Then, the cells were washed and resuspended in PI staining buffer (5 µg/ml PI and 0.25 mg/ml RNase) in PBS. DNA content was evaluated using an EPICS XL flow cytometer (Beckman Coulter Corp., Tokyo, Japan). Measurements of BrdUrd uptake was performed as described previously (26). Briefly, after Skp2-transfected cells and mock-transfected cells (2.0 × 105/plate) were incubated for 72 h in serum-free medium at 37°C and 18 h after addition of 10% FBS at 37°C, BrdUrd was added to the culture medium (10 µM), and the cultures were incubated for 30 min at 37°C. The cells were fixed in 70% ethanol at −20°C. To denature the DNA, the cells were incubated for 30 min at room temperature in 2 n HCl with 0.5% Triton X-100. After neutralization with 0.1 M sodium tetraborate (pH 8.5), the cells were incubated with anti-BrdUrd FITC (Becton Dickinson, San Jose, CA) for 30 min at room temperature and resuspended in 5 µg/ml PI. The cells were analyzed using an EPICS XL flow cytometer (Beckman Coulter Corp.). This experiment was repeated three times.

Analysis of Apoptotic Cells. After treatment with 5 µg/ml actinomycin D for 24 h, cells were harvested with 0.05% trypsin, resuspended in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2], and then incubated with FITC-conjugated Annexin V and 5 µg/ml PI (Annexin V-FITC kit; Bender Medsystems). The cells were then analyzed using EPICS XL flow cytometer (Beckman Coulter Corp.). Annexin V-positive and PI-positive cells were considered to be apoptotic. Nonstained actinomycin D-treated cells of Skp2 transfectants or mock transfectants were used as the negative controls, respectively. This experiment was repeated five times.

MTT Assay. To quantify the viable cells under treatment with actinomycin D, MTT assay was performed (27). Skp2-transfected cells and mock-transfected cells (1.0 × 105 cells/well) were seeded in 96-well plates in serum-containing medium and treated with 5 µg/ml actinomycin D for 24 h. MTT (Sigma Chemical Co., Tokyo, Japan) was added to each well (0.5 mg/ml). After incubation for 4 h at 37°C, 100 µl of α-n-propyl alcohol containing 0.1% NP40 and 4 µl HCl were added. The coloring reaction was quantitated using an automatic plate reader, Immuno-Mini NJ-2300 (Nihon InterMed, Tokyo, Japan), at 570 nm with a reference filter of 650 nm. MTT assays were carried out three times.

In Vitro Invasion Assay. The invasive potential of Skp2-transfected cells was determined by a Matrigel invasion assay using polycarbonate membranes (8.0-µm pore size) in the upper chamber of 24-well Transwell culture chambers coated with Matrigel (Becton Dickinson, San Jose, CA). Skp2-transfected cells and mock-transfected cells (1.0 × 105 cells/well) were placed in the upper chamber, and the lower chamber was filled with 750 µl of RPMI 1640 with 10% FBS as a chemoattractant. After 48 h of incubation at 37°C, the membranes were stained with May-Grunwald and Giemsa solutions. The invasive cells that had migrated through the membrane to the lower surface were counted in three different fields under a light microscope at ×100. Each experiment was performed in triplicate wells and repeated five times.

Immunofluorescence Detection. Actin filaments were stained by fixing the cells in 4% paraformaldehyde followed by incubation with rhodamine-conjugated phallolidin (Molecular Probes, Eugene, OR). Actin filaments were detected using immunofluorescence microscopy.

Statistical Analysis. Associations between the variables were tested by Student’s t test or Fisher’s exact test. Survival curves were drawn according to the Kaplan-Meier method, and the survival analysis was carried out by the Mantel-Cox test. All statistical differences were deemed significant at the level of p < 0.05. The histological type and staging of gastric carcinomas were classified on the basis of the criteria set by the Japanese Society for Cancer of the Stomach (28).

RESULTS

Clinical Significance of Skp2 Expression in Gastric Carcinoma

Skp2 Expression in Gastric Carcinoma Tissues. The gastric carcinoma tissue and normal mucosa showed variable levels of Skp2 mRNA signals by Northern blot analysis and RT-PCR (Fig. 1A). Northern blot analysis revealed that the expression of Skp2 mRNA was greater in carcinoma tissue than in normal mucosa in 21 of the 30 cases (70.0%; P < 0.05; Student’s t test). Immunohistochemical analysis revealed that Skp2 protein was predominantly expressed in the gastric carcinoma cells (Fig. 1B). We examined the Skp2 mRNA expression in several gastric carcinoma cell lines: AZ-521, KATOIII, MKN7, MKN28, MKN45, NUGC3, and NUGC4 with RT-PCR. All of them expressed Skp2 mRNA (data not shown).

Skp2 Expression Correlates Inversely with p27 Expression in Gastric Carcinoma. To perform the quantitative analysis, we evaluated the expression of Skp2 mRNA in the tumor tissues (T) by
The expression of Skp2 was measured by Northern blot analysis in the human gastric carcinoma cell line KATOIII. GAPDH was used as an internal control. Relative expression levels of Skp2 were determined using densitometry. A, five representatives of Northern blot (a) and RT-PCR (b) analysis for Skp2 in human gastric carcinoma, KATOIII, gastric carcinoma cell line; T, gastric carcinoma tissue; N, normal tissue; GAPDH, internal control. B, immunohistochemical staining of two consecutive sections of Skp2 and p27 in gastric carcinoma tissues with monoclonal antibody. a and b, Skp2 immunostaining and HE staining. c and d, strong Skp2 immunostaining and weak p27 immunostaining. e and f, weak Skp2 immunostaining and strong p27 immunostaining. a and b, ×100; c-f, ×400.

Fig. 1. Skp2 expression in human gastric carcinoma tissues and relationship between Skp2 and p27 expression. A, five representative cases of Northern blot (a) and RT-PCR (b) analysis for Skp2 in human gastric carcinoma, KATOIII, gastric carcinoma cell line; T, gastric carcinoma tissue; N, normal tissue; GAPDH, internal control. B, immunohistochemical staining of two consecutive sections of Skp2 and p27 in gastric carcinoma tissues with monoclonal antibody. a and b, Skp2 immunostaining and HE staining. c and d, strong Skp2 immunostaining and weak p27 immunostaining. e and f, weak Skp2 immunostaining and strong p27 immunostaining. a and b, ×100; c-f, ×400.

Table 1 Relationship between Skp2 mRNA and p27 protein expression in gastric carcinoma

<table>
<thead>
<tr>
<th>p27 protein</th>
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* P < 0.01, Fisher’s exact test.

The expression of Skp2 was measured by Northern blot analysis in the human gastric carcinoma cell line KATOIII. GAPDH was used as an internal control. Relative expression levels of Skp2 were determined using densitometry. A, five representatives of Northern blot (a) and RT-PCR (b) analysis for Skp2 in human gastric carcinoma, KATOIII, gastric carcinoma cell line; T, gastric carcinoma tissue; N, normal tissue; GAPDH, internal control. B, immunohistochemical staining of two consecutive sections of Skp2 and p27 in gastric carcinoma tissues with monoclonal antibody. a and b, Skp2 immunostaining and HE staining. c and d, strong Skp2 immunostaining and weak p27 immunostaining. e and f, weak Skp2 immunostaining and strong p27 immunostaining. a and b, ×100; c-f, ×400.

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The relationship between Skp2 gene expression and p27 protein expression was examined in 32 cases of gastric carcinoma. Expression of p27 protein in tumor tissues was evaluated by immunohistochemistry with anti-p27 monoclonal antibody. Fourteen of 17 cases in the high Skp2 group were in the low p27 group, and 10 of 15 cases in the low Skp2 group were in the high p27 group (Table 1). This indicated that Skp2 mRNA expression was inversely correlated with p27 protein levels in gastric carcinoma (P < 0.01). In gastric carcinoma tissues, cells positive for Skp2 showed no or very low p27 protein and vice versa, implying that there was an inverse relationship between the expression profiles of Skp2 and p27 proteins (Fig. 1B).

The Prognostic Significance of Skp2 Gene Expression in Gastric Carcinoma. As shown in Table 2, a significant difference in histological type was found between the high Skp2 mRNA expression group and the low expression group (P < 0.01). On the other hand, no significant difference was seen regarding age, sex, depth of invasion, vascular or lymphatic involvement, or lymph node metastasis. With regard to prognosis, the high Skp2 mRNA expression group showed a significantly poorer prognosis than the low expression group (P = 0.018; Fig. 2). On multivariate analysis for prognosis, Skp2 mRNA expression, lymph node metastasis, lymphatic involvement, and depth of invasion, which were found to be prognostic factors on univariate analysis, were included for the parameters. This analysis demonstrated that Skp2 mRNA expression was not an independent factor (P = 0.06).

Biological Significance of Skp2 Expression in Gastric Carcinoma

Relationship between Skp2 and p27 Expression in Skp2-transfected Carcinoma Cells. We examined the Skp2 mRNA expression in several gastric carcinoma cell lines: AZ521, KATOIII, MKN7, MKN28, MKN45, NUGC3, and NUGC4. RT-PCR analysis showed that AZ521 expressed the lowest level of Skp2 mRNA among

Table 2 Skp2 mRNA expression and clinicopathological factors in gastric carcinoma

<table>
<thead>
<tr>
<th>Skp2 T value</th>
<th>0.48 (n = 33)</th>
<th>&gt;0.48 (n = 65)</th>
<th>P*</th>
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<tr>
<td>Age (yr)</td>
<td>63.2 ± 10.9</td>
<td>66 ± 12.3</td>
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<tr>
<td>Male</td>
<td>18</td>
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<td>NS</td>
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<tr>
<td>Female</td>
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<tr>
<td>Histology</td>
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<tr>
<td>Differentiated type</td>
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<td>38</td>
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<tr>
<td>Undifferentiated type</td>
<td>24</td>
<td>27</td>
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<tr>
<td>Sensual invasion</td>
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</tr>
<tr>
<td>Absent</td>
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<td>39</td>
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<tr>
<td>Present</td>
<td>12</td>
<td>26</td>
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<tr>
<td>Vascular involvement</td>
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<tr>
<td>Absent</td>
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<td>51</td>
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<td>Present</td>
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<td>Lymphatic involvement</td>
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<td>Present</td>
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<td>41</td>
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<td>Lymph node metastasis</td>
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<td>NS</td>
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<tr>
<td>Absent</td>
<td>16</td>
<td>21</td>
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<tr>
<td>Present</td>
<td>17</td>
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* Correlation was analyzed by Fisher’s exact test. NS, not significant.

Fig. 2. Overall survival of patients with gastric carcinoma according to Skp2 mRNA expression in tumor tissues. High Skp2 mRNA expression group: T value ≥0.48 (n = 33). Low Skp2 mRNA expression group: T value ≤0.48 (n = 33). P = 0.018; Mantel-Cox method.
these cell lines (data not shown). Therefore, we used the AZ521 cell line for the subsequent assays. Three stable Skp2-transfected clones were established, and their Skp2 expression was confirmed by both Northern and Western blot analysis. All three stable Skp2-transfected clones showed a lower level of expression of p27 protein than the controls (Fig. 3). Treatment with proteasome inhibitor (MG132) blocked p27 down-regulation in Skp2-transfected cells (data not shown).

High Proliferation Activity of Skp2-transfected Carcinoma Cells. We analyzed whether Skp2 transfection altered the growth rate of gastric carcinoma cells. As shown in Fig. 4A, there was a significant difference in growth rate between Skp2-transfected cells and the mock-transfected cells at 7 days ($P < 0.05$). Both the Skp2-transfected cells and mock-transfected cells reached confluence by 10 days. To investigate how the Skp2-transfected cells acquired their high proliferation ability, we analyzed the cell cycle after serum starvation and after refeeding with serum. (Fig. 4B). The percentage of BrdUrd-positive cells (cells in S-phase) in Skp2 transfectants was significantly higher than in the mock transfectant after serum starvation for 72 h ($P < 0.01$; Fig. 4C). However, 18 h after addition of serum, there was no significant difference between them (data not shown).

Resistance to Apoptosis Induction by Actinomycin D in Skp2-transfected Carcinoma Cells. To measure the susceptibility of Skp2-transfected cells to apoptosis-inducing conditions, we performed double staining of cells with Annexin V (FITC) and PI 24 h after the addition of actinomycin D (Fig. 5A). The percentage of Annexin V- or PI-positive cells was significantly lower in Skp2 transfectants than in the mock transfectants ($P < 0.05$ and $P < 0.01$, respectively; Fig. 5B). Furthermore, to measure the number of viable cells after subjecting the cells to apoptosis-inducing conditions, we performed MTT assays 24 h after the addition of actinomycin D. The percentage of viable cells was significantly higher in Skp2 transfectants than in the mock transfectants ($P < 0.01$; Fig. 5C). These results indicate that Skp2-transfected cells may acquire resistance to apoptosis induction.

High Invasion and Motility Potential of Skp2-transfected Carcinoma Cells. We examined the invasion and motility potential of the Skp2-transfected cells using an in vitro Matrigel invasion assay (Fig. 6A). Skp2-transfected cells exhibited significantly more invasive potential than the mock-transfected cells ($P < 0.01$; Fig. 6B). To further investigate the motility potential of Skp2-transfected cells, we performed immunohistochemical staining of actin filaments and examined the reorganization of the actin cytoskeleton in Skp2 transfec-tants. Most Skp2-transfected cells formed “filopodia” (Fig. 6C). This
indicates that gastric carcinoma cells may acquire high motility by Skp2 transfection.

**DISCUSSION**

In this report, we demonstrated that Skp2 was overexpressed in gastric carcinoma cells, and Skp2 expression was inversely correlated with p27 protein levels in gastric carcinoma, and furthermore, high Skp2 mRNA expression was significantly correlated with poor prognosis in gastric carcinoma. These findings are consistent with observations in other organs reported previously (14, 19, 20, 21, 22). We consider it likely that Skp2 is related to degradation of p27 in vivo and could be a potential prognostic factor for gastric carcinoma. Unexpectedly, a significant difference in histological type was found between the high Skp2 mRNA expression group and the low expression group. The prognostic significance of Skp2 mRNA expression is considered to be independent of the histological type, because histological type was not a prognostic factor on univariate analysis. Additional studies will be required to clarify the relation of Skp2 expression to cell differentiation.

Our next experiments using Skp2 transfectants showed that Skp2 transfectants with high Skp2 levels expressed low levels of p27 protein, and that a high level of proliferation activity, resistance to apoptosis, and invasion potential of gastric carcinoma cells were elicited by Skp2 transfection. These results indicated that Skp2 over-
expression could modulate the malignant phenotype of gastric carcinoma, possibly by regulating the p27 protein level.

The relationship of p27 expression to cell proliferation and to susceptibility to apoptosis was reported elsewhere. p27 knock-out cells acquired high proliferation activity (29, 30), as we also reported previously (31), and antisense inhibition of p27 prevented cell cycle arrest in response to mitogen depletion (32). Overexpression of p27 was reported to trigger apoptosis in carcinoma cells (33). These findings are consistent with the results of our Skp2 transfection studies. Taking into consideration our results of the inverse correlation between Skp2 and p27 and the reports above, the promotion of proliferation activity and resistance to apoptosis by Skp2 transfection may be attributable to p27 degradation by Skp2 overexpression.

The relationship between p27 expression and invasion or motility potential remains unknown, although many clinical studies have indicated that low p27 expression was correlated with the depth of tumor invasion (1, 2), as we also reported previously (8). The promotion of invasion or motility by Skp2 transfection might be attributable to a different cause from p27 degradation by Skp2 overexpression. To study the molecular mechanism of the promotion of invasion by Skp2 transfection, we performed cDNA microarray assays using Human Cancer Chip Version 2.1 (TaKaRa Biochemicals, Tokyo, Japan) and identified the carcinoma-related genes whose expression level was altered by Skp2 transfection (data not shown). uPA and MMP have been demonstrated recently to be strongly involved in carcinoma invasion and metastasis by degrading the extracellular matrix (34). We also reported previously (37–40). We expected that the promotion of invasion potential of Skp2-transfected cells might be attributable to induction of production of uPA or MMP by Skp2 transfection. Unexpectedly, neither uPA nor MMP was up-regulated in Skp2-transfected cells (data not shown). In our assays of immunohistochemical staining of actin filaments, Skp2-transfected cells formed “filopodia.” The actin cytoskeleton was reported to be regulated by Rho family GTPases (Rho, Rac, and Cdc42) and to play an important role in cell locomotion via the extension of pseudopods, for example “filopodia” (41). Cdc42 participates in “filopodia” formation (42). Therefore, we considered that the promotion of invasion potential might be attributable to the promotion of motility mediated by Skp2. Skp2 expression might have influence on Rho family GTPases, especially Cdc42 activity. We are proceeding to elucidate the roles of the genes identified with the cDNA microarray assay in the modulation of the malignant phenotype of gastric carcinoma.

Recent studies suggested the role of Skp2 expression in malignant transformation. Forced expression of Skp2 in quiescent fibroblasts induced DNA synthesis (16). Skp2 cooperated with N-Ras in tumorigenesis in an in vivo model (20). Rodent fibroblasts primarily transformed by both Skp2 and H-Ras gene transfection could form colonies in soft agar and also tumors in nude mice (19). Cotransfection of Skp2 and cyclin E promoted abundant hepatocyte replication and hyperplasia of the liver in vivo (43). In our results, Skp2 overexpression modulated the malignant phenotype of the AZ521 gastric carcinoma cell line. These findings indicate that overexpression of Skp2 alone could not malignantly transform normal cells but could modulate the malignant phenotype of malignant tumors.

Recently, Mammillapalli et al. (44) reported that PTEN, a tumor suppressor, regulated the ubiquitin-dependent degradation of p27 through the ubiquitin ligase SCF<sup>Skp2</sup>. Cantley and Neel (45) showed that PTEN negatively controls the phosphoinositide 3-kinase signaling pathway for regulation of cell growth and survival by dephosphorylating the 3 position of phosphoinositides. Skp2 may function as a critical component in the PTEN/phosphatidylinositol 3-kinase pathway for the regulation of SCF<sup>Skp2</sup> and cell proliferation. Studies that clarify the oncogenic pathway leading to increased Skp2 expression and the consequences thereof will identify important targets for development of anticancer therapy.

In conclusion, we showed here that Skp2 gene overexpression could be a prognostic factor for gastric carcinoma and that Skp2 expression was correlated inversely with p27 expression in gastric carcinoma. Furthermore, we clarified that Skp2 expression could modulate the malignant phenotype of gastric carcinoma, possibly via p27 proteolysis. These findings strongly suggest that Skp2 could play an important role in gastric carcinoma progression and would be a novel molecular target for the treatment of gastric carcinoma.

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REFERENCES


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