Significance of Skp2 Expression in Primary Breast Cancer

Hideto Sonoda, Hiroshi Inoue, Kazuhiro Ogawa, Tohru Utsunomiya
Taka-aki Masuda, and Masaki Mori

Abstract

Purpose: We previously reported the p27 expression level to be an independent prognostic factor, and a high S-phase kinase-associated protein 2 (Skp2) expression level was significantly correlated with a poor prognosis in patients with gastric cancer. We herein examined the Skp2 expression in breast cancer and attempted to identify any associations between the Skp2 expression status and either the clinicopathologic variables or the patient’s prognosis.

Experimental Design: We established four Skp2-transfected breast cancer cell lines and assessed the correlations between the Skp2 and p27 expressions using real-time reverse transcription-PCR and a Western blot analysis. We then analyzed the clinicopathologic significance of Skp2 mRNA expression in 169 Japanese patients with breast cancer. An immunohistochemical analysis was also done.

Results: The p27 protein expression markedly decreased after Skp2 transfection, whereas no alteration in the p27 mRNA expression was observed. The Skp2 protein expression level as determined by immunohistochemical staining thus showed a significant correlation with the Skp2 mRNA expression ($P = 0.001$) and a significant inverse correlation with the p27 protein expression ($P = 0.042$). The patients with a high Skp2 gene expression were significantly younger than those with a low expression ($P = 0.002$). The prognosis of patients with a high Skp2 expression was significantly ($P = 0.022$) poorer than for those with a low expression. Moreover, a high expression of Skp2 was an independent variable that correlated with a shorter disease-free survival (relative risk, 3.33; 95% confidence interval, 1.296–8.578; $P = 0.013$).

Conclusions: The present results suggest that Skp2 may play an important role particularly in young breast cancer, and it is also considered to have strong independent prognostic potential and thus may prove to be a useful target for the treatment of breast cancer.

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Breast cancer incidence rates vary substantially, with the highest rates found in the United States, Canada, and Northern Europe. In Japan, breast cancer recently surpassed stomach cancer to become the most popular female cancer in 1995 (1, 2). In the past decade, breast-conserving treatment has become the standard treatment for early breast cancer, and the relapse rate has decreased thanks to advances in adjuvant therapies, such as chemotherapy, hormonal therapy, and radiation therapy. Relapses, whereas treatable, are not curable by the currently available therapy. Differences have been suggested to exist in the biological properties of each type of breast cancer.

One cyclin kinase–associated protein, p27, has been shown to have an inhibitory activity of cyclin/cyclin-dependent kinase complex during the G0 and G1 phase (3). A number of studies, including our recent report (4), have shown the protein level of p27 to markedly decrease in a variety of cancers, such as breast, colon, prostate, lung, ovary, brain tumors, and lymphomas (5–16). In those reports, the protein level of p27 was down-regulated; however, the mRNA level is not always suppressed accordingly. The reason of this discordance has been ascribed to the proteolysis system of p27. The protein level of p27 has recently been reported to mainly be regulated through the degradation by ubiquitin-dependent proteolysis (17–19), and S-phase kinase-associated protein 2 (Skp2) was identified as a cofactor that targets p27 for ubiquitination (20–22). The role of p27 in cell cycle control has been elucidated in a Skp2 knockout mice model (23). The cells from the knockout mice showed high levels of p27, free cyclin E, polyploidy, and centrosome overduplication, and these alterations were abolished in Skp2/p27 double knockout mice (24), thus suggesting that p27 is a primary target of Skp2.

A reduction in the p27 expression level is associated with a high aggressiveness and a poor prognosis in various malignant tumors, including breast cancer (5, 25). Previously, we reported that the p27 expression status was an independent prognostic factor for patients with gastric cancer (4). On the other hand, Esteva et al. reported that p27 expression was not related to the disease-free survival (26). Furthermore, we showed that Skp2
mRNA was overexpressed in cancer tissue, and the higher Skp2 expression group showed a significantly poorer prognosis in patients with gastric cancer, whereas gastric cancer cells transfected with Skp2 showed a significantly higher growth rate, resistance to apoptosis, and invasion potential than the control cells. These findings indicate that Skp2 expression can modulate the malignant phenotype of cancer, possibly via p27 proteolysis (27). In addition, Signoretti et al. have recently
showed that Skp2 is overexpressed in breast cancers that are negative for both the estrogen and HER-2 receptor and with either the primary and local recurrences or metastatic disease from breast cancers. A relationship between the Skp2 expression level and a poor prognosis was also observed (28).

In the present study, we thus analyzed the Skp2 expression status in 169 Japanese patients with breast cancers and clarified any correlations with the clinicopathologic features, including age, menopause, tumor size, the presence of lymph node metastasis and estrogen and progesterone receptors, invasion to the vessels, disease-free survival, and overall survival. A multivariate analysis using the Cox regression model was also done.

### Materials and Methods

**Clinical samples.** Fresh surgical specimens were obtained from 169 patients with primary breast cancer after informed consent was obtained. The patients had all undergone surgery at the Department of Molecular and Surgical Oncology, Medical Institute of Bioregulation, Kyushu University (Beppu, Japan) from October 1993 to October 1999. The patients with advanced stage were treated with postoperative hormonal therapy combined with radiotherapy. Chemo-therapy combined with radiotherapy was done when they had recurrent disease. Data concerning the patient characteristics, including age, menopause, tumor size, the presence of lymph node metastasis and estrogen and progesterone receptors, invasion to the vessels, disease-free survival, and overall survival. A multivariate analysis using the Cox regression model was also done.

**Cell culture.** Human breast cancer cell lines YMB-1, MCF 7, SKBR3, and CRL1500 were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). YMB-1 was maintained in DMEM, and MCF 7, SKBR3, and CRL1500 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a 5% humidified CO2 atmosphere.

**Antibodies.** Mouse monoclonal antibodies to Skp2 and p27 were purchased from the Zymed Laboratory (San Francisco, CA) and Transduction Laboratories (Lexington, KY), respectively.

**Total RNA isolation.** Frozen tissue specimens or cultured cell lines in a state of subconfluency were homogenized, and the total RNA was extracted using the modified acid/guanidine/phenol/chloroform method as described previously (29).

**Quantitative real-time PCR.** The reverse transcriptase reaction was done as described previously (29). Real-time PCR was done using the iCycler iQ detection system (Bio-Rad, Richmond, CA) and iQ SYBR Green Supermix. The reactions were done in 96-well plates with Optical-Quality 8-tube Strips (Bio-Rad). Skp2 and glyceraldehyde-3-phosphate dehydrogenase mRNA were amplified in separate tubes using the following protocol: 95°C for 10 minutes, then 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The increase in fluorescence was measured in real-time during the extension step. The following primers were used (all 5’ to 3’ direction): Skp2 sense primer, GCTGCTAAAGGCTCTGCTGCT and antisense primer, AGGCTTACTGCGAATTCC; glyceraldehyde-3-phosphate dehydrogenase sense primer, GTCAACGGATTTGCTCTGATT and antisense primer, AGTCTTCTGGTGGCAGT.

**Immunohistochemistry.** From the 169 breast cancer cases that were analyzed for their Skp2 mRNA expression level, we selected 137 specimens of which formalin-fixed, paraffin-embedded tissue was available. Immunohistochemical studies of Skp2 and p27 were done using the avidin-biotin-peroxidase method (LSAB kit, DAKO, Kyoto, Japan) as described previously (4). All sections were counterstained with hematoxylin. The primary monoclonal antibodies against Skp2 and p27 were used at dilutions of 1:500 and 1:1,000, respectively. Skp2 and p27 scores were determined by observing 1,000 cancer cells in at least five high-power fields and were classified as high (staining in

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**Table 1. Relationship among the expressions of Skp2 protein, Skp2 mRNA, and p27 protein in breast cancers (n = 137)**

<table>
<thead>
<tr>
<th></th>
<th>Skp2 mRNA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥1.02</td>
<td>≤1.02</td>
</tr>
<tr>
<td>Skp2 protein</td>
<td>Strong</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>8</td>
</tr>
<tr>
<td>p27 protein</td>
<td>Strong</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>38</td>
</tr>
</tbody>
</table>

**Table 2. Skp2 mRNA expression and clinicopathologic factors in breast cancers**

<table>
<thead>
<tr>
<th></th>
<th>≤1.02 (n = 84)</th>
<th>&gt;1.02 (n = 85)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>58.2 ± 1.3</td>
<td>52.8 ± 1.1</td>
<td>0.002</td>
</tr>
<tr>
<td>Size (cm)</td>
<td>≥2.1</td>
<td>≥2.1</td>
<td>NS (0.809)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Menopause</td>
<td>Premenopausal</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Postmenopausal</td>
<td>59</td>
<td>50</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>Present</td>
<td>37</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>47</td>
<td>46</td>
</tr>
<tr>
<td>Vascular involvement</td>
<td>Present</td>
<td>40</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>44</td>
<td>51</td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td>Present</td>
<td>42</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Progesterone receptor</td>
<td>Present</td>
<td>42</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Stage</td>
<td>I</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>49</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.
>50% of cells) or low (staining in <50% of cells) as described previously (4).

**Western blot analysis.** Total protein was extracted from samples with radioimmunoprecipitation assay buffer. Aliquots of total protein were applied to 10% acrylamide gradient gels. After electrophoresis, samples were electroblotted onto a polyvinylidene membrane (Immobilon, Millipore, Inc., Bedford, MA) at 0.5 A for 40 minutes at 4 °C. Skp2 and p27 were detected using the mouse monoclonal antibodies at a dilution of 1:2,000 and 1:2,500, respectively. The blots were developed with horseradish peroxidase–linked antimouse immunoglobulin (Promega, Inc., Madison, WI). The signals were detected using Supersignal (Pierce, Inc., Rockford, IL).

**Transfection assays.** Human Skp2 cDNA was generated by reverse transcription-PCR and subcloned into pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) as described previously (27) and then were transfected transiently into the cell lines by the LipofectAMINE method (Life Technologies, Inc., Tokyo, Japan) as described previously (30). A mock vector–transfected clone was used as a control.

**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 a survival analysis was carried out using the Mantel-Cox test. A multivariate analysis using the Cox regression model was done only on the variables with showing P < 0.05 in the univariate analyses. All statistical differences were deemed significant at the level of P < 0.05. The staging of breast cancers were classified based on the criteria established by the Union Internationale Contre le Cancer.

## Results

### Relationship between Skp2 and p27 expression in Skp2-transfected cells.

We examined the Skp2 mRNA expression in four breast cancer cell lines: YMB-1, MCF 7, SKBR3, and CRL1500 with reverse transcription-PCR. All of these cell lines expressed a low level of Skp2 mRNA (data not shown). Following Skp2 transfection, the p27 protein expression decreased in all four of the cell lines (Fig. 1), whereas there was no change in the p27 mRNA expression.

### Skp2 and p27 expression in breast cancer.

An immunohistochemical analysis in 137 breast cancer tissue specimens revealed...
that Skp2 protein was predominantly expressed in breast cancer cells. The breast carcinoma cases were then classified into four major patterns of Skp2/p27 expression (Fig. 2). Fifty-two tumors (38%) showed a strong Skp2 protein level and a weak p27 protein level. Thirty-eight tumors (28%) expressed a weak Skp2 protein level and a strong p27 protein level. In 33 tumors (24%), both weak Skp2 and weak p27 protein levels were observed. Fourteen tumors (10%) expressed both strong Skp2 and strong p27 protein levels. The Skp2 protein level thus showed a strong correlation with the Skp2 mRNA expression ($P < 0.001$), and it was also significantly reverse correlated with the p27 protein expression level ($P = 0.023$). The p27 protein expression level showed the inverse correlation with the Skp2 mRNA expression level ($P = 0.042$; Table 1).

**Table 3. Multivariate analysis on disease-free survival (n = 169)**

<table>
<thead>
<tr>
<th>Relative risk (95% confidence interval)</th>
<th>$P$</th>
</tr>
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<tbody>
<tr>
<td>Skp2 mRNA expression</td>
<td>3.334 (1.296-8.578)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>3.994 (1.361-11.715)</td>
</tr>
<tr>
<td>Lymphatic involvement</td>
<td>1.723 (0.653-4.547)</td>
</tr>
<tr>
<td>Vascular involvement</td>
<td>1.033 (0.398-2.681)</td>
</tr>
<tr>
<td>Tumor size (&gt;2.1 cm)</td>
<td>2.021 (0.757-5.393)</td>
</tr>
</tbody>
</table>

Clinical significance of Skp2 expression in breast cancer.

To perform a quantitative analysis, we evaluated the expression of Skp2 mRNA in tumor tissue specimens ($T$) by normalization as follows: $T = \text{Skp2 mRNA expression (T)}/\text{glyceraldehyde-3-phosphate dehydrogenase (T)}$. This showed that Skp2 expression is up-regulated in breast cancer. Yokoi et al. previously found that Skp2 transfection modulated the S-phase fraction than breast cancer in elderly patients (42, 43), and it also correlated with a poor prognosis (37, 40, 43). Our present data showed the Skp2 mRNA expression to be significantly higher in patients younger than 55 years (Table 2). Although no significant difference was observed, the high Skp2 mRNA expression group more frequently showed estrogen receptor--negative tumors than the estrogen receptor--positive cases ($P = 0.109$). This finding is consistent with previous observation, as reported by Signoretti et al., on 84 patients with advanced breast cancer (28), although they did not employ a multivariate analysis. We thus did a multivariate analysis; thus, high expression of Skp2 was found to be an independent prognostic variable that correlated with a shorter disease-free survival (relative risk, 3.33; 95% confidence interval, 1.296-8.578; $P = 0.013$; Table 3). In addition, the high Skp2 mRNA expression group showed a poor disease-free survival.

We previously found that Skp2 transfection modulated the malignant phenotype of cancer cells. The increase in the percentage of cells in the S phase, a high proliferation activity, resistance to apoptosis, and a high degree of invasiveness were specifically induced by Skp2 transfection in gastric cancer cells (27). The high Skp2 expression group was more frequently observed in the younger patients group. A high expression of Skp2 may thus play a more important role in breast cancers occurring in young patients.

It is important to clarify the molecular mechanisms by which a Skp2 expression is up-regulated in breast cancer. Yokoi et al. showed that Skp2, located at 5p13, often showed genomic amplification not only in cell lines but also in primary small cell lung carcinomas (44). On the other hand, Pene at al. reported that LY294002, a phosphorylation inhibitor of Akt, up-regulated the p27 protein expression, whereas the expression of both Skp2 and cyclin D1 dramatically diminished in vitro. This suggested that the biological effects of phosphatidylinositol 3-kinase activation might participate in a reduction of Skp2 protein and an increase of p27 protein (45). Rosner et al. recently indicated that TSC2 (tuberous sclerosis complex 2) deletion can shorten p27 half-life because TSC2 can bind to p27 and interfere with p27 binding to Skp2 (46). Recent studies discovered a cyclin-dependent kinase subunit 1 to be an integral part of the
ubiquitination mechanism for p27 (47, 48). In addition, the relationship among Skp2, cyclin-dependent kinase subunit 1, and p27 was also found to play a very important role in gastric carcinomaogenesis (49).

In conclusion, the prognosis of patients with a high Skp2 expression was significantly poorer than those with a low expression. Moreover, a multivariate analysis revealed that a high expression of p27 was an independent variable that correlated with a shorter disease-free survival. Thus, a Skp2 gene overexpression could therefore be a poor disease-free survival factor for breast cancer and also inversely correlated with the p27 expression in breast cancer. These findings strongly suggest that Skp2 could play an important role in breast cancer progression and may also be a novel molecular target for the treatment of breast cancer, especially in breast cancer occurring in young women.

Acknowledgments

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References

33. Tsvetkov LM, Yeh KH, Lee SJ, Sun H, Zhang H. In conclusion, the prognosis of patients with a high Skp2 expression was significantly poorer than those with a low expression. Moreover, a multivariate analysis revealed that a high expression of p27 was an independent variable that correlated with a shorter disease-free survival. Thus, a Skp2 gene overexpression could therefore be a poor disease-free survival factor for breast cancer and also inversely correlated with the p27 expression in breast cancer. These findings strongly suggest that Skp2 could play an important role in breast cancer progression and may also be a novel molecular target for the treatment of breast cancer, especially in breast cancer occurring in young women.
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