Jun Activation Domain-binding Protein 1 Expression in Breast Cancer Inversely Correlates with the Cell Cycle Inhibitor p27\textsuperscript{Kip1} 1

Maria A. Kouvaraki, George Z. Rassidakis, Ling Tian, Rakesh Kumar, Christos Kittas, and François-Xavier Claret

Departments of Molecular Therapeutics [M. A. K., L. T., F-X. C.] and Molecular Oncology [R. K.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and Laboratory of Histology and Embryology, University of Athens School of Medicine, Athens, Greece [G. Z. R., C. K.]

ABSTRACT

The Jun activation domain-binding protein 1 (JAB1), aside from being an activator protein 1 coactivator, is involved in degradation of the cyclin-dependent kinase inhibitor p27. We examined JAB1 and p27 protein expression in invasive breast carcinoma specimens and the association of this expression with clinical outcome. JAB1 was detected immunohistochemically in 43 of 53 (81%) tumors; 32 (60%) breast carcinomas showed high JAB1 expression (>50% of cells positive) and reduced or absent p27 levels (P = 0.02, Mann-Whitney U test). Tumors with high p27 expression were rarely positive for JAB1. All eight patients with JAB1-negative tumors had no evidence of relapse or disease progression at a median follow-up of 70 months. Immunohistology showed strong JAB1 expression in breast carcinoma samples but not in paired normal breast epithelial samples. Targeted overexpression of JAB1 by regulated adenovirus in breast cancer cell lines also reduced p27 levels by accelerating degradation of p27. Thus, the JAB1:p27 ratio may be a novel indicator of aggressive, high-grade tumor behavior, and control of JAB1 could be a novel target for experimental therapies.

INTRODUCTION

The human JAB1\textsuperscript{1} was originally identified as a coactivator of the gene-regulatory AP-1 proteins (Jun/Fos proto-oncogenes) involved in the control of cell proliferation (1). JAB1 (also known as CSN5) is also present in the CSN, a multiprotein complex involved in modulating signal transduction, gene transcription, and protein stability (2, 3). The role of JAB1 in human oncogenesis is under active investigation. Tomoda \textit{et al.} (4) identified a mouse JAB1 (p38\textsuperscript{Jab1}) that interacts specifically with the protein form of the CDK inhibitor p27. p27 is a universal CDK inhibitor that directly inhibits the enzymatic activity of cyclin-CDK complexes, resulting in cell cycle arrest at G\textsubscript{1} (5). p27 protein levels are increased in quiescent cells and rapidly decrease after cells are stimulated with mitogens (6). Although transcriptional regulation is possible, cellular abundance of p27 is regulated primarily at the posttranscriptional level by the ubiquitin-proteasome pathway (7). To be degraded, p27 must be phosphorylated at its Thr\textsuperscript{187} residue by the cyclin E/CDK2 complex (8–10), although a Thr\textsuperscript{187}-independent proteolytic pathway that functions during mid-G\textsubscript{1} has been suggested as well (11). JAB1 has been shown to shuttle p27 from the nucleus to the cytoplasm and to decrease the amount of p27 in the cell by accelerating p27 degradation via the ubiquitin-proteasome system (4, 12). JAB1 seems to interact with p27 in the nucleus; fusion analysis with a nuclear export signal showed that cytoplasmic transportation per se was not sufficient for p27 to be degraded (4).

Overexpression of JAB1 has been reported in human pituitary tumors (13) and in epithelial ovarian cancer (14); in the latter study, JAB1 overexpression was inversely associated with p27 levels and correlated with inferior OS (14). JAB1 expression has not been studied in breast tissue \textit{in vivo}. We used immunohistochemical analysis to examine JAB1 and p27 protein levels (15) in specimens of invasive breast carcinoma and adjacent normal breast tissue and compared those findings with clinical outcome. We hypothesized that JAB1 function as a negative regulator of p27 may have a role in breast oncogenesis. We found a strong inverse association between JAB1 and p27 expression levels in the tumors. Moreover, patients whose tumors did not express JAB1 had the highest survival rate, whereas those whose tumors had high JAB1 levels fared the worst. To further elucidate the role of JAB1 in p27 degradation in breast cancer, we infected four breast carcinoma cells lines with an adenoviral vector expressing JAB1 and found that p27 levels were significantly reduced after JAB1 gene transfer, indicating that in breast cancer JAB1 controls the activity of p27 by targeting it for degradation.

MATERIALS AND METHODS

Patients and Tissue Samples. The study group consisted of 53 women with invasive breast carcinoma (mean age, 63.2 ± 13.3 years; median age, 65 years; age range, 35–90 years) who underwent surgical treatment in the Second Department of Surgery, University of Athens School of Medicine (Athens, Greece) from November 1995 to April 1997. None of the patients had a family history of breast cancer. Patient selection was based on the availability of archived paraffin blocks for immunohistochemical studies. Six cases (11%) were stage I, 28 (53%) were stage II, 13 (25%) were stage III, and 6 (11%) were stage IV. Five tumors (9%) were grade 1, 29 (55%) were grade 2, and 19 (36%) were grade 3. All but one tumor was larger than 1 cm in maximum diameter. Histologically, 47 specimens were ductal carcinomas, 3 were lobular carcinomas, and 3 were mixed invasive carcinomas. The clinicopathological characteristics of the patients have been detailed elsewhere (15). The cutoff level for considering a tumor to be estrogen receptor or progesterone receptor positive was 10 fmol/mg.

Consecutive 5-μm paraffin-embedded sections were cut from each tumor specimen and processed for immunohistochemical analysis as described below. Tumors were surgically staged according to the American Joint Committee on Cancer tumor-node-metastasis system and graded according to the Nottingham modification of the Bloom and Richardson system.

Immunohistochemical Analysis. Monoclonal antibodies were as follows: for JAB1, clone 4D11D8 (1:400; Zymed, San Francisco, CA); for p27, clone SX53K (1:200; DAKO, Carpinteria, CA); and for Ki-67, MIB-1 (1:100; Immunotech, Westbrook, ME). The specificity of the JAB1 antibodies was tested in normal tonsil tissue samples by competition with a specific JAB1 peptide and an unspecific peptide (data not shown); results were similar to those reported elsewhere (13). The immunohistochemical method used in this study was described previously (15). Briefly, JAB1, p27, and MIB-1 antigens were retrieved by heating and incubated with the primary antibody. The immunoreaction was detected with the LSAB\textsuperscript{+} kit from DAKO. 3,3‘-diaminobenzidine was used as the chromogen, and hematoxylin was used as the counterstain. Epithelial cells of the adjacent normal breast ducts were used as an internal positive control for JAB1 and p27 expression. Expression levels of JAB1 and p27 were determined by counting at least 1000 tumor cells in 10 representative high-power fields. Tumor cells were considered JAB1 positive when staining was present, irrespective of the intensity. Based on the distribution of data (histograms) and for the purposes of a more detailed statistical
analysis, we used 50% as a cutoff to define high versus low JAB1 expression. The p27 LI was defined as the percentage of p27-positive tumor cells; we also used 50% as a cutoff for high versus low p27 expression, as has been suggested by others (16). Serial tissue sections from the same areas of the breast were used to examine JAB1, p27, and Ki-67 (a marker of cell proliferation) expression levels. The PI was defined as the percentage of MIB-1-positive tumor cells, regardless of staining intensity.

**Cell Cultures.** Of the four human breast cancer cell lines tested, BT-474 cells were maintained in RPMI 1640; both media were supplemented with 10% FCS and 1% penicillin-streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

**Cell Extracts, Tissue Samples, and Immunoblotting.** Cells in log-phase growth were collected, washed twice in cold PBS, and lysed at 4°C in lysis buffer (25 mM HEPES (pH 7.7), 400 mM NaCl, 0.5% Triton X-100, 1.5 mM MgCl2, 2 mM EDTA, 2 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, protease inhibitors (10 μg/ml leupeptin, 2 μg/ml pepstatin, 50 μg/ml antipain, 2 μg/ml aprotinin, 20 μg/ml chymostatin, and 2 μg/ml benzamidine), and phosphatase inhibitors (50 mM NaF, 0.1 mM Na3VO4, and 20 μM β-glycerophosphate)). Paired samples of normal human breast epithelium and breast carcinoma were lysed as described previously (17). Total cell lysates were resolved on 10% SDS-PAGE, transferred to nitrocellulose polyvinylidene difluoride membranes, and probed with primary polyclonal antibodies to JAB1 (Zymed) and p27 (BD-PharMingen, San Diego, CA), using enhanced chemiluminescence reagents (Amersham Pharmacia, Piscataway, NJ). Vinculin and β-actin (Sigma Chemical Co., St. Louis, MO) served as internal positive controls for all immunoblots.

**Recombinant JAB1 Adenovirus.** A recombinant adenovirus vector expressing a doxycycline-regulated (Tet-Off) form of JAB1 was constructed according to the manufacturer’s recommendations (Clontech, Palo Alto, CA), and the cloning procedure will be fully described elsewhere. Briefly, the cDNA encoding for human JAB1 (1) was fused to the Myc epitope tag fusion protein (Myc-JAB1) and inserted into pAdML/Adl restriction sites of the pTRE-shuttle vector (Clontech) to generate a pTRE-JAB1-Myc construct. Then, the Pci-SceI-IceI digestion product of the pTRE-Myc-JAB1 shuttle vector was cloned into a pAdeno-X viral DNA vector. Recombinant infectious adenoviruses were then produced by transfecting HEK 293 cells with pAdeno-X-Myc-JAB1 viral DNA and confirmed by detecting synthesis of Myc-JAB1 fusion protein by immunoblotting with anti-Myc antibodies. The resulting construct was called Ad-JAB1-Myc. Viral titer was assayed as described previously (15). Cells in log-phase growth were infected with Ad-JAB1-Myc in the absence or presence of 1 μg/ml doxycycline, a tetracycline analog, to regulate the expression of JAB1. Cells were harvested at appropriate time points, harvested, and analyzed by Western blotting with anti-Myc, and anti-p27 antibodies.

**RESULTS**

**JAB1 Expression in Breast Tumors.** JAB1 was detected in 43 of 53 (81%) breast carcinomas (Fig. 1). In 37 of the JAB1-positive tumors, JAB1 was predominantly found in the nucleus, although weak cytoplasmic immunoreactivity was also observed (Fig. 1). In the other six JAB1-positive tumors, JAB1 was predominantly found in the cytoplasm, with weaker nuclear immunoreactivity. The nucleoli were frequently negative in both expression patterns. The percentage of JAB1-positive tumor cells ranged from 20% to 98% (mean ± SD, 65.6 ± 24.5%; median, 70%). The distribution of tumors according to the percentage of JAB1-positive tumor cells is shown in Fig. 1e. Using 50% as a high/low cutoff point, 32 of the 53 (60%) tumors showed high JAB1 expression. JAB1 expression in relation to histological type, stage, grade, and hormonal status of the tumors is shown in Table 1. JAB1 was not associated with PI. JAB1 was detected in a small number of ductal epithelial cells in proximate normal and hyperplastic breast tissues.

To explore the significance of JAB1 in human breast cancer progression, we further examined whether JAB1 protein expression was different in paired normal human breast epithelium and breast carcinoma biopsy samples by immunoblotting methods. Fig. 2 shows a dramatically increased JAB1 level in six of eight tumors as compared with the paired normal tissue, which show little or no JAB1 expression.

**JAB1 Expression and p27 LI.** p27 expression was assessed in 49 of the breast tumors included in this study. p27 was detected in a highly variable proportion of tumor cells as reported previously (15). JAB1 was detected in 43 of 49 (27%) breast carcinomas. In 37 of the JAB1-positive tumors, JAB1 was predominantly found in the nucleus, although weak cytoplasmic immunoreaction was also observed (Fig. 1). In the other six JAB1-positive tumors, JAB1 was frequently negative in both expression patterns. The percentage of JAB1-positive tumor cells ranged from 0.1% to 85% (mean ± SD, 33.5 ± 24.5%; median, 34.1%; Fig. 1f). High p27 expression (p27 LI > 50%) was noted in 13 of the 49 (27%) carcinomas.

Statistical analysis revealed an inverse correlation between p27 LI and JAB1 expression (Table 2; Fig. 1g). Specifically, the mean p27 LI for tumors with high JAB1 expression was 27.2% compared with 44.5% for tumors with low JAB1 expression (P = 0.02, Mann-Whitney U test). Similarly, high JAB1 expression correlated with low p27 expression if both factors were considered categorical variables (Table 2). Five tumors showed high expression of both JAB1 and p27 proteins. p27 LI and the PI seemed to be inversely correlated, but the apparent association was not statistically significant (Spearman R = −0.2; P = 0.2).

Tumors in which JAB1 was predominantly cytoplasmic (n = 6) were associated with significantly lower p27 LI (4.5% versus 38.3%, P = 0.0045, Mann-Whitney U test; Fig. 1h).

**Association of High JAB1 Expression with Clinical Outcome.** Survival analysis of the 43 patients with information available on clinical follow-up and JAB1 and p27 expression indicated that at a median follow-up of 70 months, the 5-year PFS rate for patients with JAB1-positive tumors (n = 35) was 80% compared with 100% for patients with JAB1-negative tumors (n = 8; Fig. 3a, P = 0.08 by log-rank). The corresponding 5-year OS rates were 69% and 100%, respectively (Fig. 3b, P = 0.05 by log-rank).

**Adenoviral JAB1 Gene Transfer Reduces p27 Levels in Breast Cancer Cell Lines.** To investigate whether JAB1 overexpression in breast cancer cells would down-regulate p27 levels, we transduced the breast cancer cell lines BT-474, MDA-MD-468, MDA-MD-231, and BT-549 with JAB1 adenovirus in the absence (−) or presence (+) of doxycycline for 48 h and monitored levels of JAB-Myc and p27. Direct evidence of a functional relationship between JAB1 expression and p27 degradation is shown in Fig. 4. JAB1 overexpression accelerated p27 degradation in all cell lines tested. Down-regulation of p27 was inhibited in cells pretreated with inhibitors of the 26S proteasome lactacystin or MG132 (data not shown). These findings confirm that high expression levels of JAB1, assessed by immunohistochemical analysis, are associated with low p27 expression levels.

**DISCUSSION**

To our knowledge, this is the first report that JAB1 protein is overexpressed in most invasive breast carcinomas. Moreover, we found an inverse correlation between JAB1 and p27 expression in aggressive breast tumors (P = 0.02). Cytoplasmic localization of JAB1, although uncommon in this series, correlated with very low p27 levels (Fig. 1h). All eight of the patients with JAB1-negative tumors were alive without disease progression at a median follow-up of 70 months. These preliminary findings should be confirmed in a larger study.

---

* L. Tian et al., manuscript in preparation.
In a further investigation of the apparent role of JAB1-mediated down-regulation of p27 in breast cancer, we infected breast cancer-derived cell lines with an inducible JAB1-expressing adenoviral vector and found that p27 levels were significantly reduced after JAB1 gene transfer (Fig. 4). Taken together, these observations strongly support the concept that JAB1 mediated the down-regulation of p27, possibly by increasing the degradation of p27 through the ubiquitin/proteasome system (4). This hypothesis is supported by our in vivo findings indicating that JAB1, when strongly expressed in the cytoplasm of breast tumor tissue samples, was associated with minimal levels of nuclear p27. High expression levels of both nuclear JAB1 and p27 were found in only five tumors in our study; notably, all five were associated with axillary lymph node metastasis. In these cases, the possibility of abnormal nuclear accumulation of inactive p27/cyclin D3/CDK4–6 complexes, as has been suggested for other tumor types (18), cannot be excluded.

p27 proteolysis is probably mediated not only by JAB1 but also by the CSN complex, of which JAB1/CSN5 is a component. Other components of the CSN complex including CSN6, CSN7, and CSN8 are capable of inducing p27 down-regulation when ectopically expressed, and this function may require nuclear export of p27 (12). In addition to its roles in AP-1 transcriptional activity and p27 degrada-
tion, JAB1 may function as a cointegrator of diverse signaling pathways (19–24). However, the exact role of these mechanisms in breast oncogenesis needs further investigation.

Using immunoblotting methods, we also showed that strong JAB1 expression was altered in paired normal human breast epithelium and breast carcinoma biopsy sample, with a dramatically increased expression in six of eight tumors as compared with the adjacent normal tissue, which showed little or no JAB1 expression (Fig. 2). These findings suggest that the increased level of JAB1 protein could be closely associated with the malignancy of breast tumors. Malignant conversion of tumor is a complex process that may be regulated, at least in part, by increased expression of JAB1 and decreased expression of p27. Endogenous JAB1 is physiologically involved in the regulation of AP-1 transcriptional activity (1) and participates in COP9-mediated kinase activity in various transcription regulators (reviewed in Refs. 2 and 3). The AP-1 complex was reported to promote cellular invasion, thereby validating its components as targets for diagnosis or therapy (25–27). JAB1 may also promote the progression of breast carcinoma by activating AP-1 transcriptional proteins.

In summary, we found JAB1 to be frequently expressed and inversely correlated with p27 levels in breast cancer; this inverse asso-

Table 1  JAB1 expression and tumor characteristics in sporadic invasive breast carcinomas

<table>
<thead>
<tr>
<th>Tumor characteristics</th>
<th>Low JAB1 expression (n = 21)</th>
<th>High JAB1 expression (n = 32)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal carcinoma</td>
<td>18</td>
<td>38</td>
<td>29</td>
</tr>
<tr>
<td>Lobular carcinoma</td>
<td>2</td>
<td>33</td>
<td>1</td>
</tr>
<tr>
<td>Mixed</td>
<td>1</td>
<td>33</td>
<td>2</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>34</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>53</td>
<td>9</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>33</td>
<td>4</td>
</tr>
<tr>
<td>II</td>
<td>9</td>
<td>32</td>
<td>19</td>
</tr>
<tr>
<td>III</td>
<td>7</td>
<td>54</td>
<td>6</td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>16</td>
<td>47</td>
<td>18</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td>Estrogen receptor status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>14</td>
<td>42</td>
<td>19</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>Progesterone receptor status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>37</td>
<td>17</td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>38</td>
<td>10</td>
</tr>
</tbody>
</table>

* Chi-square test.
* Fisher’s exact test.

Table 2  JAB1 and p27 expression are inversely correlated in invasive breast carcinomas

<table>
<thead>
<tr>
<th>JAB1 level</th>
<th>p27 expression</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>27.2 ± 23.2</td>
<td>0.02a</td>
</tr>
<tr>
<td>Low</td>
<td>44.5 ± 23.2</td>
<td></td>
</tr>
</tbody>
</table>

* Mann-Whitney U test.
* Fisher’s exact test.

Fig. 2. JAB1 and p27 expression in normal breast tissue and breast tumors. Western blots of paired samples of nonneoplastic breast tissue (N) and tumor tissue (T) immunoblotted against JAB1 or vinculin (used as a loading control).

Fig. 3. Association of JAB1 expression with (a) PFS and (b) OS.
JAB1 AND p27 EXPRESSION IN AGGRESSIVE BREAST CARCINOMA

![Diagram](image)

Fig. 4. JAB1-mediated p27 degradation in breast cancer cells. Top, inducible expression of Ad-Myc-JAB1 down-regulated endogenous p27 levels in four breast cancer cell lines as indicated. Lysates were prepared from Ad-Myc-JAB1-infected cells in the absence (−) or presence (+) of doxycycline, and proteins were immunoblotted with anti-Myc and anti-p27 antibodies. Anti-β-actin antibodies were used as a loading control. Bottom, results were quantified in relative phosphorimager units.□, p27 levels; ■, JAB1 levels.

The expression was apparently due to down-regulation of p27 by JAB1. Changes in expression levels of JAB1 and p27 might contribute to deregulation of the cell cycle and might precede the progression of breast tumors. Our preliminary survival analysis indicates that JAB1 expression may be associated with inferior PFS and OS, but this early finding needs to be confirmed with a larger group of patients. Modulation of the JAB1 gene product may also provide a novel target for experimental therapies in breast cancer.

REFERENCES


Jun Activation Domain-binding Protein 1 Expression in Breast Cancer Inversely Correlates with the Cell Cycle Inhibitor p27Kip1

Maria A. Kouvaraki, George Z. Rassidakis, Ling Tian, et al.