Jab1 Expression Is Associated with Inverse Expression of p27kip1 and Poor Prognosis in Epithelial Ovarian Tumors

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ABSTRACT

Purpose: Jab1 (Jun activation domain-binding protein 1) has been described as a coactivator of AP1 transcription factor, and is a subunit of a large protein complex (called the COP9 signalosome). Recent study (K. Tomoda et al., Nature [Lond.], 398: 160–165, 1999) found that Jab1 protein can cause breakdown of p27kip1 protein in mammalian cells. To investigate whether Jab1 expression is correlated with p27kip1 protein levels as well as how it might be clinically relevant, we evaluated the expression of Jab1 in a group of epithelial ovarian tumors.

Experimental Design: Immunohistochemical analysis was performed in 80 cases of ovarian tumors (33 benign ovarian tumors and 47 ovarian carcinomas). Twenty-six of the 80 cases were evaluated by Western blot analysis.

Results: Jab1 overexpression was detected in 68.1% (32 of 47) of malignant tumors and 33.3% (11 of 33) of benign tumors. The positive ratio of Jab1 was increased from benign to malignant ovarian tumors (P = 0.002). A negative correlation between Jab1 and p27kip1 expression was found in both benign (P = 0.003) and malignant (P = 0.002) ovarian tumors. No significant correlation was observed between Jab1 overexpression and clinicopathological parameters. Kaplan-Meier survival analysis showed that Jab1 overexpression was significantly associated with poor prognosis of patients (P = 0.049).

Conclusions: Jab1 expression is inversely correlated with p27kip1 expression levels, and Jab1, as a negative regulator of p27kip1, may be associated with the progression and prognosis of epithelial ovarian tumors.

INTRODUCTION

Jab1 is originally described as a transcriptional coactivator of AP1 proteins (especially c-Jun and Jun D). It can enhance the ability of c-Jun and Jun D to activate transcription by stabilizing complexes containing these proteins at AP1 binding sites (1). In addition, Jab1 is also known as COP9 singnalosome subunit 5 (CSN5), which is a component of the COP9 signalosome regulatory complex (2, 3). Recently, it was demonstrated that Jab1 can specifically interact with the CDK1 protein p27kip1 (4). Transient coexpression of Jab1 with p27kip1 accelerated the degradation of p27kip1 in mammalian cells by translating p27kip1 from the nucleus to the cytosol, in which degradation could occur (4). The study suggested that Jab1 can act as a negative regulator of important cell cycle control proteins by targeting them for degradation.

The cell cycle progression is regulated by both the positive and negative regulators. Cyclin and CDK are positive regulators, whereas CDKI, including the INK4 and the CIP/KIP families, are negative regulators (5). p27kip1, a CIP/KIP member, causes G1 arrest by inhibiting the activities of G1 cyclin-CDK. As a negative regulator of the cell cycle, p27kip1 is a new class of tumor suppressor (6), which inhibits cyclin-CDK in a dosage-dependent manner to control cell cycle progression (7–8). Recently, decreased expression of p27kip1 has been frequently detected in human cancers (9–15), including ovarian carcinoma, which was studied by our group (16). These studies indicated that p27kip1 protein levels may be associated with the development of human cancers and appear as an important marker of cancer progression. However, to the best of our knowledge, the status of Jab1 expression in ovarian tumors, including its possible clinical significance and the correlation with p27kip1, has not been examined. Therefore, to gain better insight into the clinical relevance of Jab1, we investigated the expression of Jab1 immunohistochemically in 80 epithelial ovarian tumors and assessed whether Jab1 expression is correlated with p27kip1 protein levels, and whether Jab1 is associated with clinicopathological parameters and prognosis of epithelial ovarian carcinomas.

MATERIALS AND METHODS

Tumor Specimens. Formalin-fixed, paraffin-embedded blocks of ovarian tumor tissues from 54 patients (32 malignant and 22 benign ovarian tumors) were obtained at Department of Perinato-Gynecology of Kagawa Medical University during 1985–1996. Twenty-six fresh ovarian tumor samples (15 malignant and 11 benign ovarian tumors) were obtained at Department of Perinato-Gynecology of Kagawa Medical University.

The abbreviations used are: Jab1, Jun activation domain-binding protein 1; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; HR, hazard ratio; CI, confidence interval.

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and Department of Obstetrics and Gynecology of Takamatsu Red Cross Hospital during 1997–1998. After surgical resection, each fresh tumor specimen was immediately washed and cut out around necrotic tissue, divided into two portions: one portion was instantly frozen for protein extraction, the other portion was formalin fixed and paraffin embedded for routine and immunohistochemical investigation. Specimens consisted of 2 normal ovaries, 33 benign cystadenomas, and 47 ovarian adenocarcinomas. The median age of the 47 ovarian carcinoma patients was 49 years (range, 16–77). Sixteen patients were in stage I, 3 in stage II, 16 in stage III, and 12 in stage IV, according to the International Federation of Gynecology and Obstetrics (FIGO) classification. Histological classification of tumor was carried out according to the WHO system, 21 cases were well-differentiated (G1), 13 moderately differentiated (G2), and 13 poorly differentiated (G3, including 2 undifferentiated). Among the 47 patients with ovarian carcinoma, none received preoperative chemotherapy or radiotherapy. All received postoperative, platinum-based chemotherapy, but no radiotherapy. Follow-up data were available for all patients.

Immunohistochemistry. Paraffin sections (4-μm thickness) were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide (30 min). To reduce nonspecific binding, the sections were incubated with 10% rabbit serum for 60 min at room temperature. The sections were incubated overnight at 4°C with goat anti-Jab1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000). For each case, a corresponding section was incubated with nonimmunized goat serum as a negative control. Immunostaining was performed by the avidin-biotin peroxidase complex (ABC) method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The peroxidase activity was detected using 3,3-diaminobenzidine as the chromogen and Mayer’s hematoxylin as the counterstain. All of the samples (formalin-fixed and fresh) were handled by the same method, and the reagents were good for both.

Only cells with brown-colored nuclear staining were considered as positive. Background and cytoplasmic staining were not counted in any of the cases. A large part of benign ovarian tumors showed nuclear staining in <10% of the tumor cells. Hence, we defined overexpression of Jab1 when over 10% of the tumor cells were stained in each section. At least 20 high-power field were chosen randomly, and 2000 cells were counted. At the time of review, the investigators were not aware of the clinical outcome of the patients.

Western Blot Analysis. Approximately 0.5 g of tissue each from fresh samples were homogenized and lysed in 2.5 ml of lysis buffer [1% NP40, 150 mM NaCl, 50 mM NaF, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM Na3VO4, 10 μM Na2MnO4, 1 mM PMSF, 10 μg/ml leupeptin, 1% aprotonin]. The lysates were centrifuged at 100,000 × g for 1 h at 4°C, and the supernatant was stored at −80°C until further analysis. The extracts equivalent to 200 μg of the total protein were separated by 12% SDS-polyacrylamide gel, then transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat dried milk, 10% donkey serum and 0.1% Tween 20, then probed by polyclonal antibodies against Jab1 (1:100), and against actin (1:100) in PBS containing 5% bovine serum. After several washes with TBS, membranes were probed with a horseradish peroxidase-conjugated goat IgG (DAKO, Kyoto, Japan), and proteins were detected by an enhanced chemiluminescence system (Amerham, Tokyo, Japan).

Statistical Analysis. The associations between Jab1 and p27kip1 and clinicopathological parameters were assessed using the Pearson χ2 test and Fisher’s exact test. Multiple simultaneous comparison were made, whichever was appropriate. Associations were always considered as binomial variables, and all Ps were 2-sided. Spearman’s rank correlation was used to determine whether there was a positive or negative correlation between Jab1 and p27kip1 expression. Overall survival was calculated using the method of Kaplan-Meier, and comparison between groups was performed with the log-rank test. The HR and the CI relating to the expression of Jab1 and p27kip1 was evaluated by the Cox proportional hazard regression model. All statistical significance was set at P < 0.05. Statistical analyses were run using the JMP software version 3.2.5 (SAS Institute Inc., Cary, NC).

RESULTS

Expression of Jab1 As Well As Its Correlation with p27kip1. Jab1 overexpression was detected in 68.1% (32 of 47) of ovarian carcinomas and in 33.3% (11 of 33) of benign tumors. p27kip1 expression by our previous study (16) was in 36.2% (17 of 47) of ovarian carcinomas and 75.8% (25 of 33) of benign tumors (Table 1). The positive ratio of Jab1 expression was increased from benign to malignant tumors, showing a statistical significance (P = 0.002). The correlation between Jab1 and p27kip1 expression was investigated by Spearman’s rank correlation. A negative correlation between both proteins was identified in benign (correlation coefficient, −0.5; P = 0.003) and malignant tumors (correlation coefficient, −0.43; P = 0.002). In these cases, positive immunostaining for p27kip1 in benign tumors cells was usually negative for Jab1, and, in turn, positive immunostaining for Jab1 in malignant tumors cells was usually negative for p27kip1. No Jab1 expression was detected in normal ovarian surface epithelium in contrast with positive p27kip1 expression. (Fig. 1, a–f).

To confirm the specificity of the immunohistochemical results, Western blot analysis was carried out in 2 normal ovaries, 11 benign, and 15 malignant ovarian tumor tissues (two cases of stage I, one case of stage II, seven cases of stage III, five cases of stage IV; six cases of G1, four cases of G2, five cases of G3), in which freshly frozen materials were available. The expression of Jab1 was examined by Western blot analysis, which showed accordant result with immunohistochemistry. Jab1 and p27kip1 showed negative and positive expression,
respectively, in normal ovarian surface epithelium (data not shown). The example of Western blot analysis is shown in Fig. 2. An immunoreactive band of Jab1 at Mr 38,000 was seen in all five cases of ovarian carcinomas (Lanes 1–5), only 1 case of benign tumor showed a clear band (Lane 6), and two cases showed weakly (Lanes 8–9). Low or no p27kip1 expression was observed in malignant ovarian tumors (Lanes 1–5). Benign tumors clearly showed an immunoreactive band of p27kip1 (Lanes 6–10). Amount of actin, a housekeeping protein, was demonstrated to be rather constant among the samples.

**Correlation between Jab1 Expression and Clinicopathological Parameters.** In addition, we evaluated the association of Jab1 expression with clinicopathological parameters such as tumor grades, clinical stages, histology, lymph node status, and so forth. No significant correlation was observed between Jab1 expression and these parameters (Table 2).

**Fig. 1** Immunostaining of Jab1 and p27kip1 in ovarian tumors. Normal ovarian surface epithelium showed negative staining of Jab1 (a) and positive nuclear staining of p27kip1 (b). (Scale bars, 20 μm). Negative nuclear staining of Jab1 (c) and positive staining of p27kip1 (d) are observed in benign ovarian tumor cells. Positive nuclear staining of Jab1 (e) and negative staining of p27kip1 (f) are observed in malignant ovarian tumor cells. (Scale bars, 40 μm).

**Fig. 2** Western blot analysis of Jab1 and p27kip1 in benign and malignant ovarian tumors. Malignant tumors (Lanes 1–5) show high-level expression of Jab1, and only 1 case benign tumor showed a clear band (Lane 6), and 2 cases showed a weak band (Lanes 8–9). Low or no p27kip1 expression was observed in malignant ovarian tumors (Lanes 1–5). High-level expression of p27kip1 was observed in benign tumors (Lanes 6–10). Lane 11, NIH 3T3 cell lysate (for Jab1) and HeLa cell lysate (for p27kip1 and actin) as a positive control. kDa, Mr, in the thousands.
Correlation between Jab1 Expression and Patient Survival. The median follow-up time for all of the ovarian carcinoma patients was 24 months (range, 2–156 months). At the end point of the follow-up, 32 patients survived with a median follow-up time of 27.5 months (range, 4–156), and 15 had died of ovarian cancer after a median follow-up time of 17 months (range, 2–46). Kaplan-Meier survival analysis and Cox proportional hazard regression model showed that Jab1 overexpression has a significant effect on overall survival ($P = 0.049$; HR, 1.99; CI, 1.05–3.05; Fig. 3a). Loss of p27 kip1 expression was significantly associated with poor overall survival ($P = 0.019$; HR, 2.02; CI, 1.13–4.25; Fig. 3b). When combined phenotypes of two proteins were analyzed, patients with the Jab1-positive/p27-negative expression had an overall survival rate that was significantly lower than other phenotypes of Jab1/p27 ($P = 0.008$; data not shown).

DISCUSSION
The orderly transit of cells through the cell cycle requires a delicate balance between positive and negative regulatory factors. Any alteration in this balance can result in abnormal cell proliferation, which may contribute to cancer. Jab1 was initially described as a coactivator of AP1 transcription factor and is a subunit of a large protein complex (called the COP9 signalosome). Recent research (4) found that an increasing level of Jab1 causes an increasing breakdown of p27kip1 and indicated that Jab1 controls the activity of p27kip1 by facilitating its degradation. This finding suggested that Jab1 can act as a negative regulator of important cell cycle control proteins by targeting them for degradation.

The study of Claret et al. (1) by immunofluorescence analysis indicated that Jab1 is a nuclear protein. Through selective interaction with the Jun proteins, Jab1 can increase the specificity of target-gene activation by this large family of
related transcription factors. However, to date, the detailed biological functions of Jab1 in mammalian cells have not been identified.

In the present study, Jab1 protein expression was examined immunohistochemically in ovarian tumors. We found that Jab1 immunostaining was mainly located in the nucleus, although some weak or variable staining remained in the cytoplasm. Jab1 expression increased from normal ovarian epithelium to benign tumor to malignant tumor. Western blot analysis also showed the different Jab1 expression levels in benign and malignant ovarian tumors. These findings suggested that the alteration of Jab1 expression levels may be closely associated with the malignancy of ovarian tumors. An inverse correlation between Jab1 and p27<sup>kip1</sup> expression was found in both benign (P = 0.003) and malignant (P = 0.002) ovarian tumors. Recent study (17) reported that colocalization of p27<sup>kip1</sup> and Jab1 in the nucleus of mature ganglion cells was observed. The researchers explained that, for unknown reasons, Jab1 may be inactive and not bound to p27<sup>kip1</sup> in these cells. To our present knowledge, no other study has been performed for Jab1 expression in human tumors. The valuable comparison and definite conclusions will be obtained by a larger number of studies in the future. Our findings support the conclusion by Tomoda et al. (4) that Jab1 can specifically interact with p27<sup>kip1</sup> protein and can accelerate its degradation. Although the precise role of Jab1 in p27<sup>kip1</sup> regulation remains unclear, the current data suggest that overexpression of Jab1 seems to confer the more aggressive growth potential in ovarian carcinoma.

Inhibition of the conversion from benign to malignant tumors might be a useful strategy for the treatment of ovarian carcinoma. The present study suggested that the alteration of expression of Jab1 protein may also affect tumor development. Increased expression of Jab1 protein may enhance malignancy. Malignant conversion of tumor is a complex process, which may be regulated, at least in part, by increased expression of Jab1 and decreased expression of p27<sup>kip1</sup>. Endogenous Jab1 is physiologically involved in the regulation of AP-1 transcriptional activity (18). Jab1 has been isolated as a component of a highly conserved multimolecular complex (COP9 complex) with kinase activity toward different transcriptional regulators (2, 3, 19). AP-1 proteins have recently been reported to promote cellular invasion and to validate them as targets for diagnosis or therapy (20). Jab1 may also promote the progression of ovarian carcinoma by activating AP-1 transcriptional proteins.

The correlation between Jab1 and clinicopathological parameters as well as prognosis of patients were evaluated. No significant relationship was found for Jab1 expression with any clinicopathological parameters but Jab1 expression was significantly associated with patients’ survival (P = 0.049; Fig. 3a). We previously performed the analysis of p27<sup>kip1</sup> expression in epithelial ovarian tumors (16). The results showed that loss of p27<sup>kip1</sup> expression was significantly associated with high tumor grade (P = 0.003), lymph node positivity (P = 0.002), and poor overall survival (P = 0.019; Fig. 3b). Masciullo et al. (21) have recently reported that p27<sup>kip1</sup> expression did not correlate with any of the clinicopathological parameters and is an independent prognostic predictor. The difference can be explained by the difference in the patients’ selection. In their study, all patients were at advanced stage (III-IV), and 80.8% (80 of 99) of the cases were grade 3 tumors, whereas ours consisted of all four stages (I-IV). When Jab1 was analyzed by combined phenotypes with p27<sup>kip1</sup> or cyclin E and cdk2, we found that Jab1 may have a greater prognostic potential than when alone. Patients with the Jab1-positive/p27-negative expression had an overall survival rate that was significantly lower than other phenotypes of Jab1/p27 (P = 0.008; Fig. 3c). In addition, patients with Jab1/cyclin E/cdk2 overexpression had a significantly poorer prognosis (P = 0.002). These findings suggested that the immunohistochemical evaluation of Jab1 and p27<sup>kip1</sup> or cyclin E/cdk2 might be the most reliable indication of prognosis of epithelial ovarian carcinoma patients.

In conclusion, Jab1 protein expression is inversely correlated with p27<sup>kip1</sup> expression levels, and Jab1 is a negative regulator of p27<sup>kip1</sup> in epithelial ovarian tumors. Changes both in Jab1 and in p27<sup>kip1</sup> expression levels might contribute to the dysregulation of cell cycle and might precede the progression of ovarian tumors. Although the detailed biological functions of Jab1 in tumor cells have not been identified, our results first demonstrated that Jab1 as a negative regulator of p27<sup>kip1</sup> was associated with the progression and prognosis of epithelial ovarian tumors.

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