Prognostic Significance of Jab1 Expression in Laryngeal Squamous Cell Carcinomas

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ABSTRACT

Purpose: Jun activation domain-binding protein 1 (Jab1) is known as a coactivator of AP1 transcription factor, which contributes to tumor progression by degrading the p27kip1 protein. The purpose of this study is to investigate whether Jab1 expression is correlated with p27kip1 level and cell proliferation, as well as whether Jab1 expression is associated with clinicopathologic variables and prognosis of laryngeal squamous cell carcinoma (LSCC).

Experimental Design: Immunohistochemical and/or Western blot analysis was done in HEp-2 cells and 102 cases of LSCCs.

Results: Jab1 expression was negatively associated with p27kip1 expression and was positively associated with cell proliferation both in HEp-2 cells and LSCCs. Jab1 overexpression was detected in 51% LSCCs and was significantly associated with unfavorable clinicopathologic variables. Survival analysis revealed that Jab1 overexpression is significantly associated with short disease-free and overall survival (P = 0.0036 and P = 0.0001, respectively). When Jab1 and p27kip1 are combined, patients with Jab1+/p27kip1(−) revealed poor disease-free and overall survival (P = 0.0008 and P < 0.0001, respectively). When Jab1 expression and lymph node status are combined, patients with Jab1+/lymph node(+) revealed poorer disease-free and overall survival than others (P < 0.0001 and P < 0.0001, respectively). Furthermore, patients with the phenotype of Jab1+/p27kip1(−)/lymph node(+) revealed the worst disease-free and overall survival (P < 0.0001 and P < 0.0001, respectively). Multivariate analysis revealed that Jab1 protein is an independent prognostic indicator for overall survival.

Conclusions: These findings suggested that Jab1 protein may contribute to the tumor progression and represent a novel prognostic indicator for LSCCs.

INTRODUCTION

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 the abnormal cell proliferation and may contribute to carcinogenesis. The human Jun activation domain-binding protein 1 (Jab1) 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 is also known as COP9 signalosome subunit 5 (CSN5), which is a component of the COP9 regulatory complex (1–3). This multiprotein complex can be involved in modulating signal transduction, gene transcription, and protein stability (3, 4). p27kip1, a cyclin-dependent kinase (CDK) inhibitor, can regulate cell growth negatively by inactivating G1 stage–specific CDK-cyclin complexes, resulting in cell cycle arrest at G1 stage (5, 6). Therefore, p27kip1 protein level is increased in quiescent cells and rapidly decreases after cells are stimulated with mitogens (5). The phosphorylation of p27kip1 at T187 by the cyclin E/CDK2 complex is required for p27kip1 degradation (7, 8). The cellular abundance of p27kip1 is regulated primarily at the post-transcriptional level by the ubiquitin-proteasome pathway (9, 10). Recent studies indicated that Jab1 can interact specifically with the protein form of the CDK inhibitor p27kip1 and shuttle p27kip1 from the nucleus to the cytoplasm and to decrease the amount of p27kip1 in the cell by accelerating p27kip1 degradation via the ubiquitin-proteasome system (10, 11).

The Jab1 expression in human tumor was firstly investigated by our group, demonstrating that Jab1 expression was inversely associated with p27kip1 level and correlated with poorer overall survival of epithelial ovarian cancer (12). Subsequently, Jab1 expression has also been examined in the other kinds of tumors, including breast, pancreatic, and oral squamous cell carcinomas etc. (13–20). It is reported that Jab1 protein was mainly expressed in the invasive breast cancer (13, 14). Korbonits et al. (19) found that Jab1 was significantly associated with cervical lymph node metastasis and poor prognosis in oral squamous cell carcinoma. However, to the best of our knowledge, Jab1 expression has not been investigated in laryngeal squamous cell carcinoma (LSCC) thus far, which remains one of the most common tumors of the head and neck and the second most common respiratory cancer after lung cancer worldwide (21). The previous studies of us and others have found that p27kip1 expression was significantly associated with cell proliferation and prognosis of LSCCs (22, 23). Therefore, we hypothesized that Jab1 protein, as a negative regulator of p27kip1 protein, may play a role in the carcinogenesis of LSCCs. To gain better insight into the clinical significance of Jab1, we investigated Jab1 expression...
immunohistochemically in 102 LSCCs and assessed whether Jab1 expression is correlated with p27kip1 protein level, and whether Jab1 is associated with clinicopathologic variables and prognosis of LSCCs.

MATERIALS AND METHODS

Cell Cultures. The human laryngeal carcinoma HEp-2 cell line and NIH3T3 fibroblast cell line were maintained in Eagle’s MEM and DMEM, respectively. Both media were supplemented with 10% heat-inactivated FCS, penicillin (50 units/mL), and streptomycin (50 μg/mL). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

Immunofluorescence Histochemistry in HEp-2 Cell Line. In brief, the cultured cells were spread on a poly-d-lysine slide (Sigma, St. Louis, MO), rinsed with PBS, fixed with 4% formaldehyde for 30 minutes, washed thrice in PBS, and then permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. After washing in PBS, the cells were incubated with 10% normal goat serum (Cappel/ICN Biomedicals, Irvine, CA) for 20 minutes at room temperature. Anti-Jab1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:200 in PBS), anti-p27kip1 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:200 in PBS), anti-p27kip1 mouse monoclonal antibody (Transduction Laboratories, Lexington, KY, 1:200 in PBS), and anti-proliferating cell nuclear antigen (PCNA; PC10) mouse monoclonal antibody (Santa Cruz Biotechnology, 1:400 in PBS) were used as primary antibodies. The samples were incubated with primary antibodies for 1 hour at room temperature. For each case, a corresponding section was incubated with nonimmune goat serum as a negative control. After three washes in PBS, the sections were incubated with mixture of Alexa Fluor 594-conjugated anti-rabbit goat immunoglobulin G (Molecular Probes, Inc., Eugene, OR, 1:500) and FITC-conjugated anti-mouse IgG1 (Santa Cruz Biotechnology, 1:400 in PBS) and anti-p27kip1 mouse monoclonal antibody (Molecular Probes) was used as a nuclear marker, then examined by fluorescence microscope (Nikon, Tokyo, Japan). Microscopic data were analyzed by Meta Imaging series 4.6 System (Universal Imaging Co., West Chester, PA).

Protein Extraction and Western Blot in HEp-2 Cell Lines. The HEp-2 and NIH3T3 cells were harvested into a lysis buffer [1% v/v NP40, 150 mmol/L NaCl, 50 mmol/L NaF, 20 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 1 mmol/L Na3VO4, 10 μmol/L Na2MnO4, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 1% w/v aprotinin]. Cells were then disrupted by sonication, after which they were centrifuged to separate particular from soluble fractions. The amount of protein in each fraction was determined using a protein assay kit based on the Bradford (Bio-Rad Laboratories, Tokyo, Japan). The proteins were detected by an enhanced chemiluminescence system (Amersham).

Patients. For this retrospective study, 102 patients were chosen from a total of 458 patients who underwent surgery for primary LSCCs at the Department of Otolaryngology, the First Affiliated Hospital of China Medical University between 1988 and 1993. The patients had to fulfill the following inclusion criteria: no history of previous malignancies, primary squamous cell carcinoma of the larynx only, no previous radiotherapy or chemotherapy, and operation done by partial or total laryngectomy. The lymph nodes were confirmed as metastasis by means of conventional postoperative pathologic examination.

The main clinical and pathologic variables of the patients are shown in Table 1. Eighty-six patients were male and 16 were female; their ages ranged from 38 to 89 years (mean = 63.49 years, SD = 10.90). According to the Union International Contre Cancer Tumor-Node-Metastasis staging system (1987), 58 cases were supraglottic and 44 were glottic carcinomas. Fourteen cases were in stage I, 19 in stage II, 47 in stage III, and 22 in stage IV. There were 35 well-differentiated (G1), 30 moderately differentiated (G2), 35 poorly differentiated (G3), and 2 undifferentiated (G4) squamous cell carcinomas. The patients with T3-T4 tumors underwent postoperative adjuvant loco-regional radiotherapy (at 50 Gy). The follow-up time was 5 years for each patient ranging from 3 to 60 months (mean = 44.85 months, SD = 22.14). During the follow-up period, all cases underwent clinical and fiberoptic laryngoscopy examination every 3 months during the first year postoperation, every 6 months during the second year, followed by yearly examination. Thirty-three patients died from LSCC (12 patients as a result of local failure, 19 patients as a result of failure in regional lymph nodes, and 2 patients as a result of distant metastasis). Thirty-four patients showed recurrence. All tumor samples were obtained by

<table>
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<tr>
<th>Table 1</th>
<th>Correlation between Jab1 expression and clinicopathologic variables</th>
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biopsy (9 cases) or surgery (93 cases) before any particular therapy and were fixed for 48 hours in 10% buffered formalin and embedded in paraffin for immunohistochemical analysis.

**Immunohistochemical Analysis.** Paraffin sections (4 μm) were deparaffinized in xylene and then rehydrated through graded alcohol. No antigen retrieval procedure was used for these antibodies. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in TBS (pH 7.4) for 30 minutes. To reduce nonspecific binding, the sections were incubated with 10% goat serum in 0.1% Tween 20 (v/v) containing TBS (TTBS) for 30 minutes at room temperature. Anti-Jab1 rabbit polyclonal antibody (Santa Cruz Biotechnology, 1:400 in TBS) was incubated at 4°C overnight. For each case, a corresponding section was incubated with nonimmune rabbit serum as a negative control. Immunostaining for p27kip1 was done as published previously (22). Immunostaining was done by the avidin-biotin peroxidase complex method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Staining was visualized with 3,3-diaminobenzidine tetrachloride. The nuclei were counterstained lightly with Mayer’s hematoxylin.

All cases were scored independently by two of the authors (Y. D. and L. S.) without knowledge of patient status. At least 20 high-power fields were chosen randomly, and 2,000 cells were counted for each case. Only cells with brown-colored nuclear staining were considered as positive. The index of Jab1 was counted as a ratio of immunoreactive positive cells to the total number of cells counted. The percentage of Jab1-positive tumor cells ranged from 12% to 90% (mean ± SD, 47.7 ± 15.4%; median, 51%). Based on the distribution of data and for the purposes of a more detailed statistical analysis, we used 50% as a cutoff value to define Jab1 overexpression. This cutoff value was also used in the previous report (13). The cutoff value of p27kip1 was used as 10% according to our previous report (22).

**Statistical Analysis.** The associations among Jab1, p27kip1, PCNA, and clinicopathologic variables were assessed using the Pearson χ² test and Fisher’s exact test. Multiple simultaneous comparisons were made, whichever was appropriate. Associations were always considered as binomial variables, and all Ps were two sided. Spearman’s rank correlation was used to determine whether there was a positive or negative correlation among Jab1, p27kip1, and PCNA expression. Survival analysis was undertaken using the Kaplan-Meier method and curves were compared using log-rank test. Multivariate analysis, using the Cox’s proportional hazards model, was applied to identify which factors were independent indicators of patients’ prognosis. Statistical significance was defined as P < 0.05. All statistical analyses were done using JMP software (SAS Institute, Inc., Cary, NC).

![Fig. 1 A. nuclei of HEp-2 cells were stained with DAPI (a and e). The nuclear expression of Jab1, p27kip1, and PCNA was observed in HEp-2 cells (b, c, f, and g). Colocalization of Jab1 with p27kip1 and colocalization of Jab1 with PCNA were observed by merging (yellow; d and h). Bar, 10 μm. B. Western blot analysis of Jab1, p27kip1, and PCNA protein in HEp-2 cells.](image-url)

**Table 2** Correlation among Jab1, p27, and PCNA expression in LSCCs

<table>
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<tr>
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<th>Jab1</th>
<th>p27</th>
<th>PCNA</th>
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<tr>
<td>+</td>
<td>15</td>
<td>37</td>
<td>38</td>
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<tr>
<td>-</td>
<td>33</td>
<td>17</td>
<td>27</td>
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<tr>
<td>ρ*</td>
<td>0.372</td>
<td>0.0001</td>
<td>0.198</td>
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<tr>
<td>P</td>
<td>0.0057</td>
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*ρ, Spearman correlation coefficient.

p27 data are from refs. 22 and 24.

PCNA data are from refs. 22 and 24.
RESULTS

**Jab1 Expression and its Correlation with p27kip1 and Cell Proliferation in HEp-2 cells.** Immunofluorescence double staining has been done in HEp-2 cells using anti-Jab1, anti-p27kip1, and anti-PCNA antibodies. We found that Jab1 protein is localized in the nuclei of HEp-2 cells. Interestingly, cells with Jab1 expression are usually negative for p27kip1 expression. In turn, cells with p27kip1 expression are usually negative for Jab1 expression. A few cases with colocalization of Jab1 and p27kip1 were found by merging (yellow; Fig. 1A:a-d). However, all of the cells with Jab1 expression are closely associated with the expression of PCNA identified by merging (yellow; Fig. 1A:e-h).

To further confirm the specificity of the immunohistochemical result, the Western blot analysis has been carried out in HEp-2 and NIH3T3 cells which used as positive control. The expression of Jab1, PCNA, and p27kip1 was examined by Western blot analysis, and each blot showed a single specific band at the right molecular weight in both HEp-2 and NIH3T3 cells (Jab1: 38 kDa, PCNA: 36 kDa, p27kip1: 27 kDa, β-actin: 43 kDa; Fig. 1B).

**Jab1 Expression and its Correlation with p27kip1, PCNA, and Clinicopathologic Variables in LSCCs.** Jab1 expression was rarely detected in normal epithelium adjacent to the LSCC in contrast with positive p27kip1 expression (Fig. 2A and E).

**Fig. 2** The expression of Jab1 and p27kip1 in LSCCs. Normal epithelium showed negative staining of Jab1 (a) and positive nuclear staining of p27kip1 (c). Increased Jab1 expression was accompanied with decreased p27kip1 expression in the representative LSCCs (b-d and f-h). Bar, 20 μm.

**Fig. 3** Disease-free survival curves (A) and overall survival curves (B) according to Jab1 expression. Disease-free survival curves (C) and overall survival curves (D) according to Jab1/p27kip1 expression.
Jab1 expression was predominantly localized in the nuclei of carcinoma cells of LSCCs. Fifty-one percent (52 of 102) of the tumors were shown an overexpression pattern for Jab1 protein (Fig. 2B-D). Jab1 negative section tended to be p27kip1 positive (Fig. 2B and F), and Jab1 positive section tended to be p27kip1 negative (Fig. 2D and H). There were certain numbers of intermediate cases as well (Fig. 2C and G). The expression of p27kip1 and PCNA in LSCCs has been reported by our previous studies (data are from refs. 22, 24, respectively; Table 2). The correlation between Jab1 and p27kip1 or PCNA expression was investigated by Spearman’s rank correlation. The results show that Jab1 expression was negatively associated with p27kip1 expression (Spearman’s $\rho = -0.372$, $P = 0.0001$; Table 2; Fig. 2B-D and F-H) and was positively associated with PCNA expression (Spearman’s $\rho = 0.198$, $P = 0.0457$; Table 2).

In addition, we evaluated the association of Jab1 expression with clinicopathologic variables. Jab1 overexpression was significantly associated with tumor size ($P < 0.001$), lymph node metastasis ($P = 0.005$), and advanced stage.

**Fig. 4** Disease-free survival curves (A) and overall survival curves (B) according to lymph node status. Disease-free survival curves (C) and overall survival curves (D) according to Jab1/lymph node status. Disease-free survival curves (E) and overall survival curves (F) according to Jab1/p27kip1/lymph node status.
(P = 0.001), but it is not associated with patients’ age (P = 0.981), sex (P = 0.529), tumor site (P = 0.076), and tumor grade (P = 0.639; Table 1).

**Correlation between Jab1 Expression and Patients’ Survival.** By using the Kaplan-Meier analysis, patients with Jab1 overexpression are significantly associated with short disease-free and overall survival (P = 0.0036 and P = 0.0001, respectively; Fig. 3A and B). Our previous study indicated that p27kip1 is an independent prognostic factor in LSCC (22); thus, the combined phenotypes of two proteins were analyzed subsequently. Patients with the phenotype of Jab1 (+)/p27kip1(—) revealed poorer disease-free and overall survival than others (P = 0.0008 and P < 0.0001, respectively; Fig. 3C and D). It is reported that lymph node metastasis is an important prognostic factor in LSCC (25–27); thus, we investigated the correlation between lymph node metastasis and patients prognosis in LSCC. The results showed that lymph node metastasis is significantly associated with short disease-free and overall survival (P = 0.0003 and P = 0.0002, respectively; Fig. 4A and B) and is an independent prognostic factor for disease-free and overall survival by multivariate analysis (P = 0.041 and P = 0.027; Table 3). Therefore, a subset analysis was done by combining Jab1 expression with the lymph node status. The results revealed that patients with the phenotype of Jab1(+)/lymph node(+) have poorer disease-free and overall survival than others (P < 0.0001 and P < 0.0001, respectively; Fig. 4C and D). Furthermore, patients with the phenotype of Jab1(+)/p27kip1(—)/lymph node(+) have the worst disease-free and overall survival. The 5-year disease-free survival rate for patients with the phenotype of Jab1(+)/p27kip1(—)/lymph node(+) was 29% compared with others (65%) and the 5-year overall survival rate were 29% compared with others (75%; Fig. 4E and F, P < 0.0001 and P < 0.0001, respectively). Multivariate analysis using the Cox’s proportional hazards model showed that Jab1 protein is an independent prognostic indicator for patients’ overall survival (P = 0.013; Table 3).

**DISCUSSION**

In the current study, we investigated Jab1 expression both in HEP-2 cells and in a series of 102 LSCC tissues. Immunostaining of Jab1 was mainly localized in the nuclei of HEP-2 cells and LSCCs, and Jab1 expression was negatively associated with p27kip1 expression and positively associated with cell proliferation identified by PCNA expression, a proliferating cell marker, expressed specifically in the cell nucleus from late G1 to S phase. These findings in LSCCs are consistent with our previous study in epithelial ovarian cancer and the studies in other cancers (12, 13, 17, 18). The study of Clare et al. (1) by immunofluorescence analysis indicated that Jab1 is a nuclear protein, and it seems to interact with p27kip1 in the nucleus, where fusion analysis with a nuclear export signal showed that cytoplasmic transportation per se was not sufficient for p27kip1 to be degraded (10). A recent study (28) reported that colocalization of p27kip1 and Jab1 in the nuclei of mature ganglion cells was observed. The researcher explained that, for unknown reasons, Jab1 may be inactive and not bound to p27kip1 in these cells. In the current study, colocalization of Jab1 and p27kip1 was also observed occasionally by merging image in HEP-2 cells. This fact can be explained by the above opinion. Additionally, we hypothesize that these cells labelled for both Jab1 and p27kip1 may reflect to the status of Jab1-binding to p27kip1 in the nuclei. Then, the complex begins to migrate from the nuclei to the cytoplasm, where p27kip1 will be degraded subsequently by ubiquitin/proteasome pathway.

Advances in the cell cycle study have revealed that interaction among cyclins, CDKs, and CDK inhibitors play an elemental part in cell cycle progression. As one of the negative regulators of the cell cycle, p27kip1 protein is a new class of tumor suppressor, which inhibits cyclin-CDK in a dosage-dependent manner to control cell cycle progression (29, 30). Proteins whose activity is required only at certain stages of the cell cycle can be inactivated by selective protein turnover, and target proteins are often flagged for destruction by phosphorylation. Recent studies indicated that p27kip1 is a substrate of the ubiquitin/proteasome system, and its breakdown depends on the phosphorylation status (7, 9, 31). Some studies indicated that p27kip1 mRNA levels do not fluctuate during the cell cycle and p27kip1 protein levels are regulated at the translational and post-translational levels by the ubiquitin/proteasome pathway (8, 9, 32–35). Jab1 has been implicated in promoting cell proliferation by facilitating relocation of p27kip1 from the nucleus to the cytoplasm, thereby accelerating the degradation of p27kip1 by the ubiquitin/proteasome pathway (11). It is reported that p27kip1 levels can be significantly reduced after Jab1 gene transfer into the cells of breast cancer (13). Jab1 may promote the tumor progression by activating AP-1 transcriptional proteins, and the AP-1 complex may promote cellular

**Table 3** Multivariate Cox model analysis of disease-free survival and overall survival

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<th>Characteristics</th>
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<th>Overall survival</th>
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<td></td>
<td>Hazard ratio</td>
<td>95% Confidence interval</td>
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<td>Age (≥60/≤60)</td>
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<td>0.70-1.31</td>
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<tr>
<td>Sex (male/female)</td>
<td>0.76</td>
<td>0.53-1.14</td>
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<td>Tumor site (supraglottic/glottic)</td>
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<tr>
<td>Tumor grade (G3,G4/G1,G2)</td>
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<td>0.95-1.77</td>
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<td>Tumor size (T3,T4/T1,T2)</td>
<td>1.44</td>
<td>0.99-2.17</td>
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<td>Lymph node (+/-)</td>
<td>1.43</td>
<td>1.01-2.05</td>
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<tr>
<td>Jab1 (+50%≤50%)</td>
<td>1.31</td>
<td>0.93-1.88</td>
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invasion, thereby validating its components as targets for diagnosis or therapy (36–38). The previous studies suggested that loss of p27kip1 expression is associated with proliferation activity and aggressiveness of cancer (22, 39–42). A recent study found that Jab1 overexpression is significantly associated with tumor progression in oral squamous cell carcinoma (16). The proliferative activity of tumor cells is correlated with progression and prognosis of LSCCs (24, 43). In the current study, 51% LSCCs showed Jab1 overexpression and was significantly associated with the proliferation of tumor cells, tumor size, lymph node metastasis, and advanced stage. Therefore, these results suggest that Jab1 protein, as a negative regulator of p27kip1, might contribute to the progression of LSCCs.

With regard to the possible role of Jab1 protein on tumor patients’ prognosis, it is still unclear thus far. Currently, prognostic evaluation is mainly based on the traditional method including the clinical stage, tumor site, and histopathologic grade. Recent studies have suggested that other factors, such as molecular and cellular characteristics of the primary tumors, may improve our ability to prognosticate (44). Sui et al. (12) first reported that Jab1 overexpression is correlated with poor overall survival of epithelial ovarian cancer. In one study of breast cancer, the 5-year overall survival for patients with Jab1 positive was 69% compared with 100% for patients with Jab1 negative (13). Also, Jab1 overexpression was associated with poor prognosis of oral squamous cell carcinoma by a recent report (16). In our study, patients with Jab1 overexpression are significantly associated with short disease-free and overall survival. The 5-year overall survival for patients with Jab1 positive was 48% compared with 84% for patients with Jab1 negative, and multivariate analysis showed that Jab1 protein is an independent prognostic indicator for overall survival. When Jab1 and p27kip1 were combined, patients with the phenotype of Jab1(+)/p27kip1(−) showed poor disease-free and overall survival. The previous studies indicated that lymph node metastasis can be used as a prognostic factor of LSCC (25–27), which is supported by the present study. Therefore, a subset combined analysis has been done by Jab1 expression and lymph node status. The results revealed that patients with Jab1(+)/lymph node(+) were significantly associated with poor disease-free and overall survival. More interestingly, patients with the phenotype of Jab1(+)/p27kip1(−)/lymph node(+) showed the worst prognosis. The 5-year overall survival for patients with Jab1(+)/p27kip1(−)/lymph node (+) was 29% compared with others (75%). Therefore, the evaluation of Jab1 protein, alone and/or combined with p27kip1 as well as lymph node metastasis, may further provide new information for patients’ prognosis. Also, such information could provide better planning of appropriate treatment strategies, especially for the determination of the neck dissection as well as for the better management after a surgery.

In conclusion, we showed large variations of Jab1 expression and showed a close correlation with p27kip1 and cell proliferation in LSCCs. Jab1, as a negative regulator of p27kip1, was associated with the progression and prognosis of LSCCs. If our results are confirmed in a larger study, Jab1 protein may provide a novel target for experimental therapies in human tumors.

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