Reduction of p12^{DOC-1} Expression Is a Negative Prognostic Indicator in Patients with Surgically Resected Oral Squamous Cell Carcinoma

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ABSTRACT

**Purpose:** p12^{DOC-1} is a growth suppressor that negatively regulates cyclin-dependent kinase 2 (CDK2) activities. Expression of p12^{DOC-1} is reduced and/or lost in tumor tissues. The purpose of this study is to correlate in vivo the expression of p12^{DOC-1} in oral cancer tissues by immunohistochemistry with clinical and pathological parameters.

**Experimental Design:** Twenty-five cases of normal oral mucosa and 127 cases of oral squamous cell carcinomas were evaluated. Patients’ charts were reviewed for clinical, pathological, and 10-year survival data. Because p12^{DOC-1} is a growth suppressor and associates with CDK2, parallel immunostaining was done for proliferating cell nuclear antigen and CDK2 to evaluate cell proliferation and potential correlation with CDK2.

**Results:** Our results showed that strong p12^{DOC-1} staining was uniformly seen in normal oral mucosa. p12^{DOC-1} staining was reduced or absent in 81 cases (63.8%) of oral squamous cell carcinomas. Decreased p12^{DOC-1} staining (<25% of cells stained) correlated with tumor mode of invasion (P = 0.001) and higher proliferating cell nuclear antigen (P = 0.0028) and CDK2 (P = 0.0020) expression.

**Conclusions:** These results allow us to conclude that reduction of p12^{DOC-1} protein expression is a frequent event in oral cancers. Intratumor immunohistochemical evaluation of p12^{DOC-1} expression can be an adjunctive prognostic indicator for patients with oral cancer.

INTRODUCTION

Abnormalities in various components of the cell cycle-regulatory machinery have been found in various types of human cancer including head and neck/oral cancer. The cell cycle is governed by CDKs, the activities of which are regulated by binding with the cyclins (1, 2), while negatively regulated by CKIs (1). The CDKs integrate mitogenic and growth inhibitory signals and coordinate cell cycle transitions (3). Passage from G1 into S phase is regulated by the activities of cyclin D-, cyclin E-, and cyclin A-associated kinases. B-type cyclin-associated kinases regulate G2-M transition. Two families of CKIs have been identified. INK4 family members (p15\textsuperscript{INK4b}, p16\textsuperscript{INK4a}, p18, and p19) bind cyclin D-dependent kinases, specifically CDK4 and CDK6. Universal kinase inhibitor protein family members, including p21\textsuperscript{WAF1/CIP1/CAP20}, p27\textsuperscript{Kip1}, and p57\textsuperscript{Kip2}, bind and inhibit their target CDKs. The cyclins, CDKs, and CKIs are frequently altered in cancer or disrupted secondarily by oncogenic events (4). In head and neck/oral cancer, cyclin D1 is frequently amplified and overexpressed (5, 6). In addition, altered expression of p21\textsuperscript{WAF1/CIP1/CAP20}, p15, and p16\textsuperscript{INK4a} has been reported (7–10).

p12^{DOC-1} is a growth suppressor identified and cloned from the Syrian hamster oral cancer model using a subtractive hybridization-based approach (11). It is a highly conserved cellular gene. The full-length human and mouse p12^{DOC-1} cDNAs are 1.6 kb and 1.2 kb, respectively. The identity of human and rodent p12^{DOC-1} polypeptides is 97%, whereas the mouse and hamster p12^{DOC-1} protein sequences are identical. Ectopic expression of doc-1 gene in keratinocytes is associated with growth suppression and increased doubling time (11). The human DOC-1 cDNA was cloned recently (12), and the gene has been mapped to chromosome 12q24 and partially characterized. The full-length human DOC-1 cDNA is 1634 bp in length encoding 115 amino acids (M, 12,400; isoelectric point, 9.62). We have shown that p12^{DOC-1} is a specific CDK2-associated...

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The abbreviations used are: CDK, cyclin-dependent kinase; CKI, CDK inhibitor; PCNA, proliferating cell nuclear antigen.
protein that negatively regulates CDK2 activities by sequestering the monomeric pool of CDK2, effectively reducing intracellular active CDK2 (13). Although the p21\textsuperscript{NAP1/LIP/CAP30} family of CDK inhibitors is universal for all of the CDKs and the p16\textsuperscript{INK4a} family is specific for CDK4 and CDK6, p12\textsuperscript{DOC-1} is a specific CDK2-associated protein that suppresses CDK2 activities (13). The present study uses immunohistochemistry to evaluate the clinical behavior of the p12\textsuperscript{DOC-1} protein in normal and tumor oral mucosal tissues. Western blot analysis was used to evaluate levels of p12\textsuperscript{DOC-1} on a subset of paired normal and tumor oral mucosa. We also examined the associations between the intratumor immunohistochemical findings and specific clinical and pathological parameters, cell proliferation, and 10-year patient survival status.

**MATERIALS AND METHODS**

**Patients.** A total of 127 cases of oral squamous cell carcinomas were examined. These patients underwent surgery at the Department of Oral and Maxillofacial Surgery II, Okayama University Dental School (Okayama, Japan) from April 1989 to May 1998, and clinical follow-up data were available. All of the patients received no prior therapy, such as chemotherapy or radiation therapy, before surgery. Surgery is the first treatment of choice at Okayama University Dental School. The clinical course of patients was followed until death or the end of 1998. The tissues, obtained at the time of biopsy, were formalin-fixed and paraffin-embedded for the immunohistochemical study. The patients were staged according to the Tumor-Node-Metastasis (TNM) system (14). The grade of tumor differentiation was determined according to the criteria proposed by the WHO. Mode of invasion was classified according to Jakobsson’s classification (15). The follow-up range is from 10 months to 120 months (10 years). Median follow-up time is 55.7 months. Normal oral mucosal tissue came from tumor-free normal donors consenting to the use of tissues for this study.

**Antibodies.** The polyclonal antisera (p12\textsuperscript{DOC-1}/Ab3), raised against GST-p12\textsuperscript{DOC-1}, were used for immunohistochemical studies. The polyclonal antisera to this protein were generated by Research Genetics, Inc. (Huntingville, AL). Antibody used for PCNA and CDK2 immunodetection was obtained from DAKO (Carpinteria, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

**Immunohistochemistry.** Four-μm sections were cut from the specimens, deparaffinized in xylene, and rehydrated in graded alcohol. Endogenous peroxidase was blocked by incubating tissue sections in 0.1% hydrogen peroxide in absolute methanol for 20 min. For p12\textsuperscript{DOC-1} staining, we have found that heat treatment optimizes the detection of p12\textsuperscript{DOC-1} using the p12\textsuperscript{DOC-1}/Ab3 antibody. Before applying primary antibodies, sections were heated in an autoclave for 15 min in a citrate buffer (pH 6.0). The p12\textsuperscript{DOC-1}/Ab3 antibody was used at concentration of 2 μg/ml, and the incubation time was 16 h at 4°C. Monoclonal antibody to PCNA and CDK2 were used at a 1:100 dilution, and the incubation time was 16 h at 4°C. Immunodetection was performed using the Envision system (DAKO). Peroxidase activity was visualized by applying diaminobenzidine chromogen, containing 0.05% hydrogen peroxidase. The sections were then counterstained with methyl green, dehydrated, and mounted. Negative control staining was carried out by substituting nonimmune mouse or rabbit serum for primary antibodies. In addition, for p12\textsuperscript{DOC-1} detection, a GST-p12\textsuperscript{DOC-1} fusion protein is routinely used to immunodeplete the p12\textsuperscript{DOC-1} antibody as a control. Monolayers of the human keratinocyte cell line HaCaT, grown on glass slides, were used as a positive control in each experiment.

**Immunohistochemical Assessment.** All of the immunostaining was evaluated in a coded manner without knowledge of the clinical and pathological parameters. The degree of p12\textsuperscript{DOC-1}, PCNA, and CDK2 staining was scored independently by two investigators (S. S. and M. M.). The agreement rate between the two examiners was 97.6% (124 of 127). The newly produced polyclonal antibody raised against GST-p12\textsuperscript{DOC-1} was used in these studies (p12\textsuperscript{DOC-1}/Ab3). This p12\textsuperscript{DOC-1} antibody predominantly stained the nuclei of p12\textsuperscript{DOC-1}-producing cells. For each section, 10 high-power fields were randomly chosen, and a total of at least 1000 epithelial cells were evaluated. The results were expressed as the percentage of cells that stained for p12\textsuperscript{DOC-1}. To evaluate reproducibility, 25% of the slides were chosen randomly and scored twice. The p12\textsuperscript{DOC-1} staining in these samples showed a wide range of expression levels, from 0–80%. p12\textsuperscript{DOC-1} staining was predominantly nuclear and variable in intensity. Although all of the normal oral mucosal showed p12\textsuperscript{DOC-1} staining, malignant epithelium showed weak to undetectable p12\textsuperscript{DOC-1} staining; often less than 25% of the tumor cells were stained. By this distribution analysis, we assigned a tissue (normal or tumor) to be p12\textsuperscript{DOC-1}-positive or -negative at the 25% epithelial cell-staining level. We considered samples positive if more than 25% of the cells had moderate or strong staining. To assess the PCNA and CDK2 staining, the “labeling index” was calculated as the ratio of the number of PCNA- or CDK2-positive cells to the 1000 total cells examined, expressed as a percentage.

**Western Blot Analyses.** Protein was extracted from the tissue and subjected to Western blot analysis with the p12\textsuperscript{DOC-1} polyclonal antibody (p12\textsuperscript{DOC-1}/Ab3). Tissues were snap-frozen in liquid nitrogen and stored at −80°C. The samples were homogenized in lysis buffer [150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 2% SDS, and 50 mM Tris-HCl (pH 8.0)] with protease inhibitors (50 mM sodium fluoride, 200 μM sodium orthovanadate, 2 μg/ml of aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μM clast-lactacycin β-lactone) and boiled for 5 min in Laemmli-loading buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.2% DT T, 20% glycerol, and 0.001% bromphenol blue]. Protein concentration was measured with the Bio-Rad DC Kit (Bio-Rad, Hercules, CA). Fifty μg of protein from each sample was subjected to electrophoresis in a SDS-polyacrylamide gel (15%) and then transferred to polyvinylidene difluoride membrane (Bio-Rad). The membrane was incubated with the anti-p12\textsuperscript{DOC-1} antibody at a dilution of 1:1000. Horseradish peroxidase-conjugated rabbit antimouse antibody was used as the secondary antibody. Signals were visualized using the enhanced chemiluminescence nonradioactive method according to the manufacturer’s instruction (Amer sham Pharmacia Biotech, Piscataway, NJ). The Oct-1 antibody to assess the effectiveness of nuclear/cytoplasmic separation of the human diploid keratinocyte HaCaT cells was obtained from Santa Cruz Biotechnology (sc-232).
Statistics. Associations between p12^DOC-1^ expression and other clinicopathological measures were calculated using contingency table methods and tested for significance using Pearson’s \( \chi^2 \) test. Survival curves were calculated using the Kaplan-Meier method and the log-rank test. Univariate associations between clinicopathological parameters and post-treatment patient survival were evaluated using Cox proportional hazards regression. Statistical significance was considered at the value of \( P < 0.05 \).

RESULTS

Clinical and Pathological Cohort Data. The characteristics of the 127 patients included in this study are shown in Table 1. There were 82 males (64.6%) and 45 females (35.4%), with an overall mean age of 66.6 years (range, 23–93 years). Fifty-two patients (40.9%) were classified as WHO grade I, 44 (34.6%) were classified as grade II, and 31 (24.4%) were classified grade III. Clinical stages were classified according to Tumor-Node-Metastasis system (14). There were 17 cases (13.4%) in T1, 62 cases (48.8%) in T2, 15 cases (11.8%) in T3, and 33 cases (26.0%) in T4. As for the N stages, 91 cases (71.7%) were in N0, 10 cases (7.9%) in N1, 25 cases (19.7%) in N2, and 1 case (0.8%) in N3. There were 17 cases (13.4%) of stage I, 45 cases (35.4%) in stage II, 20 cases (15.7%) in stage III, and 45 cases (35.4%) in stage IV. The follow-up range is from 10 months to 120 months (10 years). Median follow-up time is 55.7 months. Normal oral mucosa tissue came from tumor-free normal donors consenting to the use of tissues for this study.

Western Blot Analysis of p12^DOC-1^ Expression in Normal and Malignant Oral Epithelium. Direct comparison of p12^DOC-1^ expression in normal oral mucosa and corresponding tumor tissue was possible in four paired cases (Fig. 1).

Table 1  p12^DOC-1^ expression and clinicopathological parameters

<table>
<thead>
<tr>
<th>Total</th>
<th>p12^DOC-1^-positive</th>
<th>p12^DOC-1^-negative</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>35</td>
<td>11 (31.4%)</td>
<td>24 (68.6%)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>92</td>
<td>35 (38.0%)</td>
<td>57 (62.0%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>82</td>
<td>31 (37.8%)</td>
<td>51 (62.2%)</td>
</tr>
<tr>
<td>Female</td>
<td>45</td>
<td>15 (33.3%)</td>
<td>30 (66.7%)</td>
</tr>
<tr>
<td>Clinical T stage^a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 and T2</td>
<td>79</td>
<td>28 (35.4%)</td>
<td>51 (64.6%)</td>
</tr>
<tr>
<td>&gt;T3</td>
<td>48</td>
<td>18 (37.5%)</td>
<td>30 (62.5%)</td>
</tr>
<tr>
<td>Clinical N stage^a</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>91</td>
<td>41 (45.1%)</td>
<td>50 (54.9%)</td>
</tr>
<tr>
<td>N1</td>
<td>36</td>
<td>5 (13.9%)</td>
<td>31 (86.1%)</td>
</tr>
<tr>
<td>Stage^b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I + II</td>
<td>62</td>
<td>25 (40.3%)</td>
<td>37 (59.7%)</td>
</tr>
<tr>
<td>III + IV</td>
<td>65</td>
<td>21 (32.3%)</td>
<td>44 (68.2%)</td>
</tr>
<tr>
<td>Tumor differentiation^b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade II</td>
<td>96</td>
<td>39 (40.6%)</td>
<td>57 (59.4%)</td>
</tr>
<tr>
<td>Grade III</td>
<td>31</td>
<td>7 (22.6%)</td>
<td>24 (77.4%)</td>
</tr>
<tr>
<td>Mode of invasion^c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–3</td>
<td>60</td>
<td>31 (51.7%)</td>
<td>29 (48.3%)</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>15 (22.4%)</td>
<td>52 (77.6%)</td>
</tr>
</tbody>
</table>

\(^a\) Union Internationale Contre le Cancer-TNM classification of malignant tumors (1997).
\(^b\) WHO histological typing of oral and oropharyngeal tumors (1971).
\(^c\) Jakobsson, P. A., et al., Ref. 15.
Clinical Correlates of p12DOC-1 Immunostaining. We proceeded to examine whether tumor expression of p12DOC-1 was associated with various clinical parameters that may be of prognostic value. These included mode of invasion, cervical lymph node involvement, patient age, gender, tumor grade, and stage (Table 1). There is an inverse association of p12DOC-1 expression with mode of invasion and cervical lymph node involvement. Low p12DOC-1 expression strongly associates with mode of invasion ($P = 0.001$) and cervical lymph node involvement ($P = 0.001$). However, no association is observed between p12DOC-1 and patient’s age, gender, tumor grade of differentiation, and clinical staging. For patient survival analysis, there is a positive association of p12DOC-1 staining of the tumor and patient survival after 10 years. Survival data shown in Fig. 4 showed the Kaplan-Meier survival curves for groups with high and low p12DOC-1 expression. The probabilities of the patient’s survival after 5 years were 50.5% for low p12DOC-1-staining tumors and 73.9% for high p12DOC-1-staining tumors. The difference is statistically significant ($P = 0.0214$).

Table 3 presents the univariate survival analysis of clinicopathological data and p12DOC-1 immunohistochemical staining in the 127 cases of oral cancer. T stage ($P = 0.0319$), tumor differentiation ($P = 0.0078$), mode of invasion ($P = 0.0001$), and p12DOC-1 expression ($P = 0.0323$) were correlated to clinical survival. p12DOC-1 expression is a strong independent predictor of survival ($P = 0.0323$; relative risk $= 2.333$). Interestingly, PCNA-labeling indices did not reach statistical significance when its effect on survival was examined ($P = 0.0523$).

DISCUSSION

Several recent functional studies (11–13) support the growth suppressor role of p12DOC-1. Ectopic expression of p12DOC-1 in culture cells can lead to growth suppression, arresting cells in $G_0$ of the cell cycle, and suppression of DNA replication (13). The mechanism of action for p12DOC-1 appears to be through its association with CDK2 and suppressed activity of CDK2, resulting in pRB hypophosphorylation (13), p12DOC-1 acts on the biochemical pathway of CDK2 activation at or before the association of cyclin E and A by specific association with the monomeric nonphosphorylated form CDK2 (13). The mechanism of action of p12DOC-1 is apparently different from that of p16INK4a and p21$^{WAF1/CIP1/CAP20}$ families of CDK inhibitors. In addition to inhibition of CDK2 activity, p12DOC-1 also associates with DNA polymerase-α/primase in its NH$_2$ terminus, resulting in a direct interface with DNA replication and, hence, cell proliferation (16).

Our data suggest that the majority of human oral cancers (63.8%) exhibit either a loss or significant reduction of p12DOC-1. The mechanism of the tumor-associated loss of p12DOC-1 expression is currently not clear. Thus far, we have not been able to detect any intragenic mutations of the human DOC-1 gene (12). Recent data suggest that although transcriptional activity of DOC-1 can be detected in both normal and tumor cell lines and tissues (12), p12DOC-1 protein can only be detected in normal tissues and cell lines (13), suggesting a role of post-translational regulation. We are currently examining post-translational regulatory mechanisms in the regulation of...
Alternatively, the expression of p12^DOC-1 could be cell cycle-dependent. If so, the rapidly proliferating tumor cells are likely to express lower levels of p12^DOC-1 than normal cells. This possibility is also being explored.

Our data showing the consistent reduction and/or loss of p12^DOC-1 expression in oral cancer tissues support the hypothesis that the reduction/loss of p12^DOC-1 may contribute to carcinogenesis. This is a large case study examining the expression of human p12^DOC-1 in resected human oral cancers. In our study, the reduction of p12^DOC-1 expression in oral cancer is 63.8%. Functional activities of cell cycle regulators are inactivated by various mechanisms during tumor development, such as point mutation in p53 and homozygous deletion or methylation of p16^ink4a. A number of tumor suppressors have been shown to be inactivated in head and neck/oral cancer (17, 18). Thus far, mutation of p53 is the most common genetic alteration in oral cancer. Somers et al. (19) and Burns et al. (20) reported 77% and 33% of head and neck/oral cancer have a mutated p53, respectively. p16^ink4a alteration in oral cancers is another frequent event. Reed et al. (21) reported over 80% whereas Pande et al. (22) reported 66% of oral cancers completely lacked p16^ink4a expression. In our study, p12^DOC-1 was expressed in all of the cases with normal epithelium, whereas 63.8% of oral cancers examined exhibited reduced p12^DOC-1 expression. These results suggest that p12^DOC-1 could be one of the commonly inactivated growth suppressors in primary oral cancers.

An inverse association was found to exist between p12^DOC-1 and levels of PCNA and CDK2 expression in the

Table 2  Relationship between p12^DOC-1 expression and PCNA-CDK2 labeling index in 127 cases of oral cancer tissues

<table>
<thead>
<tr>
<th>p12^DOC-1 expression</th>
<th>PCNA LI^a</th>
<th>CDK2 LI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (n = 46)</td>
<td>16.5 ± 1.7</td>
<td>5.6 ± 1.0</td>
</tr>
<tr>
<td>Negative (n = 81)</td>
<td>23.4 ± 1.4</td>
<td>9.6 ± 0.8</td>
</tr>
</tbody>
</table>

^a LI, labeling index (mean ± SE).

![Fig. 3 Immunostaining of p12^DOC-1 (A and D), CDK2 (B and E), and PCNA (C and F) in normal (A, B, and C) and malignant (D, E, and F) human oral mucosal tissues. Magnification, ×200.](image)

![Fig. 4 Kaplan-Meier curve of time of 5-year survival status (free of treatment failure) for oral cancer patients, with and without intratumor p12^DOC-1 expression level.](image)
Many parameters have been used for prediction of prognosis for oral cancer, e.g., increased PCNA immunoreactivity, usually associated with progression of malignancy of oral cancer, significantly associates with poor patient survival (24–26). Our data show that p12DOC-1 expression negatively associates with cell proliferation, lymph node metastasis, and histological mode of invasion. Our study also examines the association of p12DOC-1 expression in tumors with patient survival. In the Cox proportional hazards analysis, intratumor expression of p12DOC-1 is significantly associated with greater patient survival. Similar results are seen in the Kaplan-Meier survival analysis, suggesting that p12DOC-1 expression quantification could be an important prognostic indicator for oral cancer.

REFERENCES


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