Altered p16/MTS1/CDKN2 and cyclin D1/PRAD-1 Gene Expression Is Associated with the Prognosis of Squamous Cell Carcinoma of the Esophagus

Hiroya Takeuchi, Soji Ozawa, Nobutoshi Ando, Chih-Horng Shih, Kazuo Koyanagi, Masakazu Ueda, and Masaki Kitajima

ABSTRACT

The p16/MTS1/CDKN2 gene and the cyclin D1/PRAD-1 gene cooperatively regulate cyclin-dependent kinase 4-mediated phosphorylation of pRB in the cell cycle of normal cells. p16/CDKN2 gene and cyclin D1/PRAD-1 gene alterations have been detected in squamous cell carcinoma cell lines and in several primary squamous cell carcinomas of the esophagus. We immunohistochemically assessed p16 and cyclin D1 expression in 111 squamous cell carcinomas of the esophagus after evaluation of the antibodies against p16 and cyclin D1 protein using four squamous cell carcinoma cell lines.

Loss of p16 expression was detected in 56 of 111 cases (50%). The mean number of metastatic lymph nodes without p16 expression was significantly higher than the number of nodes with p16 expression (P = 0.04). The postoperative survival rate for patients without p16 expression was significantly lower than that of patients with p16 expression (P = 0.04). Cyclin D1 overexpression was found in 28 of the 111 cases (25%) and correlated with distant organ metastasis after curative surgery (P = 0.05). The survival rate of patients with cyclin D1 overexpression was significantly lower than that of patients without cyclin D1 overexpression (P = 0.01). A positive correlation between the loss of p16 expression and cyclin D1 overexpression was observed (P = 0.03).

The loss of p16 expression and overexpression of cyclin D1 may be useful prognostic indicators in patients with squamous cell carcinomas of the esophagus. It may be possible to select more suitable treatment for patients with squamous cell carcinomas of the esophagus by evaluating the status of p16 and cyclin D1 expression.

INTRODUCTION

Molecular oncology studies have recently focused on the relationship between tumorigenesis and deregulation of the cell cycle. Gene abnormalities in cell cycle regulators that function in the transition from G1 to S phase have been associated with tumorigenesis and the malignant potential of tumors. The p16 (MTS1/CDKN2) gene, located at the 9p21 chromosomal region, was identified as a growth suppressor gene in 1993 (1). The p16 protein controls S-phase entry from G1 phase in the cell cycle by binding to CDK4 and inhibiting CDK4-mediated phosphorylation of pRB, which may activate the E2F transcription factor (2). Homozygous deletions and point mutations of the p16/CDKN2 gene are frequently detected in various primary tumors and cell lines (3–12). Homozygous deletions of p16/CDKN2 were found in 83% of ESCC cell lines (10), and hypermethylation of the CpG island in the 5’ region and point mutations, in addition to homozygous deletions, were detected at very high levels, with no expression of the p16 protein in primary ESCCs (13).

Growth and DNA synthesis of ESCC and other cell lines were inhibited by microinjection of p16/CDKN2 DNA and by p16/CDKN2 gene delivery using a vector (14, 15), suggesting that an abnormality in p16/CDKN2 function may cause loss of control of cell growth and, thereby, tumorigenesis. Several investigations have shown an association between p16/CDKN2 gene alterations and the clinicopathological features of the tumor (16, 17). Inactivation of p16/CDKN2 has been correlated with a worsened prognosis in malignant melanoma, leukemia, pancreatic cancer, and non-small cell lung cancer (5, 6, 18–20), but the association between p16/CDKN2 alterations and the clinical characteristics and prognosis of ESCC remains unclear.

The cyclin D1/PRAD-1 gene has been mapped to 11q13, and cyclin D1 protein accelerates S-phase entry from G1 phase by activating CDK4-mediated phosphorylation of the pRB (21–23). Amplification of cyclin D1/PRAD-1 has been detected in human primary ESCCs with overexpression of cyclin D1 (24–26) and has been considered as a possible prognostic factor associated with distant organ metastasis in patients with ESCC (24, 25).

ESCC has one of the highest malignant potentials of any tumor and often has a poor outcome. Direct invasion to adjacent organs, lymph node metastasis, and distant organ metastasis
have been evaluated as useful indicators for predicting outcome in ESCC. Furthermore, the malignant potential or tumor proliferation of ESCC may be reflected by the abnormalities in p16/CDKN2 and/or cyclin D1/PRAD-1.

In addition, correlations between the abnormalities in p16/CDKN2 and RB and between the abnormalities in cyclin D1/PRAD-1 and RB have been found in ESCC (14, 26) and other tumors (20, 27-30), but the association between p16/CDKN2 and cyclin D1/PRAD-1 alterations in ESCC has not been investigated. Following the finding that p16 and cyclin D1 are an inhibitor and accelerator, respectively, of CDK4-mediated phosphorylation of pRB (31), interest has focused on the relationship between these two genes.

Alterations in the p16/CDKN2 and cyclin D1/PRAD-1 genes disrupt p16 and cyclin D1 expression, causing loss of p16 expression and overexpression of cyclin D1 (13, 17, 24). Immunohistochemistry has been successfully used to detect the gene alterations in both p16/CDKN2 and cyclin D1/PRAD-1 (20, 24, 27, 32). On the basis of these findings, we immunohistochemically detected p16 and cyclin D1 expressions in esophageal squamous cell lines and in 111 ESCCs in this study. We investigated whether aberrant p16 and cyclin D1 expressions correlated with clinicopathological factors and postoperative prognosis in patients with ESCC. In addition, the relationship between aberrant p16 and cyclin D1 expression was evaluated.

**MATERIALS AND METHODS**

**Cell Lines.** Human ESCC cell lines of the TE series (TE1, TE6, TE8, and TE9), were kindly provided by Dr. Nishihira (School of Medicine, Tohoku University, Sendai, Japan).

**Patients.** The subjects of this study were 111 patients (median age, 61 years; range, 40-83 years; 98 men and 13 women) with squamous cell carcinoma of the thoracic esophagus, who had undergone curative surgery at Keio University Hospital (Tokyo, Japan) between 1990 and 1994. TTE was performed in 84 cases, and THE was performed in 27. Pathological examination was performed according to the Guidelines for the Clinical and Pathological Studies on Carcinoma of the Esophagus of the Japanese Society for Esophageal Diseases and the tumor-node-metastasis classification (33). Patients were followed in the outpatient clinic, and diagnostic examinations, consisting of chest X-ray, computed tomography, and ultrasound, were performed every 6 months to detect recurrences. The maximum follow-up period was 73 months, and the mean observation period was 29 months.

**Materials.** Monoclonal mouse antihuman p16 antibody PMG175-405 and monoclonal mouse antihuman cyclin D1 antibody PMG124-326 were obtained from PharMingen (San Diego, CA) for immunohistochemistry and Western blotting. Nonspecific mouse IgG1 was used as a negative control reagent. The detection reaction used the DAKO A/S (Glostrup, Denmark) labeled streptavidin-biotin kit.

**Immunohistochemistry.** The cell lines and surgically resected tumor specimens were fixed in 10% formalin and embedded in paraffin by conventional techniques. Three-μm sections were freshly cut and deparaffinized in xylene.Slides that contained the most invasive lesion of the tumor were selected and treated with an antigen retrieval step in Target Unmasking Fluid purchased from PharMingen at 90°C for 10 min. The sections and cell lines were reacted with antibody PMG175-405 at 5 μg/ml at room temperature for 1 h for p16 staining and antibody PMG124-326 at 5 μg/ml at 4°C overnight for cyclin D1 staining. Negative control slides were treated with nonspecific mouse IgG1 under equivalent conditions. The DAKO labeled streptavidin-biotin kit provided the secondary reagents. Slides were developed with diaminobenzamidine and counterstained with hematoxylin.

**Western Blotting.** Cell lines were lysed, and approximately equal amounts of total cellular protein were run on 10–20% SDS-polyacrylamide gradient gels for p16 and 10% gels for cyclin D1. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom). p16 protein was detected using anti-p16 monoclonal antibody PMG175-405. Anti-cyclin D1 monoclonal antibody PMG124-326 was used to detect cyclin D1 protein. Secondary antibody incubation was with a hors eradish peroxidase conjugated to goat antimouse antibody, followed by detection with enhanced chemiluminescence reagents (Amersham). The blot was exposed to autoradiography film.

**Evaluation.** We made reference to previously described criteria for assessing immunohistochemical results (19, 20, 27, 34–36). p16 staining was considered positive if more than 80% of the tumor cells showed nuclear staining. If less than 80% of the tumor cells showed nuclear staining, the specimen was considered to be negative. A squamous cell carcinoma cell line (TE1) was used as a positive control. Inflammatory cells and stromal cells served as internal positive controls. Nonspecific mouse IgG1 was used as a negative control. For cyclin D1 staining, if strong nuclear staining was recognized in more than 10% of the tumor cells, the specimen was considered to be positive. If strong nuclear staining was seen in less than 10% of the tumor cells, the specimen was judged to be negative.
RESULTS

Statistical Analysis. Patient groups were compared using the $\chi^2$ test and Student's $t$ test. The cumulative survival rates for patient groups were calculated using the Kaplan-Meier method and compared by using the Mantel-Cox test. Life table analysis was performed using the stepwise logistic regression model.

RESULTS

p16 and Cyclin D1 Expression in Esophageal Cancer Cell Lines. Immunohistochemical staining and Western blot analysis of ESCC cell lines (TE1, TE6, TE8, and TE9) were performed with the anti-p16 antibody and the anti-cyclin D1 antibody (Table 1 and Fig. 1). p16 expression was detected only in TE1, and the other cell lines were considered to be negative for p16 by both immunohistochemical staining and Western blot. Cyclin D1 overexpression was detected in TE6, TE8, and TE9, as a $M_s$ 36,000 protein by Western blot. Positive immunohistochemical cyclin D1 staining was detected in TE6, TE8, and TE9.

p16 and Cyclin D1 Expression in Primary Human Esophageal Carcinomas. Among the 111 thoracic ESCC cases, 55 (50%) were positive for p16 by immunohistochemistry, whereas 56 (50%) were negative for p16 (Fig. 2A). There were no significant differences in clinicopathological back-
Table 2  Association between p16 expression and clinical and pathological background factors

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<td>-</td>
<td>40 (71%)</td>
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* NS, not significant.

* a Student's t test.

* b NS, not significant.

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<th>No. of cases (%)</th>
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</table>

but this difference did not reach statistical significance. The mean number of metastatic lymph nodes in TTE patients without p16 expression was significantly higher than that in TTE patients with p16 expression (P = 0.04; Table 3). The postoperative survival rate for patients without p16 expression was significantly lower than that for patients with p16 expression (P = 0.04; Fig. 3A).

Twenty-eight (25%) of the 111 thoracic ESCC cases were positive for cyclin D1 (overexpression), whereas 83 (75%) were negative for cyclin D1 (no overexpression) upon immunohistochemical analysis (Fig. 2B). Cyclin D1 overexpression did not correlate with any of the clinicopathological background factors assessed (Table 4). The rate of distant organ metastasis with cyclin D1 overexpression was higher than with normal cyclin D1 expression (P = 0.05; Table 5). The postoperative survival rate for patients with cyclin D1 overexpression was significantly lower than that for patients with normal cyclin D1 expression (P = 0.01; Fig. 3B).

**Correlation between Loss of p16 Expression and Cyclin D1 Overexpression.** Eight of the 111 cases (29%) were positive for both p16 and cyclin D1 (overexpression), 20 (71%) were negative for p16 and positive for cyclin D1, 47 (57%) were positive for p16 and negative for cyclin D1, and 36 (43%) were negative for both p16 and cyclin D1 (Table 6). More cyclin D1-positive cases were negative (n = 20) than positive (n = 8) for p16 expression. A positive correlation was observed between the loss of p16 expression and cyclin D1 overexpression (P = 0.03). The postoperative survival rate of the p16-positive/cyclin D1-negative group was significantly higher than that of the other groups (P < 0.05), except for the p16-negative/cyclin D1-negative group (P = 0.06; Fig. 3C).

The prognostic value of the loss of p16 expression and cyclin D1 overexpression was compared with that of other predictive factors, such as the pN factor, pT factor, age, histological differentiation, and vascular invasion. Life table analysis based on the stepwise logistic regression model was performed for these background factors and both p16 and cyclin D1 expression (Table 7). Cyclin D1 overexpression (P = 0.01) and the loss of p16 expression (P = 0.04) were found to be of value in predicting the outcome of ESCC.

**DISCUSSION**

Here, we immunohistochemically evaluated aberrant p16 and cyclin D1 expressions using paraffin-embedded specimens of squamous cell carcinoma of the esophagus. The loss of p16 expression and cyclin D1 overexpression had a significant impact on the outcome of ESCC.
expression correlated with lymph node metastasis, and cyclin D1 overexpression correlated with distant organ metastasis. These changes in gene expression were found to be valuable prognostic indicators for ESCC. In addition, we demonstrated a positive correlation between the loss of p16 expression and cyclin D1 overexpression.

Inactivation of the p16/CDKN2 gene is frequently recognized as a result of homozygous deletion, hypermethylation of

Table 4  Association between cyclin D1 overexpression and clinical and pathological background factors

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<td>-</td>
<td>22 (89%)</td>
<td>61 (73%)</td>
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</tbody>
</table>

⁴ NS, not significant.

According to the Guidelines for Clinical and Pathologic Studies on Carcinoma of the Esophagus of the Japanese Society for Esophageal Diseases. Lu, upper intrathoracic esophagus; Im, middle intrathoracic esophagus; Ei, lower intrathoracic esophagus.

³ PT₁, tumor invades lamina propria or submucosa; PT₂, tumor invades muscularis propria; PT₃, tumor invades adventitia.

⁵ pN₀, no regional lymph node metastasis; pN₁, regional lymph node metastasis.

Each pathological findings was judged according to the Guidelines for the Clinical and Pathologic Studies on Carcinoma of the Esophagus of the Japanese Society for Esophageal Diseases.

Fig. 3  Cumulative survival curves after curative surgery. A, patients with and without p16 expression. B, patients with and without cyclin D1 expression. C, patients with p16 and/or cyclin D1 expression. ○, p16+/cyclin D1− (n = 47); □, p16+/cyclin D1+ (n = 8); △, p16−/cyclin D1− (n = 36); ●, p16−/cyclin D1+ (n = 20).
Altered Expression of p16 and cyclin D1 in ESCC

Table 5  Incidence of distant organ recurrence after surgery

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* X2 test.  
b NS, not significant.

Table 6  Relationship between p16 and cyclin D1 expression

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<tr>
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<td>83 (100%)</td>
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* P = 0.03, X2 test.

Studies on ESCC have shown that cyclin D1 overexpression, evaluated by immunohistochemical staining, results from cyclin D1/PRAD-1 amplification (30). We made reference to previously published criteria for immunohistochemical cyclin D1 staining (40). cyclin D1/PRAD-1 amplification was previously recognized in the TE6, TE8, and TE9 cell lines (10, 14), and our results from cyclin D1 staining and Western blot correlated with those of this earlier study. In addition, we immunohistochemically examined cyclin D1 staining in 15 cases in which cyclin D1/PRAD-1 amplification had already been detected by slot blot analysis (amplification, eight cases; no amplification, seven cases; Ref. 25). Our cyclin D1 staining results coincided with cyclin D1/PRAD-1 alterations in 12 cases (data not shown). These results suggest that the antibody is useful for evaluating cyclin D1 overexpression due to gene amplification.

Shinozaki et al. (25) reported that amplification of cyclin D1/PRAD-1, evaluated by slot blot analysis, was found in 28 of 122 cases (23%) of ESCC and that cyclin D1/PRAD-1 amplification, as a prognostic factor for ESCC, correlated with distant organ metastasis. cyclin D1/PRAD-1 amplification and overexpression were detected in 38% of ESCC patients in another report (24). Here, we found cyclin D1 overexpression in 28 of the 111 patients (25%). Cyclin D1 overexpression correlated with distant organ metastasis and survival rate after surgery. These results are consistent with reports by other investigators (24, 25).

Some investigators have reported that cyclin D1/PRAD-1 expression in normal epithelium. Geradts et al. (34) reported that an antigen retrieval step was not necessary for p16 staining using the polyclonal antibody, but we achieved enhanced staining with less background using Target Unmasking Fluid (PharMingen) in an antigen retrieval step (39). Other approaches, such as the microwave and enzyme methods, were also attempted, but the Target Unmasking Fluid method was found to be superior.

In primary squamous cell carcinoma of the esophagus and of the head and neck, p16/CDKN2 alterations have been identified at various rates (39–79%; Refs. 12, 13, and 32). These results may reflect contamination with normal tissue or the heterogeneity of the malignant tissue. We detected loss of p16 expression in 56 of the 111 patients (50%) in this study, indicating the presence of various gene abnormalities, including homozygous deletion, hypermethylation, and mutation. Maesawa et al. (13) reported that homozygous deletions were recognized in 5 of 31 patients (16%) with primary ESCCs, de novo methylation was seen in 6 patients (19%), and a mutation was seen in 1 patient (3.2%).

The CpG island, and point mutation in ESCC (12, 13). Examination of p16 expression should detect all p16/CDKN2 alterations because nearly all of these gene alterations cause loss of p16 expression in ESCC (13) and other carcinomas (17, 32, 37, 38). We used an immunohistochemical assay to evaluate p16 expression because studies based on homogenized resected specimens may be affected by contamination with normal tissue (stromal cells or normal mucosal cells). In addition, the heterogeneity of malignant tissues, as exemplified by coincident cancer pearls, do not show nuclear staining in squamous cell carcinoma of the head and neck (32, 37). Geradts et al. (27) and other investigators have recommended immunohistochemical assay for detecting p16/CDKN2 alterations (17, 19, 20, 28, 34).

We used the monoclonal anti-p16 antibody that other investigators have used for immunostaining (37). In studies on ESCC cell lines, p16 expression was detected only in TE1 cells by both immunohistochemical staining and Western blot. p16/CDKN2 is reportedly the wild type in TE1, whereas homozygous deletions of p16/CDKN2 have been recognized in TE6, TE8, and TE9 (10, 14). Our findings on p16 expression coincided with the description of p16/CDKN2 alterations in these studies, and they confirm the usefulness of the monoclonal anti-p16 antibody. We defined 80% as a cutoff value for p16-positive staining because about 80% of cells in normal epithelium demonstrated nuclear staining. We recognized weak nuclear staining in normal squamous epithelial cells, other than basal cells of the epithelium and highly keratinized cells of the upper layer (data not shown). Several studies have used 100% as a cutoff for p16 staining (20, 27), but we could not use this criterion in the present study because keratinized cells, such as cancer pearls, do not show nuclear staining in squamous cell carcinoma and are thus indistinguishable, in this regard, from
amplification occurred with pRB expression in some carcinomas and in ESCC (26, 28). On the other hand, a negative correlation was found between p16/CDKN2 and RB gene alterations in cancer cell lines and primary carcinomas (17, 27–30). The same results have been reported in both ESCC cell lines and primary squamous cell carcinomas (14, 20). Here, we observed a positive correlation between the loss of p16 expression and cyclin D1 overexpression. These results are compatible with the correlations of cyclin D1 amplification with pRB expression and of p16/CDKN2 alterations with the RB abnormality. The correlations among these three cell cycle regulators are interesting with reference to ESCC oncogenesis. The mechanism underlying the correlation between the loss of p16 expression and cyclin D1 overexpression has not been identified, but these results suggest that accumulation of many kinds of gene alterations occurs during oncogenesis and tumor progression. Furthermore, the p16/CDKN2 and cyclin D1/PRAD-1 alterations may be linked because these cell cycle regulators are associated with CDK4-mediated phosphorylation of pRB.

Here, the mean number of metastatic lymph nodes associated with p16-negative tumors was significantly higher than that for p16-positive tumors. Maesawa et al. (13) reported that p16/CDKN2 alterations correlated with lymph node metastasis and advanced-stage ESCC. Similar findings have been observed in the other primary carcinomas (5, 17–20, 41). The association of p16/CDKN2 alterations with advanced-stage ESCC may be due to more invasive and aggressive proliferation of cancer cells with p16/CDKN2 alterations, as compared to p16/CDKN2 wild type. p16/CDKN2 alterations may not be directly related to the metastatic potential of a tumor. However, tumor growth, which is significantly faster in metastatic organs, may have an effect on prognosis. The same mechanism of tumor proliferation and metastasis is possible for cyclin D1/PRAD-1. Cyclin D1 overexpression due to gene amplification, which accelerates tumor growth, may be associated with distant organ metastasis.

In addition, it is also possible that the gene alterations in cell cycle regulators, i.e., p16 and cyclin D1, may have other functions that are associated with metastasis of cancer cells, which have yet to be elucidated (13). However, these clinical characteristics and metastatic potential may be accounted for by other, as yet unknown genes on 9p21 and 11q13 or may be a result of cooperative interactions among several oncopgenes and tumor suppressor genes. This issue requires further investigation.

In multivariate analysis, cyclin D1 was found to be more valuable than p16 in predicting outcome. Cyclin D1 overexpression was independent of clinicopathological factors for outcome, but the loss of p16 expression was associated with lymph node metastasis in this study. p16/CDKN2 alterations may be a prognostic factor for lymph node metastasis in ESCC. Evaluating aberrant p16 and cyclin D1 expression in endoscopic biopsy specimens may allow the selection of more suitable treatments for patients with ESCC.

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Altered Expression of pl6 and cyclin D1 in ESCC


Altered p16/MTS1/CDKN2 and cyclin D1/PRAD-1 gene expression is associated with the prognosis of squamous cell carcinoma of the esophagus.


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