p12$^{CDK2-AP1}$ is associated with tumor progression and a poor prognosis in esophageal squamous cell carcinoma

YUKIHARU HIYOSHI[1], MASAYUKI WATANABE[1], KOUTARO HIRASHIMA[1,2], RYUICHI KARASHIMA[1], NOBUTAKA SATO[1], YU IMAMURA[1], YOUHEI NAGAI[1], NAOYA YOSHIDA[1], EIICHIRO TOYAMA[1], NAOKO HAYASHI[1] and HIDEO BABA[1]

[1]Department of Gastroenterological Surgery, Graduate School of Medical Sciences, [2]Department of Surgical Pathology, Kumamoto University Hospital, 1-1-1 Honjo, Kumamoto 860-8556, Japan

Received February 10, 2009; Accepted April 2, 2009

DOI: 10.3892/or_00000403

Abstract. p12 CDK2-associating protein 1 (p12$^{CDK2-AP1}$) is a growth suppressor that negatively regulates cyclin-dependent kinase 2 (CDK2) activities. In addition, p12$^{CDK2-AP1}$ has also been shown to interfere in DNA replication. A reduction of p12$^{CDK2-AP1}$ expression is known to be a negative prognostic indicator in patients with oral squamous cell carcinoma. To elucidate the role of p12$^{CDK2-AP1}$ expression in esophageal squamous cell carcinoma (ESCC), we immunohistochemically examined the expression of p12$^{CDK2-AP1}$ protein in 120 resected ESCC specimens and determined its association with the clinicopathological characteristics and prognosis. Of the 120 ESCCs, 79 (65.8%) showed positive staining (≥25% of cancer cells showing p12$^{CDK2-AP1}$ expression), while 41 (34.2%) lacked the staining (<25% of cancer cells showing p12$^{CDK2-AP1}$ expression). Negative staining for p12$^{CDK2-AP1}$ was found to be significantly associated with advanced lesions [depth of tumor (P=0.001), lymph node metastasis (P<0.001), pathological stage (P<0.0001) and venous invasion (P<0.0001)], and a poor prognosis (disease-free survival and overall survival: log-rank P<0.05). The rate of lymph node metastasis in patients with p12$^{CDK2-AP1}$ negative-T1 ESCC was significantly higher than that in patients with p12$^{CDK2-AP1}$ positive one (P<0.05). These results suggest the down-regulation of p12$^{CDK2-AP1}$ to be related to tumor aggressiveness and a poor prognosis in patients with ESCC.

Introduction

p12 CDK2-associating protein 1 (p12$^{CDK2-AP1}$), originally named deleted in oral cancer-1 (DOC-1), therefore acts as a growth suppressor by negatively regulating the activity of cyclin-dependent kinase 2 (CDK2) (1). This gene is highly conserved and located on chromosome 12q24. The protein is a 115-aa polypeptide and is ubiquitously expressed in normal tissues (2). CDK2 activity is thought to play a key role in late G1 to S phase progression by phosphorylating and inactivating the retinoblastoma (Rb) protein. Phosphorylated and inactive Rb allows the transcription of genes under the control of E2F, which are required for DNA replication (3). Therefore, the down-regulation of p12$^{CDK2-AP1}$ is expected to result in an unregulated cell-cycle progression. In addition, p12$^{CDK2-AP1}$ has also been shown to interact with polymerase-α-primase, which is a principal polymerase in eukaryotic DNA replication (4). The interaction between p12$^{CDK2-AP1}$ and polymerase-α-primase was shown to result in direct interference in DNA replication. Recently, it has been shown that p12$^{CDK2-AP1}$ also mediates the growth suppressing signal from TGF-ß (5). In human oral squamous cell carcinomas (OSCCs), a loss or reduction of p12$^{CDK2-AP1}$ expression is associated with increased tumor invasion, lymph node metastases, and decreased survival (6). Taken together, p12$^{CDK2-AP1}$ negatively regulates cell-cycle progression and cell proliferation, and it can be a target for silencing or down-regulation in tumorigenesis. Although previous studies have demonstrated the roles of p12$^{CDK2-AP1}$ as a tumor suppressor gene in OSCC (6-8) and colorectal cancer (9,10), there have so far been few reports showing the p12$^{CDK2-AP1}$ expression in esophageal squamous cell carcinoma (ESCC).

The aims of this study were to investigate the immunohistochemical expression of p12$^{CDK2-AP1}$ in ESCC and to clarify the significance of down-regulation of this molecule in ESCC.

Materials and methods

Patients. The study included 120 patients with ESCC who underwent a curative surgical resection at Kumamoto University Hospital between January 1997 and October 2007. All of these patients underwent an esophagectomy with lymph node dissection. None of these patients underwent an endoscopic mucosal resection, palliative resection, pre-operative chemotherapy or radiotherapy, and none of them

Correspondence to: Dr Hideo Baba, Department of Gastroenterological Surgery, Graduate School of Medical Science, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
E-mail: hdobaba@kumamoto-u.ac.jp

Key words: p12, CDK2AP1, esophageal cancer

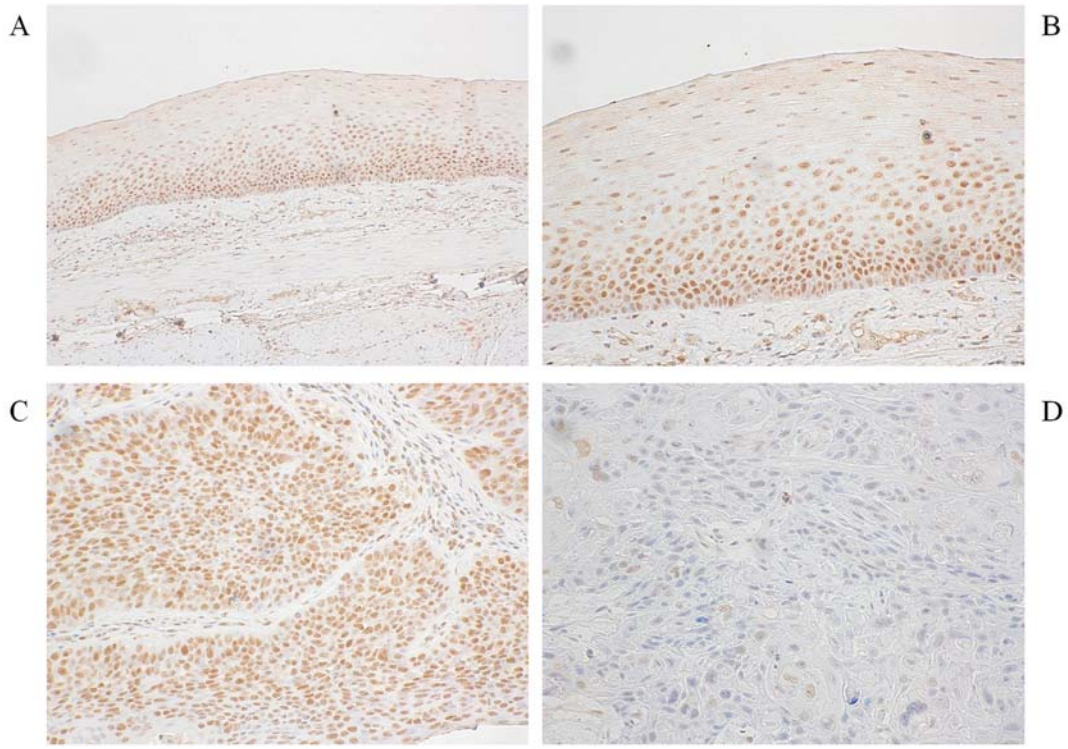

Figure 1. Immunohistochemical staining of p12$^{CDK2-AP1}$. Original magnification: x100 (A), x200 (B-D). (A and B) Expression of p12$^{CDK2-AP1}$ in normal esophageal epithelium. (C) Positive staining of p12$^{CDK2-AP1}$ of ESCC is indicated. p12$^{CDK2-AP1}$ is clearly shown in the nuclei of ESCC. (D) Negative staining of p12$^{CDK2-AP1}$ of ESCC is indicated.

had either synchronous or metachronous multiple cancers in other organs. The clinical data, including age, gender, tumor location, lymph node metastasis, T classification (11), TNM stage (11) and histological grading (12) were available for all 120 patients. Patients were periodically (every 1-3 months) examined on an outpatient basis to make sure they did not have disease recurrence. The mean follow-up period for the 120 patients was 28.6 months (range, 1-133 months). Informed consent for the research was obtained from each patient. The study design was approved by the ethics review board of the university.

Immunohistochemistry. The polyclonal rabbit anti-p12 antibody reactive with human p12$^{CDK2-AP1}$ was kindly provided by Dr Yong Kim and Dr David T.W. Wong (University of California Los Angeles, CA, USA). The antibody was diluted 1:500 with 0.1 M phosphate-buffered saline (PBS; pH 7.4). The paraffin blocks were cut into 4-μm thick sections and mounted on slides. The sections were deparaffinized in xylene and dehydrated with graded ethanol washes. The sections were then autoclaved in 10 mmol/l citrate buffer, pH 6.0, at 121˚C for 15 min. After cooling for 20 min, the sections were rinsed with distilled water. They were incubated with 3% hydrogen peroxide for 5 min at 25˚C to block endogenous peroxidase activity, rinsed with distilled water, and washed with PBS. They were incubated with anti-p12 antibody overnight at 4˚C. All sections were washed 3 times with PBS for 5 min. For linking, all sections were incubated with horseradish peroxidase-labeled polymer (Envision™ + Kit, Dako) for 60 min at 25˚C and washed 3 times with PBS for 5 min. Thereafter they were incubated with 3,3'-diaminobenzidine tetrahydrochloride, and applied as a 0.02% solution containing 0.005% H_2O_2 in 0.05 M Tris-HCl (pH 7.6) at 25˚C for 10 min. Thereafter, they were rinsed gently with distilled water and washed in flowing water for 5 min. Finally, the sections were counterstained lightly with hematoxylin, dehydrated in graded ethanol and then xylene, and mounted with cover slips. All slides were examined by light microscopy.

The samples were positive for p12$^{CDK2-AP1}$ when more than 25% of the cells at the invasive front exhibited moderate or strong staining as reported previously (6). The staining assessment was independently carried out by an experienced pathologist without any knowledge of either the clinical or survival data.

Statistical analysis. p12$^{CDK2-AP1}$ expression in ESCC was assessed to identify any association with the clinicopathological parameters using the χ^2 two-tailed test or Fisher's exact test. The disease-free survival and overall cancer-specific survival curves were constructed using the Kaplan-Meier method, and the log-rank test was used to evaluate the statistical significance of the differences. A statistical analysis was performed using the StatView™ software program, version 5.0 (SAS Institute, Cary, NC, USA). A two-sided significance level of $P<0.05$ was used for all statistical analyses.

Results

Positive staining for p12$^{CDK2-AP1}$ was observed in the nuclei near the basal layer in the normal esophageal epithelium (Fig. 1A and B). Of the 120 ESCCs, 79 (65.8%) showed positive staining, while 41 (34.2%) lacked any staining (Fig. 1C and D).

Table I. Relationship between the p12$^{CDK2-AP1}$ expression and clinicopathological characteristics in 120 patients with ESCC.

Parameters	Total (n=120)	p12$^{CDK2-AP1}$ expression		P-value
		Negative (n=41)	Positive (n=79)	
Age				
≤65	64	19	45	0.36
>65	56	22	34	
Gender				
Male	105	34	71	0.42
Female	15	7	8	
Location				
Upper	18	7	11	0.098
Middle	57	14	43	
Lower	45	20	25	
Histological grading				
Well	50	18	32	0.87
Mod-por	70	23	47	
T classification				
Tis-T1	70	15	55	0.0010
T2-T3	50	26	24	
N status				
N0	66	13	53	0.00046
N1	54	28	26	
Stage				
0-I	50	6	44	<0.0001
II-III	70	35	35	
Lymph nodes				
Negative	95	30	65	0.35
Positive	25	11	14	
Venous invasion				
Negative	59	9	50	<0.0001
Positive	61	32	29	

N status indicates lymph node metastasis status. P-values were calculated using the χ^2 test.

The relationships between p12$^{CDK2-AP1}$ expression and clinicopathological characteristics in 120 patients with ESCC are summarized in Table I. The expression of p12$^{CDK2-AP1}$ was significantly correlated to T classification (P=0.001), lymph node metastasis (P<0.001), TNM stage (P<0.0001) and venous invasion (P<0.0001). On the other hand, no significant relationships were observed between p12$^{CDK2-AP1}$ expression and age, gender, tumor location, histological grading and lymphatic invasion. Table II shows the relationships between p12$^{CDK2-AP1}$ expression and lymph node metastasis in 65 patients with T1 stage ESCC. The rate of lymph node metastasis in patients with p12$^{CDK2-AP1}$ negative-T1 stage ESCC was significantly higher than that in patients with p12$^{CDK2-AP1}$ positive one (60 vs. 22%, P=0.013). In addition, a similar significant association was also seen in patients with T1sm (limited to the submucosa) ESCC. The rate of lymph node metastasis in patients with p12$^{CDK2-AP1}$ negative-T1sm ESCC was significantly higher than that in patients with p12$^{CDK2-AP1}$ positive one (75.0 vs. 29.6%, P=0.014).

Patients with p12$^{CDK2-AP1}$-negative tumors had a significantly shorter disease-free survival and overall cancer-specific survival than patients with p12$^{CDK2-AP1}$ positive tumors (P=0.0156 and 0.0023, respectively; Fig. 2). However, the p12$^{CDK2-AP1}$ expression was not shown to be an independent prognostic factor in a multivariate analysis using the Cox model (data not shown).

Discussion

This is the first report to demonstrate the relationship between the tumor suppressor gene p12$^{CDK2-AP1}$ expression and clinicopathological characteristics and prognosis of ESCC. In addition, we also demonstrated the relationship between p12$^{CDK2-AP1}$ expression and lymph node metastasis in patients with superficial ESCC.

Shintani *et al* (6) immunohistochemically evaluated p12 expression of human OSCC by 25% cut-off and demonstrated that the majority of OSCCs (63.8%) exhibit either a loss or significant reduction (<25%) of p12$^{CDK2-AP1}$. The present study used the 25% cut-off as reported previously (6) and demonstrated that of the 120 cases of ESCCs examined, 41 cases (34.2%) showed <25% of cells stained for p12$^{CDK2-AP1}$. Comparison of the p12$^{CDK2-AP1}$ expression and the clinicopathological characteristics in 120 patients with ESCC revealed significant correlations between p12$^{CDK2-AP1}$ expression and depth of tumor, lymph node metastasis, pathological stage and venous invasion. In addition, patients with low p12$^{CDK2-AP1}$ expression in the tumors had a poorer prognosis than patients with high p12$^{CDK2-AP1}$ expression. Like previous reports demonstrating the role of p12$^{CDK2-AP1}$ as a tumor suppressor gene (6-10), the current data suggest that p12$^{CDK2-AP1}$ also acts as a tumor suppressor gene in ESCC and loss of p12$^{CDK2-AP1}$ may have important implication during the progression of ESCC.

Lymph node metastasis is a frequent event in ESCC and it has been reported that the rate of metastasis was 3-6% in intramucosal carcinomas and 21-24% in submucosal carcinomas, respectively (13,14). However, more resent reports demonstrated that the lymph nodes may be involved in up to 10% of intramucosal carcinomas and in up to 50% of submucosal carcinoma (15-17). In the present study, 11.5% (3/26) of T1m carcinomas and 43.6% (17/39) of T1sm carcinomas had lymph node metastasis. These findings are consistent with the latter. Several reports have tried to identify histopathological parameters predicting the presence of positive nodes in patients with superficial carcinoma of esophagus (T1m/T1sm) by classifying the depth of invasion into six levels (m1, intraepithelial tumors; m2, tumors invading the lamina propria; m3, tumors in contact with or invading

Table II. Relationship between the p12$^{CDK2-AP1}$ expression and lymph node metastasis in 65 patients with T1 stage ESCC.

T classification	N status	Total	p12$^{CDK2-AP1}$ expression		P-value
			Negative	Positive	
T1m-T1sm	N0	45	6	39	0.013
	N1	20	9	11	
T1m	N0	23	3	20	1
	N1	3	0	3	
T1sm	N0	22	3	19	0.014
	N1	17	9	8	

N status indicates lymph node metastasis status. P-values were calculated using the χ^2 test and Fisher's exact test. T1m, limited to the mucosa or muscularis mucosae. T1sm, limited to the submucosa.

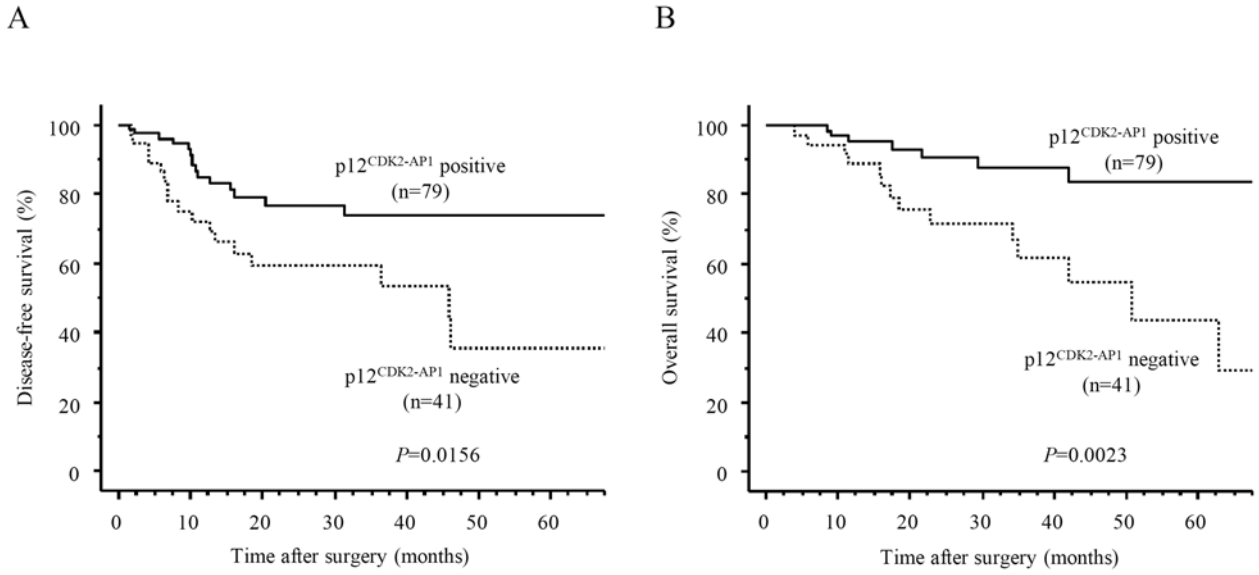

Figure 2. Relationship between p12$^{CDK2-AP1}$ expression and the survival rate in patients with ESCC. (A) Disease-free survival. (B) Overall cancer-specific survival.

the muscularis mucosa; sm1, tumors invading the most superficial 1/3 of the submucosa; sm2/3, tumors invading deeper than sm1 level) (16,18-22). Eguchi *et al* (21) demonstrated that lymph node metastasis was found in 0, 6, 18, 53 and 54% of m1, m2, m3, sm1 and sm2/3 lesions, respectively. Shimada *et al* (22) demonstrated that it was found in 0, 0, 6, 32 and 39% of each type of lesion, respectively. Furthermore, several biological markers predicting lymph node metastasis of ESCC have been identified. For example, Han *et al* (23) demonstrated that p53 and vascular endothelial growth factor C (VEGF C) expressions were correlated with lymph node metastasis in patients with ESCC and suggested that immunohistochemical analysis of these molecules could be useful for determining preoperative lymph node metastasis. The present study demonstrated that the rate of lymph node metastasis in patients with p12$^{CDK2-AP1}$ negative-T1sm ESCC was significantly higher than that of patients with p12$^{CDK2-AP1}$ positive specimens. These findings suggest the loss of p12$^{CDK2-AP1}$ expression to be associated with lymphatic spread of cancer cells and the investigation of p12$^{CDK2-AP1}$ expression can therefore be a useful predictor of lymph node metastasis in superficial ESCC. Li *et al* (24) demonstrated that over-expression of CDK2 might promote abnormal proliferation of cells during colorectal carcinogenesis and could facilitate lymph node metastasis. Based on the fact that p12$^{CDK2-AP1}$ negatively regulates the activity of CDK2, loss of p12$^{CDK2-AP1}$ might act an important role in lymph node metastasis of ESCC.

In this study of ESCC, loss of p12$^{CDK2-AP1}$ expression was correlated with tumor aggressiveness and poor prognosis. In addition, it was associated with the lymphatic spread of cancer cells in superficial ESCC. Therefore, the assessment of p12$^{CDK2-AP1}$ may be a useful tool for assessing tumor aggressiveness and the prognosis in patients with ESCC.

Acknowledgements

We thank Dr Yong Kim, Dr David T.W. Wong (University of California Los Angeles, CA, USA) and Dr S. Shintani (Showa University, Tokyo, Japan) for kindly providing us with the anti-p12 antibody.

References

1. Shintani S, Ohyama H, Zhang X, *et al*: p12(DOC-1) is a novel cyclin-dependent kinase 2-associated protein. Mol Cell Biol 20: 6300-6307, 2000.
2. Tsuji T, Duh FM, Latif F, *et al*: Cloning, mapping, expression, function, and mutation analyses of the human ortholog of the hamster putative tumor suppressor gene Doc-1. J Biol Chem 273: 6704-6709, 1998.
3. Sherr CJ and McCormick F: The RB and p53 pathways in cancer. Cancer Cell 2: 103-112, 2002.
4. Matsuo K, Shintani S, Tsuji T, *et al*: p12(DOC-1), a growth suppressor, associates with DNA polymerase alpha/primase. FASEB J 14: 1318-1324, 2000.
5. Hu MG, Hu GF, Kim Y, *et al*: Role of p12(CDK2-AP1) in transforming growth factor-beta1-mediated growth suppression. Cancer Res 64: 490-499, 2004.
6. Shintani S, Mihara M, Terakado N, *et al*: Reduction of p12DOC-1 expression is a negative prognostic indicator in patients with surgically resected oral squamous cell carcinoma. Clin Cancer Res 7: 2776-2782, 2001.
7. Todd R, McBride J, Tsuji T, *et al*: Deleted in oral cancer-1 (doc-1), a novel oral tumor suppressor gene. FASEB J 9: 1362-1370, 1995.
8. Kohno Y, Patel V, Kim Y, *et al*: Apoptosis, proliferation and p12(doc-1) profiles in normal, dysplastic and malignant squamous epithelium of the Syrian hamster cheek pouch model. Oral Oncol 38: 274-280, 2002.
9. Yuan Z, Sotsky Kent T and Weber TK: Differential expression of DOC-1 in microsatellite-unstable human colorectal cancer. Oncogene 22: 6304-6310, 2003.
10. Sotsky Kent T, Yuan Z, Miller A and Weber TK: Deleted in oral cancer-1 expression upregulates proapoptosis elements in microsatellite-unstable human colorectal cancer. Ann Surg Oncol 11: 192-196, 2004.
11. Sobin LH and Wittekind CH (eds): International Union Against Cancer (UICC): TNM classification of malignant tumors. 6th edition. Wiley, New York, 2002.
12. Hamilton SR and Aaltonen LA: World Health Organization classification of tumours. Tumours of the Digestive System. IARC Press, Lyon, 2000.
13. Sabik JF, Rice TW, Goldblum JR, *et al*: Superficial esophageal carcinoma. Ann Thorac Surg 60: 896-902, 1995.
14. Rice TW, Zuccaro G Jr, Adelstein DJ, Rybicki LA, Blackstone EH and Goldblum JR: Esophageal carcinoma: depth of tumor invasion is predictive of regional lymph node status. Ann Thorac Surg 65: 787-792, 1998.
15. Tajima Y, Nakanishi Y, Ochiai A, *et al*: Histopathologic findings predicting lymph node metastasis and prognosis of patients with superficial esophageal carcinoma: analysis of 240 surgically resected tumors. Cancer 88: 1285-1293, 2000.
16. Matsubara T, Ueda M, Abe T, Akimori T, Kokudo N and Takahashi T: Unique distribution patterns of metastatic lymph nodes in patients with superficial carcinoma of the thoracic oesophagus. Br J Surg 86: 669-673, 1999.
17. Fujita H, Sueyoshi S, Yamana H, *et al*: Optimum treatment strategy for superficial esophageal cancer: endoscopic mucosal resection versus radical esophagectomy. World J Surg 25: 424-431, 2001.
18. Tachibana M, Yoshimura H, Kinugasa S, *et al*: Clinicopathological features of superficial squamous cell carcinoma of the esophagus. Am J Surg 174: 49-53, 1997.
19. Endo M, Yoshino K, Kawano T, Nagai K and Inoue H: Clinicopathologic analysis of lymph node metastasis in surgically resected superficial cancer of the thoracic esophagus. Dis Esophagus 13: 125-129, 2000.
20. Araki K, Ohno S, Egashira A, Saeki H, Kawaguchi H and Sugimachi K: Pathologic features of superficial esophageal squamous cell carcinoma with lymph node and distal metastasis. Cancer 94: 570-575, 2002.
21. Eguchi T, Nakanishi Y, Shimoda T, *et al*: Histopathological criteria for additional treatment after endoscopic mucosal resection for esophageal cancer: analysis of 464 surgically resected cases. Mod Pathol 19: 475-480, 2006.
22. Shimada H, Nabeya Y, Matsubara H, *et al*: Prediction of lymph node status in patients with superficial esophageal carcinoma: analysis of 160 surgically resected cancers. Am J Surg 191: 250-254, 2006.
23. Han U, Can OI, Han S, Kayhan B and Onal BU: Expressions of p53, VEGF C, p21: could they be used in preoperative evaluation of lymph node metastasis of esophageal squamous cell carcinoma? Dis Esophagus 20: 379-385, 2007.
24. Li JQ, Miki H, Ohmori M, Wu F and Funamoto Y: Expression of cyclin E and cyclin-dependent kinase 2 correlates with metastasis and prognosis in colorectal carcinoma. Hum Pathol 32: 945-953, 2001.