Low Expressions of p27Kip1 and High Expressions of ki-67 are Potentially Poor Prognostic Factors in Esophageal Squamous Cell Carcinoma Patients

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Abstract **Objectives** To evaluate the prognostic significance of ki67 and p27kip1 in esophageal squamous cell carcinoma (ESCC). **Methods** Tissue blocks from 98 patients with ESCC who underwent transthoracic subtotal esophagectomy with lymph nodes dissection at our institution between 1999 and 2000, were available for this study. Immunohistochemistry using monoclonal antibodies against p27kip1 and ki-67 was used to examine protein expression. **Results** ki-67 was high expressed in 36 cases (36.7%), and p27kip1 was expressed in 41 cases (41.8%). Analysis with Kaplan-Meier survival, patients with ki-67 high expression (P=0.007) or p27kip1 negative expression alone, or (P=0.024), both in combination (P=0.001), had a worse prognosis. The relationship of these findings to clinicopathological parameters revealed that there were a higher rate of lymph node metastasis in ki-67 high expression and p27kip1 negative cases. **Conclusion** Our results suggested that over expression of ki-67, particularly with low expression of p27kip1 is potentially correlated to poor prognostic factor in esophageal squamous cell carcinoma patients. However, to verify the prognostic significance of these factors, a multivariate analysis of a larger number of patients should be undertaken.

Key Words Ki-67; P27kip1; Esophageal squamous cell carcinoma; Immunohistochemistry

Esophageal squamous cell carcinoma (ESCC) occurs rather frequently in the Chinese, rapid tumor growth with early lymphatic and hematogensus spreading discourages the efforts of multiple therapeutic modalities for ESCC patients. More than half of the patients with resectable ESCC have regional disease at diagnosis, and most of them died of tumor recurrence within 1-2 years after surgical resection. Therefore, it is important to find a method for identifying cancer cell proliferation and spreading, and to improve survival for ESCC patients.

In the present study, we analyzed the expression of ki-67 and p27kip1 immunohistochemically to evaluate whether their expression correlated with 5-year survival in ESCC, either individually or in combination. We also studied the relation between the expression of these proteins and other clinicopathological parameters.

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MATERIALS AND METHODS

Patients and tissues

From January 1999 to June 2000, 109 patients with ESCC who were surgically treated in Surgery Oncology Department of First Hospital in Xi´an Jiaotong University,104 patients underwent transthoracic subtotal esophagectomy with lymph node dissection. Those who died within 3 months after surgery and those died of another causes other than ESCC within 5 years were excluded from this study. Thus, the subjects were 98 patients, consisting of 27 females and 71 males whose ages ranged from 49 to 72 years old, with a mean of 56.7±8.4 years old. They had not undergone chemotherapy or radiotherapy preoperatively.

Postoperatively, adjuvant treatment was given in case of lymph node metastasis. A median of 26 lymph nodes was removed in each patient. Radiotherapy was given to 62 patients, radiation and chemotherapy to 15 patients, and another 21 patients received chemotherapy only.

The specimens were evaluated blindly by two experienced pathologists for histopathological diag-

nosis according to the criterion by World Health Organization.

Surgical resection

Transthoracic subtotal esophagectomy was done in all patients. Complete resection was defined as no macroscopic evidence of tumor in the field after surgery. Esophagogastrostomy was stapled in the left thoracic cavity for tumors located below the carina and hand-sewn in the neck for those located above carina. In all patients a similar lymph node dissection was done. In the mediastinum included paratracheal lymph nodes, infracarinal lymph nodes, and periesophageal lymph nodes. In the abdominal field, right and left paracardiac lymph nodes, lymph nodes along lesser curvature, and celiac lymph nodes were removed.

Immunohistochemistry

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Paraffin blocks were cut at 4mm thickness. Sections were deparaffinized with xylene, rehydrated in graded alcohol. 0.3%H_2O_2 was then applied to block endogenous peroxidase activity. The sections were then incubated with mouse monoclonal anti-p27kip1 protein (1:100, 2.5ug IgG1 /ml, sigma Co. USA.), mouse monoclonal anti-ki67 antigen (1:100, 0.2ug IgG1/ml, sigma Co. USA.) for 30 min at room temperature. The sections were then incubated with biotin-labeled secondary antibody (1:30) and streptavidin-biotin-peroxidase (1:30) for 20 min each. Tissue was stained for 5 min with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) freshly prepared in 0.05M Tris buffer at pH 7.6, containing 0.024% H_2O_2, Immunostaining was performed in accordance with the manufacturer´s instructions. The sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted. Negative control staining was carried out by substituting non-immune mouse serum for primary antibodies. Only distinct nuclear staining was considered positive. Counting 2000 cells, when the staining tumor cells >10%, which was considered as positive for p27kip1 according to the criteria used in the earlier studies[1].

Statistical analysis

Between the expression of those antigens and the clinicopathological parameters were determined by Fisher´s exact test. Kaplan-Meier survival analysis with log-rank test was used to evaluate the relationships between expressions of those antigens and survival distributions. Cox´s proportional hazard analysis was used for multivariate analysis. P-value less than 0.05 were considered statistically significant.

RESULTS

Clinicopathological findings

Of 98 patients with ESCC, 46 cases were well differentiated, 33 were moderately differentiated, and 19 were poorly differentiated. According to the 1997 UICC-TNM classification postoperative staging was based on pathologic examination of the resected s pecimens, including the dissected lymph nodes. Wall penetration of a tumor for clinical T categorization was based on the deepest invasion evident pathologic examination of the resected specimens.

Immunohistochemistry

The results were summarized in Table 1 and Fig.1. p27kip1 were expressed in the nuclei of lymphocytes, fibroblasts, and normal mucosae epithelial cells, which served as internal positive control. In neoplastic lesions, p27kip1 immunostaining was mostly confined to the cell nuclei and was stronger than in nomal (Fig.1a and b), whereas, the cytoplasmic staining was found in few cases. 41 cases (41.8%) were positive for p27kip1.

Very few cells were positive for ki-67 in non-neoplastic esophageal tissues. In tumors, distinct nuclear staining was observed. For survival analysis purposes, we classified all the tumors into low (0–49% of tumor cell nuclei positive), and high proliferation (50-100%) groups. According to this criterion, 36 cases (36.8%) showed high expression of ki-67 (Fig. 1c and d). There was no definite correlation between p27kip1 and ki-67 expression. Comparing the expression of two proteins to clinicopathological parameters, ki-67 expression increased and p27kip1 expression decreased parallel to the grade of ESCC (Table 1).

Prognostic significance of ki67 and p 27

Patients with p27kip1 positive tumors had a significantly better prognosis than those with p27kip1 negative tumors (P=0.024, Fig.2a). Patients with ki-67 high expression tumors had a significantly worse prognosis than those with ki-67 low expression tumors (P=0.007, Fig. 2b). Moreover, comparing the ki-67 high expression/p27kip1 negative

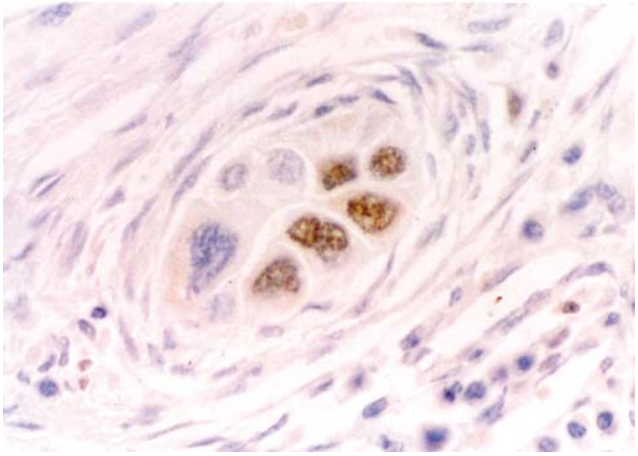

Fig.1a Immunohistochemistry, p27kip1 expression in nucleus of esophageal squamous cell carcinoma (×400)

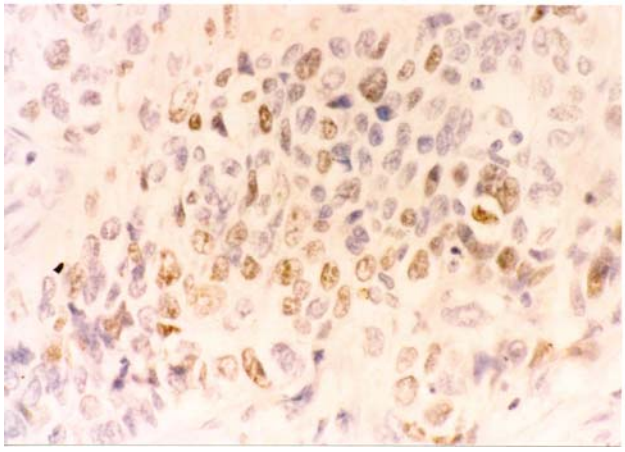

Fig.1b Immunohistochemistry, p27kip1 expression of esophageal squamous cell carcinoma (×200)

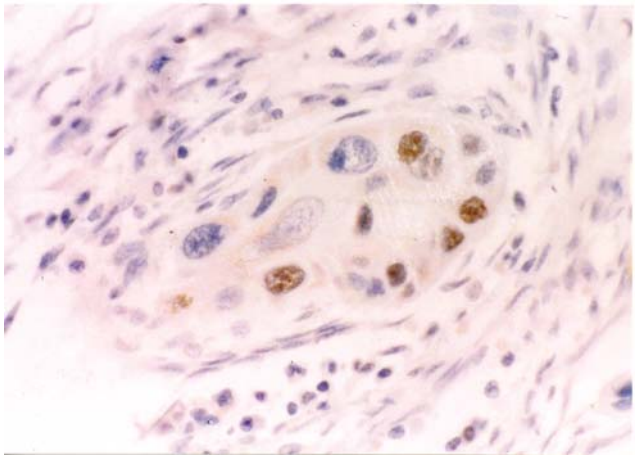

Fig.1c Immunohistochemistry, ki-67 expression in nucleus of esophageal squamous cell carcinoma (×400)

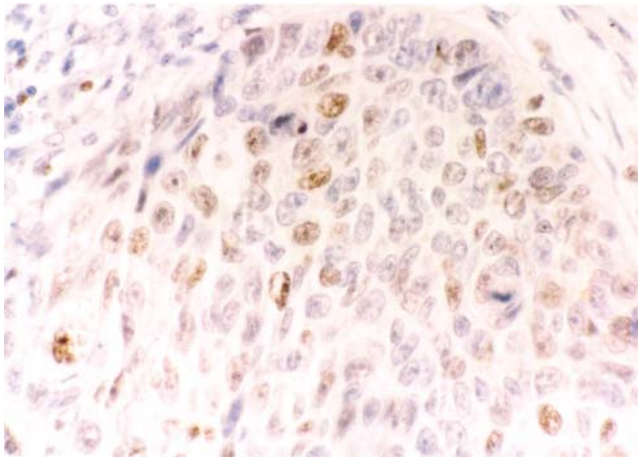

Fig.1d Immunohistochemistry, ki-67 expression of esophageal squamous cell carcinoma (×200)

Table 1 Clinicopathological characteristics of patients according to the results of immunohistochemistry

	n	P27	P	Ki-67	P
		Positive(%)		High expresson(%)	
Gender					
Female	27	12(44.4)	0.82	14(33.3)	0.065
Male	71	29(40.8)		22(38.0)	
Differentiation					
Well	46	26(56.5)	0.001**	9(19.6)	0.026*
Moderately	33	13(39.3)	0.031***	14(42.4)	0.000**
Poorly	19	2(10.5)		13(68.4)	
pT classification					
1 and 2	81	37(45.6)	0.111	28(34.6)	0.409
3 and 4	17	4(23.5)		8(47.1)	
pN classification					
0	64	29(45.3)	0.394	18(28.1)	0.027
1	34	12(35.3)		18(52.9)	

Abbreviations: *, well vs moderately; **, well vs poorly; ***, moderately vs poorly

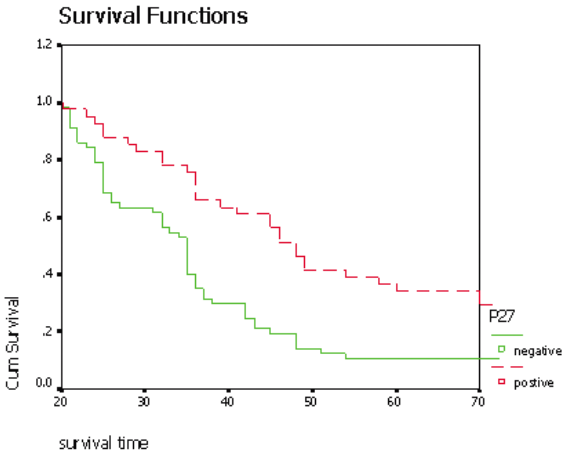

Fig. 2a

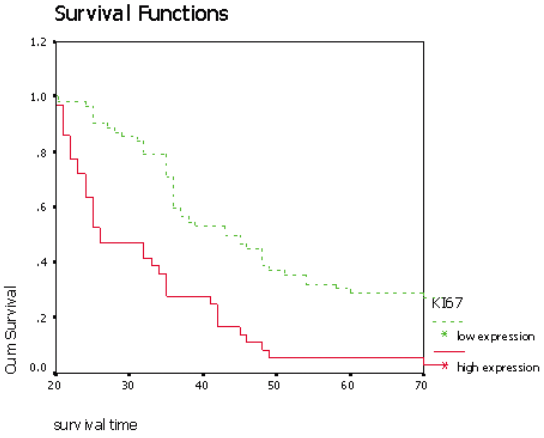

Fig. 2b

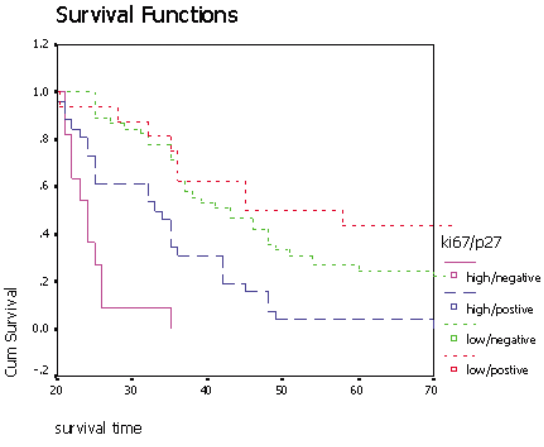

Fig. 2c

Fig.2 Kaplan-Meier survival curves of esophageal squamous cell carcinoma patients. In (2c), "ki67 status/p27 status" was indicated, and (high/-) group had worse prognosis than (high/+) group (*P*=0.001), (low/-) group (*P*=0.016) and (low/+) group (*P*=0.12).

group with the other combined three groups, the former had a significantly worse prognosis (P = 0.001, Fig.2c).

Multivariate prognostic factor analysis

The results of 98 patients were summarized in Table 2. pT and pN classifications and ki-67 expression were significant factors. However, p27kip1 expressions were not significant factors. Therefore, we analyzed the correlation p27kip1 status and other factors. This analysis revealed that ki-67 and p27kip1 status correlate with pN classification (*P*= 0.003, Table 3).

DISCUSSION

p27Kip1 is capable of blocking cell cycle progression from G1 to S phase by binding cyclin D/cdk4, cyclin E/cdk2 and cyclin A/cdk2 complex. Thus, the loss of p27kip1 protein expression is thought to reflect rapid cell growth and may participate in the development of tumors through unbridled cell proliferation. In this study, we found the reduced expression of p27kip1 protein in 58.2% of ESCC, which is comparable to those obtained by other studies [2]. These results indicate that loss of p27kip1 protein expression is a common event and may play an important role in the pathogenesis of ESCC.

It has been shown that patients of colorectal [3], prostate, oral squamous cell carcinoma, hepatocellular carcinoma[4–6], or non-small cell lung cancer with low or absent expression of p27kip1 protein had poor prognosis. In contrast, two groups in Japan reported that increased expression of p27kip1 protein was associated with an advanced clinical stage and poor survival in patients with ESCC[7]. In the present study, patients with p27kip1 negative tumors had unfavorable prognosis. In order to confirm this, we performed multivariate analysis. However, the results showed that p27kip1 was not significant factor. Instead, it was reconfirmed that high expression of ki-67 and was very important. It would be speculated that low expression of p27kip1 in ESCC might be associated with high proliferation activity of tumor cells as well as lymph node metastasis.

ki-67 is a cell proliferation-associated antigen, which is expressed in all stages of the cell cycle except G0[8] and is regarded as an indicator of biological aggressiveness [9]. Expression of ki-67 could be observed in different human malignancies [10–12].

Table 2 Cox´s proportional hazard analysis of prognostic factors in ESCC patients

Factors	Hazard ratio	95%CI	*P*
P27kip1	1.7	0.9–3.3	0.13
Ki-67	1.9	1.0–3.8	0.048
differentation	1.1	0.5–2.5	0.81
pT	2.3	1.2–4.4	0.015
pN	3.5	1.7–7.2	0.0008

Table 3 Relationship between combination of ki67 and p27kip1 status (ki67/p27kip1) and cinicopathological factors

	n	pN		*P*
		N0	N1	
(Low / +) group	16	5	11	0.003
Other groups combined	82	59	23	

Prognostic significance of ki-67 positivity has also been demonstrated in several types of tumours such as breast cancer, ovarian cancer and oesophageal cancer[13-14]. Yamanaka et. al[15] found that a high proliferation index of ki-67 was associated with poor outcome in patients with non-Hodgkin's lymphomas of Waldeyer´s ring and nasal cavity. However, there exist controversial studies regarding the clinical application of this antigen. Several reports have shown that ki-67 does not have any prognostic value in terms of predicting outcome. Roychowdhury et al.[16] did not observe any association of ki-67 with prognosis in nasopharyngeal carcinoma. Stoll et al.[17] studied 107 patients with OSCC and oropharyngeal carcinoma and found that ki-67 index was not able to predict survival or recurrence-free survival of the patients. In accordance with our study, we observed a significant expression of ki-67 in ESCC, and this study revealed a correlation between ki-67 index and histological grade of differentiation. (P=0.000; Table 1).

Our results suggested that ki-67 over-expression in ESCC, particularly with low p27kip1 expression, is potentially correlated to poor prognosis. The most significant finding was that the combination of proliferation and cell cycle markers yielded considerably better information regarding prognosis than did any of the individual parameters alone. Larger scale analysis is required to verify the prognostic significance of these factors.

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