Original Article

Immunohistochemical analysis of nuclear survivin expression in esophageal squamous cell carcinoma

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RUNNING TITLE: Nuclear survivin expression in ESCC

ABSTRACT

Purpose. Despite advances in the treatment of esophageal carcinoma, the prognosis for this disease remains poor. Therefore, it is important to obtain a better understanding of the molecular basis of esophageal carcinogenesis. The purpose of this study was to clarify the roles of survivin in esophageal squamous cell carcinoma (ESCC).

Methods. One hundred twenty-two ESCC surgical specimens resected from 1989 to 1999 were examined. Survivin expression was assessed by immunohistochemistry. Tumor cells were considered survivin-positive if the immunoreactivity was confined to the nucleus, and a scoring method was applied.

Results. Survivin-positive immunostaining was detected in 68 patients (56%). There was a significant association between survivin expression and pN (P=0.0472). Moreover, the overall survival rate was worse in patients with survivin-positive tumors than in patients with survivin-negative tumors (P=0.0189).

Conclusion. The overexpression of survivin was associated with the overall survival rate and poor prognosis in patients with ESCC. Survivin **may** be targeted during cancer therapy because of its selective expression in malignant tissue.

KEY WORDS: survivin, esophageal squamous cell carcinoma, immunohistochemistry, carcinogenesis, prognosis

INTRODUCTION

Despite advances in surgical technique and perioperative management that have improved survival to some extent, esophageal squamous cell carcinoma (ESCC) remains a disease with a poor prognosis. The overall 5-year survival rate generally remains less than 50%, even with the use of multimodality therapy.[1-3] Recently, there has been a better understanding of the molecular basis of esophageal carcinogenesis, and prognostically important biologic markers have been identified. Survivin is a member of the inhibitors of apoptosis protein family. Survivin participates in the complex network regulating programmed cell death and cell division.[4-6] Survivin protein is commonly detected in fetal tissues, but not in normal adult tissues. Survivin is overexpressed in several human cancers, which suggests that the reactivation of the survivin gene contributes to carcinogenesis.[4] Overexpression of survivin has been associated with parameters of aggressiveness and poor prognosis in several solid tumors;[4,7-9] however, conflicting data have also been reported.[10] The aim of the present study was to investigate the association of survivin expression with surgical data, response to chemotherapy and prognosis in a large group of primary ESCC patients who underwent surgical resection. Immunohistochemical studies were performed on 122 surgical specimens of ESCC. Clinical and histopathologic factors were obtained from a retrospective review of patient records.

MATERIALS AND METHODS

Patients and esophageal specimens. We examined all complete ESCC surgical specimens resected from 1989 to 1999. We included only the patients who had no evidence of metastasis to other organs and who did not receive prior anticancer

treatment. Cases of in-hospital death were excluded. Surgical specimens from 122 patients who had undergone radical esophagectomy at the Department of Surgical Oncology at Hokkaido University, Hokkaido Gastroenterology Hospital, and Teine Keijinkai Hospital were included in the current study. Data was collected from the patients' clinical records. For each patient, one section from the deepest point of tumor invasion was selected for evaluation. The specimens were examined histologically after staining with hematoxylin and eosin, and the clinicopathologic stage was determined according to the TNM classification system of the International Union Against Cancer.[11] The 122 patients included in the study consisted of 105 men and 17 women. The median patient age was 62.3 years (range, 38 to 82 years). A relatively large number of patients had early stage disease (78 patients, 64%). Sixty-one patients (50%) had lymph node metastases, and 19 patients (16%) had distant nodal metastases. The study population had the following performance status (PS): PS0, 107 patients; PS1, 14 patients; and PS2, 1 patient. The median follow-up period was 29 months.

All specimens were fixed in 10% formalin and embedded in paraffin wax. One of the deepest sections from each tumor was selected for evaluation. Serial 4 micrometer-thick sections were examined by immunohistochemistry.

Immunohistochemistry. Each slide was deparaffinized in xylene, rehydrated and washed in PBS for 15 min. Antigen retrieval was achieved by treating the slides in a pressure cooker containing 1.5 l of boiling water. Slides were treated in the pressure cooker for 7 minutes in 10mM citrate buffer at pH 6.0. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min. Non-specific binding was blocked by incubating the slides with 10% normal goat serum (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) for 30 min. The slides were incubated overnight at 4 ℃ with the

primary antibody. Polyclonal rabbit anti-human survivin antibody (diluted 1:20; Alpha Diagnostic International, San Antonio, TX, USA) was used under previously described conditions.[9] After washing, sections were treated with biotinylated secondary antibody (Histofine Simple Stain MAX-PO (MULTI), Nichirei, Tokyo, Japan) for 30 min. Then, sections were incubated with peroxidase-conjugated streptavidin for 30 min. Visualization of the immunoreaction was conducted with 3, 3'-diaminobenzidine (DAB and DAB H_2O_2; Ventana DAB Universal Kit; Ventana-Bio Tek Solutions, Tokyo, Japan) for 5 min. Finally, sections were counterstained with haematoxylin. As a negative control, nonimmune purified rabbit serum was used for the primary antibody.

Assessment of immunoreactivity. Survivin immunoreactivity was observed in the nucleus and cytoplasm of cancer cells. Tumor cells were considered survivin-positive if the immunoreactivity was observed nucleus by the previous study.[12] To quantitate survivin expression in the various samples, a scoring method was applied.[8,13] The mean percentage of positive tumor cells was determined from at least 1000 tumor cells that were counted systematically at ×400 magnification (Olympus Optical Co, Ltd, Tokyo, Japan) in 5 visual fields. The percentage of positive tumor cells was assigned to one of the following categories: 0, <5%; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, >75%. The intensity of nuclear survivin immunostaining was scored as follows: (a) weak, 1+; (b) moderate, 2+; and (C) intense, 3+. For tumors with heterogeneous staining, the predominant pattern was taken into account for scoring. The percentage of positive tumor cells and the staining intensity were multiplied to produce a weighted score for each case. Cases with a weighted survivin score <1 were considered to be negative. The current study was performed in a retrospective manner. All specimens were evaluated by 3 investigators (S. M., M. M., and T. I.), who were blinded to the patients' clinical

information.

Statistical analysis. Either the chi-square test or Fisher's exact test was used to analyze the correlation between survivin expression and patient parameters, including histopathologic findings. The Kaplan-Meier method was used to generate survival curves. Survival differences were analyzed with the log-rank test, based on the status of survivin expression. Univariate and multivariate analyses of survivin immunoreactivity and clinicopathological features were performed using the Cox proportional hazard regression model. Probability values less than 0.05 were considered statistically significant. All analyses were performed using statistical analysis software (Statview J version 5.0; SAS Institute Inc. Cary, NC).

RESULTS

We performed immunohistochemical analysis on 122 ESCC specimens. Survivin immunoreactivity was observed in the nucleus and cytoplasm of cancer cells, as seen in a previous study.[12, 13] Fifty-four specimens (44.3%) were immunoreactive for nuclear survivin in less than 5% of cells; 20 specimens (16.4%) were immunoreactive in 5-25% of cells; 22 specimens (18.0%) were immunoreactive in 26-50% of cells; 18 specimens (14.7%) were immunoreactive in 51-75% of cells; 8 specimens (6.6%) were immunoreactive in more than 75% of cells. According to the criteria of the current study, 68 specimens (55.7%) were positive for survivin (Fig. 1).

Associations between survivin over-expression and clinical factors are shown in Table 1. The average age of survivin-positive patients was 62.9 years (range, 47-82 years), and the average age of survivin-negative patients was 61.7 (range, 38-78 years). Of the 68 patients (55.7%) who were positive for survivin, 62 (91.2%) were men and 6 (8.8%)

were women. The over-expression of survivin was not related to gender (P=0.0674) or age (P=0.1684). Survivin immunoreactivity had a statistically significant relationship to the p-N classification (P=0.0472) of clinicopathological features, as determined by the chi-square test (Table 1). However, there was no statistically significant relationship with p-T classification (P=0.1326) and p-Stage (P=0.1543). The percentage of survivin-positive patients increased as the p-T classification; 16 specimens (29.6%) were immunoreactive in p-T1; 7 specimens (53.8%) were immunoreactive in p- T2; 30 specimens (69.8%) were immunoreactive in p- T3; and 9 specimens (75%) were immunoreactive in p- T4. Similarly the percentage of survivin-positive patients increased as the p-Stage advanced; 16 specimens (42.1%) were immunoreactive in p-Stage I; 22 specimens (57.9%) were immunoreactive in p-Stage II; 18 specimens (60.0%) were immunoreactive in p-Stage III; and 12 specimens (63.2%) were immunoreactive in p-Stage IV. Moreover, the overall 5-year survival rate, as determined by the Kaplan-Meier method, was worse in patients with survivin-positive tumors than in patients with survivin-negative tumors (P=0.0189) (Fig. 2). Univariate analyses that were performed with the Cox proportional hazards model identified the following factors as negative predictors: survivin positivity (P=0.0213), p-T classification (P<0.0001), p-N classification (P<0.0001) and p-M classification (P=0.0440). Multivariate analyses indicated that T classification (P=0.0090) and N classification (P=0.0004) were independent prognostic factors. Survivin positivity was not an independent factor (P=0.2373) (Table 2).

DISCUSSION

Recently, several reports have suggested that survivin is **over** expressed in many types of human tumors.[7, 14-20] **Overexpression of survivin may lead to promote cell survival.** Because survivin can directly inhibit the activities of caspase-3 and caspase-7, one role of survivin may be the prevention of apoptosis.[21-23] Thus, overexpression of survivin may increase the malignant potential of a tumor. Immunohistochemical expression of the first-characterized anti-apoptotic gene, Bcl-2, which regulates an apoptotic pathway different from that of survivin, has been reported in ESCC.[24, 25] **But there were few previous reports about nuclear survivin expression in cancer cells.** Therefore, we performed an immunohistochemical analysis of survivin in 122 ESCC specimens. **We believe that this manuscript is the first report about a large group analysis of nuclear survivin expression to clinicopathological status in ESCC.**

Our analysis provides suggestions for the biological function of survivin in ESCC. We found survivin expression in both the nucleus and cytoplasm of ESCC specimens. In normal squamous cell epithelium of the esophagus, survivin expression was occasionally recognized **weakly**; this expression was mainly localized in the cytoplasm of the basal layer. But, in high-grade dysplasia and aggressive tumor tissues, survivin expression was often recognized in the nucleus as well as the cytoplasm. **And nuclear survivin expression found more intensively at invasive tumor cells.** These differences in staining may be related to another function of survivin, cell proliferation. Previous studies have suggested that nuclear survivin expression is more important than cytoplasmic expression in clinicopathological studies.[12, 21] So, we adopted scoring methods to quantify nuclear survivin immunoreactivity. In this study, the ratio of nuclear survivin immunopositivity was associated with overall survival rate, and the

overexpression of survivin was significantly associated with a poor prognosis (P=0.0189). **We think that** the poor prognosis was caused by a significant association between nuclear survivin expression and lymph node metastasis (P=0.0472) (Table 1). Interestingly, the percentage of patients who overexpressed survivin tended to increase with pathological T-classification and stage progression. These data suggest that survivin overexpression is implicated in the resistance to apoptotic stimuli, so that survivin overexpression **may** reflect the tumor progression and malignancy in ESCC. **Survivin may be targeted during cancer therapy because of its selective expression in malignant tissue.**

We conclude that overexpression of survivin was not a independent prognostic factor, but it may be effective to predict poor prognosis in patients with ESCC.

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Table 1 Relationship between clinicopathologic features and survivin expression in surgical specimens of esophageal squamous cell carcinoma [a]

Variables		Survivin Positive (n=68)	Survivin negative (n=54)	P value[b]
Gender	Male	62	43	0.0674
	Female	6	11	
Age	≥65	27	15	0.1684
	<65	41	39	
p-Stage	I, II	38	37	0.1543
	III, IV	30	17	
p-Grade	G1	18	14	0.9458
	Others	50	40	
p-T classification	T1, T2	26	28	0.1326
	T3, T4	42	26	
p-N classification	N0	28	32	0.0472*
	N1	40	22	
p-M classification	M0	54	47	0.2678
	M1	14	7	
Tumour size	≥4.5cm	36	28	0.9047
	<4.5cm	32	26	
Adjuvant therapy	Yes	25	24	0.3901
	No	43	30	

[a] TNM classification system of the International Union Against Cancer.
[b] The P value was calculated by chi-square test.
* Significant

Table 2 Univariate and multivariate analyses of survivin expression and pathologic parameters in patients undergoing curative resection of esophageal squamous cell carcinoma

Univariate

Factor	Hazard ratio (95% confidence interval)	P value
Survivin {(-)/(+)}	0.494 (0.271-0.900)	0.0213*
Gender (male/female)	4.070 (0.984-16.835)	0.0527
Age (≥65 yrs/<65 yrs)	1.285 (0.702-2.353)	0.4163
p-Grade (1 / others)	0.769 (0.401-1.474)	0.4285
p-T classification (3,4 / 1,2)	4.016 (2.107-7.653)	<0.0001*
p-N classification (1/0)	6.064 (2.886-12.743)	<0.0001*
p-M classification (1/0)	2.658 (1.357-5.208)	0.0440*
Tumour size (<4.5cm/ ≥4.5cm)	0.769 (0.401-1.474)	0.0924
Adjuvant therapy (No/Yes)	1.091 (0.591-2.011)	0.7812

Multivariate

Survivin {(-)/(+)}	0.690 (0.372-1.277)	0.2373
p-T classification (3,4 / 1,2)	0.387 (0.190-0.789)	0.0090*
p-N classification (1/0)	0.234 (0.106-0.520)	0.0004*
p-M classification (1/0)	0.746 (0.364-1.527)	0.4219
Tumour size (<4.5cm/ ≥4.5cm)	1.224 (0.618-2.425)	0.5618

*Significant

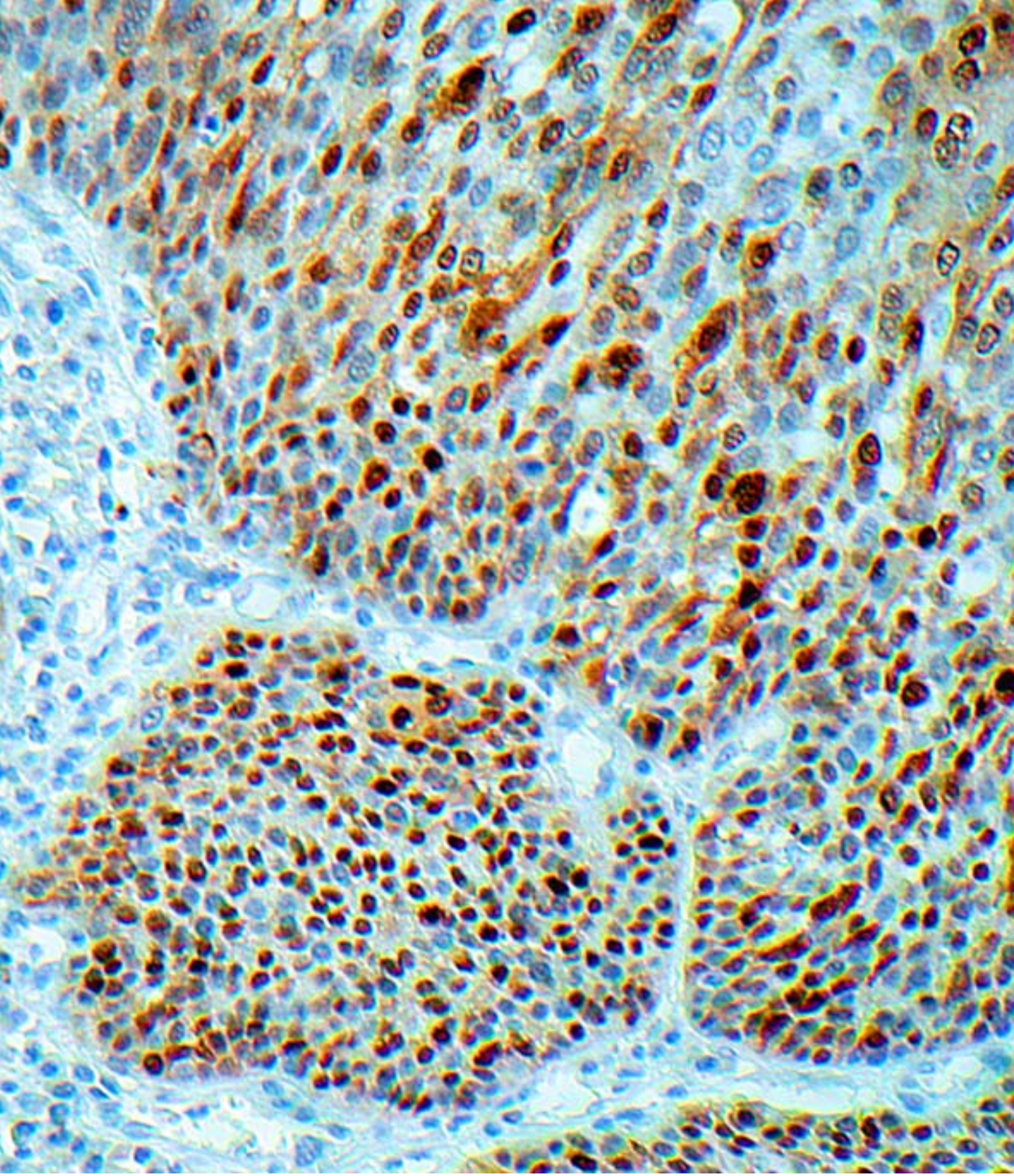
FIGURE LEGENDS

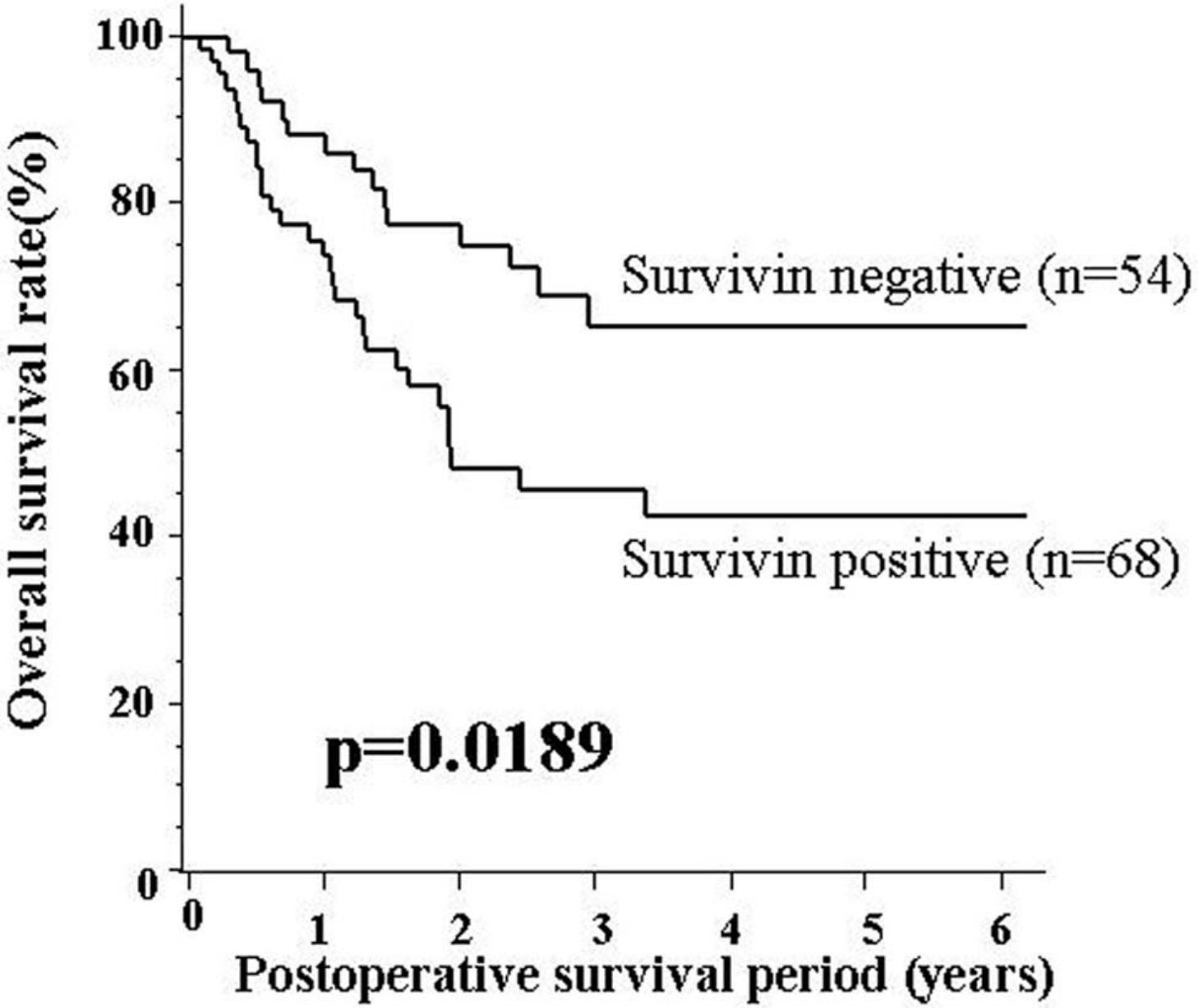
Figure 1

Immunohistochemical staining of survivin showing diffuse nuclear and cytoplasmic staining of ESCC tumor cells. (DAB with hematoxylin counterstain, original magnification ×200)

Figure 2

Comparison of overall survival curves for patients with survivin-positive and -negative tumors out of 122 patients who underwent radical esophagectomy.




100
80
60
40
20
0
Overall survival rate(%)
Survivin negative (n=54)
Survivin positive (n=68)
p=0.0189
0
1
2
3
4
5
6
Postoperative survival period (years)