E2F5 as an Independent Prognostic Factor in Esophageal Squamous Cell Carcinoma

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Abstract. *Background: E2F Transcription Factor 5 Protein (E2F5) is considered to act primarily as a transcriptional repressor in the cell cycle. However, its expression and role in esophageal squamous cell carcinoma (ESCC) have not been investigated. We examined whether the expression of E2F5 is related to the clinicopathological features and prognosis of patients with ESCC. Materials and Methods: The expression of E2F5 was analyzed by immunohistochemistry in 64 primary tumor samples obtained from patients with ESCC who had undergone curative esophagectomy between 1998 and 2009. According to the expression of E2F5 in tumor cells, cases were divided into E2F5-positive (27 cases) and -negative groups (37 cases). The relationship of various clinicopathological features and prognosis with the E2F5 status, were analyzed. Results: In the clinicopathological analysis, the proportion of poorly-differentiated tumors was significantly higher in the E2F5-positive group than in the E2F5-negative group (p=0.027). The 5-year survival rate of the E2F5-positive group was 39.3%, which was significantly poorer than that of the E2F5-negative group (83.8%) (p=0.006). In multivariate analysis, the expression of E2F5 was one of the most important independent prognostic factors after radical esophagectomy. Conclusion: The expression of E2F5 in ESCC may be correlated with a worse prognosis of patients with ESCC.*

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Esophageal carcinoma is one of the most malignant tumor types. The epidemiology of esophageal carcinoma differs depending on the geographic area. In Eastern countries, squamous cell carcinoma is the predominant cellular type of esophageal cancer, while esophageal adenocarcinoma has been increasing in Western countries (1, 2). In recent years, advances in surgical techniques, perioperative management, preoperative and postoperative chemotherapy, and radiotherapy have reduced mortality and improved the prognosis of patients with esophageal squamous cell carcinoma (ESCC) (3, 4). However, the long-term outcomes of ESCC after esophagectomy remain poor compared with those of other types of carcinomas. It is important to develop more useful prognostic factors to predict for outcome of patients with ESCC after esophagectomy.

The E2 promoter binding factor (E2F) family, which is a group of transcription factors involved in the regulation of cellular proliferation, consists of eight members (E2F1-E2F8) (5-7), which are divided into activators and repressors. E2F1-3 are transcriptional activators and their expression has been reported to correlate with the uncontrolled proliferation of cells in human cancer such as lung, ovarian, breast and gastrointestinal (8-11). On the other hand, E2F4-8 are thought to be transcriptional repressors that may have oncogenic influences on cellular proliferation (12). E2F5 has been said to act as a repressor, binding to pocket proteins (p130, p107 and pRb) in the G_1 phase of the cell cycle (13, 14). The expression of E2F5 is related to human cancers such as breast, colon, ovarian, hepatocellular carcinoma and osteogenic sarcoma (15-19). However, the value of E2F5 expression in ESCC has not been previously evaluated, to our knowledge.

In our study, we evaluated the immunochemical expression of E2F5 in patients with ESCC who underwent radical esophagectomy. Furthermore, we investigated the prognostic impact of E2F5 expression by comparing the results of cases with different clinicopathological parameters.

Materials and Methods

Patients and primary tissue samples. ESCC tumor samples were obtained from 64 patients with macroscopically-proven primary ESCC resected curatively between 1998 and 2009 at the Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto, Japan. All patients underwent curative R0 resection. The samples were embedded in paraffin after 24 h of formalin fixation. The cases selected in this study had not developed synchronous tumors or multiple metachronous tumors, and neither preoperative chemotherapy nor radiotherapy was given. All patients gave their written informed consent for the use of their tissues. Nineteen patients (30.0%) died of cancer recurrence; there were no operation-related deaths. The median survival time was 18.0 months (ranging between 4 and 51 months). The median follow-up period was 47.6 months (ranging between 4 and 157 months). Staging was principally based on the International Union Against Cancer (UICC)/TNM Classification of Malignant Tumors (seventh edition) (20).

Immunohistochemistry. Paraffin-embedded tumor tissue was sectioned into 4-μm-thick slices and subjected to immunohistochemical staining for E2F5 and Ki-67 using the avidin-biotin-peroxidase complex method. Briefly, paraffin sections were de-waxed in xylene and hydrated through a graded series of alcohols. Antigen retrieval was performed by heating the samples in Dako REAL Target Retrieval Solution (Glostrup, Denmark) for 40 min at 98˚C. Endogenous peroxidase activity was quenched by incubating the sections for 30 min in 0.3% H_2O_2. The sections were then treated with protein blocker and incubated for 60 min at room temperature and overnight at 4˚C with antibody to E2F5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or Ki-67 (Santa Cruz Biotechnology). The avidin-biotin-peroxidase complex system (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA, USA) was used for color development with diaminobenzidine tetrahydrochloride at room temperature for 30 min. The sections were counterstained with hematoxylin. Finally, the sections were dehydrated through a graded series of alcohols, cleared in xylene and mounted.

Immunohistochemically-stained samples for E2F5 were graded for staining intensity and the proportion of positively-stained tumor cells. E2F5 was mostly localized in the cytoplasm (Figure 1). The staining intensity of the cytoplasm was scored as 0 (not reactive), 1 (weakly-reactive), 2 (moderately-reactive) or 3 (strongly-reactive). Figure 2 shows panels of the four immunohistochemical staining intensity scores for E2F5. The proportion of positive tumor cells was scored as 0 (0 to 10%), 1 (11 to 33%), 2 (34 to 66%) or 3 (67% or more). The total score was calculated by adding the scores of each case (mean±SD=3.11±1.18). Total scores of 4 or more were defined as the E2F5-positive group and scores of 3 or less were defined as the E2F5-negative group.

The proliferative activity reflected by Ki-67 by immunohistochemistry was estimated quantitatively by counting immunoreactive tumor cells. Only distinct immunoreactive tumor cell nuclei were counted. Ki-67-stained cells were quantified in five selected fields with the highest proliferative activity at ×400 magnification. The labeling index of each case was calculated as the number of positive cells divided by the total number of examined cells in all the examined fields.

Statistical analysis. Statistical analysis was carried out using Student's *t*-test for comparisons between two groups, and Fisher's exact test to investigate the correlations between clinicopathological features and E2F5. Survival was calculated using the Kaplan-Meier method, and differences in survival were examined using the log-rank test. Multivariate analysis was performed using the Cox proportional hazard model to identify independent predictors of survival. Differences were considered significant when the *p*-value was less than 0.05. Statistical analyses were performed using StatView-J 5.0 (SAS Institute Inc., Cary, NC).

Results

Out of 64 cases, 27 patients (42.2%) were E2F5-positive and 37 (57.8%) were E2F5-negative after scoring (Table I). We compared eight clinicopathological features between the two groups. There were no significant differences between the two groups in terms of age, gender, depth of invasion (pT), lymph node metastasis (pN), lymphatic invasion, venous invasion and stage. On the other hand, in terms of histological grade, the proportion of poorly-differentiated tumors was significantly higher in the E2F5-positive group than in the E2F5-negative group (Table I). To evaluate the association of E2F5 with Ki-67 expression, immunohistochemical analysis of Ki-67 was performed. The proportion of Ki-67-positive cells varied widely between the tumors. The minimum Ki-67 labeling index was 5.3% and the maximum was 53.7% (median=30.3%, mean±SD=28.3%±12.2%). However, there were no significant differences in Ki-67 labeling index according to E2F5 status (Table I).

Next, we assessed which of the nine factors influenced survival after curative resection of ESCC. In univariate analysis of survival after curative esophagectomy, five factors, namely pT, pN, lymphatic invasion, venous invasion and E2F5, were found to be significant prognostic factors (p=0.007, 0.032, 0.033, 0.015 and 0.006, respectively) (Table II). The 5-year survival rate was 39.3% for the E2F5-positive group and 83.8% for the E2F5-negative group (Figure 3). In multivariate analysis, pT, pN, lymphatic invasion and expression of E2F5 were independent prognostic factors (p=0.019, 0.014, 0.040, <0.001) (Table III). Expression of E2F5 was the strongest prognostic factor of all clinicopathological features. These findings suggest that the expression of E2F5 might be a valuable prognostic factor for patients with ESCC.

Discussion

In recent years, the development of chemotherapy, radiotherapy and surgical techniques has led to improvement in the prognosis of esophageal cancer, which is said to be one of the most malignant tumor types. Operation-related death and complications of surgery have decreased (21). However, the long-term prognosis of esophageal cancer remains unsatisfactory, even after radical esophagectomy. The 5-year survival rates are reported to be less than 50% after esophagectomy. In Japan, ESCC is the most common histological type (92.2%), with adenocarcinoma being quite

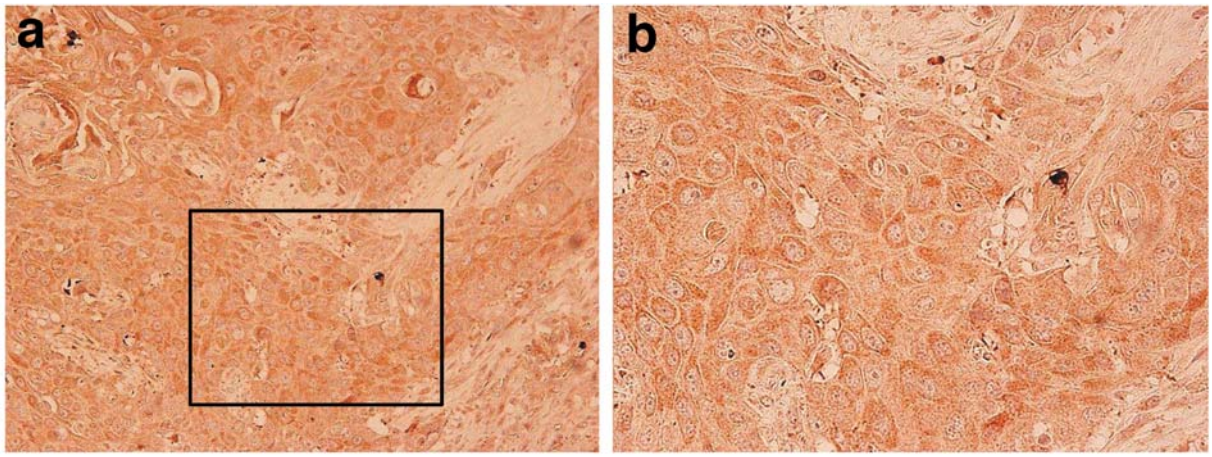

Figure 1. *E2F transcription factor 5 protein (E2F5) expression pattern in human esophageal squamous cell carcinoma (ESCC). Immunohistochemical staining with E2F5 antibody of primary tumor samples of human ESCC. a: Typical example of E2F5 expression in the cytoplasm of ESCC (original magnification, ×200). b: Detail of the area shown in (a) (original magnification, ×400). E2F5 antibody reaction demonstrates diffuse fine granular staining in the cytoplasm.*

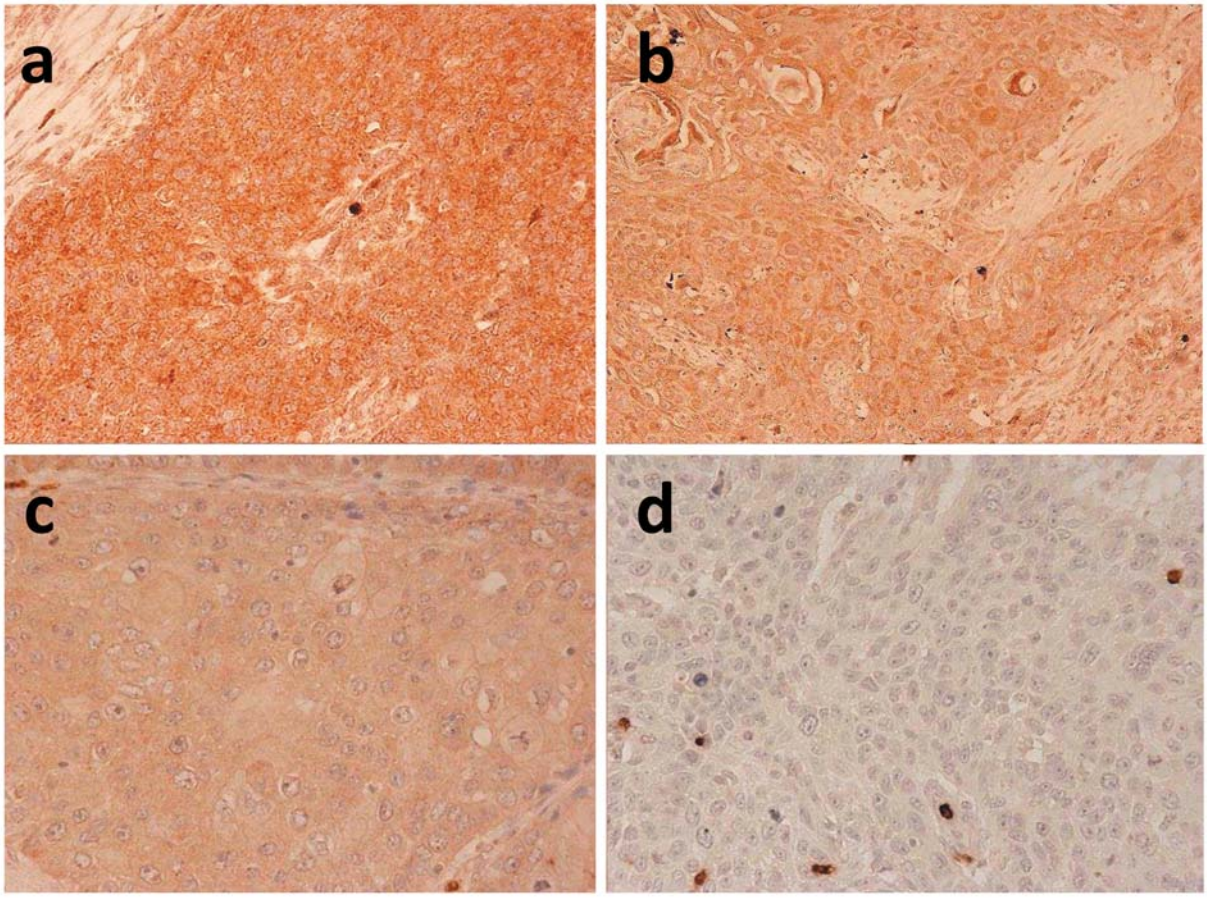

Figure 2. *Immunohistochemical staining of primary human esophageal squamous cell carcinoma (ESCC) samples with E2F transcription factor 5 protein (E2F5) antibody (×400). E2F5 expression of strong intensity (intensity score 3) (a), moderate intensity (intensity score 2) (b), weak intensity (intensity score 1) (c) and no staining (intensity score 0) (d).*

Table I. *Associations between the clinicopathological features of esophageal squamous cell carcinoma and E2F transcription factor 5 protein (E2F5) status*.

Variable		E2F5		
		Positive (n=27)	Negative (n=37)	*p*-Value
Age	<60 years	9	10	0.586
	≥60 years	18	27	
Gender	Male	23	31	0.879
	Female	4	6	
Histological grade	Well- or moderately-differentiated	15	30	0.027*
	Poorly-differentiated	12	7	
Depth of invasion (pT)	pT1	13	19	0.800
	pT2-3	14	18	
Lymph node metastasis (pN)	pN0	15	17	0.448
	pN1-3	12	20	
Lymphatic invasion	Negative	13	19	0.800
	Positive	14	18	
Venous invasion	Negative	13	22	0.369
	Positive	14	15	
pStage	I	12	13	0.451
	II-III	15	24	
Ki-67 labeling index (%)+		28.1±14.2	26.6±11.6	0.644

Data are the mean±SD. Student's *t*-test was used for two-group comparisons. *$p<0.05$: Fisher's exact test. pT: Pathological T stage; pN: pathological N stage; pStage: pathological stage.

Table II. *The 5-year survival rate of patients with esophageal squamous cell carcinoma according to various clinicopathological parameters*.

Variable		n	5-Year survival rate (%)	*p*-Value
Age	<60 years	19	73.1	0.185
	≥60 years	45	56.5	
Gender	Male	54	66.5	0.541
	Female	10	76.2	
Histological grade	Well- or moderately-differentiated	45	74.7	0.193
	Poorly-differentiated	19	53.4	
Depth of invasion (pT)	pT1	32	81.8	0.007*
	pT2-3	32	53.8	
Lymph node metastasis (pN)	pN0	32	78.6	0.032*
	pN1-3	32	56.3	
Lymphatic invasion	Negative	32	78.4	0.033*
	Positive	32	57.4	
Venous invasion	Negative	35	81.6	0.015*
	Positive	29	50.4	
pStage	I	25	76.5	0.155
	II-III	39	61.9	
E2F5	Negative	37	83.8	0.006*
	Positive	27	39.3	

*$p<0.05$: Log-rank test. pT: Pathological T stage; pN: pathological N stage; pStage: pathological stage.

rare (3.0%) (22). Patients with ESCC should be specifically targeted in an effort to improve their overall prognosis.

The E2F family was discovered as a group of factors that bind with the promoter of E2 genes on adenoviral DNA in 1986 by Nevins (23). It is now well-known that the E2F family act as transcriptional factors associated with pocket proteins such as pRb, p107 and p130, and play an important role in cellular proliferation. Therefore, E2F members may have the potential to act as oncogenic factors. E2F1-3 are transcriptional activators regulated by pocket proteins, which

Table III. *Prognostic factors of esophageal squamous cell carcinoma according to multivariate analysis*

Variable		RR	95% CI	*p*-Value
Depth of invasion (pT)	pT1	1		
	pT2-3	3.475	1.227-9.842	0.019*
Lymph node metastasis (pN)	pN0	1		
	pN1-3	3.538	1.292-9.689	0.014*
Lymphatic invasion	Negative	1		
	Positive	2.820	1.048-7.587	0.040*
Venous invasion	Negative	1		
	Positive	2.105	0.786-5.637	0.131
E2F5	Negative	1		
	Positive	6.518	2.29-18.549	<0.001*

95% CI: 95% Confidence interval; RR: risk ratio; E2F5: E2F transcription factor 5 protein; pT: pathological T stage; pN: pathological N stage; *$p<0.05$: Cox's proportional hazards model.

are predominantly nuclear. E2F4 and E2F5 are transcriptional repressors binding with pocket proteins in the G_1 phase, which are primarily found in the cytoplasm. While E2F1-5 functions depend on pocket proteins, E2F6-8 are thought to be transcriptional repressors that act independently of pocket proteins, owing to their lack of pocket protein-binding domains (6, 7).

E2F5 is one of the transcriptional repressors that does depend on pocket proteins (13, 14, 24). To our knowledge, our immunohistochemical study is the first to report the expression of E2F5 in human ESCC tissue. Several studies have previously reported on the expression or amplification of *E2F5* in other types of human solid carcinoma, but there are few reported studies that assessed the correlation between E2F5 status and the prognosis of malignant tumors. Kothandaraman *et al*. demonstrated that the expression of E2F5 in tissues and serum of human epithelial ovarian cancer might improve the diagnosis of malignancy (17). Fuchs *et al*. suggested that *E2F5* genes may be one of the markers of human osteogenic sarcoma, along with another 21 genes (19). Lassmann *et al*. detected DNA amplification of E2F5 (8p22 – q21.3) in sporadic colorectal cancer (16).

In our study, the expression of E2F5 was correlated with histological grade, but not with other clinicopathological factors. Jiang *et al*. found that E2F5 expression was significantly higher in human primary hepatocellular carcinoma than in normal liver tissues by immunohistochemistry. They also reported that E2F5 intensity tended to positively correlate with tumor grade (18). Umemura *et al*. showed that the expression of E2F5 was associated with higher histological grade in human breast cancer and that the expression of E2F5 led to worse clinical outcomes with shorter disease-free survival (15). Their and our results suggest that the expression of E2F5 is closely linked with the differentiation of cancer cells and is related to prognostic significance. They also reported a significant

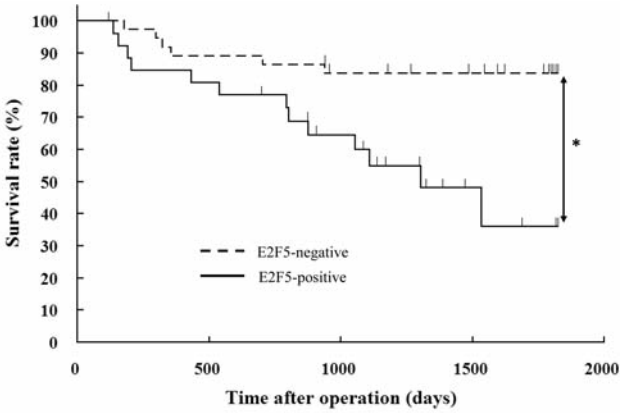

Figure 3. *Survival curve of patients with esophageal squamous cell carcinoma using the Kaplan-Meier method. The patients were classified into two groups: E2F transcription factor 5 protein (E2F5)-positive group and E2F5-negative group. Statistical analysis: log-rank test (*p<0.05).*

correlation between E2F5 status and Ki-67 labeling index. Ki-67, a known proliferation marker, is expressed in late G_1, S, G_2 and M phases. Several studies assessed the cell proliferative activity of ESCC or other carcinomas using the Ki-67 labeling index (25). We examined the association between E2F5 status and Ki-67 labeling index, but no significant relationship was found between the two. One of the reasons for this may be the difference in methods of assessment in immunohistochemistry, although further studies will be needed with a larger sample size.

Our prognostic analysis demonstrated that patients in the E2F5-positive group had a significantly worse outcome than those in the E2F5-negative group, as well as there being other prognostic factors such as pT, pN and lymphatic invasion. Our result suggests that E2F5 may play an oncogenic role and be related to the invasiveness of cancer cells in ESCC.

In conclusion, we investigated whether E2F5 expression in patients with ESCC is associated with various clinicopathological factors and prognosis by immunohistochemical analysis. The expression of E2F5 in the cytoplasm was correlated with the histological grade of ESCC, and was one of the most significant prognostic factors. The expression of E2F5 may be an indicator of poor clinical outcome after esophagectomy and a novel therapeutic target for the future treatment of ESCC.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Acknowledgements

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