Clinicopathological and Prognostic Significance of FOXM1 Expression in Esophageal Squamous Cell Carcinoma

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Abstract. *Background: Esophageal squamous cell carcinoma (ESCC) has a poor prognosis because invasion and metastasis are prevalent. To improve diagnosis, it is important to identify and characterize tumor-specific molecular markers in ESCC. FOXM1 is overexpressed and correlates with pathogenesis in a variety of human malignancies. We aimed to investigate the clinical significance of FOXM1 overexpression in ESCC. Patients and Methods: FOXM1 expression was assessed in ESCC specimens from 174 curatively-resected cases. The relationships between FOXM1 expression, clinicopathological parameters, and prognoses were examined. Results: Immunohistochemical analysis showed that 94 (54.0%) tumors were positive for FOXM1 expression. FOXM1 positivity did not correlate with any clinicopathological parameter. However, FOXM1-positive cases had poorer prognoses than FOXM1-negative ones (p=0.0037, log-rank test). In multivariate analysis, the following were independent prognostic factors: pT, pN, neoadjuvant chemotherapy, and FOXM1 expression (hazard ratio=1.69, 95% confidence interval=1.06-2.75, p=0.027). Conclusion: FOXM1 may be a novel prognostic factor in patients with ESCC who undergo curative resection.*

Esophageal cancer is one of the most aggressive diseases of the gastrointestinal tract (1). In Japan and other East Asian countries, the majority of esophageal cancer diagnoses are esophageal squamous cell carcinoma (ESCC). Despite improvements in surgical technique, chemotherapy, and radiation therapy, the mortality rate of ESCC remains high and

its prognosis remains poor because of the high prevalence of invasion and metastasis (2). To improve survival, it is important to identify and characterize tumor-specific molecular markers in ESCC that may contribute to its carcinogenesis.

FOXM1 is a member of the Forkhead family of transcription factors (3, 4). FOXM1 acts in the cell cycle by regulating the transition from the G_1 to the S phase, as well as the progression to mitosis (4-6). FOXM1 is predominantly expressed in fetal tissues, but its expression may be maintained in proliferating adult tissues (5, 6). Overexpression of FOXM1 has been observed in cancer of the liver, breast, prostate, brain, cervix, colon, lung, and stomach (7-14). These findings link FOXM1 to the tumorigenesis and progression of several kinds of malignancies. However, the relationship of FOXM1 to ESCC prognosis remains unclear. In the present study, we investigated whether FOXM1 could be used as an independent biomarker to predict prognosis in patients with ESCC.

Patients and Methods

Patients and treatments. The present study included 174 patients with pathologically-confirmed primary ESCC (Table I) who underwent curative surgical resection at Osaka University Hospital between 2001 and 2007. The study population included 19 women and 155 men; the median age was 64 years (range=46 to 81 years). All patients underwent subtotal esophagectomy *via* right thoracotomy with two- or three-field lymphadenectomy. Non-curative resection was excluded, and curative (R0) resection was achieved for all patients. No patients died of postoperative complications. The 63 patients with lymph node metastasis at initial diagnosis received neoadjuvant chemotherapy (NAC), which consisted of two courses of 5-fluorouracil, cisplatin, and adriamycin. After surgery, patients were surveyed every three months by physical examination and serum tumor markers (squamous cell carcinoma antigen, carcinoembryonic antigen), every six months by computed tomographic scanning and abdominal ultrasonography, and every year by endoscopy until tumor recurrence. Patients with tumor recurrence received chemotherapy or chemoradiotherapy as long as they were able to tolerate it. The mean overall survival (OS) was 46.3 months, and the mean recurrence-free survival (RFS) was 42.8 months.

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Table I. *Clinical characteristics of 174 patients with esophageal squamous cell carcinoma.*

Parameter	Patients, n (%)
Age, years	64 (46-81)[a]
Gender, male/female	155 (89.0)/19 (11.0)
Histology[b], poor/mod/well	42 (24.1)/93 (53.4)/39 (22.4)
pT[c], 0/1/2/3/4	0 (0)/50 (28.7)/27(15.5)/84 (48.3)/13 (7.5)
pN[c] N0/N1/N2/N3	54 (31.0)/56 (32.2)/37 (21.3)/27 (15.5)
pStage[c] 0/I/II/III/IV	0 (0)/33 (18.9)/41 (23.6)/74 (42.5)/26 (14.9)

[a]Data presented as median (range). [b]Poorly, moderately, and well-differentiated squamous cell carcinoma. [c]pT, pN, pStage (pathological classification) according to the seventh edition of the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) classification.

Immunohistochemical analysis. FOXM1 expression was evaluated by immunohistochemistry of 4-μm-thick sections of 10% formalin-fixed and paraffin-embedded tissue blocks, as described previously (12). For staining, tissue slides were de-paraffinized in xylene and then rehydrated using graded ethanol. For antigen retrieval, slides were autoclaved in 10 mM citrate buffer (pH 6.0) at 110°C for 20 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 20 min. Non-specific binding was blocked with 10% normal serum for 20 min. Subsequently, tissue slides were incubated overnight with FOXM1 antibody (sc502, dilution 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C in a moist chamber. Sites of antibody binding were visualized with the ABC peroxidase detection system (Vector Laboratories, Burlingame, CA, USA). Finally, sections were incubated in 3,3'-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 for 1 min and counterstained with 0.1% hematoxylin. One representative slide with the deepest tumor invasion was selected from each patient and subjected to immunohistochemistry. The percentage of cancer cells stained with the antibody was then determined. FOXM1 staining for each ESCC sample was defined as positive when more than 10% of the cancer cells in a section were immunoreactive with the FOXM1 antibody; it was defined as negative when 10% or fewer of the cancer cells in a section were positive.

Statistical analysis. Statistical analysis was performed using JMP software (JMP version 9.0.2; SAS Institute, Cary, NC, USA). The relationship between FOXM1 expression and various clinicopathological parameters was assessed using the χ^2 test. RFS and OS were assessed using the Kaplan–Meier method and compared using the log-rank test. All parameters found to be significant in univariate analysis using the Cox proportional hazards model were entered into multivariate survival analysis. *p*-Values <0.05 were considered significant; each *p*-value was derived from a two-tailed test.

Results

FOXM1 expression in ESCC. A total of 174 samples (Table I) that contained both cancerous and non-cancerous lesions were evaluated for FOXM1 expression by immunohisto-chemistry. Out of these, 94 (54.0%) were positive for FOXM1 expression; staining was mainly cytoplasmic, with

Table II. *Correlation between FOXM1 expression and clinicopathological parameters.*

Parameters	FOXM1 expression		
	Positive (%)	Negative (%)	*p*-Value
Age, years			
<65	46 (26.4)	43 (24.7)	0.53
≥65	48 (27.6)	37 (21.3)	
Gender			
Male	84 (48.3)	71 (40.8)	0.90
Female	10 (5.8)	9 (5.2)	
Histology[a]			
Poor, moderate	74 (42.5)	61 (35.1)	0.70
Well	20 (11.5)	19 (10.9)	
Neoadjuvant chemotherapy			
Yes	35 (20.1)	28 (16.1)	0.76
No	59 (33.9)	52 (29.9)	
pT[b]			
T1-2	38 (21.8)	39 (22.4)	0.27
T3-4	56 (32.2)	41 (23.6)	
pN[b]			
N0	24 (13.8)	30 (17.2)	0.089
N1-3	70 (40.2)	50 (28.4)	
pStage[b]			
I, II	36 (20.7)	38 (21.8)	0.22
III, IV	58 (33.3)	42 (24.1)	

[a]Poorly, moderately, and well-differentiated squamous cell carcinoma. [b]pN, pT, pStage (pathological classification) according to the seventh edition of the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) classification.

faint nuclear staining in tumor cells (Figure 1A). The remaining 80 (46.0%) samples were negative for FOXM1 expression (Figure 1B). In contrast, none of the samples of normal squamous epithelium exhibited substantial FOXM1 staining, although some basal cells exhibited faint nuclear immunostaining (Figure 1C). FOXM1-positive cells were detected in various parts of the tumors, including the surface, central, and deep areas of the esophagus.

Correlation between FOXM1 expression and clinico-pathological parameters. Table II lists the correlations between FOXM1 expression and various clinicopathological parameters. No significant correlations were observed between FOXM1 expression and other parameters, including age, sex, histology, use of NAC, or depth of tumor invasion (Table II).

Correlation between FOXM1 expression and survival. The total 5-year OS rate was 52.7%. Patients with FOXM1-positive tumors exhibited poorer OS than those with negative tumors (5-year OS 42.8% *versus* 64.8%, *p*=0.0037; Figure 2A). Similarly, patients with FOXM1-positive tumors exhibited poorer 5-year RFS than those with FOXM1-
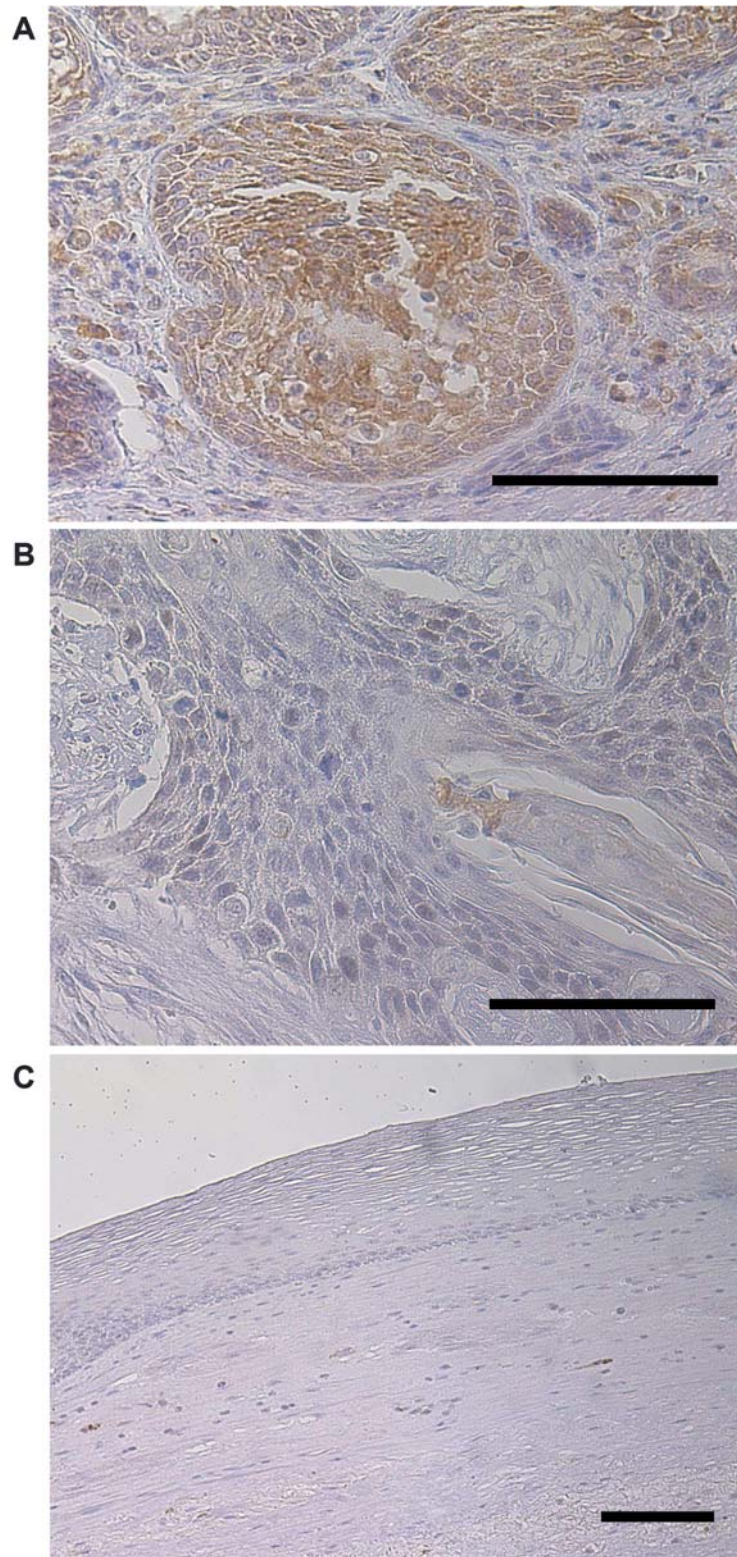
Figure 1. *FOXM1 expression determined by immunohistochemical staining. A: Representative FOXM1-positive esophageal squamous cell carcinoma exhibiting staining mainly in the cytoplasm of tumor cells (magnification ×200). B: Representative FOXM1-negative esophageal squamous cell carcinoma exhibiting almost no staining of tumor cells (magnification ×200). C: Representative normal squamous epithelium that was negative for FOXM1 expression except in a few basal cells (magnification ×100). Scale bars, 100 μm.*
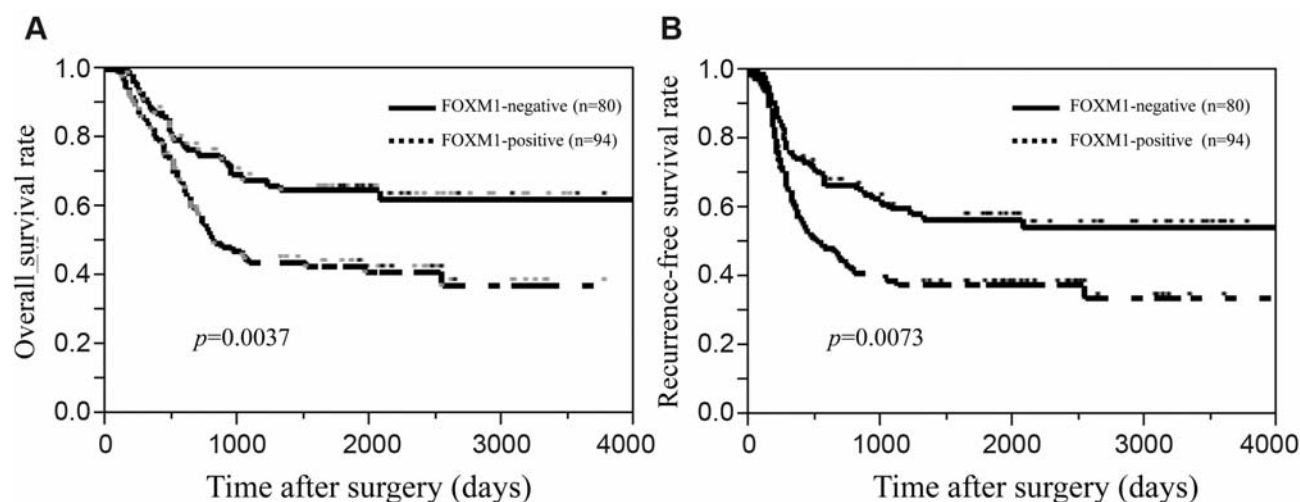
Figure 2. *Survival curves according to FOXM1 expression. A: Overall survival curve according to FOXM1 expression for all patients plotted by the Kaplan–Meier method. B: Recurrence-free survival curves according to FOXM1 expression for all patients. Differences between the two groups were evaluated using the log-rank test.*

Table III. *Univariate and multivariate analysis of overall survival using Cox's proportional hazard model.*

Parameter	Number of cases	Univariate		Multivariate	
		HR (95% CI)	p-Value	HR (95% CI)	p-Value
Age, ≥65 years *vs*. < 65 years	89 *vs*. 85	1.13 (0.73-1.76)	0.57		
Sex, female *vs*. male	19 *vs*. 155	1.02 (0.47-1.93)	0.96		
Histology, poor, moderate *vs*. well[a]	135 *vs*. 39	1.54 (1.87-2.91)	0.14		
pT (T3, 4 *vs*. T1, 2)[b]	97 *vs*. 77	2.48 (1.56-4.05)	<0.0001	1.69 (1.04-2.82)	0.033
pN (N1-3, N0)[b]	120 *vs*. 54	3.56 (2.01-6.93)	<0.0001	2.77 (1.54-5.42)	0.0004[b]
Neoadjuvant chemotherapy, yes *vs*. no	63 *vs*. 111	2.36 (1.52-3.66)	0.0001	1.97 (1.26-3.10)	0.0031
FOXM1 expression, positive *vs*. negative	94 *vs*. 80	1.95 (1.24-3.15)	0.0034	1.69 (1.06-2.75)	0.027

[a]Poorly, moderately, and well differentiated squamous cell carcinoma. [b]pT, pN, (pathological classification) according to the seventh edition of the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) classification. HR: Hazard ratio; CI: confidence interval.

negative tumors. In univariate analysis, the following were significantly associated with OS: pT [hazard ratio (HR)=2.48, 95% confidence interval (CI)=1.56-4.05, $p<0.0001$], pN (HR=3.56, 95% CI=2.01-6.93, $p<0.0001$), NAC (HR=2.36, 95% CI=1.52-3.66, $p=0.0001$), and FOXM1 expression (HR=1.95, 95% CI=1.24-3.15, $p=0.0034$) (Table III). The four parameters that showed statistical significance ($p<0.05$) in univariate analysis were entered into multivariate analysis. Multivariate analysis revealed that pN was the poorest prognostic factor (HR=2.77, 95% CI=1.54-5.42, $p=0.0004$), followed by NAC (HR=1.97, 95% CI=1.26-3.10, $p=0.0031$), pT (HR=1.69, 95% CI=1.04-2.82, $p=0.033$), and positive FOXM1 expression (HR=1.69, 95% CI=1.06-2.75, $p=0.027$) (Table III).

Discussion

In the present study, we investigated the expression of FOXM1 in ESCC tissues. To our knowledge, this is the largest series of samples analyzed for FOXM1 expression in ESCC to date. Our analysis revealed that FOXM1 expression in ESCC is an independent prognostic indicator for OS. This finding is consistent with previous reports (7, 11, 12, 15, 16). In our series, patients with advanced ESCC received NAC. Thus, NAC became a strong prognostic factor for OS. As far as we are aware, there is just one report on the association between FOXM1 and ESCC in clinical samples (17). In that study, Hui *et al*. reported that FOXM1 overexpression was associated with pathological stage, but not with prognosis of patients with

ESCC. However, it might be premature to conclude that FOXM1 is not associated with the prognosis of patients with ESCC. The report by Hui *et al*., assessed only 64 patients, and may have been too small to reveal an association between FOXM1 expression and prognosis. Notably, although that study did not find an association between FOXM1 expression and prognosis, it did show a positive association between FOXM1 expression and pathological stage.

FOXM1 is a proliferation-associated transcription factor with important roles in cell proliferation, differentiation, and apoptosis (5, 6, 18). However, the mechanism by which FOXM1 signaling induces tumor growth is not well-understood. Multiple pathways crosstalk with the FOXM1 pathway, including the phosphatidylinositol 3-kinase/protein kinase B (Akt) (19, 20), nuclear factor-κB (21), sonic hedgehog (22), extracellular signal-regulated kinase (23), cyclooxygenase-2 (24), epidermal growth factor receptor (25, 26), vascular endothelial growth factor (27, 28), avian myelocytomatosis virus oncogene cellular homolog (c-MYC) (29, 30), p53 (31, 32), and hypoxia-inducible factor-1 pathways (33). Thus, these reports strongly suggest that FOXM1 is centrally-involved in tumor aggressiveness. In our analysis FOXM1 expression was associated not only with OS but also RFS, this phenomenon was consistent with these mechanisms.

Overexpression of FOXM1 in tumor cell lines is correlated with resistance to apoptosis and to premature senescence induced by oxidative stress, which is strongly implicated in resistance to chemotherapy (34). Recent studies show that FOXM1 is overexpressed in a variety of human cancer types and is crucially-implicated in tumorigenesis (3, 8-10, 35, 36). Furthermore, down-regulation of FOXM1 leads to inhibition of cell growth, migration, and invasion in several cancer types (36-38). These results suggest that FOXM1 may play a crucial role in the development and progression of human cancer. Therefore, although more studies are required, inactivation of FOXM1 may represent a promising strategy for developing novel and selective anticancer therapies.

In conclusion, here we examined the expression of FOXM1 protein in ESCC specimens and investigated correlations between FOXM1 overexpression and clinicopathological characteristics. Patients that were positive for FOXM1 expression had worse prognoses. Thus, evaluation of FOXM1 expression might help identify a subset of patients with ESCC who need more intensive treatment.

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