


ORIGINAL ARTICLE

FOXM1: A potential indicator to predict lymphatic metastatic recurrence in stage IIA esophageal squamous cell carcinoma

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Keywords

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Abstract

Background: Previous studies have elucidated that FOXM1 may predict poor prognosis in patients with multiple solid malignant tumors. In this study we explored the differential expression of FOXM1 in stage IIA esophageal squamous cell carcinoma (ESCC) and investigated its prognostic value.

Methods: Immunohistochemistry (IHC) and Western blot were used to detect FOXM1 expression in ESCC. Correlations between FOXM1 expression and clinicopathological variables, and five-year lymphatic metastatic recurrence (LMR) and overall survival (OS) of patients were analyzed.

Results: FOXM1 was aberrantly expressed in ESCC. Statistical analysis revealed a close relationship between FOXM1 expression and tumor size ($P = 0.024$), depth of invasion ($P = 0.048$), and degree of differentiation ($P = 0.043$). The five-year LMR of patients in the FOXM1 overexpression group was significantly increased compared to the low expression group ($P = 0.001$). The five-year OS of patients in the FOXM1 overexpression group was significantly reduced compared to the low expression group ($P = 0.007$). Log-rank tests demonstrated that large tumor size ($P = 0.044$), poor differentiation degree ($P = 0.005$), deep invasion ($P = 0.000$), and FOXM1 overexpression ($P = 0.007$) may indicate poor prognosis in stage IIA ESCC. Cox multivariate regression analysis revealed that all of these variables were independent predictors of unfavorable outcome ($P < 0.05$).

Conclusion: FOXM1 could be a predictor of lymphatic metastatic recurrence in stage IIA ESCC after Ivor Lewis esophagectomy.

Introduction

Esophageal carcinoma, a common cancer of the digestive tract, is the sixth leading cause of cancer-related death worldwide.^{1,2} In China, the mortality rate of esophageal cancer ranked fourth among all cancers, with standardized mortality rates steadily increasing by an average of $1.06/10^5$ per year from 1991 to 2012. In 2012, the mortality rate was $16.77/10^5$ and the standardized mortality rate was $7.75/10^5$.³ There are two main histopathological types of this disease: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. ESCC is the predominant type in China, comprising approximately 90% of all diagnosed esophageal cancer cases in some areas.^{4,5} Given

improvements at diagnostic level, an increasing number of individuals are now diagnosed at earlier stage; however, prognosis is still not optimistic, with a five-year overall survival (OS) rate of only 20–30%.^{5–7} Lymphatic metastatic recurrence (LMR) is the main reason for treatment failure and poor prognosis.^{8–10} Even in stage IIA ESCC, the five-year survival rate is only 30–50% after complete resection, and many patients may eventually die from LMR.¹¹ To improve the OS of ESCC patients, it is necessary to control locoregional LMR. Several indexes are associated with LMR, including tumor node metastasis (TNM) staging, but the specificity and sensitivity cannot satisfy our clinical requirements. Therefore, we still need to investigate useful

biological markers to predict the risk of LMR for individuals with ESCC to subsequently improve prognosis.

FOXM1 is an important transcriptional factor of the Forkhead box family, characterized by an evolutionarily conserved DNA binding domain called the winged-helix domain.^{12,13} Previous studies have demonstrated that FOXM1 is aberrantly expressed in various malignant tumors, such as prostate and breast adenocarcinoma, cervical and nasopharyngeal carcinoma, and ESCC.^{14–18} FOXM1 regulates the cell cycle by influencing the phase transitions from G1 to S and G2 to M and participates in cell proliferation.^{19–21} In addition, FOXM1 also participates in the migration and invasion of several tumor cells and promotes cancer metastasis.^{22–24} Recently, studies have demonstrated that FOXM1 is overexpressed in ESCC and may predict poor prognosis in patients.²⁵ Nevertheless, the association between FOXM1 expression and LMR still needs to be elucidated.

In the present study we sought to validate the relationships between FOXM1 expression and clinicopathological parameters, and the five-year LMR and OS rates of stage IIA ESCC patients after Ivor Lewis esophagectomy with two-field lymphadenectomy. We explored whether FOXM1 can predict the LMR of patients.

Methods

Patients and specimens

Eight pairs of frozen ESCC tissues and corresponding noncancerous esophageal tissues (> 5 cm from the

margin of tumor) were collected from Shandong Provincial Hospital Affiliated to Shandong University from June 2016 to 2017. In addition, 178 formalin-fixed paraffin-embedded tumor specimens were harvested from patients who underwent Ivor Lewis esophagectomy with two-field lymphadenectomy from January 2007 to December 2009.

The inclusion criteria were: (i) curative (R0) resection of mid-thoracic ESCC; (ii) > 12 (13–27) lymph nodes dissected; (iii) no administration of preoperative adjuvant chemotherapy or postoperative treatment; and (iv) all patients were pathologically diagnosed as stage IIA (T2-3N0M0) after surgery. Patients with hematogenous recurrence were excluded. Detailed clinical data of the patients is presented in Table 1.

Approval was obtained from the Research Ethic Committee of Shandong Provincial Hospital Affiliated to Shandong University. Informed consent was obtained from each patient or their relatives.

Immunohistochemistry (IHC) and immunohistochemical score (IHS)

Immunohistochemistry (IHC) was used to examine FOXM1 expression using the streptavidin-peroxidase method. Anti-FOXM1 rabbit polyclonal antibodies (Abcam, Cambridge, MA, USA) were diluted at 1:100. The primary antibodies were replaced by phosphate-buffered saline (PBS) as a negative control. The secondary biotinylated antibody kit was purchased from ZSGB Biotech (Beijing, China).

Table 1 Correlation of FOXM1 expression with clinicopathological features of 178 stage IIA ESCC patients

Parameters	Cases (178)	FOXM1		P†	Five-year OS (%)	P‡	Five-year LMR (%)	P‡
		Low expression	Overexpression					
Gender				0.543		0.240		0.317
Male	99	40	59		49.4		64.6	
Female	79	36	43		38.4		55.7	
Age (years)				0.548		0.779		0.582
> 50	96	43	53		40.6		64.6	
≤ 50	82	33	49		46.3		56.1	
Tumor Size (cm)				0.024		0.044		0.020
< 3	83	43	40		53.0		49.4	
≥ 3	95	33	62		34.7		70.5	
Depth of invasion				0.048		0.000		0.000
T2	80	41	39		58.8		42.5	
T3	98	35	63		30.6		75.5	
Differentiation degree				0.043		0.005		0.014
Low	67	22	45		31.3		70.1	
Mid-high	111	54	57		50.5		55.0	
FOXM1						0.007		0.001
Low expression		76			52.6		47.4	
Overexpression			102		36.3		70.6	

Bold values indicate $P < 0.05$. † χ^2 test. ‡Log-rank test. ESCC, esophageal squamous cell carcinoma; FOXM1, Forkhead box M1; LMR, lymphatic metastatic recurrence.

Two pathologists who were blinded to the clinicopathological data evaluated all sections. The immunohistochemical score (IHS) was measured by combining the quantity score (percentage of positive stained cells in five fields) with the staining intensity score. The quantity score was rated as follows: 0 (< 5%), 1 (5–25%), 2 (26–50%), and 3 (> 50%). The staining intensity was scored as 0 (absent), 1 (weak), 2 (moderate), and 3 (strong). The total score was classified into low expression (0–4) and overexpression (5–9).

Western blot analysis

Protein was extracted from tissue samples, and the concentration was determined using a bicinchoninic acid kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein (40 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane filters (Millipore, Billerica, MA, USA). Briefly, 5% non-fat dry milk was used to block the non-specific binding. Membranes were incubated overnight at 4°C with primary antibodies anti-FOXM1 (1:500) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1: 5000; Abcam, Cambridge, MA, USA). After washing, the membranes were incubated with corresponding secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit antibodies, 1: 5000; ZSGB Biotech, Beijing, China) for one hour at room temperature. Finally, the protein levels were quantified by an enhanced chemiluminescence (ECL) detection system (Amersham Imager 600; General Electric, Fairfield, CT, USA).

Follow-up

All patients underwent a routine examination every three to six months. The examinations mainly consisted of physical examination, B ultrasonography of the abdomen, chest and upper abdomen computed tomography (CT) scan, positron emission tomography (PET), bone scintigraphy, and cerebral CT. If progressive lymph node enlargement was observed in postoperative imaging, biopsy was the first choice to identify whether metastatic lymph node recurrence was involved. When mediastinal lymph node enlargement was identified in CT scans but a biopsy was difficult to obtain, a PET-CT scan was taken. Follow-up of this study ended in December 2015; the longest follow-up period was six years.

Statistical analysis

The χ^2 test was used to analyze the relationship between FOXM1 expression and clinicopathological variables. Survival and recurrence curves were calculated using the Kaplan–Meier method. A log-rank test was used to

compare the differences between FOXM1 expression and the survival and recurrence status of patients. Cox regression analysis was used to evaluate independent prognostic factors. A statistically significant difference was defined with a two-tailed *P* value < 0.05. All statistical analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA).

Results

FOXM1 overexpression in esophageal squamous cell carcinoma (ESCC)

Immunohistochemical assays were used to detect FOXM1 expression in ESCC (Fig 1). FOXM1 expression was mainly observed in the cytoplasm as a yellow or brownish-yellow stain. In some cases it was also detected in the nuclei of tumor cells. Low or undetected FOXM1 expression was observed in noncancerous tissues. According to IHS standard, we divided all patients into two groups: 102 cases (57.3%) of overexpression, and 76 (42.7%) of low expression. We also validated the differential expression of FOXM1 by Western blot analysis in eight pairs of ESCC and noncancerous tissues. FOXM1 was obviously overexpressed in ESCC compared to noncancerous tissues (*P* < 0.05), which was consistent with IHC results (Fig 2).

FOXM1 expression and clinicopathological parameters

We used the chi-square test to detect the relationship between FOXM1 expression and the clinicopathological parameters of patients. FOXM1 expression was significantly associated with tumor size (*P* = 0.024), tumor differentiation degree (*P* = 0.043), and depth of invasion (*P* = 0.048).

FOXM1 expression and prognosis

After thorough follow-up, 108 cases (60.7%) were diagnosed as LMR within five years. The five-year OS rate of 178 patients was 41.0%. The median survival time was 49.5 months (9–72). The five-year LMR and OS rates of patients in the overexpression group were 70.6% and 36.3%, respectively. In the low expression group, the five-year LMR rate was only 47.4%, and the five-year OS rate reached 52.6%. Kaplan–Meier analysis revealed that the five-year OS rate was obviously reduced in patients with FOXM1 overexpression (Fig 3). In contrast, the five-year LMR rate was correspondingly increased in this group. Univariate analysis showed that tumor size (*P* = 0.044), depth of invasion (*P* = 0.000), differentiation degree (*P* = 0.005), and FOXM1 expression (*P* = 0.007) were

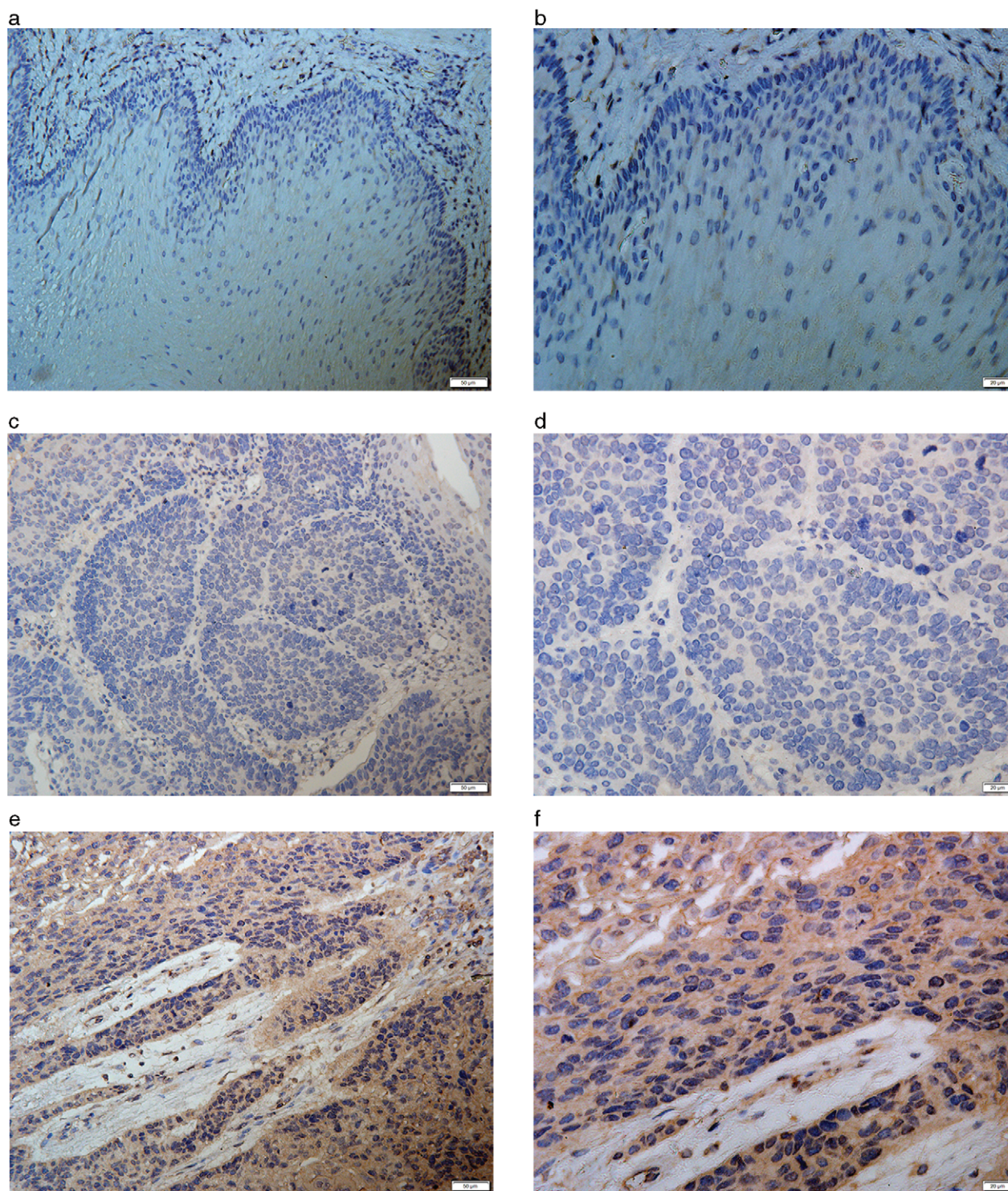


Figure 1 Immunohistochemical staining of FOXM1 in esophageal squamous cell carcinoma (ESCC) and noncancerous tissues. Representative (a,b) negative, (c,d) low, and (e,f) strong positive expression of FOXM1 in ESCC tissue (x200, x400, respectively).

significant prognostic factors. In contrast, gender ($P = 0.240$) and age ($P = 0.779$) did not reach statistical significance. To exclude confounding factors, Cox

proportional hazards model analysis was performed. Multivariate analysis revealed that tumor size ($P = 0.034$), differentiation degree ($P = 0.024$), depth of invasion ($P = 0.000$),

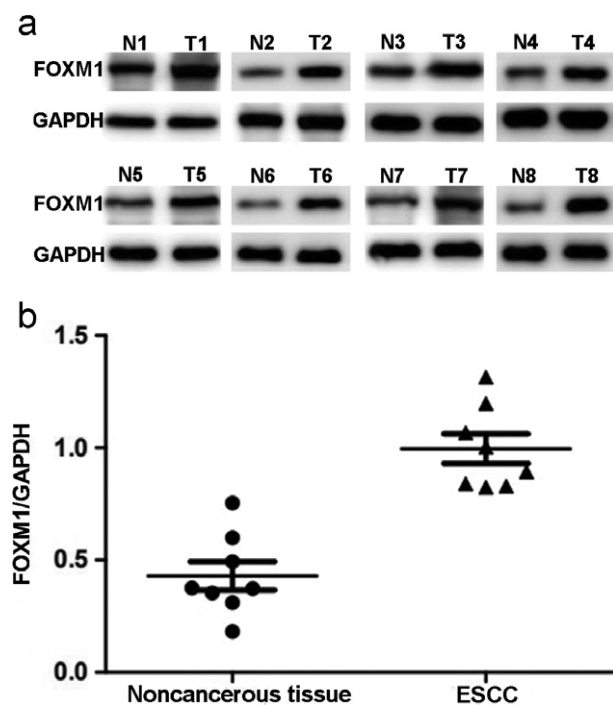


Figure 2 Western blot analysis of FOXM1 protein expression in non-cancerous and tumor tissues. (a) Bands of FOXM1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and (b) quantitative analysis of FOXM1/GAPDH in noncancerous and tumor tissues.

and FOXM1 expression ($P = 0.016$) were all independent prognostic factors (Table 2). These results indicated that stage IIA ESCC patients with FOXM1 overexpression tend to exhibit a high risk of LMR.

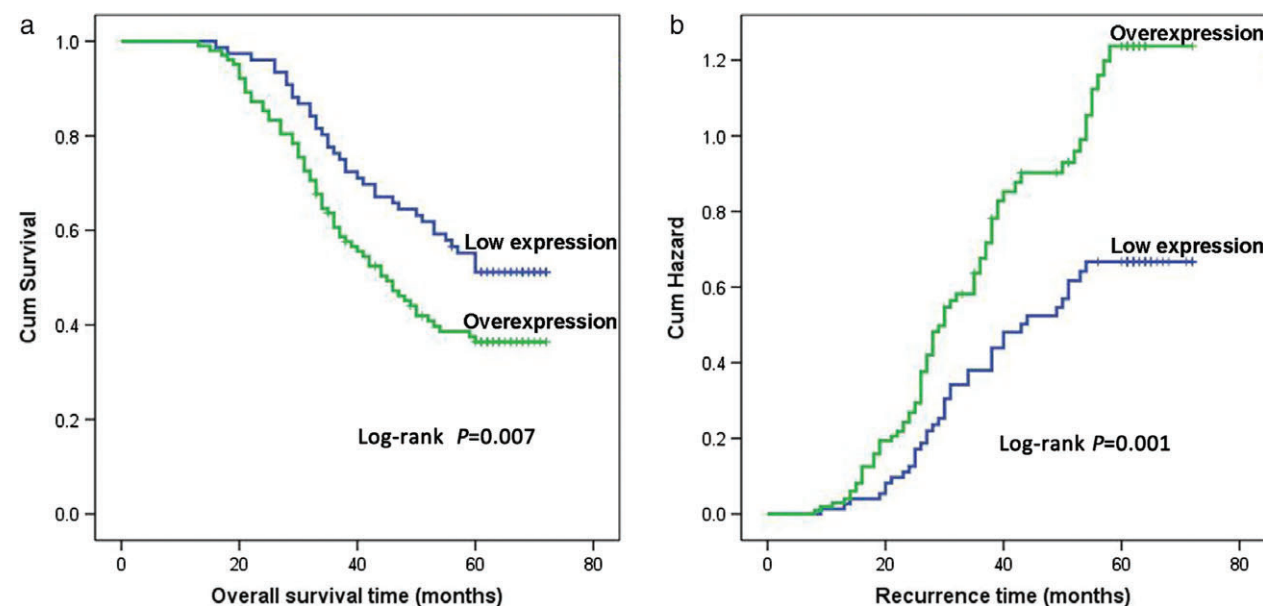


Figure 3 Kaplan–Meier analysis and log-rank test of FOXM1 for five-year (a) overall survival and (b) lymphatic metastatic recurrence of 178 stage IIA esophageal squamous cell carcinoma (ESCC) patients.

Discussion

It is widely accepted that surgery is the first choice for the treatment of localized ESCC.^{26,27} However, the overall survival of ESCC patients remains unsatisfactory. Even in stage IIA ESCC, the five-year OS rate is only 30–50%. Locoregional LMR is the main recurrence pattern and the most common reason for treatment failure. In contrast to other digestive tract components, the esophagus has its own unique histological structure involving rich lymphatic drainage and anastomosis in the submucosa, which may lead to early lymph node metastasis and postoperative LMR.^{28–30} In 1991, Yoshinaka *et al.* reported that approximately 47% of ESCC patients experienced lymphatic metastasis when the submucosa (T1b) was invaded.³¹

Evidence indicates that postoperative adjuvant chemotherapy could significantly improve the five-year survival rate in advanced tumors, but adjuvant chemotherapy has not been demonstrated to be advantageous in early stage ESCC because of significant adverse effects.^{32–34} Whether adjuvant therapy should be administered to stage IIA ESCC patients remains controversial. According to the National Comprehensive Cancer Network (NCCN) esophageal cancer guidelines, patients may not accept adjuvant therapy after complete tumor resection.³⁵ In China, patients tend to receive primary surgery if tumors can be completely resected. Ivor Lewis esophagectomy with two-field lymph node dissection is the main surgical modality for early stage ESCC.^{27,36} Thus, pathological stage IIA ESCC patients typically do not receive adjuvant therapy.

Table 2 Multivariate Cox regression analysis of prognostic factors of 178 stage IIA ESCC patients

Parameters	Five-year OS		Five-year LMR	
	HR (95% CI)	<i>P</i>	HR (95% CI)	<i>P</i>
Gender (male vs. female)	1.271 (0.845–1.912)	0.250	1.246 (0.841–1.846)	0.273
Age (> 50 vs. ≤ 50 years)	0.865 (0.578–1.293)	0.479	0.837 (0.567–1.236)	0.372
Tumor size (≥ 3 cm vs. < 3 cm)	0.472 (0.236–0.946)	0.034	0.445 (0.233–0.852)	0.014
T status (T2 vs. T3)	3.781 (1.847–7.739)	0.000	4.325 (2.200–8.503)	0.000
Differentiation degree (low vs. mid-high)	1.591 (1.064–2.377)	0.024	1.483 (1.003–2.192)	0.048
FOXM1 (low expression vs. overexpression)	1.661 (1.100–2.508)	0.016	1.877 (1.252–2.813)	0.002

Bold values indicate $P < 0.05$. CI, confidence interval; ESCC, esophageal squamous cell carcinoma; HR, hazard ratio; LMR, lymphatic metastatic recurrence; OS, overall survival.

However, previous research has demonstrated that some individuals experience postoperative LMR, which is the main cause of death of ESCC patients.³⁷ Therefore, to improve OS, it is crucial to determine the indicators to predict prognosis in stage IIA ESCC patients and administer treatment according to the different prognostic indications. At present, the commonly used TNM staging cannot accurately and sensitively evaluate the prognosis of ESCC patients. Therefore, we argue that it is of great significance to investigate biological markers to predict the potential for recurrence and administer adjuvant therapy to prevent lymphatic metastasis and further improve prognosis.

Upregulation of FOXM1 is related to tumorigenesis and the progression of various solid tumors and might serve as a novel prognostic biomarker.^{13,38} Takata *et al.* reported that ESCC patients with FOXM1 positive expression exhibited poor prognosis.²⁵ Hui *et al.* reported that low cytoplasmic FOXM1 levels correlated with early stage ESCC, and nuclear FOXM1 expression was observed in young ESCC patients.¹⁴ However, reports about the relationship between FOXM1 expression and postoperative LMR in stage IIA ESCC patients are not available. In the present study, we confirmed that FOXM1 is overexpressed in ESCC. In addition, stage IIA ESCC patients with FOXM1 overexpression may exhibit a high risk of LMR and poor prognosis after Ivor Lewis esophagectomy with two-field lymph node dissection.

The underlying mechanism of FOXM1-mediated tumor invasion and metastasis remains unclear. Tumor metastasis is a multistep and complex process involving local invasion, intravasation, extravasation, formation of micrometastases, and colonization.³⁹ The epithelial-mesenchymal transition (EMT) and matrix metalloproteinases (MMPs) exert a profound influence on the processes of tumor invasion and metastasis. During the process of EMT, tumor cells acquire a more peculiar mesenchymal phenotype and gain the ability to metastasize.^{40,41} MMP-2 and MMP-9 are directly associated with angiogenesis and degradation of basement membrane collagen, leading to metastasis.⁴² Recently, numerous studies have demonstrated that FOXM1 plays an

important role in the activation of EMT and MMPs.^{23,24,43,44} Wang *et al.* reported that downregulation of FOXM1 by small interfering RNA could inactivate MMP-2, MMP-9, and VEGF, subsequently resulting in the inhibition of cell growth, migration, invasion, angiogenesis, and metastasis of pancreatic cancer.⁴⁵ They also elucidated that FOXM1 downregulation may attenuate the proliferation and aggressiveness of breast cancer cells by modulating uPA, uPAR, MMP-2, MMP-9, and VEGF expression.¹⁸ In subsequent studies, we will attempt to elucidate the detailed mechanisms of FOXM1 for regulating ESCC metastasis and detect the associations between FOXM1 expression and EMT markers and MMPs in vitro. In addition, xenograft mouse models can be used to validate the role of FOXM1 in tumor metastasis in vivo.

Some limitations of this study should be noted. First, the number of samples is relatively small. In addition, all participants are Han Chinese with a similar genetic background. Thus, a larger sample size and multicenter randomized studies are needed to confirm the prognostic value of FOXM1.

In conclusion, we identified that FOXM1 is aberrantly expressed in ESCC. Patients with FOXM1 overexpression may exhibit an increased risk of LMR and poor prognosis. FOXM1 may serve as a potential biomarker to predict the LMR in stage IIA ESCC after Ivor Lewis esophagectomy with two-field lymph node dissection. Some regimes, including postoperative adjuvant therapy and targeted drug treatment, may serve as complementary treatments to control locoregional LMR and subsequently improve the survival of patients with an otherwise poor prognosis.

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Disclosure

No authors report any conflict of interest.

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