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ORIGINAL ARTICLE

Overexpression of IFITM3 predicts poor prognosis in stage IIA esophageal squamous cell carcinoma after Ivor Lewis esophagectomy

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Esophageal squamous cell carcinoma; esophagectomy; interferon-induced transmembrane protein 3; Ivor Lewis; prognosis.

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Abstract

Background: Recent research has shown that IFITM3 plays an important role in the tumorigenesis of many malignancies. We investigated the clinicopathological variables and prognostic value of IFITM3 in stage IIA esophageal squamous cell carcinoma (ESCC) patients.

Methods: Immunohistochemistry and Western blot analysis were used to examine IFITM3 expression in tumor specimens. The relationships between IFITM3 expression and clinicopathological variables, as well as the five-year survival and recurrence status of patients, were analyzed.

Results: *IFITM3* was aberrantly expressed in tumor tissue. Statistical analysis showed a close correlation of IFITM3 expression with T tumor status ($P = 0.004$). Additionally, IFITM3 overexpression, advanced T status, poor degree of differentiation, and large tumor size were not only associated with poor survival but were high lymphatic metastatic recurrence predictors in ESCC patients ($P < 0.05$).

Conclusion: Our data indicated that IFITM3 overexpression may predict poor prognosis in stage IIA ESCC patients after Ivor Lewis esophagectomy.

Introduction

Esophageal carcinoma (EC) is one of the most common aerodigestive tract malignant tumors and is the seventh leading cause of mortality; it was estimated that 17 460 new cases and 15 070 deaths occurred in 2012.[1,2] Esophageal squamous cell carcinoma (ESCC) is the major histological type. Epidemiological research has shown strong links between low economic social status and ESCC occurrence in some developing countries, and China has the highest incidence.[3,4] Despite significant improvements at diagnostic level and with therapeutic modalities, the prognosis of ESCC patients remains poor, with >75% of patients dying within five years, regardless of treatment modality.[4–6]

To date in China, there is no general treatment standard for ESCC. National Comprehensive Cancer Network (NCCN) EC guidelines are often referenced in clinical practice. These guidelines suggest that patients should not receive adjuvant therapy after complete tumor resection. In China, pathological stage IIA ESCC patients do not typically receive adjuvant therapy after surgery. Nevertheless, previous research has indicated that patients at this stage tend to exhibit different survival outcomes and nearly 30% experience lymphatic metastatic recurrence within three years.[7,8] In our opinion, there are some deficiencies in the NCCN guidelines. It is necessary to further stratify patients at this stage based on different recurrence and survival outcomes. Postoperative adjuvant therapy should be administered to patients at high risk of recurrence or poor survival probability to improve overall survival (OS). However, although the prognosis of ESCC patients is clinically considered to be tumor node metastasis (TNM) stage-specific, TNM stage cannot always accurately and sensitively predict ESCC patient prognosis.[9,10] Therefore, determining the biological markers to predict prognosis in stage IIA ESCC patients is of great clinical significance.

IFITM3, also known as 1-8U, is an important member of the interferon (IFN)-inducible transmembrane protein family.[11] It is reported to be upregulated and is associated with carcinogenesis and progression in in many human

malignancies, such as gastric and breast cancers, colorectal tumors, and gliomas.[12–15] IFITM3 likely exerts a profound influence on cell proliferation, migration, and invasion by modulating the Wnt/β-catenin signaling pathway and is implicated in the G0/G1 checkpoint to control the cell cycle of tumors.[14] In addition, our previous retrospective study demonstrated that IFITM3 overexpression might predict a high risk of lymphatic metastatic recurrence in pN0 ESCC.[16]

In this study, we sought to revalidate aberrant IFITM3 expression in tumor tissues and adjacent normal mucosa (ANM) in stage IIA ESCC. We explored the relationship between IFITM3 expression and clinicopathological characteristics, OS, and lymph node metastatic recurrence in such patients. We also explored whether the *IFITM3* gene can predict prognosis in stage IIA ESCC patients after Ivor Lewis esophagectomy.

Methods

Patients and specimens

Mid-thoracic ESCC patients who underwent Ivor Lewis esophagectomy with two-field lymphadenectomy at Shandong Provincial Hospital Affiliated to Shandong University, China, from February 2008 to October 2010 were eligible for this study. One hundred and eighty-five patients were enrolled, including 110 men and 75 women, ranging from 40 to 75 years of age (clinicopathological data is listed in Table 1). The standard procedure of Ivor Lewis esophagectomy with two-field lymphadenectomy has been described in a previous study.[8]

The inclusion criteria were as follows: (i) all patients achieved complete tumor resection (R0), and the proximal and distal incisal tumor margins, as well as lateral margin, were pathologically examined without residual foci;[17] (ii) 18 ± 5.8 (range 12–25) lymph nodes were intraoperatively dissected; (iii) patients were at pathological stage IIA (pT2-3N0M0) according to criteria of the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) staging system;[18] and (iv) patients had no history of previous malignancies or other severe diseases that may influence the outcome of our follow-up. Patients were not eligible if preoperative neoadjuvant chemotherapy or postoperative adjuvant treatment had been administered.

Esophageal squamous cell carcinoma tissue and ANM (more than 5 cm from the margin of ESCC) were collected from the surgical specimens of all patients. ANM did not exhibit tumor infiltration, deterioration, or necrosis upon macroscopic and microscopic examination.

The Ethic Committee of Shandong Provincial Hospital Affiliated to Shandong University approved this study. Written informed consent was obtained from each participant.

Immunohistochemistry and immunohistochemical evaluation

IFITM3 protein expression was examined by the streptavidin-peroxidase immunohistochemical method.

Table 1 Correlations between IFITM3 expression and clinicopathological variables, survival, and recurrence in stage IIA ESCC patients

		IFITM3 expression level						
Variables	No. of patients N = 185	Low (*n* = 77)	High (*n* = 108)	*P*†	5-year survival rate (%)	*P*‡	5-year recurrence rate (%)	*P*‡
Age (years)				0.718		0.292		0.116
<50	40	18	22		55.0		60.0	
≥50	145	59	86		42.1		76.6	
Gender				0.878		0.161		0.123
Male	115	47	68		41.7		76.5	
Female	70	30	40		50.0		67.1	
Tumor size (mm)				0.630		0.012		0.007
<30	57	22	35		57.9		61.4	
≥30	128	55	73		39.1		78.1	
Differentiation degree				0.236		0.013		0.006
Poor	47	16	31		34.0		85.1	
Well/ moderate	138	61	77		48.6		68.8	
T status				0.004		0.003		0.003
T2	59	34	25		61.0		59.3	
T3	126	43	83		37.3		79.4	
IFITM3 overexpression						0.003		0.012
Yes			108		35.2		78.7	
No		77			58.4		64.9	

†$\chi 2$ test; ‡log rank test. ESCC, esophageal squamous cell carcinoma.

Formalin-fixed and paraffin-embedded surgical specimens were sequentially cut into 4 μm sections. The sections were then dewaxed, and antigen retrieval and hydrogen peroxide incubation were performed in sequence. Rabbit anti-IFITM3 monoclonal antibodies (GeneTex, San Antonio, TX, USA) were used at a dilution of 1:200 and incubated at 4°C overnight. Monoclonal primary antibody was replaced by phosphate buffered saline (PBS) as a negative control. Further experimental steps were followed according to the instructions of a secondary biotinylated antibody kit purchased from ZSGB Biotech (Beijing, China).

Two independent pathologists blinded to the clinical data evaluated the sections. The final immunohistochemical score (IHS) was calculated by combining the proportion with the staining intensity. The proportion was scored as follows: 0 (0–10%), 1 (11–25%), 2 (26–50%), 3 (51–75%), and 4 (75–100%). The staining intensity was scored as: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). In this study, IHS ≥ 8 was considered overexpression.[16]

Western blot analysis

The 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 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane filters (Millipore, Billerica, MA, USA). Five percent non-fat dry milk that resolved in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) was then used to block non-specific binding for one hour at room temperature. Membranes were incubated with IFITM3 primary antibodies (1:1000 dilution; GeneTex) and the same dilution of β-actin (1:1000; Abcam, Cambridge, MA, USA) overnight at 4°C. Following washing, the membranes were incubated with corresponding secondary antibody conjugated with horseradish peroxidase anti-rabbit immunoglobulin G (1:5000; ZSGB Biotech) for one hour at room temperature. Finally, the protein levels were quantified using an enhanced chemiluminescence (ECL) detection system (Amersham Imager 600; General Electric, Fairfield, CT, USA).

Follow-up

Patients were examined every three to six months after surgery. Follow-up was strictly carried out, including a thorough physical examination, chest and upper abdomen computed tomography (CT) scan, abdominal ultrasound, bone scintigraphy, and cerebral CT.[19] Positron emission tomography (PET) was administered to specific patients if necessary. Examinations were compared to preoperative imaging data. If there was progressive lymph node enlargement, a biopsy was the first method used to identify possible mediastinal lymph node recurrence. Patients with mediastinal lymph node enlargement identified in CT scans were advised to undertake PET-CT examination if biopsy was difficult to achieve. A total of 34 patients were diagnosed with lymphatic metastatic recurrence by PET-CT.

Statistical analysis

The $\chi 2$ test was used to analyze the relationship between IFITM3 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 IFITM3 expression and survival, as well as expression and recurrence. Cox regression analysis was used to evaluate the independent prognostic factors. A significant statistical difference was defined with a two-tailed P value <0.05. All statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

IFITM3 expression analysis in specimens

Immunohistochemical assay was used to detect IFITM3 protein expression. Positive expression was presented as yellow or brownish-yellow staining in the cytoplasm of malignant cells. Significant immunoreaction of positive expression was detected in malignant cells, while low or undetected IFITM3 protein expression occurred in ANM (Fig 1). Furthermore, according to IHS criteria, we divided all specimens into two groups: 108 cases (58.4%) were categorized as overexpressed, and the remainder as (41.6%) low expression.

To verify the aberrant upregulation of IFITM3, we further analyzed the expression levels in ESCC tissues and their ANMs using Western blot analysis (Fig 2). IFITM3 expression in tumor tissues was higher than in ANM ($P < 0.05$), which was consistent with immunohistochemistry results.

IFITM3 expression and clinicopathological variables

According to the eligibility criteria, 185 ESCC patients of different ages, gender, tumor sizes, degrees of differentiation, T status, and IFITM3 expression levels were enrolled in this study (Table 2). χ^2 analysis demonstrated a significant correlation between IFITM3 expression level and T

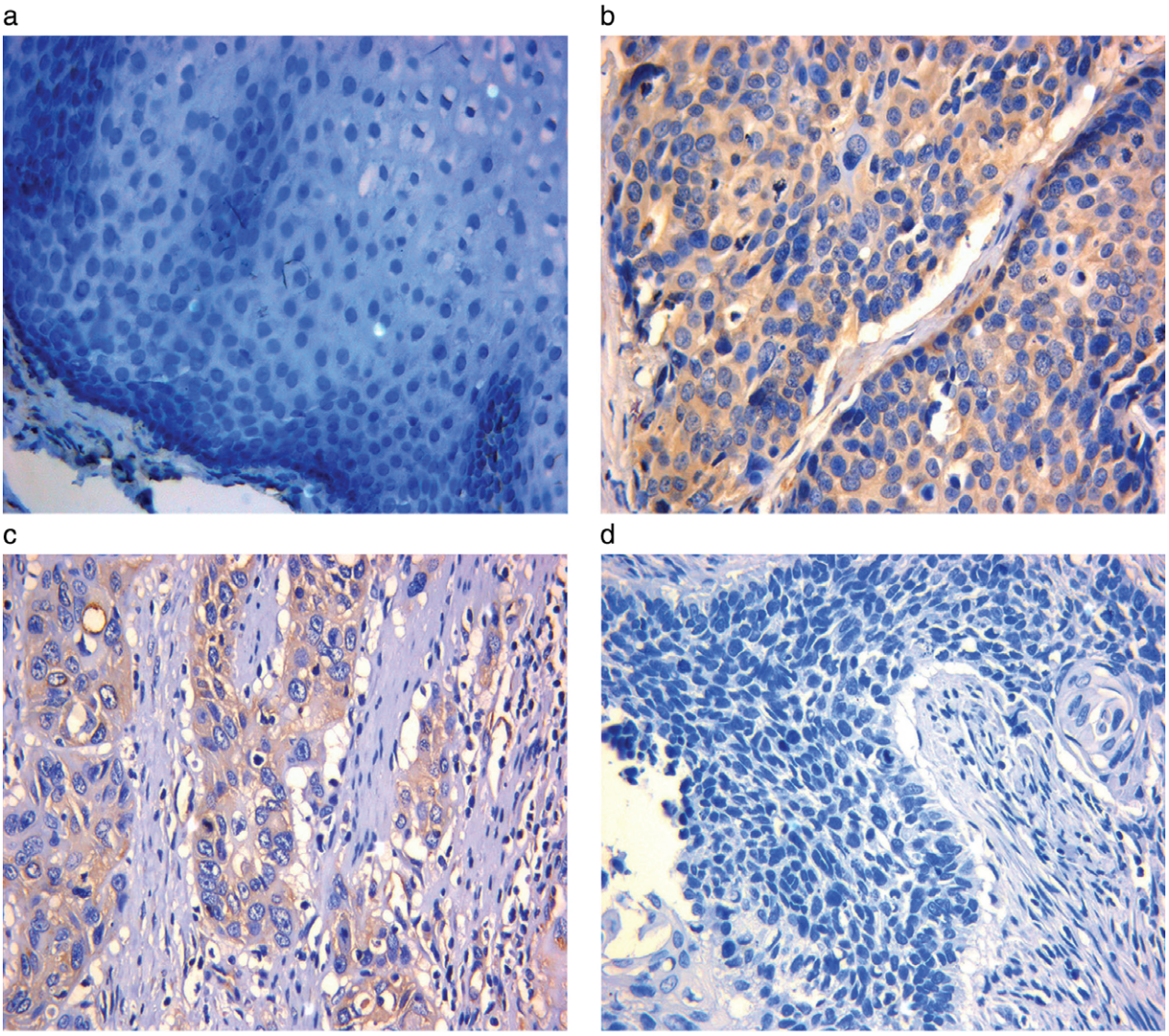

Figure 1 Immunohistochemistry assay of IFITM3 in esophageal squamous cell carcinoma (ESCC) tissue and adjacent normal esophageal mucosa (ANM). (**a**) ANM shows no IFITM3 protein expression (×400). (**b**) Strong positive immunoreaction of IFITM3 in the cytoplasm of ESCC tissue (×400). (**c**) Moderate and (**d**) extremely low IFITM3 expression in ESCC tissue (×400).

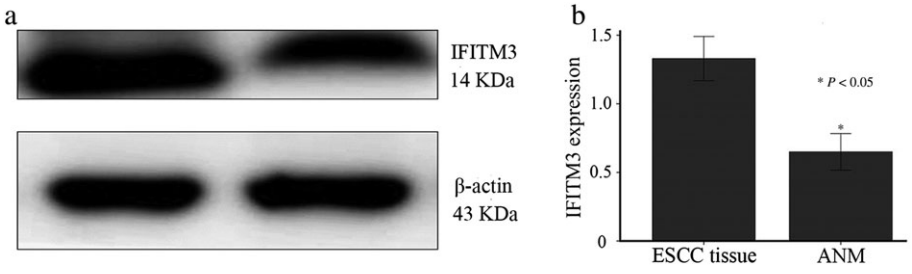

Figure 2 Western blot analysis of IFITM3 protein expression in esophageal squamous cell carcinoma (ESCC) tissue and adjacent normal mucosa (ANM). (**a**) *Lane 1* and *2* β-actin protein as a control and *Lane 3* and *4* IFITM3 protein expression in ESCC tissues and ANM, respectively. (**b**) Relative expression of IFITM3 protein in ESCC tissue and ANM.

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

	Survival status		Recurrence status	
Variables	HR (95% CI)	*P*	HR (95% CI)	*P*
Age (≥50 vs. <50)	1.031 (0.606–1.754)	0.910	1.148 (0.721–1.827)	0.562
Gender (male vs. female)	1.389 (0.902–2.141)	0.136	1.315 (0.900–1.921)	0.157
Tumor size (≥30 mm vs. <30 mm)	1.761 (1.109–2.797)	**0.016**	1.630(1.104–2.407)	**0.014**
T status (T2 vs. T3)	1.863 (1.158–2.999)	**0.010**	1.677 (1.126–2.497)	**0.011**
Differentiation degree (poor vs. well/moderate)	1.559 (1.015–2.395)	**0.042**	1.518 (1.040–2.216)	**0.030**
IFITM3 (overexpression vs. low expression)	1.825 (1.197–2.782)	**0.005**	1.548 (1.088–2.203)	**0.015**

B, regression coefficient; CI, confidence interval; ESCC, esophageal squamous cell carcinoma HR, hazard ratio; SE, standard error; Wald, Wald value. Statistical significance ($P < 0.05$) are shown in bold.

status ($P = 0.004$). Conversely, the relationships between IFITM3 expression and age, gender, tumor size, and degree of differentiation did not reach statistical significance ($P > 0.05$).

IFITM3 expression and prognosis

After thorough follow-up, the five-year survival and lymphatic metastatic recurrence rates of stage IIA ESCC patients with IFITM3 overexpression were 35.2% and 78.7%, respectively. However, in patients with low IFITM3 expression, the survival rate was 58.4% and the lymphatic metastatic recurrence rate was only 64.9% (Table 1).

Kaplan–Meier analysis showed that the five-year survival rate was obviously decreased in patients with IFITM3 overexpression ($P = 0.003$) (Fig 3). Additionally, the recurrence rate was correspondingly increased in this group ($P = 0.012$) (Fig 4). Thus, compared to patients with low expression, ESCC patients with IFITM3 overexpression might have a poorer prognosis. We also found that tumor size, degree of differentiation, and T status may also influence OS and recurrence in ESCC patients ($P < 0.05$) (Figs 3–4). Multivariate Cox regression revealed that IFITM3 expression, tumor size, degree of differentiation, and T status were all independent prognostic factors ($P < 0.05$) (Table 2).

Discussion

Different to other digestive tracts, the intramural lymphatic plexuses of the esophagus run longitudinally and are richly anastomotic in the submucosa, which may to a large extent contribute to the early and extensive lymphatic metastasis of esophageal cancer.[20] In 1991, Yoshinaka *et al.* reported that approximately 47% of ESCC patients might experience lymph node metastasis when the submucosa (T1b) was invaded.[21] Thus, regardless of the treatment modality selected, a high incidence of locoregional lymphatic recurrence occurs.

The NCCN EC guidelines suggest that patients should not receive adjuvant therapy after complete tumor resection, and individuals with advanced T status (above T2) should receive neoadjuvant chemotherapy before surgery. However, in China, patients tend to receive primary surgery if tumors can be completely resected. Ivor Lewis esophagectomy via a thoracoabdominal two-field lymph node dissection is the main surgical modality. Thus, ESCC patients in pathological stage IIA usually do not receive adjuvant therapy.

However, previous research has reported that some patients will experience postoperative tumor relapse or metastasis,[7,8] deemed to be the main lethal cause for ESCC patients.[22,23] Therefore, in order to improve OS, it is crucial to determine the indicators to predict survival in stage IIA ESCC patients, and then administer treatment according to the different prognostic inclinations. Although prognosis for ESCC patients is pathological TNM stage-specific, a more sensitive biomarker is needed as TNM stage it is not always sensitive enough to determine prognosis. Thus, it is necessary to determine further biological markers.

IFITM3 is an important member of the IFN-inducible transmembrane protein family. It has been reported to exert a profound influence on cell proliferation, migration, and invasion by modulating the Wnt/β-catenin signaling pathway and is implicated in the G0/G1 checkpoint to control the cell cycle of many human malignant tumors. Hu *et al.* reported that IFITM3 overexpression was correlated with growth, migration, invasion, and metastasis in gastric cancer.[14] Li *et al.* demonstrated that IFITM3 overexpression was an important independent prognostic factor for disease-free interval and was upregulated in nodal metastasis of colon tumors.[15] Our previous retrospective research demonstrated that IFITM3 may act as a biomarker to predict lymphatic metastatic recurrence in pN0 ESCC; however, no studies have reported the prognostic value of IFITM3 in ESCC.

In our study, immunohistochemical analysis revealed that more than 50% of patients displayed IFITM3

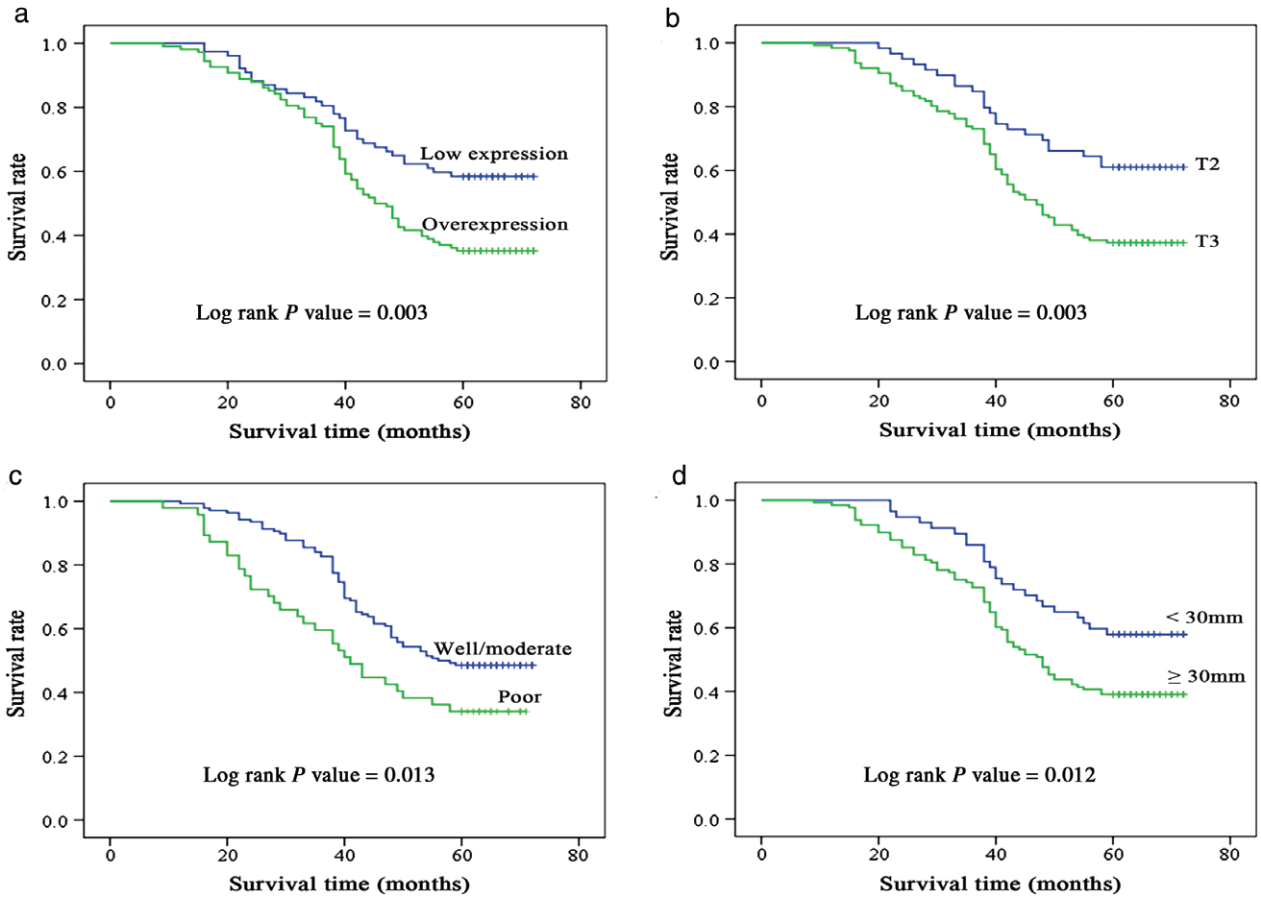

Figure 3 Overall survival of stage IIA ESCC patients according to (**a**) IFITM3 expression level, (**b**) T status, (**c**) degree of differentiation, and (**d**) tumor size.

overexpression, and weak or undetected staining was found in controlled ANM. The experimental results were confirmed by Western blot analysis. These outcomes revalidated our conclusion that IFITM3 may exhibit aberrant expression in ESCC tissues and their mucosae, as demonstrated in our earlier research. Moreover, based on statistical analysis of protein levels (immunohistochemistry and Western blot analysis), IFITM3 overexpression was associated with poor survival and high recurrence rates in patients with stage IIA ESCC. In order to identify independent prognostic factors, we performed multivariate Cox regression analysis. The results showed that tumor size, degree of differentiation, IFITM3 protein expression, and T status were independent prognostic factors. Thus, IFITM3 could serve as a biomarker to stratify the prognosis of patients with stage IIA ESCC.

One hundred and eighty-five mid-thoracic ESCC patients who received Ivor Lewis esophagectomy and were pathologically staged as IIA ESCC were enrolled in this study. After thorough follow-up, the five-year survival rate was barely 44.9% and the recurrence rate was as high as 73.0%. This is not an encouraging result, suggesting that the routine treatment modality used in our institute (Ivor Lewis) may not be consistent with current ESCC treatment. We believe that ESCC patients with a poor prognosis should receive targeted adjuvant therapy. Although no randomized trial has yet demonstrated that postoperative chemotherapy or radiotherapy can improve survival in patients with stage IIA EC,[24–26] our previous study demonstrated positive results and we believe that postoperative adjuvant radiotherapy can significantly reduce lymphatic metastatic recurrence and improve prognosis in ESCC patients.[7,8,27] Considering all of these findings, we believe that it is necessary that stage IIA patients with IFITM3 overexpression receive postoperative adjuvant radiotherapy, which will act as a compensatory mechanism to control the metastatic recurrence of locoregional lymph nodes and improve survival.

This study was retrospective and had a limited sample size. Although this is the first time that IFITM3 expression has been used to predict survival and recurrence in ESCC patients, replication studies with different parameters and prospective and multicenter randomized studies are needed to verify this prognostic significance.

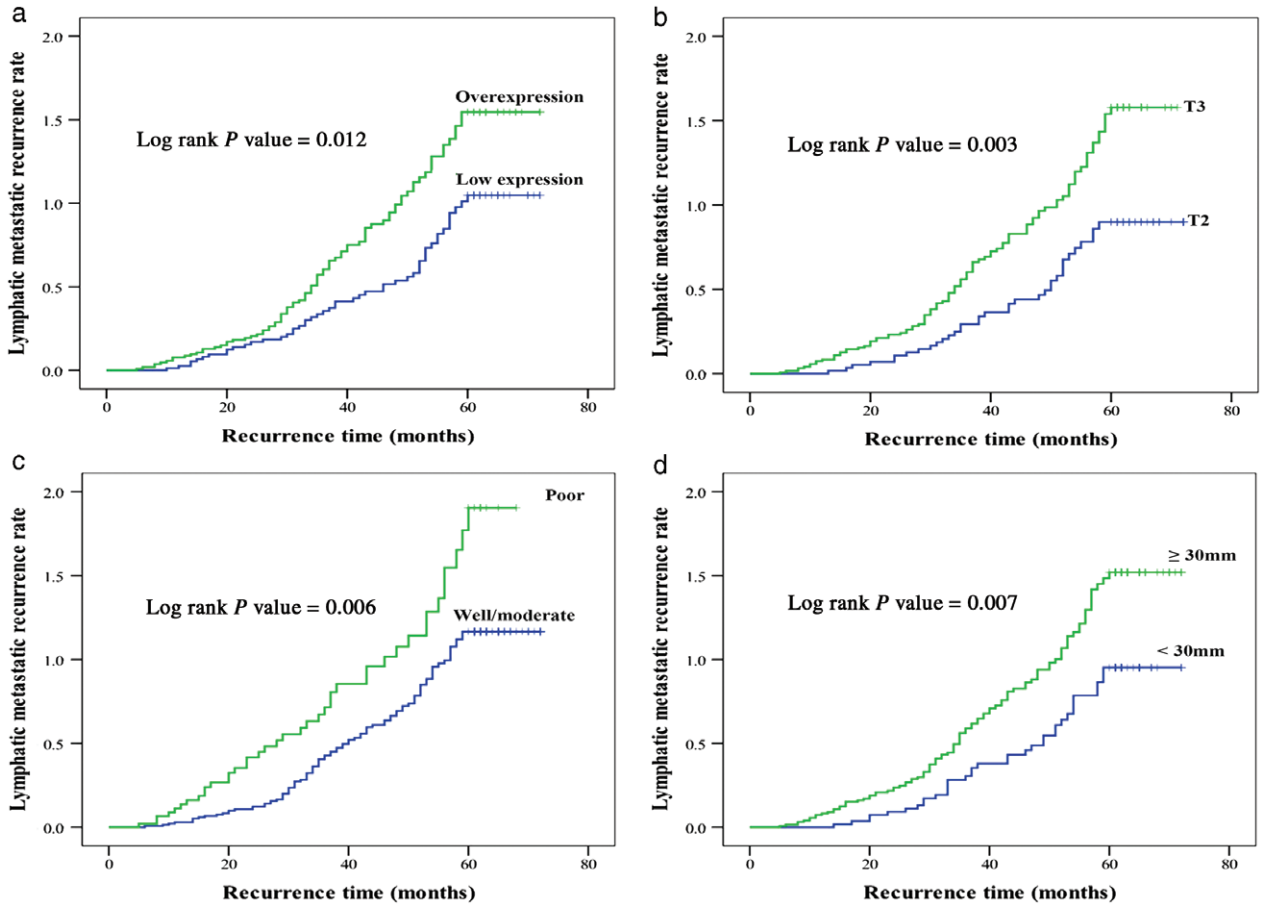

Figure 4 Lymphatic metastatic recurrence in stage IIA esophageal squamous cell carcinoma (ESCC) patients according to (**a**) IFITM3 expression level, (**b**) T status, (**c**) degree of differentiation, and (**d**) tumor size.

Our results demonstrate that IFITM3 expression has a close relationship with prognosis in ESCC patients. IFITM3 overexpression may predict poor prognosis in stage IIA ESCC patients after Ivor Lewis esophagectomy.

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Disclosure

No authors report any conflict of interest.

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