Overexpression of IFITM3 predicts high risk of lymphatic metastatic recurrence in pN0 esophageal squamous cell carcinoma after Ivor-Lewis esophagectomy?

Yang Jia, Miao Zhang, Wenpeng Jiang, Zhiping Zhang, Shiting Huang, Zhou Wang

Background: Recent researches have shown that the aberrant expression of IFITM3 was implicated in lymph node metastasis of many malignancies. Our research aimed to investigate the expression of IFITM3 in pathological N0 (pN0) esophageal squamous cell carcinoma (ESCC) and its relationship with lymph node metastatic recurrence. **Methods**: Immunohistochemistry (IHC) was used to examine the expression profile of IFITM3 in 104 pairs of samples. Each pair consisted of ESCC tissue and its adjacent normal mucosa (ANM). And this aberrant expression was verified by reverse transcription-polymerase chain reaction (RT-PCR) with 20 cases of tumor specimens with strong immunostaining and their mucosal tissues. In addition, 20 cases of low expression tissues and their ANMs were also evaluated. Moreover, the correlation between IFITM3 expression level and clinicopathological variables as well as recurrent status was analyzed. **Results**: Both IHC and RT-PCR demonstrated that IFITM3 expression level was significantly higher in tumor tissue than ANM. Statistical analysis showed a significant correlation of IFITM3 expression with T status of esophageal cancer ($p = 0.015$). In addition, IFITM3 overexpression ($p = 0.010$) and advanced T status ($p = 0.008$) were both associated with high rate of lymph node metastatic recurrence. Multivariable Cox regression analysis further suggested that the T status ($p = 0.000$) and IFITM3 expression ($p = 0.004$) were independent risk factors in pN0 ESCC. **Conclusions**: Even pN0 ESCC patients still will experience lymphatic metastatic recurrence. IFITM3 gene could be a predictor of lymphatic metastatic recurrence in pN0 ESCC after Ivor-Lewis esophagectomy.

Overexpression of IFITM3 Predicts High Risk of Lymphatic Metastatic Recurrence in pN0 Esophageal Squamous Cell Carcinoma after Ivor-Lewis Esophagectomy?

Abstract

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Methods: Immunohistochemistry (IHC) was used to examine the expression profile of IFITM3 in 104 pairs of samples. Each pair consisted of ESCC tissue and its adjacent normal mucosa (ANM). And this aberrant expression was verified by reverse transcription-polymerase chain reaction (RT-PCR) with 20 cases of tumor specimens with strong immunostaining and their mucosal tissues. In addition, 20 cases of low expression tissues and their ANMs were also evaluated. Moreover, the correlation between IFITM3 expression level and clinicopathological variables as well as recurrent status was analyzed.

Results: Both IHC and RT-PCR demonstrated that IFITM3 expression level was significantly higher in tumor tissue than ANM. Statistical analysis showed a significant correlation of IFITM3 expression with T status of esophageal cancer ($p = 0.015$). In addition, IFITM3 overexpression ($p = 0.010$) and advanced T status ($p = 0.008$) were both associated with high rate of lymph node

metastatic recurrence. Multivariable Cox regression analysis further suggested that the T status (p = 0.000) and IFITM3 expression (p = 0.004) were independent risk factors in pN0 ESCC.

Conclusions: Even pN0 ESCC patients still will experience lymphatic metastatic recurrence. IFITM3 gene could be a predictor of lymphatic metastatic recurrence in pN0 ESCC after Ivor-Lewis esophagectomy.

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43 Introduction

44 Esophageal carcinoma (EC) is the sixth leading cause of mortality among various malignant
45 tumors worldwide. Esophageal adenocarcinoma and esophageal squamous cell carcinoma
46 (ESCC) are the two most common histological type. Both of them have an obviously geographic
47 distribution characteristic, and China is a country with high incidence of ESCC(Ferlay et al.
48 2010). Despite of the improvement of diagnostic level and utilization of combined treatment
49 modalities in recent years, the prognosis of ESCC patients remains poor. Even in pN0 ESCC, the
50 5-year survival rate is only approximately 70% after complete resection, and lymphatic
51 metastatic recurrence is the main reason that account for the failure of operation(Eloubeidi et al.
52 2002; Visbal et al. 2001). Therefore, in order to improve the long-term survival of ESCC patients,
53 it is of great clinical significance to control the locoregional lymph node metastatic recurrence
54 after surgery.

Clinically, the number of metastasis-positive lymph node is usually used to evaluate the risk of lymphatic metastatic recurrence in locally advanced disease(Law & Wong 2001). However, there is no reliable index existing to predict this rate in pN0 ESCC. Although some molecules were previously reported to be used to stratify the recurrent risk in pN0 ESCC(Li et al. 2009; Song et al. 2012), actually none of them is proved to be universally accepted and commonly used. Therefore, sensitive biological markers that may predict this recurrent risk are urgently needed.

Interferon-induced transmembrane protein 3 (IFITM3), also known as 1-8U, is one of the important members of the IFN-inducible transmembrane protein family. IFITM3 likely exerts profound influences on cell proliferation, migration, invasion through the modulation of Wnt/β-catenin signaling pathway and is implicated in G0/G1 checkpoint to control the cell cycle of tumors(Hu et al. 2014; Yang et al. 2013; Zhao et al. 2013). It has been reported to be overexpressed in many human malignancies, such as gastric cancer, colorectal tumor, breast cancer, glioma, as well as oral squamous cell carcinoma.

Previous research has shown that IFITM3 is upregulated in gastric cancer, which correlates with tumor invasion and metastasis(Hu et al. 2014). Moreover, it has also been demonstrated to have a close relationship with the prognosis of colon cancer and confirmed to be an independent risk fatcor for disease-free interval(Li et al. 2011). However, the clinicopathological significance and prognostic value of IFITM3 in ESCC patients remains unknown.

In this study, we want to validate the relationship between the expression level of IFITM3 and clinicopathological characteristics as well as the recurrent risk of ESCC patients who had

undergone Ivor-Lewis esophagectomy. We also aimed to explore whether IFITM3 gene can predict lymphatic metastatic recurrence or not in pN0 ESCC.

Materials & Methods

Patients

From January 2008 to January 2010, patients with midthoracic ESCC who underwent Ivor-Lewis esophagectomy with two-field lymphadenectomy in our department (Provincial Hospital Affiliated to Shandong University, China) were eligible for this study. There were 104 patients, including 83 men and 21 women, ranging from 40 to 75 years (Clinicopathological data is listed in **Table 1**).

All patients met the following inclusion criteria: (1) According to 2009 Union for International Cancer Control (UICC) standard for midthoracic ESCC, Ivor-Lewis esophagectomy with two-field lymph node dissection was conducted to achieve completely resection (R0), the proximal and distal incisal margins as well as lateral margin were pathologically examined without residual foci(Arai et al. 2012). At the same time, average lymph nodes dissected was 18 ± 5.8 (ranging from 12 to 25); (2) Patients enrolled in the study were restaged after surgery according to TNM staging (UICC, 2009) for esophageal cancer; (3) Without history of previous malignancies or other severe diseases that may influence the outcome of our follow up; (4) Patients were not eligibled if preoperatively neoadjuvant chemotherapy or postoperatively adjuvant treatment was administered.

Surgical procedure of Ivor-Lewis esophagectomy

There were four thoracic surgeons working together to perform this kind of surgery, and the thoracic operation was made by two surgeons. The patient was placed in the 40°–45° left lateral decubitus position. After a right anterolateral thoracotomy, the chest was entered through the fourth intercostal space. The azygos vein arch was divided, and the esophagus was dissected from the esophagogastric junction to the apex of the chest. When the tumor invasion obviously extended outside the esophagus, the thoracic duct was routinely ligated above the diaphragm.

At the same time, an upper midline abdominal incision was made by another two surgeons, and the abdomen was explored. During mobilization of the stomach, care was taken to preserve the right gastroepiploic vessels and arcades. The left gastric artery and vein were isolated and doubly ligated at their origin. Pyloroplasty was not routinely performed. Then, the hiatus was enlarged and the stomach was pulled into the chest. An endto-side esophagogastric anastomosis was performed within the apex of the chest, and the stomach was secured into the mediastinum.(Chen et al. 2009a; Chen et al. 2009b)

Specimens

The ESCC tissue and ANM (more than 5 cm from the margin of ESCC) were collected from surgical specimen of each selected patient. At the same time, the ANM was required to have no tumor infiltration, deterioration as well as necrosis from both the macroscopic and microscopic examination.

113 This study was approved by the Ethic Committee of Provincial Hospital Affiliated to
114 Shandong University and the approval number is 2008081. Written informed consent was
115 obtained from all the participants.

116 **Immunohistochemistry**

117 The streptavidin-peroxidase immunohistochemical method was used to examine the
118 IFITM3 protein expression. Formalin-fixed and paraffin-embedded surgical specimens were
119 sequentially cut into 4μm sections. Then the sections were dewaxed, antigen retrieval and
120 hydrogen peroxide incubation. Rabbit anti-IFITM3 monoclonal antibodies (GeneTex, USA)
121 were used at a dilution of 1:200 and incubated at 4℃ overnight. The monoclonal primary
122 antibody was replaced by phosphate-buffered saline (PBS) as negative control. Further
123 experimental steps were followed according to the instructions of secondary biotinylated
124 antibody kit that purchased from ZSGB Biotech (BeiJing, China).

125 The expression of IFITM3 protein was determined according to Sakakura's criteria. And
126 two pathologists blinded to the clinical data were invited to evaluate the IHC section
127 independently. The outcome was calculated by combining the proportion with the staining
128 intensity. The proportion was scored as follows: 0 (0 – 10 %), 1 (11 – 25 %), 2 (26 – 50 %), 3
129 (51 – 75 %), and 4 (75 – 100 %). The staining intensity was scored: 0 (negative), 1 (weak), 2
130 (moderate), and 3 (strong). The final immunohistochemical score (IHS) was defined as the
131 proportion score × staining intensity score. In this study, IHS ≥ 8 was considered to be
132 overexpression.

RNA Extraction and RT-PCR

Total RNA was extracted from fresh frozen tissue by using Trizol (Invitrogen) according to the manufacture protocols. The purity of RNA was measured by UV spectrophotometer (NanoDrop 2000) and the OD 260/280 value ranging from 1.8 to 2.0 can be used to reverse transcription. The detailed RT-PCR procedure was followed by CWBio two-step RT-PCR kit (JiangSu, China). The primer sequence of IFITM3 gene were: 5'-CAAGGAGGAGCACGAGG-3' (forward primer) and 5'-TTGAACAGGGACCAGACG-3' (reverse primer). β-actin was used as internal control and primer sequence of β-actin were: 5'-AGAGCCTCGCCTTTGCCGATCC-3' (forward primer) and 5'-ATACACCCGCTGCTCCGGGTC-3' (reverse primer). The PCR products of IFITM3 were further separated on 1% agarose gel electrophoresis. Azure C2000 (Azure Biosystems, USA) was used for electrophoresis gelatin image formation analysis.

Follow-Up

According to our plan, patients were examined every 3 – 6 months after surgery, and the checklist has been described in our previous study(Akhtar et al. 2014). We compared the imaging data preoperatively and postoperatively in detail to differentiate whether recurrence occurred or not. If the lymph nodes were swollen or the minor axis of them was more than 1 cm, clinical diagnosis of lymphatic recurrence can be made. And then for the patients with cervical and superficial swollen lymph nodes, fine-needle aspiration biopsy was conducted to make pathological diagnosis. In addition, some recurrent patients were also diagnosed with PET-CT.

Statistical Analysis

χ^2 test was used to analyze the relationship between IFITM3 expression and clinicopathological variables. The lymph node metastatic recurrent curves were calculated by Kaplan–Meier method and Log-rank test was used to compare the differences between the two curves. Cox regression analysis were performed to evaluate risk factors. A significant satatistical difference was defined if the two-tailed *p* value less than 0.05. All statistical analysis was performed by using SPSS version 17.0 (Chicago, IL, USA).

Results

IFITM3 Expression Analysis in ESCC and ANM

Immunohistochemistry assay was used to detect the expression level of IFITM3 protein. Overexpression was presented as yellow or brownish yellow staining in the cytoplasm of tumor cell. Just as shown in **Fig. 1C**, the significant immunoreaction of positive expression can be readily differentiated. However, there was low or undetected staining in ANM (**Fig. 1A**). Then two pathologists blinded to score the IHC sections independently, the sections with IHS more than 8 were defined as overexpression (**Fig. 1C**) and others were low expression (**Fig. 1B**). Further, according to this criteria, we divided all the ESCC specimens into two groups: 59 cases (56.7%) were categorized to overexpression group and 45 cases (43.3%) were in low expression group.

170 To verify this aberrant upregulation of IFITM3, we examined the mRNA expression level
171 by RT-PCR with 20 pairs of specimens that randomly selected from overexpression group and
172 20 pairs of tissues that originated from low expression group. The result showed that the mRNA
173 expression level was consistent with protein expression that IHC demonstrated (**Fig. 2**).

174 **IFITM3 Expression and Clinicopathological Characteristics**

175 According to the eligible criteria that mentioned above, in total of 104 cases of ESCC
176 patients enrolled in this research with different age, gender, tumor size, differentiation degree, T
177 status and IFITM3 expression level (**Table 1**). χ^2 analysis demonstrated that the expression level
178 of IFITM3 had a close relationship with T status of tumor ($p = 0.015$). In contrast, there were no
179 statistical differences between expression level and age, gender, tumor size as well as
180 differentiation degree ($p > 0.05$).

181 **IFITM3 Expression and Lymphatic Metastatic Recurrence**

182 Through thorough 3-year follow-up, total 42 cases (40.4%) were confirmed first lymph
183 node metastatic recurrence within 3 years, in which 30 patients (71.4%) were detected IFITM3
184 overexpression. In low IFITM3 expression group, the 3-year lymphatic recurrence rate was only
185 26.7%. Conversely, in overexpression group, this rate can reach up to 50.8% (**Table 1**). Kaplan–
186 Meier analysis showed that lymph node metastatic recurrent rate was significantly increased for
187 patients with IFITM3 overexpression (**Fig. 3)** and Log-rank test calculated that these two
188 recurrent curves had significant statistical difference ($p = 0.010$). In view of this, we can

conclude that ESCC patients with IFITM3 overexpression may have a higher recurrent risk of lymphatic metastasis.

Cox Regression Analysis of Risk Factors

As is shown in **Table 1**, T status ($p = 0.008$) and expression of IFITM3 ($p = 0.010$) were both lymph node metastatic recurrent risk factors in pN0 ESCC after Iver-Lewis esophagectomy. Then we performed Multivariate Cox regression analysis to identify variables related closely to the prognosis of ESCC patients. These results revealed that advanced T status of tumor ($p = 0.000$) and IFITM3 overexpression ($p = 0.004$) were independent risk factors (**Table 2**). As is shown in **Fig. 3**, patient with early T status and low expression of IFITM3 may have lower lymphatic metastatic recurrent risk.

Discussion

ESCC is one of the most common neoplasms in China with high incidence of lymph node metastatic recurrence, especially in mediastinum, neck and abdominal cavity(Chen et al. 2007). Even in pN0 ESCC, more than 40% of individuals can be detected micro-metastasis (Wang et al. 2004). Surgery today is still considered to be the first-line treatment modality for ESCC patients with resectable lesions(Hulscher et al. 2002; Olsen et al. 2011), but the overall survival can not satisfy our expections and nearly half of them still will experience tumor relaps(Eloubeidi et al. 2002; Korst et al. 1998; Rice et al. 2001; Visbal et al. 2001).

To date, in China, there is no general treatment standard for ESCC; the NCCN esophageal

cancer guidelines are often referenced in clinical practice. These guidelines suggest that patients ought not receive adjuvant therapy after complete tumor resection, but individuals with advanced T status (above T2) should accept neoadjuvant chemotherapy before surgery. But in China, patients tend to receive primary surgery if tumors can be completely resected and Ivor-Lewis esophagectomy via a thoracoabdominal two-field lymph node dissection is the main surgical modality. Compared with three-field lymph node dissection, the advantage of Ivor-Lewis esophagectomy is that the latent surgical trauma and complications can be effectively controled; but the cervical lymph node dissection cannot be accomplished simultaneously. Therefore, for patients with high risk of lymphatic metastasis, we think postoperative adjuvant therapy may act as a compensation to control the lymphatic recurrence after Ivor-Lewis esophagectomy.

In this study, we first found the differential expression of IFITM3 in tumor tissues and their ANMs as well as the important clinicopathological significance of IFITM3. Our result was consistent with previous researches which demonstrated that IFITM3 overexpressed in many hunman malignancies, such as gastric cancer, colorectal cancer, oral squamous cell carcinoma, glioma, breast cancer. These findins suggested that IFITM3 may play important roles and maybe a molecular marker in pN0 ESCC.

Regarding to the prognostic value of IFITM3, previous studies have drawn the contradictory conclusions in different cancers, which reflecting the complexity of IFITM3 in different tumor microenvironment. For gastric cancer, Hu et al(Hu et al. 2014) thought that IFITM3 overexperssion correlated with the lymph node metastasis. Li et al demonstrated that it

was an important independent prognostic factor for disease-free interval and upregulated in nodal metastasis of colon tumor(Li et al. 2011). Conversely, Yang et al did not find a association between IFITM3 expression and lymph node metastasis in breast cancer(Yang et al. 2013). And El-Tanani et al(El-Tanani et al. 2010) even drew the opposite conclusion, and they deemed that IFITM3 may inhibit the proliferation, development and metastasis of cancer through reducing the expression of osteopontin. However, to our knowledge no study has demonstrated the prognostic significance of IFITM3 in ESCC. This question attracted us to explore whether IFITM3 could be as a biomarker to evaluate the recurrent risk of lymph node metastasis in ESCC. Here data in our study showed that the high incidence of lymphatic metastatic recurrence in pN0 ESCC was associated with advanced T status and IFITM3 overexpression. Strikingly, multivariate Cox regression analysis showed that IFITM3 expression and T status were independent risk factors. The findings strongly suggested that IFITM3 could serve as a biomarker to stratify the risk of lymph node metastatic recurrence and then play an important role in the process of treatment modality selection in pN0 ESCC.

Total 104 patients with midthoracic ESCC in this study were received Ivor-Lewis esophagectomy with two-field lymph node dissection. And all of them had undergone theoretic R0 resection and pathologically comfirmed pN0 after surgery. But through thorough follow-up, 40.4% patients showed first recurrence of lymph node metastasis within 3 years. On account of this fact, for patients with high risk of lymphatic metastatic recurrence, we think it has important clinical significance to accept adjuvant therapy to control the lymph node recurrence. And

248 previous study have demonstrated that postoperative adjuvant radiotherapy can significantly
249 reduce the lymphatic metastatic recurrence in ESCC. Combined with the finding in this study
250 and our previous researches, we think it is indispensible for pN0 ESCC patients with IFITM3
251 overexpression to receive postoperative adjuvant radiotherapy to control the locoregional lymph
252 node metastatic recurrence.

253 However, this study is retrospective and consists of limited sample size. Although this is the
254 first time for demonstrating that IFITM3 is a predictor for lymph node metastatic recurrence of
255 ESCC patients, replication studies with different horizons, prospective and multicentric
256 randomized studies are also needed to certificate this prognostic significance.

257 Conclusions

258 Our study demonstrated that IFITM3 expression has a close relationship with lymphatic
259 metastatic recurrence. And it could serve as an important biomarker to predict the lymph node
260 metastatic recurrence in pN0 ESCC after Ivor-Lewis esophagectomy.

261 **Conflict of interest** The authors declare that they have no conflicts of interest.

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Table 1(on next page)

Correlation of IFITM3 expression with clinicopathological characteristics.

Table1:

Correlation of IFITM3 expression with clinicopathological characteristics of pN0 ESCC patients.

Variables of patients	No. of patients	IFITM3 Expression		p value[a]	3-year recurrence rate (%)	p value[b]
		High	Low			
		59	45			
Age (years)				0.335		0.550
≥ 50	83	45	38		42.2	
< 50	21	14	7		33.3	
Gender				0.365		0.889
Male	78	42	36		41.0	
Female	26	17	9		38.5	
Tumor size (cm)				0.418		0.193
≥ 5cm	40	25	15		47.5	

< 5cm	64	34	30		35.9	
T status				0.015		**0.008**
T1+T2	41	30	11		24.4	
T3	55	24	31		49.1	
T4a	8	5	3		62.5	
Differentiation degree				0.249		0.111
Low	25	17	8		52.0	
Moderate-High	79	42	37		36.7	
IFITM3 overexpression						**0.010**
Yes		59			50.8	
No			45		26.7	

[a] χ^2 test

[b] Log-rank test

Table 2(on next page)

Multivariate Cox regression analysis.

Table2:

Multivariate Cox regression analysis of risk factors in pN0 ESCC.

	B	SE	Wald	*p* value	HR	95%CI	
						Lower	Upper
Age	0.040	0.453	0.008	0.930	1.041	0.429	2.526
Gender	0.041	0.389	0.011	0.915	1.042	0.487	2.232
Tumor size	0.226	0.324	0.489	0.484	1.254	0.665	2.365
T status	0.904	0.253	12.783	**0.000**	2.470	1.505	4.054
Differentiation	0.311	0.350	0.790	0.374	1.365	0.687	2.712
IFITM3 overexpression	1.040	0.360	8.357	**0.004**	2.828	1.398	5.723

1 B regression coefficient; SE standard error; Wald Wald value; HR hazard ratio; CI confidence interval

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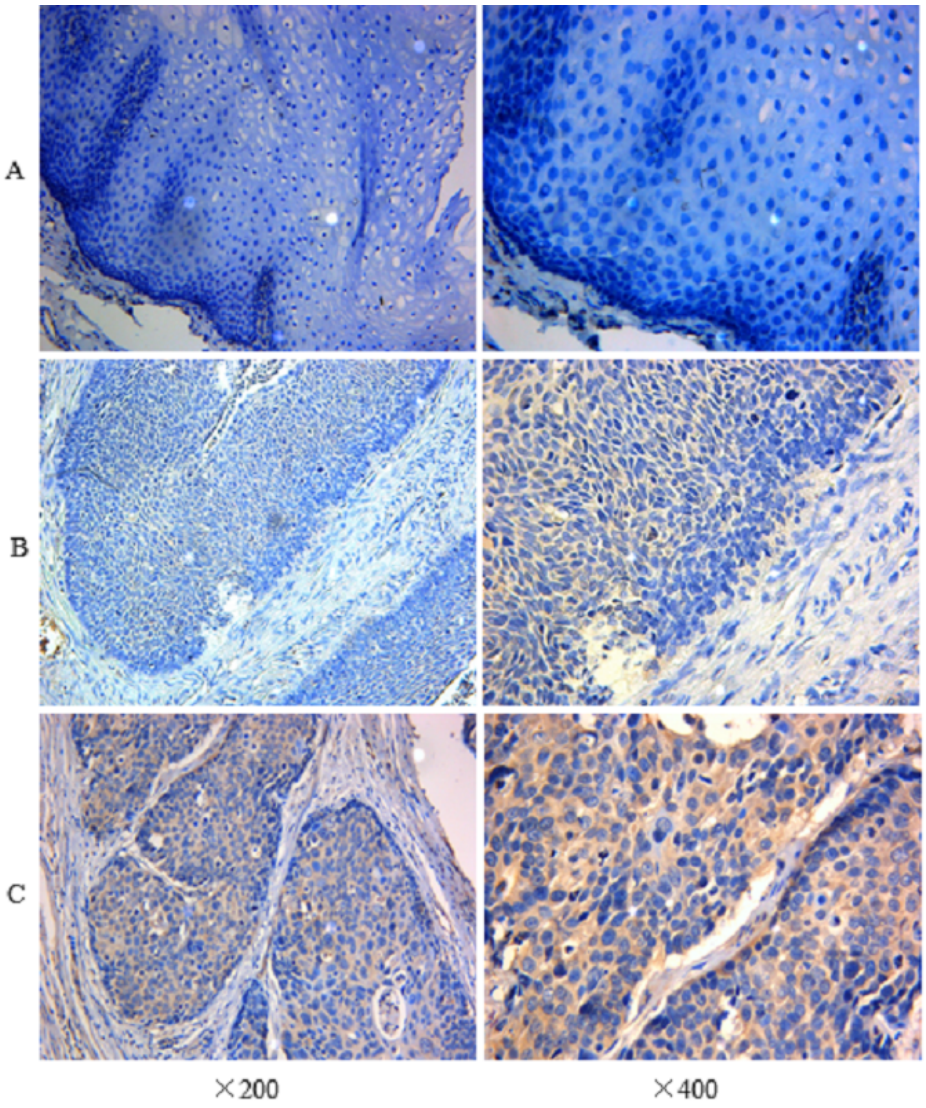
1

Immunohistochemistry assay of IFITM3 in ESCC tissue and ANM.

Figure 1: Immunohistochemistry assay of IFITM3 in ESCC tissue and ANM. (A) Negative expression of IFITM3 in ANM (×200, ×400). (B) Low expression in ESCC tissue (×200, ×400). (C) Strong positive immunocreation of IFITM3 in the cytoplasm of ESCC tissue (×200, ×400).



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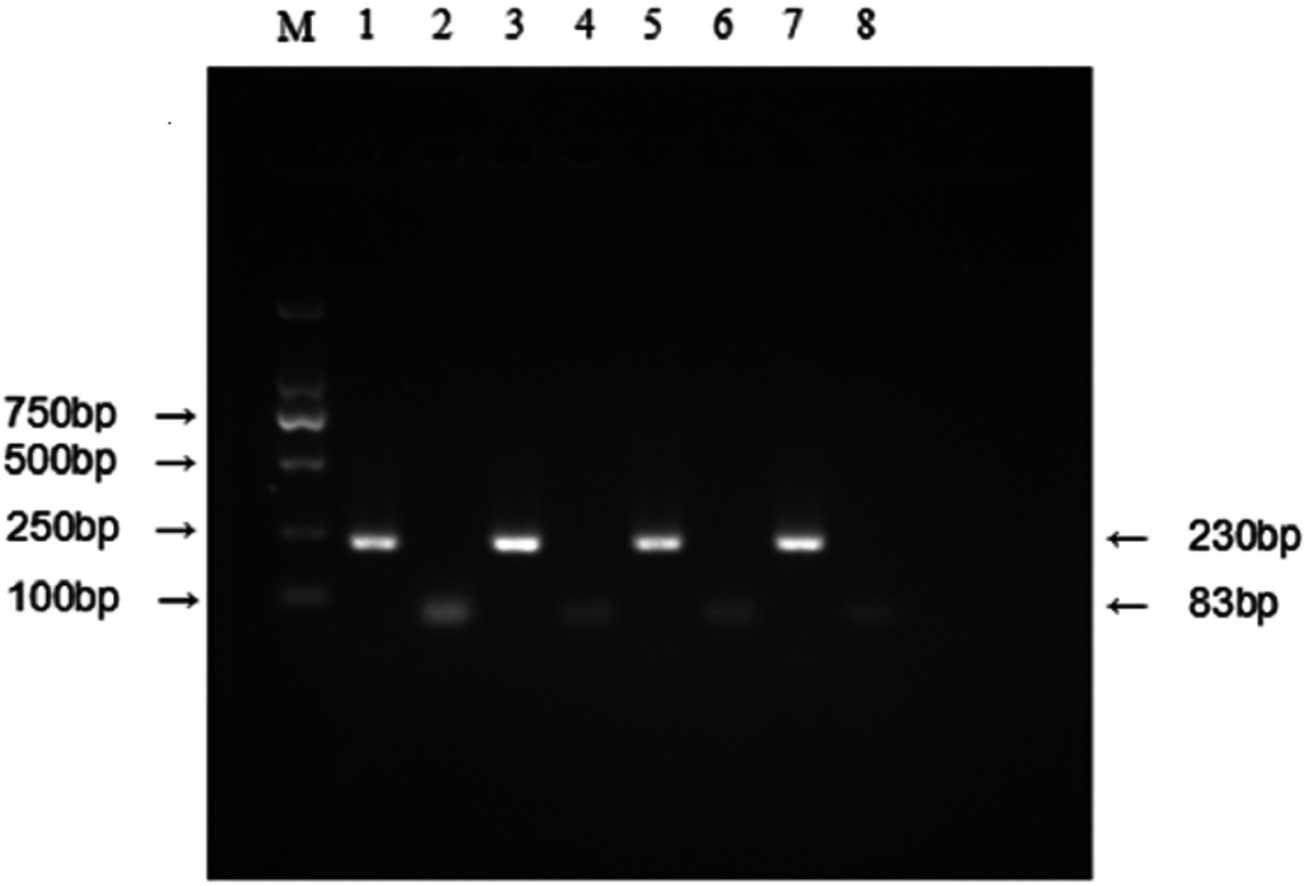
Relative expression level of IFITM3 mRNA was detected by RT-PCR.

Figure 2: (M) Molecular marker. (1) and (2) Respectively represent the mRNA expression level of β-actin and IFITM3 in tumor tissues with IFITM3 protein overexpression; (3) and (4) Represent the mRNA expression level of β-actin and IFITM3 in their ANMs. (5) and (6) Respectively represent the mRNA expression level of β-actin and IFITM3 in low IFITM3 protein expressed tumor tissues; (7) and (8) Represent this expression level of β-actin and IFITM3 in their ANMs.



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Lymphatic metastatic recurrent curves for patients with different IFITM3 expression level and T status.

Figure 3: Lymphatic metastatic recurrent curves for patients with different IFITM3 expression level and T status. (A) and (B) Respectively represent patients with IFITM3 overexpression (p = 0.010) and advanced T status (p = 0.004).