Overexpression of IFITM3 Predicts the 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 studies have shown that the aberrant expression of IFITM3 is implicated in the 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). This aberrant expression was verified by reverse transcription-polymerase chain reaction (RT-PCR) with 20 tumor specimens with strong immunostaining and their mucosal tissues. Inaddition, 20 samples of low expression tissues and their ANMs were evaluated. Moreover, the correlations between the IFITM3 expression level and the clinicopathological variables, recurrent status and overall survival (OS) of patients were analyzed. **Results**: Both IHC and RT-PCR demonstrated that the IFITM3 expression level was significantly higher in tumor tissue than in ANM. Statistical analysis showed a significant correlation of IFITM3 expression with the T status of esophageal cancer ($p = 0.015$). Inaddition, IFITM3 overexpression was demonstrated to be not only an important risk factor of lymphatic metastatic recurrence but a significant prognostic factor in pN0 ESCC ($p < 0.005$). **Conclusions**: Even pN0 ESCC patients will still experience lymphatic metastatic recurrence. The IFITM3 gene could be a predictor of lymphatic metastatic recurrence in pN0 ESCC after Ivor-Lewis esophagectomy.

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Abstract

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Results: Both IHC and RT-PCR demonstrated that the IFITM3 expression level was significantly higher in tumor tissue than in ANM. Statistical analysis showed a significant correlation of IFITM3 expression with the T status of esophageal cancer ($p = 0.015$). In addition,

19   IFITM3 overexpression was demonstrated to be not only an important risk factor of lymphatic

20   metastatic recurrence but a significant prognostic factor in pN0 ESCC ($p < 0.005$).

21   **Conclusions**: Even pN0 ESCC patients will still experience lymphatic metastatic recurrence.

22   The IFITM3 gene could be a predictor of lymphatic metastatic recurrence in pN0 ESCC after

23   Ivor-Lewis esophagectomy.

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Introduction

43   Esophageal carcinoma (EC) is the sixth leading cause of mortality among various malignant

44   tumors worldwide. Esophageal adenocarcinoma and esophageal squamous cell carcinoma

45   (ESCC) are the two most common histological types. Both have an obvious characteristic

46   geographic distribution, and China is a country with high incidence of ESCC (Ferlay et al. 2010).

47   Despite the improvement in the diagnostic level and utilization of combined treatment modalities

48   in recent years, the prognosis of ESCC patients remains poor. Even in pN0 ESCC, the 5-year

49   survival rate is only approximately 70% after complete resection, and lymphatic metastatic

50   recurrence is the main reason for the failure of the operation (Eloubeidi et al. 2002; Visbal et al.

51   2001). Therefore, to improve the long-term survival of ESCC patients, it is of great clinical

52   significance to control locoregional lymph node metastatic recurrence after surgery.

53   Clinically, the number of metastasis-positive lymph nodes is usually used to evaluate the

54   risk of lymphatic metastatic recurrence in locally advanced disease (Law & Wong 2001).

55   However, no reliable index has been used to predict this rate in pN0 ESCC. Although some

56   molecules were previously used to stratify the recurrent risk in pN0 ESCC (Li et al. 2009; Song

57   et al. 2012), none has been proven to be universally accepted and commonly used. Therefore,

58   sensitive biological markers that may predict this recurrent risk are urgently needed.

59   Interferon-induced transmembrane protein 3 (IFITM3), also known as 1-8U, is one of the

60   important members of the IFN-inducible transmembrane protein family. IFITM3 likely exerts

61   profound influences on cell proliferation, migration, and invasion through the modulation of the

62   Wnt/β-catenin signaling pathway and is implicated in the G0/G1 checkpoint to control the cell

63   cycle of tumors (Hu et al. 2014; Yang et al. 2013; Zhao et al. 2013). It is overexpressed in many

64   human malignancies, such as gastric cancer, colorectal tumor, breast cancer, glioma, and oral

65   squamous cell carcinoma.

66   Previous research has shown that IFITM3 is upregulated in gastric cancer, which is

67   correlated with tumor invasion and metastasis (Hu et al. 2014). Moreover, it has also been

68   demonstrated to have a close relationship with the prognosis of colon cancer and was confirmed

69   to be an independent risk factor for disease-free interval (Li et al. 2011). However, the

70   clinicopathological significance and prognostic value of IFITM3 in ESCC patients remains

71   unknown.

72   In this study, we sought to validate the relationships between the expression level of

73   IFITM3 and the clinicopathological characteristics, lymphatic metastatic recurrence risk and

74   overall survival of ESCC patients who had undergone Ivor-Lewis esophagectomy. We aimed to

75   explore whether the IFITM3 gene can predict lymphatic metastatic recurrence in pN0 ESCC.

Materials and Methods

Patients

78   From January 2008 to January 2010, patients with midthoracic ESCC who underwent Ivor-

79   Lewis esophagectomy with two-field lymphadenectomy in our department (Provincial Hospital

80   Affiliated to Shandong University, China) were eligible for this study. In total, this study

81   enrolled 104 patients, including 83 men and 21 women, with ages ranging from 40 to 75 years

82   (Clinicopathological data are listed in **Table 1**).

83   All patients met the following inclusion criteria: (1) According to 2009 Union for

84   International Cancer Control (UICC) standard for midthoracic ESCC, Ivor-Lewis

85   esophagectomy with two-field lymph node dissection was conducted to achieve complete

86   resection (R0), and the proximal and distal incisal margins as well as lateral margin were

87   pathologically examined without residual foci (Arai et al. 2012). At the same time, the average

88   number of lymph nodes dissected was 18 ± 5.8 (ranging from 12 to 25); (2) Patients enrolled in

89   the study were restaged after surgery according to TNM staging (UICC, 2009) for esophageal

90   cancer; (3) Without history of previous malignancies or other severe diseases that may influence

91   the outcome of our follow-up; (4) Patients were not eligible if preoperative neoadjuvant

92   chemotherapy or postoperative adjuvant treatment was administered.

93   **Surgical procedure of Ivor-Lewis esophagectomy**

94   Four thoracic surgeons worked together to perform this type of surgery, and the thoracic

95   operation was performed by two surgeons. The patient was placed in the 40°–45° left lateral

96   decubitus position. After a right anterolateral thoracotomy, the chest was entered through the

97   fourth intercostal space. The azygos vein arch was divided, and the esophagus was dissected

98   from the esophagogastric junction to the apex of the chest. When the tumor invasion obviously

99   extended outside the esophagus, the thoracic duct was routinely ligated above the diaphragm.

100   At the same time, an upper midline abdominal incision was made by another two surgeons,

101   and the abdomen was explored. During mobilization of the stomach, care was taken to preserve

102   the right gastroepiploic vessels and arcades. The left gastric artery and vein were isolated and

103   doubly ligated at their origin. Pyloroplasty was not routinely performed. Then, the hiatus was

104   enlarged and the stomach was pulled into the chest. An endto-side esophagogastric anastomosis

105   was performed within the apex of the chest, and the stomach was secured in the mediastinum

106   (Chen et al. 2009a; Chen et al. 2009b).

107   **Specimens**

108   The ESCC tissue and ANM (more than 5 cm from the margin of ESCC) were collected

109   from surgical specimens of each selected patient. At the same time, the ANM did not exhibit

110   tumor infiltration, deterioration or necrosis upon macroscopic and microscopic examination.

111   This study was approved by the Ethics Committee of Provincial Hospital Affiliated to

112   Shandong University, and the approval number was 2008081. Written informed consent was

113   obtained from all the participants.

114   **Immunohistochemistry**

115   The streptavidin-peroxidase immunohistochemical method was used to examine the

116   expression of the IFITM3 protein. Formalin-fixed and paraffin-embedded surgical specimens

117   were sequentially cut into 4-μm sections. Then, the sections were dewaxed, antigen retrieval and

118   hydrogen peroxide incubation. Rabbit anti-IFITM3 monoclonal antibodies (GeneTex, USA)

119   were used at a dilution of 1:200 and incubated at 4℃ overnight. The monoclonal primary

120   antibody was replaced by phosphate-buffered saline (PBS) as a negative control. Further

121   experimental steps were followed according to the instructions of a secondary biotinylated

122   antibody kit purchased from ZSGB Biotech (BeiJing, China).

123   The expression of the IFITM3 protein was determined according to Sakakura's criteria.

124   Two pathologists blinded to the clinical data were invited to evaluate the IHC sections

125   independently. The outcome was calculated by combining the proportion with the staining

126   intensity. The proportion was scored as follows: 0 (0 − 10%), 1 (11 − 25%), 2 (26 − 50%), 3 (51

127   – 75%), and 4 (75 – 100%). The staining intensity was scored: 0 (negative), 1 (weak), 2

128   (moderate), and 3 (strong). The final immunohistochemical score (IHS) was defined as the

129   proportion score × staining intensity score. In this study, IHS ≥ 8 was considered to represent

130   overexpression.

131   **RNA Extraction and RT-PCR**

132   Total RNA was extracted from fresh frozen tissue using Trizol (Invitrogen) according to the

133   manufacturer's protocols. The purity of RNA was measured by UV spectrophotometer

134   (NanoDrop 2000) and the OD 260/280 value ranging from 1.8 to 2.0 was used to reverse

135   transcription. The detailed RT-PCR procedure was followed by a CWBio two-step RT-PCR kit

136   (JiangSu, China). The primer sequences of the IFITM3 gene were 5'-

137   CAAGGAGGAGCACGAGG-3' (forward primer) and 5'-TTGAACAGGGACCAGACG-3'

138   (reverse primer). β-actin was used as internal control, and the primer sequences of β-actin were

139   5'-AGAGCCTCGCCTTTGCCGATCC-3' (forward primer) and 5'-

140   ATACACCCGCTGCTCCGGGTC-3' (reverse primer). The PCR products of IFITM3 were

141   further separated on 1% agarose gel electrophoresis. Azure C2000 (Azure Biosystems, USA)

142   was used for electrophoresis gelatin image formation analysis.

143   **Follow-Up**

144   According to our plan, patients were examined every 3 – 6 months after surgery, and the

145   checklist was described in our previous study (Akhtar et al. 2014). We compared the imaging

146 data preoperatively and postoperatively in detail to differentiate whether recurrence occurred or

147 not. If the lymph nodes were swollen or the minor axis was more than 1 cm, the clinical

148 diagnosis of lymphatic recurrence was made. Fine-needle aspiration biospy and PET-CT were

149 also assisted to make the diagnosis of recurrence. The follow-up was ended in July 2013, and the

150 complete 3-year follow-up data was reviewed.

151 **Statistical Analysis**

152 The χ^2 test was used to analyze the relationship between IFITM3 expression and

153 clinicopathological variables. The recurrence curves and survival curves were calculated by the

154 Kaplan–Meier method. Univariate log-rank test and Cox regression analysis were respectively

155 performed to evaluate the recurrence risk and prognostic factors. A satatistically significant

156 difference was represented by a two-tailed p value less than 0.05. All statistical analyses were

157 performed using SPSS version 17.0 (Chicago, IL, USA).

158 # Results

159 **IFITM3 Expression Analysis in ESCC tissue and ANM**

160 The immunohistochemistry assay was used to detect the expression level of the IFITM3

161 protein. Overexpression was presented as yellow or brownish yellow staining in the cytoplasm of

162 the tumor cell. As is shown in **Fig. 1C**, the significant immunoreaction of positive expression can

163 be readily differentiated. However, there was low or undetected staining in ANM (**Fig. 1A**).

164 Furthermore, according to the criteria of IHS, we divided all the specimens into two groups: 59

165   cases (56.7%) were categorized as the overexpression group (**Fig. 1C**) and 45 cases (43.3%)

166   were in the low expression group (**Fig. 1B**).

167   To verify this aberrant upregulation of IFITM3, we examined the mRNA expression level

168   by RT-PCR with 20 pairs of specimens randomly selected from the overexpression group and 20

169   pairs of tissues that originated from the low expression group. The results showed that the

170   mRNA expression level was consistent with protein expression as demonstrated by IHC (**Fig. 2**).

171   **IFITM3 Expression and Clinicopathological Characteristics**

172   According to the inclusion criteria mentioned above, a total of 104 ESCC patients were

173   enrolled in this study with different ages, genders, tumor sizes, degrees of differentiation, T

174   status and IFITM3 expression levels (**Table 1**). χ^2 analysis demonstrated that the expression level

175   of IFITM3 had a close relationship with T status of tumor ($p = 0.015$). In contrast, there were no

176   significant differences between expression level and age, gender, tumor size and degree of

177   differentiation. ($p > 0.05$)

178   **IFITM3 Expression and Lymphatic Metastatic Recurrence**

179   Through thorough follow-up, a total of 42 cases (40.4%) were confirmed to have first

180   lymph node metastatic recurrence within 3 years, in which IFITM3 overexpression was detected

181   in 30 patients (71.4%). In the low IFITM3 expression group, the 3-year lymphatic recurrence

182   rate was only 26.7%. Conversely, in the overexpression group, this rate reached up to 50.8%

183   (**Table 1**). As is shown in **Fig. 3**, Kaplan–Meier analysis showed that the recurrence rate was

184 significantly increased in patients with IFITM3 overexpression, and the log-rank test calculated

185 that these two curves were significantly different ($p = 0.010$).

186   In addition to the expression level of IFITM3, T status of tumor ($p = 0.008$) was also

187 elucidated to be associated with the lymphatic recurrence in pN0 ESCC (**Table 1**). Multivariate

188 Cox regression analysis revealed that these two variables were independent recurrence risk

189 factors ($p < 0.05$, **Table 2**). Patients with early T status and low expression of IFITM3 may have

190 a lower recurrence risk of lymphatic metastasis (**Fig. 3**).

191 **IFITM3 Expression and Overall Survival**

192   As is shown in our data, the 3-year OS rate of patients with IFITM3 protein overexpression

193 and low expression were respectively 64.4% and 88.9%. **Fig. 4** demonstrated that the long

194 survival of patients was associated with moderate-high differentiation, early T status of tumor

195 and low IFITM3 expression. Univariate analysis and multivariate analysis revealed that these are

196 independent and significant prognostic factors ($p < 0.05$, **Table 3**).

197 **Discussion**

198   ESCC is one of the most common neoplasms in China, with a high incidence of lymph node

199 metastatic recurrence, especially in the mediastinum, neck and abdominal cavity (Chen et al.

200 2007). Even in pN0 ESCC, more than 40% of individuals exhibit micro-metastasis (Wang et al.

201 2004). Surgery is still considered to be the first-line treatment modality for ESCC patients with

202 resectable lesions (Hulscher et al. 2002; Olsen et al. 2011), but the overall survival is not ideal

203 and nearly half of patients will still experience tumor relapse (Eloubeidi et al. 2002; Korst et al.

204 1998; Rice et al. 2001; Visbal et al. 2001).

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

206 cancer guidelines are often referenced in clinical practice. These guidelines suggest that patients

207 should not receive adjuvant therapy after complete tumor resection, but individuals with

208 advanced T status (above T2) should receive neoadjuvant chemotherapy before surgery.

209 However, in China, patients tend to receive primary surgery if tumors can be completely resected

210 and Ivor-Lewis esophagectomy via a thoracoabdominal two-field lymph node dissection is the

211 main surgical modality. Compared with three-field lymph node dissection, the advantage of Ivor-

212 Lewis esophagectomy is that the latent surgical trauma and complications can be effectively

213 controlled; however, cervical lymph node dissection cannot be accomplished simultaneously.

214 Therefore, for patients with a high risk of lymphatic metastasis, we believe that postoperative

215 adjuvant therapy may act as a compensatory mechanism to control lymphatic recurrence after

216 Ivor-Lewis esophagectomy.

217   In this study, we first found the differential expression of IFITM3 in tumor tissues and their

218 ANMs, as well as the important clinicopathological significance of IFITM3. Our results were

219 consistent with previous researches that demonstrated that IFITM3 is overexpressed in many

220 hunman malignancies, such as gastric cancer, colorectal cancer, oral squamous cell carcinoma,

221 glioma, and breast cancer. These findings suggested that IFITM3 may play important roles and

222 maybe a molecular marker in pN0 ESCC.

223   Regarding to the prognostic value of IFITM3, previous studies have elicited contradictory

224   conclusions for different cancers, which reflects the complexity of IFITM3 in different tumor

225   microenvironments. For gastric cancer, Hu et al (Hu et al. 2014) suggested that IFITM3

226   overexpression is correlated with lymph node metastasis. Li et al demonstrated that it was an

227   important independent prognostic factor for disease-free interval and is upregulated in the nodal

228   metastasis of colon tumors (Li et al. 2011). Conversely, Yang et al did not find an association

229   between IFITM3 expression and lymph node metastasis in breast cancer (Yang et al. 2013). El-

230   Tanani et al (El-Tanani et al. 2010) even drew the opposite conclusion; they deemed that IFITM3

231   may inhibit the proliferation, development and metastasis of cancer by reducing the expression

232   of osteopontin. However, to our knowledge, no study has demonstrated the prognostic

233   significance of IFITM3 in ESCC. This question led us to explore whether IFITM3 could be a

234   biomarker to evaluate the risk of lymph node metastatic recurrence in ESCC. The data from this

235   study showed that the high incidence of lymphatic metastatic recurrence in pN0 ESCC was

236   associated with advanced T status and IFITM3 overexpression. At the same time, the expression

237   level of IFITM3 was also elucidated to be an important prognostic factor. The findings strongly

238   suggested that IFITM3 could serve as a biomarker to stratify the risk of recurrence and survival,

239   and then play an important role in the selection of a treatment modality in pN0 ESCC.

240   A total of 104 patients with midthoracic ESCC in this study underwent Ivor-Lewis

241   esophagectomy with two-field lymph node dissection. All of these patients had undergone

242   theoretic R0 resection and had pathologically confirmed pN0 after surgery. However, during the

243 follow-up period, 40.4% patients showed the first lymphatic metastatic recurrence within 3 years.

244 For patients with a high risk of lymphatic metastatic recurrence, we believe that these findings

245 have important clinical significance in choosing to accept adjuvant therapy to control lymph

246 node recurrence. Previous studies have demonstrated that postoperative adjuvant radiotherapy

247 can significantly reduce the lymphatic metastatic recurrence in ESCC. Combined with findings

248 in this study and our previous studies, we believe that it is indispensible for pN0 ESCC patients

249 with IFITM3 overexpression to receive postoperative adjuvant radiotherapy to control the

250 metastatic recurrence of locoregional lymph nodes and in turn improve the survival.

251 However, this study was retrospective and had a limited sample size. Although this is the

252 first time that IFITM3 was demonstrated to be a predictor for lymph node metastatic recurrence

253 of ESCC patients, replication studies with different parameters and prospective and multicenter

254 randomized studies are also needed to verify this prognostic significance.

Conclusions

256 Our study demonstrated that IFITM3 expression has a close relationship with lymphatic

257 metastatic recurrence. This could serve as an important biomarker to predict the lymph node

258 metastatic recurrence in pN0 ESCC after Ivor-Lewis esophagectomy.

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

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Correlation of IFITM3 expression with clinicopathological characteristics

Table1: Correlation of IFITM3 expression with clinicopathological characteristics of pN0 ESCC patients

Variables	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
≥ 5	40	25	15		47.5	

< 5	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	

1   [a] χ^2 test

2   [b]   Log-rank test

Multivariate Cox regression analysis

Table 2: 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

Univariate and multivariate analyses of overall survival

Table 3: Univariate and multivariate analyses of overall survival for 104 cases of pN0 ESCC patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*	HR	95% CI	*p*
Gender						
Male versus Female	1.095	0.440-2.727	0.846	_		
Age (years)						
≥50 versus <50	1.388	0.478-4.028	0.546	_		
Tumor size (cm)						
≥5 versus <5	2.076	0.960-4.490	0.063	_		
T status						
T1+T2 versus T3+T4a	3.082	1.162-8.175	**0.024**	3.979	1.443-10.974	**0.008**
Differentiation degree						
Moderate-High versus Low	3.125	1.445-6.758	**0.004**	2.346	1.056-5.212	**0.036**
IFITM3 overexpression						
Yes versus No	3.644	1.373-9.673	**0.009**	4.260	1.548-11.723	**0.005**

2   Statistical analysis was performed using the proportional hazard model (Cox). Data considered significant ($p < 0.05$) in the univariate

3   analysis were examined in the multivariate analysis

4   HR hazard ratio; CI confidence interval

5   *p* values < 0.05 in bold font were considered significant

1

Immunohistochemistry assay of IFITM3 in ESCC tissue and ANM

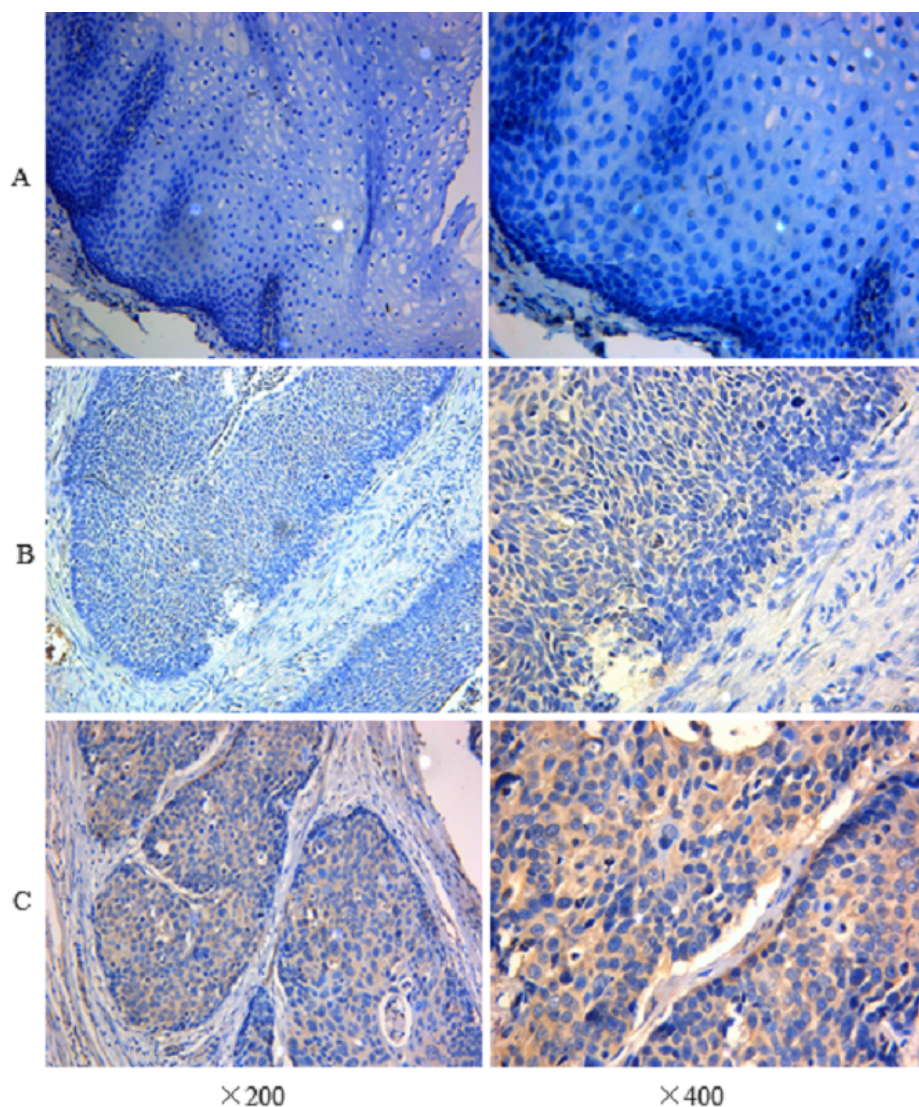
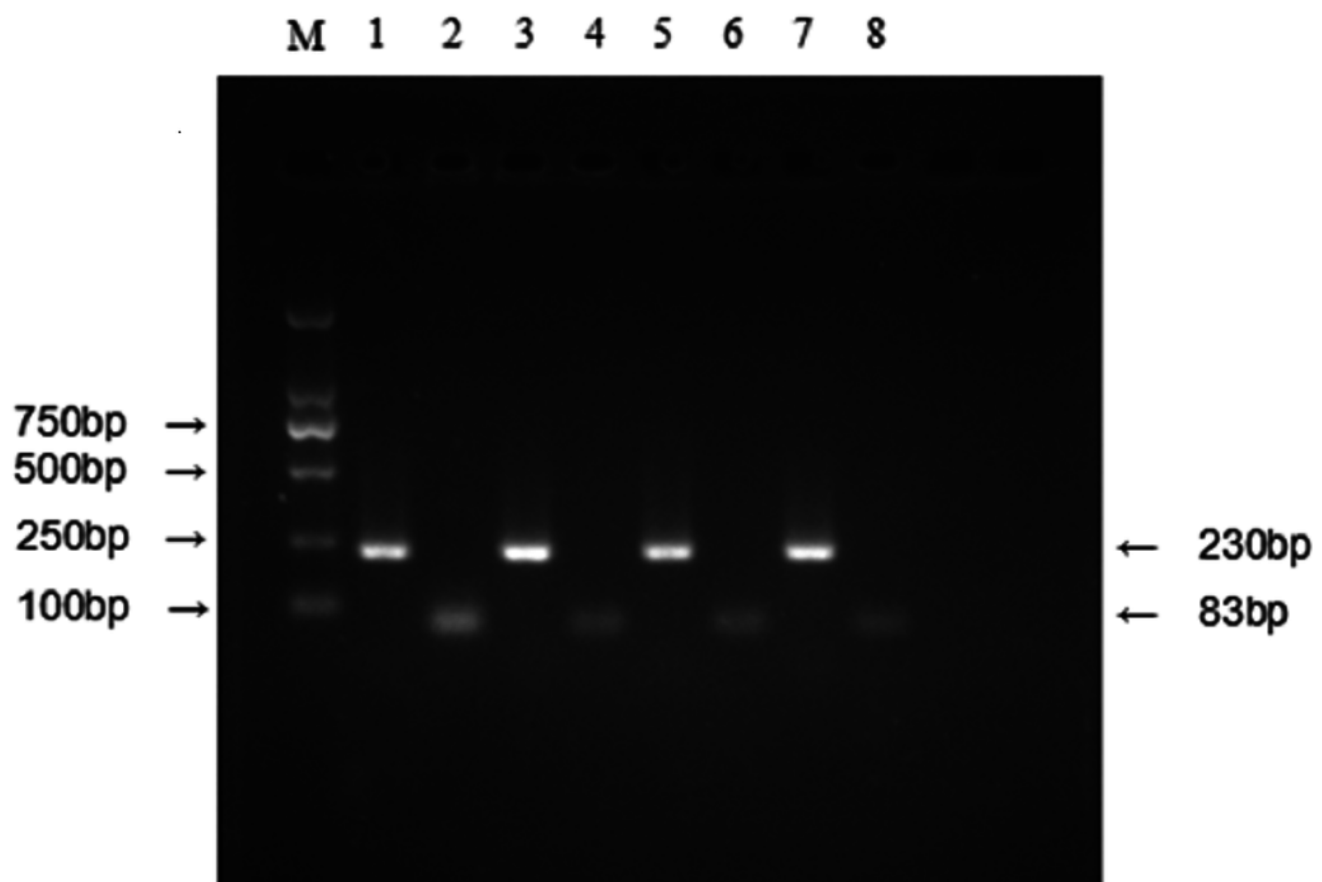
Figure 1: Immunohistochemistry assay of IFITM3 in ESCC tissue and ANM. (A) Negative expression of IFITM3 in ANM (×200, ×400). (B) Low expression in ESCC tissue (×200, ×400). (C) Strong positive immunocreation of IFITM3 in the cytoplasm of ESCC tissue (×200, ×400)

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

3

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

Figure 3: Lymphatic metastatic recurrence 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)

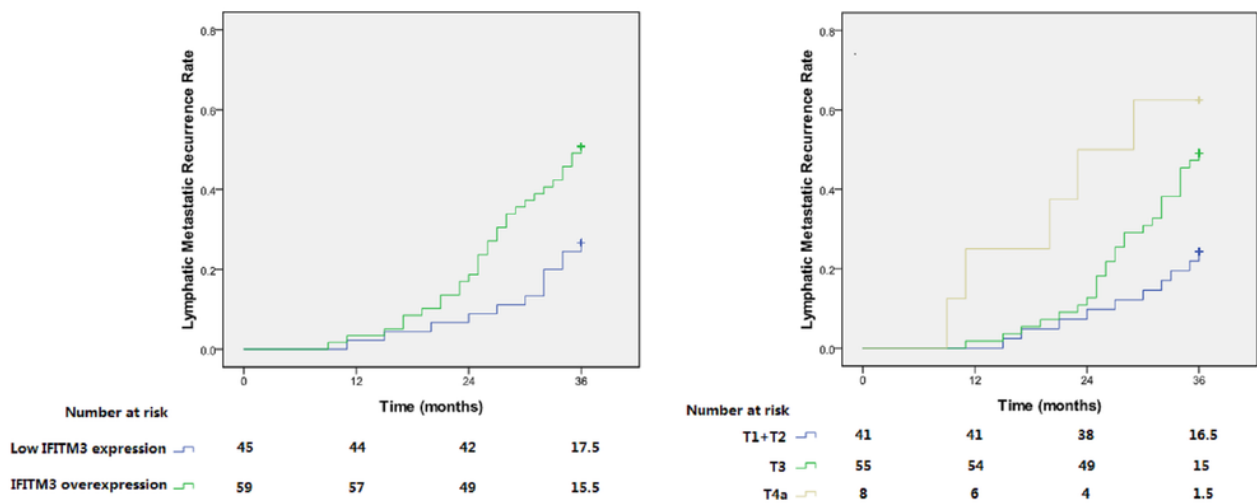
4

Kaplan–Meier analysis of overall survival of ESCC patients

Figure 4: Kaplan–Meier analysis of overall survival of ESCC patients. Overall survival of patients with pN0 ESCC according to (A) IFITM3 expression level; (B) T status of tumor; (C) differentiation degree