

# High Expression of GRP78 Promotes Invasion and Metastases in Patients with Esophageal Squamous Cell Carcinoma

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## Abstract

**Background** Esophageal squamous cell carcinoma (ESCC) has a poor prognosis due to its high frequency of metastasis and invasion. Recent studies have suggested glucose-regulated protein 78KD (GRP78) may play important roles in progression and development of malignant tumors. However, the mechanisms of invasion and metastasis of ESCC in relation to GRP78 still remain obscure.

**Aim** The aim of this study was to investigate the effect of GRP78 on invasion and metastasis of ESCC and to explore its potential mechanism.

**Methods** GRP78 expression levels in ESCC tissues were examined by immunohistochemistry. RT-PCR and western blot were used to test the relative expression of GRP78 in non-metastatic and high-metastatic ESCC cells. In vitro

and in vivo studies were both performed to investigate the role of GRP78 in invasion and metastasis of ESCC cells. The expression of metastasis-related proteins was examined by western blot in GRP78-depleted cells.

**Results** The expression of GRP78 is correlated with invasion, metastasis and poor prognosis in ESCC patients. GRP78 expression was significantly higher in highly metastatic cells compared with ESCC non-metastatic cells. In addition, down-regulation of GRP78 significantly inhibited the metastatic potential of ESCC cells in both in vitro and in vivo studies. The expression of MMP-2 and MMP-9 were down-regulated in GRP78-depleted ESCC cells.

**Conclusions** The present study demonstrated that GRP78 plays important roles in invasion and metastasis of ESCC, indicating that GRP78 might be used as a potential prognostic and therapeutic marker in patients with ESCC by modulating the expression of MMP-2 and MMP-9.

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**Keywords** ESCC · GRP78 · Invasion · Metastasis

## Abbreviations

ESCC Esophageal squamous cell carcinoma  
siRNA Small interfering RNA  
NC Negative control  
GRP78 Glucose-regulated protein 78KD

## Introduction

Human esophageal squamous cell carcinoma (ESCC) ranks as the ninth most common tumor and the sixth leading cause of death from cancer worldwide [1]. Although the mortality of ESCC has significantly decreased because of the advances in surgery, radiotherapy and chemotherapy,

approximately 70 % of ESCC patients have a poor prognosis due to early lymph node (LN) metastasis and invasion of neighboring organs at diagnosis [2].

The development and progression of ESCC is believed to be a complex process involving the interaction of a number of genes. To date, some genetic alterations have been reported to be related to the development of ESCC, such as the overexpression of cyclinD1 and c-Myc and mutations of p53 and p16 [3, 4]. In addition, some genes were also reported to be involved in the metastasis of ESCC. For example, matrix metalloproteinases (MMPs) play key roles in the metastasis of ESCC [5, 6]. Recent evidence also indicates the epithelial–mesenchymal transition (EMT) is also involved in the invasion and metastasis of ESCC, for example, E-cadherin and twist [7, 8]. However, the underlying mechanisms responsible for the invasion and metastasis of ESCC remain poorly understood.

An increasing body of evidence has revealed that glucose-regulated protein GRP78 is involved in tumor development and progression. GRP78 has been shown to be overexpressed in many human cancers, such as hepatocellular carcinoma, colon cancer, lung cancer, gastric cancer and prostate cancer [9–13]. GRP78 was also found to be elevated in ESCC compared to normal tissues [14]. Several studies have also shown that the expression of GRP78 is related to invasion and metastasis of human cancers [15, 16]. Overexpression of GRP78 is related to increased LN metastasis and poor prognosis in patients with gastric cancer [12], and knockdown of GRP78 has been found to decrease the invasion and ECM degradation in hepatocellular carcinoma cells [12]. These findings demonstrate that GRP78 may take part in the tumor metastasis. However, the role of GRP78 in invasion and metastasis in ESCC remains unknown.

In this study, we found that GRP78 was overexpressed in tissues from ESCC patients with LN metastasis compared with the tissues from patients without LN metastasis, and the expression of GRP78 was significantly higher in the highly metastatic ESCC cell lines than that in the non-metastatic ESCC cell lines that we constructed by repeated transwell assays. Moreover, the potential for metastasis and invasion was decreased both *in vivo* and *in vitro* when GRP78 was down-regulated. We also show that down-regulation of GRP78 could, at least in part, modulate the expression of MMP2 and MMP9 in ESCC cell lines.

## Methods

### Patients and Clinical Samples

One hundred thirteen human ESCC specimens were obtained from patients who underwent potentially curative

surgery at Xijing Hospital of Digestive Diseases, the Fourth Military Medical University, between Oct 2008 and June 2009. None of these patients had received radiation or chemotherapy before surgery.

### Immunohistochemical Staining

Tissue sections were fixed with 10 % formaldehyde, embedded in paraffin and sectioned to 5- $\mu$ m thick. The specimens were then incubated with anti-GRP78 polyclonal antibody (1:100, Santa Cruz, CA, USA) at 4 °C over night. The negative control was treated with PBS instead of anti-GRP78 antibody. After being washed three times with PBS, the specimens were incubated with secondary antibody for 30 min. The tissue sections were examined by two independent pathologists blinded to the clinical data. The samples were scored as 0, 1, 2 or 3 based on the staining intensity. The immunoreactivity proportion was ranked as 0 (0 %), 1 (0–30 %), 2 (30–60 %) or 3 (>60 %). The two scores were added to obtain the final results: negative (–), 0–2; weak, positive (+), 3–6.

### Cell Culture

The esophageal carcinoma cell (ESCC) lines EC109 and EC9706 were obtained from the Chinese Academy of Medical Science. The cells were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS) at 37 °C in humidified air containing 5 % carbon dioxide. The cells were used in the logarithmic phase of growth throughout the experiment.

### Selection of Highly and Non-invasive Cell Sublines Using Transwell Chambers

To select highly and non-invasive cell sublines, EC9706 cells and EC109 cells were seeded on a matrigel (BD Biosciences, San Jose, CA, USA)-coated, 8  $\mu$ m-pore transwell (Coring, USA) after being starved for 24 h. The next day, cells on the underside of the membrane and cells remaining on the top of membrane were collected separately. The collected cells were expanded and then re-seeded into another matrigel coated transwell. Such selection rounds were repeated ten times, resulting in sublines with highly metastatic subpopulations named EC109-P and EC9706-P. The non-metastatic sublines were named EC109-N and EC9706-N.

### RNA Extraction and Quality Real-Time PCR (qRT-PCR)

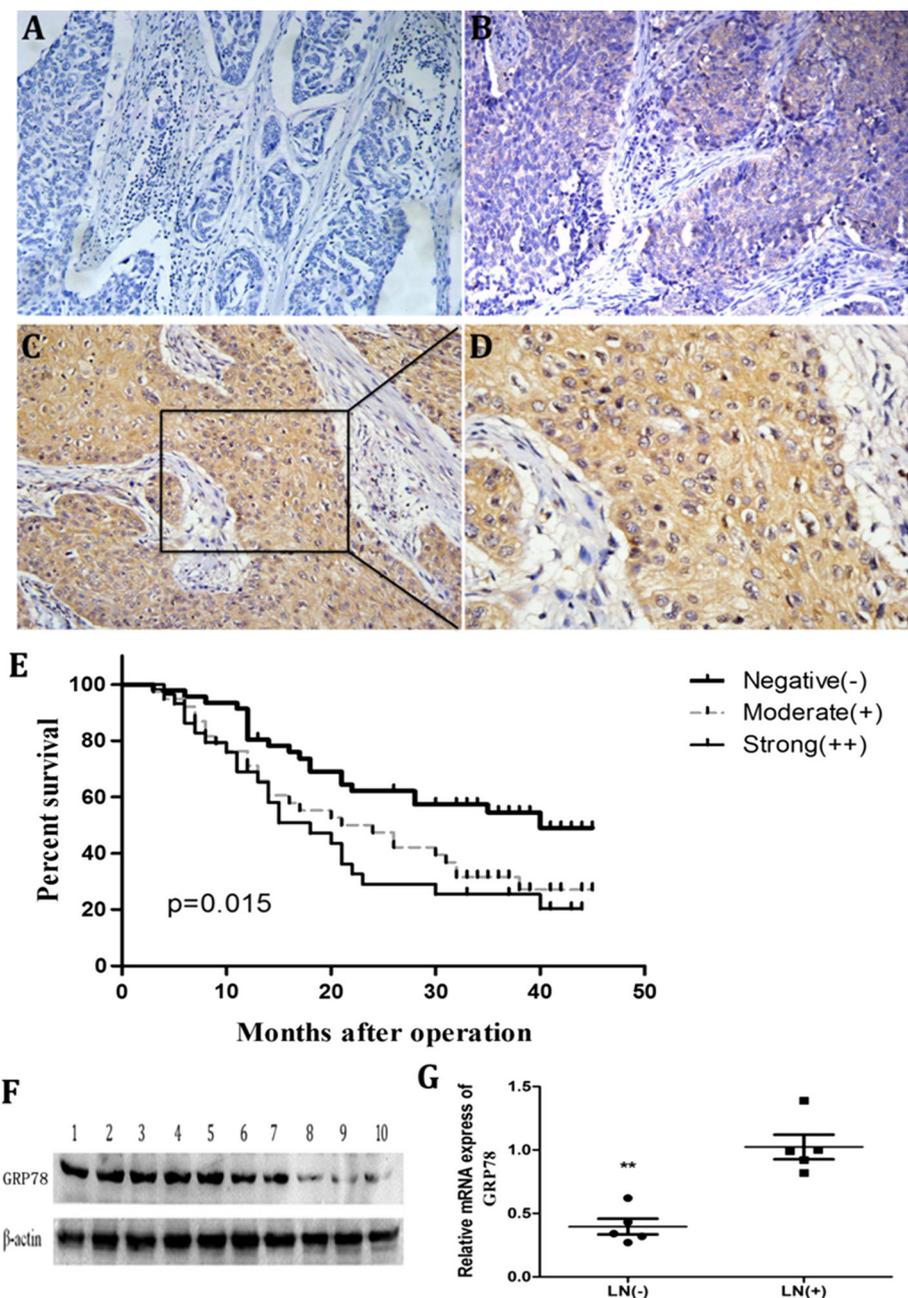
Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and synthesized into cDNA using the

PrimeScript RT reagent Kit (TaKaRa, Dalian, China). The qRT-PCR primers for GRP78 were designed by Takara. The primers were GRP78 forward: 5'-GAGAAGGAGGAGGCTGAATACCG-3', reverse: 5'-GTGATGTTGTAGGTGTCTGCG-3'; GAPDH forward: 5'-GCACCGTCAAGGCTGAGAAC-3', reverse: 5'-TGGTGAAGACGCCAGTGA-3'. Real-time PCR was performed using SYBR Premix *Ex Taq* II (TaKaRa) and measured in a LightCycler 480 system (Roche, Basel, Switzerland). The levels of GAPDH were used as an internal control. All of the reactions were run in triplicate.

### Lentivirus-Mediated siRNA Construction and Cell Transfection

The lentivirus-mediated small interfering RNA for GRP78 (siGRP78) and the negative control RNA (siNC) were designed and synthesized by Genepharma (Shanghai, China). The GRP78 siRNA sequence was: F: 5'-GGUACUGCUUGAUGUAUGUTT-3', R: 5'-ACAUACAUCAAGCAGUACCTT 3'; The control siRNA sequence was: F: 5'-UUCUCCGAACGUGUCACGUTT-3', R: 5'-ACGUGACACGUUCGGAGAATT-3. For transfection experiments, EC109-P and

**Fig. 1** Immunohistochemical-staining of GRP78 in human esophageal squamous cell carcinoma (ESCC) and overall survival time of the patients shows that increased expression of GRP78 is associated with lymph node metastasis in ESCC. **a** Representative images for negative staining of GRP78 (magnification  $\times 200$ ). **b** Representative images for weak staining of GRP78 (magnification  $\times 200$ ). **c** Representative images showing the strong staining of GRP78 (magnification  $\times 200$ ). **d** Representative images showing the strong staining of GRP78 (magnification  $\times 400$ ). **e** Correlation of GRP78 expression with overall survival time in patients with ESCC by Kaplan–Meier analysis,  $p = 0.015$ . **f** Western blot analysis was performed on tumor tissues with lymph node metastasis (1–5) and tumor tissues without lymph node metastasis (6–10) using antibodies against GRP78, with  $\beta$ -actin as internal control. **g** qRT-PCR analysis was performed for tumor tissues with lymph metastasis (1–5) and tumor tissues without lymph metastasis and GAPDH as internal control,  $p = 0.0079$



EC9706-P were plated in six-well plates and siRNAs were transfected using Lipofectamine<sup>TM</sup> 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. For transfection, target cells ( $1 \times 10^5$ ) were cultured in 24-well plates, grown to 50–60 % confluency and transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). In lentiviral vector transfection, cells were transfected with 60  $\mu$ l  $1 \times 10^8$  TU/ml LV4-NC or LV4-GRP78 using 5  $\mu$ g/ml polybrene according to the manufacturer's protocol. After the transfection, the cells were selected by 1  $\mu$ g/ml puromycin for one week. The expression of GRP78 in the cells was further confirmed by western blot analysis and qRT-PCR.

### Wound and Healing Assay

Cells were seeded into six-well plates and grown to confluence. The cell layer was scratched with a 100- $\mu$ l pipette tip and washed three times to remove non-adherent cells. The cells were overlaid with serum-free media for 24 h and then washed three times with PBS. Wound width was measured at 0 and 24 h after wounding by using a light microscope (Olympus BX51, Olympus). The experiments were repeated three times for reproducibility.

### Transwell Migration and Invasion Assays

For transwell migration assays, 50,000 cells were seeded per well into transwell inserts (8  $\mu$ m pore size, Corning, USA). For invasion assays, 100,000 cells were seeded into matrigel-coated chambers (BD Biosciences, USA). After incubation at 37 °C for 24 h, the wells were washed with PBS three times and then fixed with methanol for 1 h and stained with 1 % crystal violet for 30 min. The cells on the top side of the membrane were scraped off, and the cells on the underside of the membrane were counted by a light microscope (Olympus BX51, Olympus) at 200 $\times$  magnification in ten randomly selected fields.

### In Vivo Metastasis and Immunohistochemistry

A total of  $1 \times 10^6$  cells in 0.2 ml PBS were injected subcutaneously into the tail vein of nude male mice (5–6 weeks old). Each experimental group contained six mice. The mice were sacrificed six weeks after injection, and their lung tissues were examined for metastases. The lung tissues were made into serial sections before HE staining and observed under a light microscope. The expression of GRP78 in lung tissues was examined by immunostaining analysis as described previously. All studies were performed according to the regulations of an IACUC protocol.

### Statistical Analysis

The results are presented as mean  $\pm$  SD. Comparisons between groups were made using Student's *t* test, one-way ANOVA test and Pearson chi-square ( $\chi^2$ ) tests or Fisher's exact tests (where applicable). The survival curves of the patients were drawn using the Kaplan–Meier method and the log-rank test was used for survival analysis. The SPSS16.0 software program (Chicago, IL, USA) was used for statistical analysis.  $p < 0.05$  was considered as statistically significant.

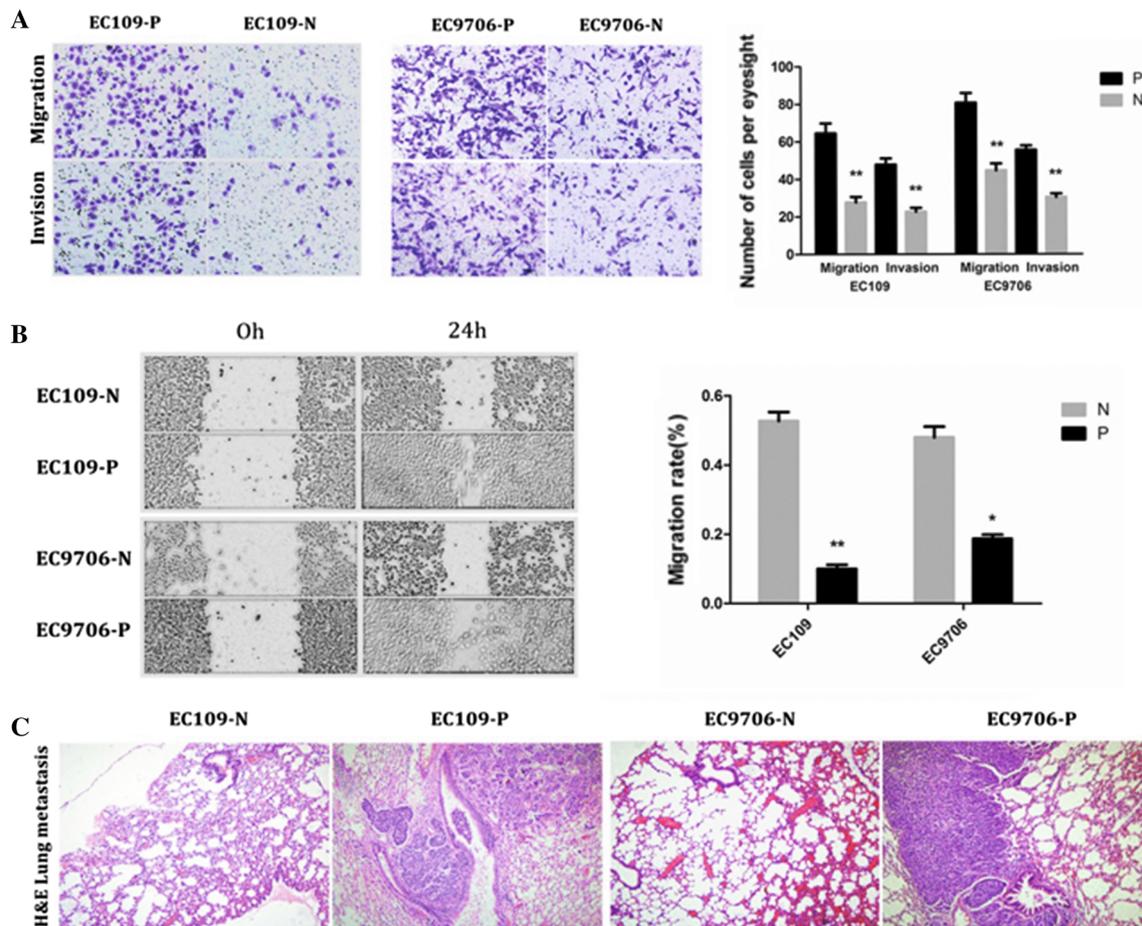
### Results

#### The Overexpression of GRP78 Is Associated with Lymph Node Metastasis in ESCC

The expression of GRP78 was examined in tissue sections from 113 ESCC patients by immunohistochemical staining, and GRP78 was found to be positively stained in the

**Table 1** Relationship between GRP78 expression and clinical features in patients with esophageal squamous cell carcinoma (ESCC)

Parameter	Negative	GRP78 expression		<i>p</i> value
		Moderate	Strong	
Age (years)	57.87 $\pm$ 7.86	59.02 $\pm$ 6.86	58.86 $\pm$ 8.69	
Gender				0.606
Male	32	29	23	
Female	14	9	6	
Tumor size (cm)				0.208
<4	19	10	7	
$\geq$ 4	27	28	22	
Location				0.606
Upper	13	7	3	
Middle	21	20	13	
Lower	12	11	13	
Differentiation				0.345
Well	25	19	10	
Moderate	15	13	16	
Poor	6	6	3	
AJCC stage				0.024
0–I	18	7	4	
II–IV	28	31	25	
Lymphatic invasion				0.002
Negative	37	17	10	
Positive	9	21	19	
LN metastasis				<0.001
Negative	31	13	7	
Positive	15	25	22	



**Fig. 2** The metastatic characteristics of highly and non-metastatic cells. EC109 and EC9706 cells were established by repeated transwell migrations as described previously. After ten rounds of selection, the highly metastatic sublines EC109-P and EC9706-P and the non-metastatic sublines EC109-N and EC9706-N were generated. In vitro migration and invasion activity of EC109 and EC9706 sublines were measured. Migration and invasion activities were measured in vitro and in vivo. **a** Representative photos of migrated and invasive cell numbers, magnification  $\times 200$ . *Bar graphs* represent the average number of cells on the underside of the membrane ( $*p < 0.05$ ;  $**p < 0.01$  by Student's *t* test). Number of transwell migrated cells in EC109-P or EC9706-P were compared to EC109-N ( $64.5 \pm 17.0$  vs.

$27.7 \pm 9.15$ ,  $44.5 \pm 5.52$  vs.  $20.8 \pm 3.43$ ,  $p < 0.01$ ) or EC9706-N ( $80.7 \pm 16.47$  vs.  $44.6 \pm 11.67$ ,  $55.5 \pm 7.50$  vs.  $30.4 \pm 6.06$ ,  $p < 0.01$ ), respectively. **b** Migration activities were measured with wound and healing analysis; magnification  $\times 40$ . *Bar graphs* represent the average migration rate of the cells ( $*p < 0.05$ ;  $**p < 0.01$  by Student's *t* test). The migration rate was significantly decreased in EC109-P-siGRP78 compared to EC109-P ( $0.53 \pm 0.045$  vs.  $0.10 \pm 0.020$ ,  $p < 0.01$ ) and EC9706-P-siGRP78 compared to EC9706-P ( $0.48 \pm 0.053$  vs.  $0.19 \pm 0.020$ ,  $p < 0.01$ ). **c** H&E staining of lungs isolated from mice that received intravenous tail injections of EC109-N, EC9706-N, EC109-P and EC9706-P cells; magnification  $\times 100$

cytoplasm of ESCC (Fig. 1a–d). The positive staining rate of GRP78 in the series is 59.29 % (67/113). An analysis of the relationship between the level of GRP78 expression and patients' clinical features revealed that GRP78 had a significantly higher positive staining rate in ESCC patients with LN invasion and metastasis than those without LN invasion, metastasis and stage ( $p < 0.05$ ). However, GRP78 expression had no significant correlations with age, sex, location and differentiation (Table 1;  $p > 0.05$ ). Patients with weak or strong positive GRP78 expression

**Table 2** The incidence of metastasis in mice that were injected with highly- and non-metastatic cells

Cell lines	Lung metastasis		<i>p</i> value
	Negative	Positive	
EC109-N	16	4	<0.05
EC109-P	3	17	
EC9706-N	14	6	<0.05
EC9707-P	5	15	

showed significantly poorer survival than patients with negative GRP78 expression ( $p < 0.01$ , Fig. 1e). The log-rank test revealed that the average survival lengths of patients with negative, weak or strong expression of GRP78 were  $32.63 \pm 1.99$ ,  $26.44 \pm 2.71$ ,  $20.07 \pm 2.32$  months, respectively ( $p = 0.014$ ). To further investigate the expression of GRP78 in ESCC patients, western blot analysis and a qRT-PCR assay were used for five tumor tissue samples with LN metastasis and five tumor samples without LN metastasis. The results revealed that GRP78 protein and mRNA was present at a higher level in tumor samples with LN metastasis compared with those without LN metastasis (Fig. 1f, g). Taken together, these results indicated that ESCC metastasis is associated with significantly increased expression of GRP78.

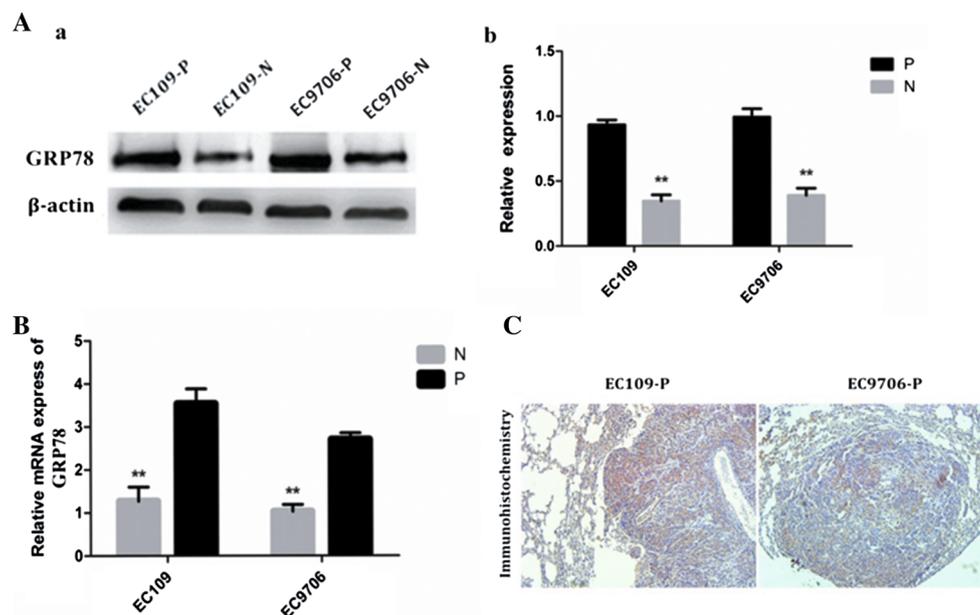
### Establishment and Characterization of Metastatic Sublines from EC109 and EC9706 Cells

To establish an ESCC metastasis model, highly metastatic cell lines EC109-P and EC9706-P and non-metastatic cell lines EC109-N and EC9706-N were selected from EC109 and EC9706 cells using repeated transwell assays. Cells were obtained after ten rounds of selection, and the metastatic ability of the cells was tested by transwell,

wound healing and in vivo assays. As shown in Fig. 2a, transwell assays showed the migration ability and invasion ability of EC109-P and EC9706-P were greater than that of EC9706-N and EC109-N. As shown in Fig. 2b, wound healing assays also showed similar results. Metastatic potential was also tested in nude mice in vivo. As shown in Fig. 2c, most of the mice displayed obvious lung metastasis after injection of EC109-P and EC9706-P cells, but only a few metastatic ESCC cells were detected in the lungs after injection of EC109-N and EC9706-N cells (Table 2).

### The Expression of GRP78 Is Specifically Upregulated in Highly Metastatic ESCC Cells

To confirm the role of GRP78 expression in highly- and non-metastatic cells, western blot analysis was employed and showed that GRP78 expression was markedly higher in EC109-P and EC9706-P cells compared with the matched non-metastatic cell lines EC109-N and EC9706-N (Fig. 3a). qRT-PCR also confirmed that highly metastatic cells had a higher mRNA level of GRP78 compared with the non-metastatic cells (Fig. 3b). Immunohistochemical analysis of lung metastasis formed from EC109-P and EC9706-P cells in nude mice confirmed GRP78 expression



**Fig. 3** Expression of GRP78 in highly- and non-metastatic cell lines. **a** The protein levels of GRP78 in highly metastatic cells (EC109-P and EC9706-P) and parental non-metastatic cells (EC109-N and EC9706-N) were examined by western blotting.  $\beta$ -actin was used as an internal control. The relative expression of GRP78 is significantly decreased in EC109-P compared to EC109-N ( $0.93 \pm 0.065$  vs.  $0.35 \pm 0.083$ ,  $p = 0.01$ ) and in EC9706-P compared to EC9706-N ( $0.99 \pm 0.110$  vs.  $0.39 \pm 0.095$ ,  $p = 0.02$ ). **b** The mRNA levels of GRP78 in highly- and non-metastatic cell lines were examined by

qRT-PCR and GAPDH was used as an internal control ( $*p < 0.05$ ;  $**p < 0.01$  by Student's  $t$  test). The relative expression of GRP78 is significantly decreased in EC109-P compared to EC109-N ( $1.27 \pm 0.571$  vs.  $3.53 \pm 0.602$ ,  $p = 0.01$ ) and EC9706-P compared to EC9706-N ( $1.03 \pm 0.276$  vs.  $2.7 \pm 0.264$ ). **c** Immunohistochemical analysis of lung metastasis tissues formed from EC109-P and EC9706-P cells confirmed the high positive rate of GRP78 expression

was at a high level (Fig. 3c). All of the results indicated that GRP78 might be involved in invasion and metastasis of ESCC cells.

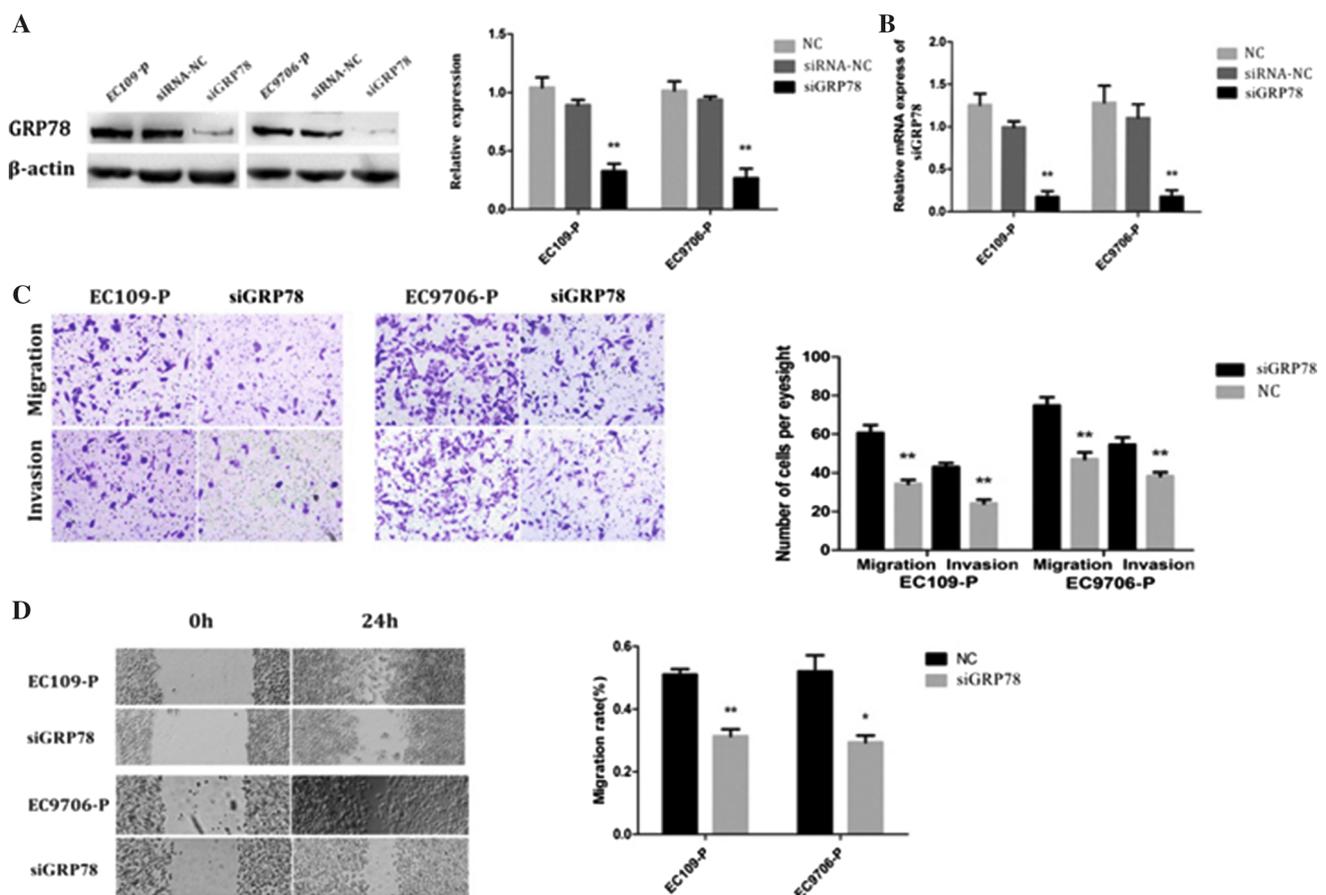
### Depletion of GRP78 Expression Inhibits Tumor Metastasis and Invasion in ESCC Cells

To further investigate whether GRP78 alters the capacity of ESCC cells for invasion and migration, GRP78 siRNA was transfected into EC109-P and EC9706-P cells. The down-regulation of GRP78 was confirmed by western blot analysis and qRT-PCR (Fig. 4a, b). As shown in Fig. 4c, the results of transwell assays showed that the migratory ability of GRP78 siRNA-transfected cells was significantly

reduced compared with control cells, and a reduction in the cells' invasion ability was also confirmed by invasion assay. In addition, wound and healing assays also showed similar results to the transwell assay (Fig. 4d). These results indicated that down-regulation of GRP78 could significantly inhibit ESCC cell invasion and migration in vitro.

### Depletion of GRP78 Expression Inhibits ESCC Metastasis and Invasion In Vitro

To confirm whether inhibition of GRP78 would affect the ability of ESCC cells to metastasize in vivo, the siGRP78-transfected cells and control cells were injected into nude



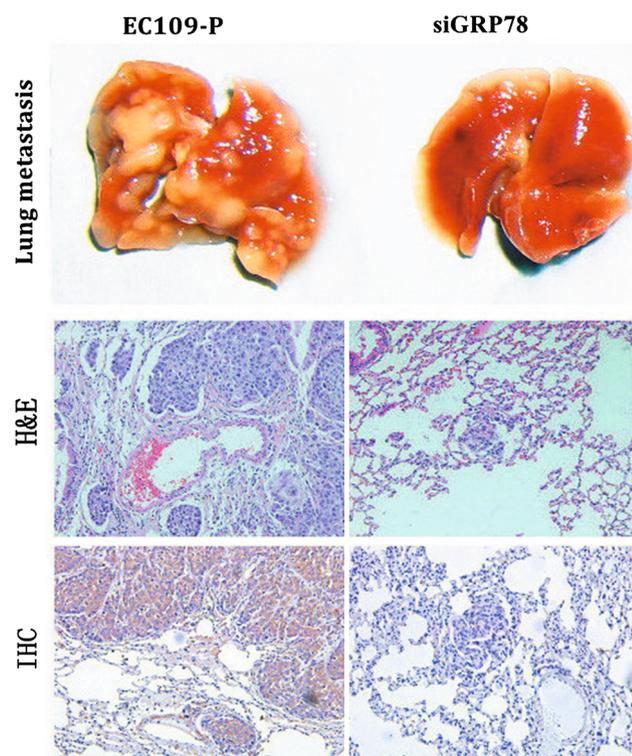
**Fig. 4** Suppression of GRP78 by siRNA and its effect on tumor cell invasion and metastasis in vitro. **a** Suppression of GRP78 by siRNA in cell lines EC109-P and EC9706-P were validated by western blot and qRT-PCR ( $*p < 0.05$ ;  $**p < 0.01$  by one-way ANOVA analysis, EC109:  $F = 31.907$ ,  $p = 0.01$ ; EC9706:  $F = 37.688$ ,  $p < 0.01$ ). **b** Suppression of GRP78 by siRNA in cell lines EC109-P and EC9706-P was validated by qRT-PCR ( $*p < 0.05$ ;  $**p < 0.01$  by one-way ANOVA analysis, EC109:  $F = 35.234$ ,  $p < 0.01$ ; EC9706:  $F = 14.842$ ,  $p < 0.05$ ). **c** Representative images of cell invasion and migration assay (magnification  $\times 200$ ). *Bar graphs* represent the average number of cells on the underside of the membrane ( $*p < 0.05$ ;  $**p < 0.01$  by Student's *t* test). The number of transwell

migrated cells was significantly decreased in EC109-P-siGRP78 compared to EC109-P ( $60.6 \pm 13.099$  vs.  $34.4 \pm 6.535$ ,  $43.0 \pm 6.532$  vs.  $24.2 \pm 6.338$ ,  $p < 0.01$ ) and EC9706-P-siGRP78 compared to EC9706-P ( $74.9 \pm 13.370$  vs.  $47.0 \pm 11.255$ ,  $54.5 \pm 11.937$  vs.  $38.4 \pm 5.891$ ,  $p = 0.02$ ). **d** Migration activities were measured with wound and healing analysis; magnification  $\times 40$ . *Bar graphs* represent the average migration rate of the cells ( $*p < 0.05$ ;  $**p < 0.01$  by Student's *t* test). The migration rate was significantly decreased in EC109-P-siGRP78 compared to EC109-P ( $0.51 \pm 0.03$  vs.  $0.313 \pm 0.0379$ ,  $p = 0.03$ ) and EC9706-P-siGRP78 compared to EC9706-P ( $0.52 \pm 0.0889$  vs.  $0.293 \pm 0.0379$ ,  $p = 0.015$ )

**Table 3** The incidence of metastasis in mice that were injected with control and siGRP78 cells

Cell line	Lung metastasis		<i>p</i> value
	Negative	Positive	
EC109-control	5	15	<0.05
EC109-siGRP78	13	7	

mice through the tail vein. The results showed that few lung metastases were found in mice injected with siGRP78-transfected cells compared with the controls (Table 3). Histological analyses revealed that the number and the size of metastatic nodules in the lungs of mice were significantly smaller in the controls. Moreover, immunohistochemical analysis of lung metastasis tissues formed from siGRP78-transfected cells confirmed GRP78 expression was lower compared to the controls (Fig. 5). Taken together, these results confirmed that down-regulation of GRP78 could inhibit the metastasis of ESCC cells in vivo.



**Fig. 5** Inhibition of metastasis of ESCC in vivo by GRP78 siRNA. Tumor metastatic model was established by injection of ESCC cells through the tail vein ( $n = 8$  per group). Representative photos of lungs harvested at six weeks post injection are shown. Note that downregulation of GRP78 significantly reduced the number and size of the metastatic nodules in the lungs. Representative H&E-stained lung sections from the two groups of mice showing metastatic cancer cells in the lungs are shown. Immunohistochemical analysis of lung metastasis tissues formed from EC109-P and siGRP78 cells showing the expression of GRP78 are also shown

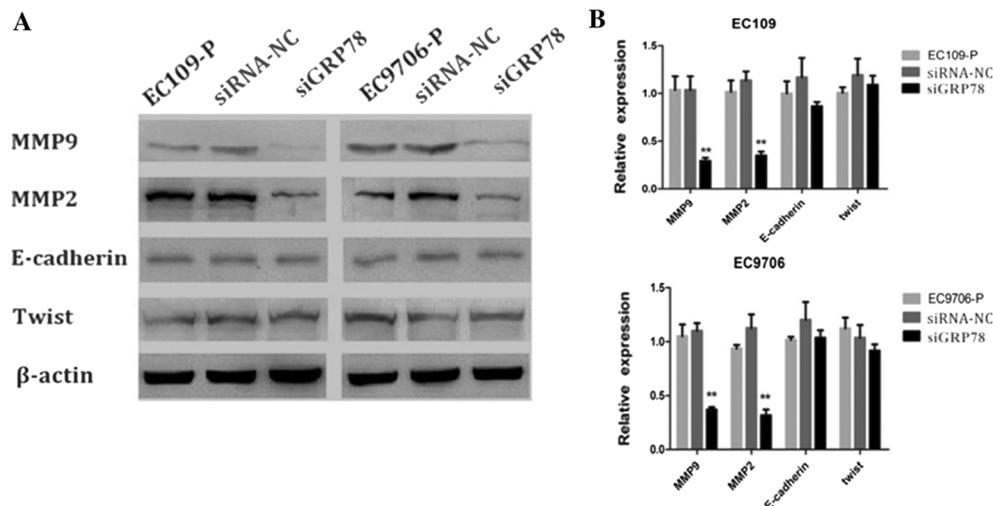
## Depletion of GRP78 Altered the Expression of Metastasis-Related Proteins

Previous studies have reported that GRP78 is involved in metastasis of tumors through multiple mechanisms [15]. To further investigate the role and mechanisms of GRP78 in metastasis and invasion of ESCC, the expression of metastasis-related genes such as E-cadherin, twist, MMP2 and MMP9 was examined in EC109-P and EC9706-P cells in which GRP78 had been knocked down. Western blot analysis showed the expression of MMP2 and MMP9 were downregulated when GRP78 was decreased, whereas the expression of E-cadherin and twist was not significantly changed when GRP78 was depleted (Fig. 6). These results indicated that down-regulation of the MMPs might suppress the metastasis in GRP78 down-regulated cells. This finding suggests that down-regulation of GRP78 might suppress metastasis by affecting the expression of MMP-2 and MMP-9 in ESCC cells.

## Discussion

As one of the most aggressive tumors, ESCC metastasis is a life-threatening problem with few satisfactory clinical treatments available. Cancer cells migrate from their primary location to lymph or blood vessels during metastasis, and LN metastasis often occurs at early stages [17]. In our study, immunohistochemistry results in 113 ESCC patients reveal that the expression of GRP78 was higher in patients with LN metastasis than in those without LN metastasis. Additionally, patients with positive GRP78 expression had a better five-year survival rate than patients with negative GRP78 expression. Moreover, western blot analysis and qRT-PCR both showed that GRP78 is overexpressed in tumor tissues with LN metastasis compared with tumor tissues without LN metastasis. To our knowledge, this is the first report that demonstrates that GRP78 may be involved in ESCC patient metastasis.

Heat-shock proteins (HSPs) are major regulators of the unfolded protein response (UPR) due to their roles as molecular chaperones. The expression of the glucose regulated protein GRP78, which is a member of the HSP family and is also referred to as BiP (immunoglobulin heavy chain-binding protein), can be enhanced under certain stress conditions including hypoxia, glucose deprivation, acidosis and so on [18]. GRP78 is localized in the endoplasmic reticulum (ER) and possesses corresponding functions, such as controlling protein folding and assembly,  $\text{Ca}^{2+}$  binding and regulating ER stress signaling [19]. A number of studies have demonstrated that GRP78 is significantly overexpressed in a variety of tumors including ESCC, suggesting that GRP78 may play an important role



**Fig. 6** Effect of down-regulation of GRP78 by siRNA on metastasis-related molecules. Cells were transfected with control siRNA or siRNA targeting GRP78. Total cell lysates were immunoblotted for E-cadherin, twist, MMP-9, and MMP-2,  $\beta$ -actin was used as an internal control. siRNA-NC: cells were transfected with control siRNA; siGRP78: cells were transfected with siRNA targeting GRP78. Expression of E-cadherin ( $F = 0.653$ ,  $p > 0.5$ ), twist

( $F = 0.632$ ,  $p > 0.5$ ), MMP-9 ( $F = 23.250$ ,  $p < 0.01$ ) and MMP-2 ( $F = 22.034$ ,  $p < 0.001$ ) in EC109-P cells was examined by western blotting. Expression of E-cadherin ( $F = 0.314$ ,  $p > 0.05$ ), twist ( $F = 0.961$ ,  $p > 0.05$ ), MMP-9 ( $F = 28.081$ ,  $p < 0.001$ ) and MMP-2 ( $F = 27.141$ ,  $p < 0.001$ ) in EC9706-P cells was examined by western blotting (one-way ANOVA analysis,  $n = 3$ , \* $p < 0.05$ ; \*\* $p < 0.01$ )

in tumor biology. However, most of these studies focus only on the roles of GRP78 in promoting tumor growth and drug resistance [20, 21], and few papers have investigated the metastatic role of GRP78 in tumors. In addition, the function of GRP78 in ESCC remains unclear.

Tumor cell migration and invasion are crucial steps in metastasis. Metastasis is a multistep process of complex interactions between tumor cells and the host. To metastasize, tumor cells must have the capability of transversing the basement membrane and stromal matrix. This process involves cell detachment, matrix dissolution and cell migration. The cells then must enter into the blood stream, survive in the circulatory system, exit from blood vessels and finally, settle down in different organs [22]. The expression level of GRP78 was measured in highly metastatic and non-metastatic cells, and we found that the protein and mRNA level of GRP78 in highly metastatic cells was higher than that in non-metastatic cells. To further examine whether GRP78 contributes to ESCC cells' invasion and metastasis, we used GRP78 siRNA vectors to knockdown the expression of GRP78, which resulted in a decrease of invasion and metastasis capability in vivo and in vitro. These results showed that downregulation of GRP78 expression inhibited the invasion and migration of ESCC cells.

It has been reported that GRP78 is involved in metastasis by regulating ECM degradation and the activity of MMPs in hepatocellular carcinoma. Moreover, mounting evidence has demonstrated the involvement of EMT in

cancer metastasis. The role of GRP78 in modulating MMPs and EMT-related genes in ESCC, however, has not been reported. To explore the possible mechanisms by which GRP78 induces ESCC cell invasion and migration, the expression of metastasis-related genes MMP-2, and MMP-9 and EMT-related genes E-cadherin and twist were examined by western blot. The results showed that MMP-2 and MMP-9 were significantly decreased in GRP78-deleted cells. However, the expression of E-cadherin and twist was not significantly affected. MMP-2 and MMP-9, which are both MMP gelatinases, are involved in the proteolysis of the major components of the basement membrane and are reported to mediate the invasion and metastasis in tumor development [23–25]. These results indicated that downregulation of MMP-2 and MMP-9 might suppress the metastatic potential in cells in which GRP78 is down-regulated.

In summary, for the first time, our study reveals that GRP78 is involved in metastasis of ESCC and is associated with the prognosis of ESCC patients. Our findings show that downregulation of GRP78 restricts cancer cell invasion and migration and that the role of GRP78 in metastasis may be associated with the regulation of the expression of MMPs, especially down-regulation of MMP-2 and MMP-9. Taken together, these results suggest that targeting GRP78 may provide appealing therapeutic strategies for the treatment of ESCC patients with metastasis, and GRP78 may serve as a cellular target for the development of novel therapeutic approaches.

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**Conflict of interest** None.

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